

Nucleosome assembly alters the accessibility of the antitumor agent duocarmycin B₂ to duplex DNA

Tingting Zou^a, Seiichiro Kizaki^a, Ganesh N. Pandian^b, Hiroshi Sugiyama^{a,b*}

Abstract: To evaluate the reactivity of antitumor agents in a nucleosome architecture, we conducted *in vitro* studies to assess the alkylation level of duocarmycin B₂ on nucleosomes with core and linker DNA using sequencing gel electrophoresis. Our results suggested that the alkylating efficiencies of duocarmycin B₂ were significantly decreased in core DNA and increased at the histone-free linker DNA sites when compared with naked DNA condition. Our finding that nucleosome assembly alters the accessibility of duocarmycin B₂ to duplex DNA could advance their design as antitumor agents.

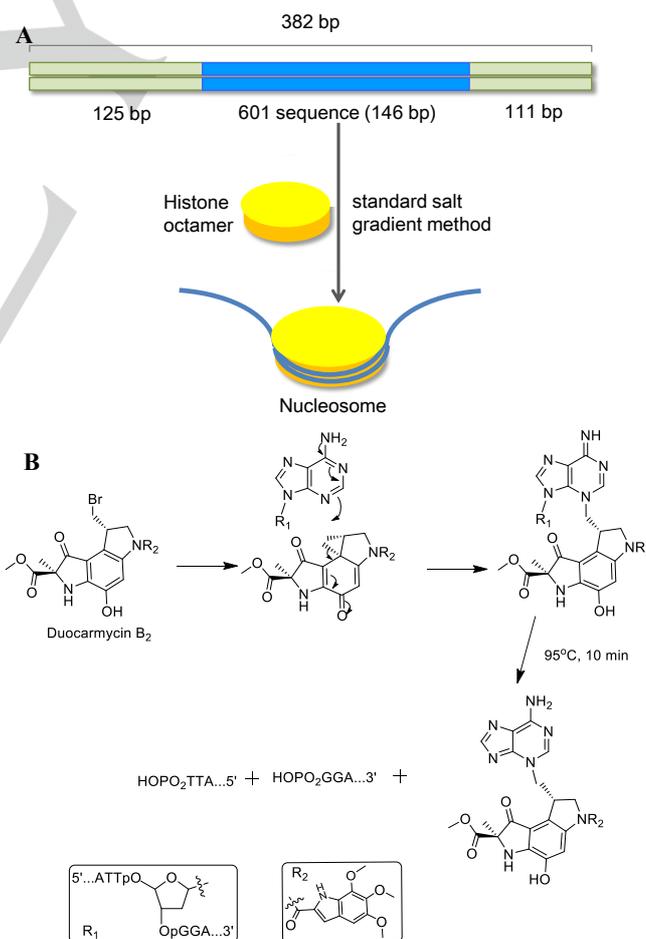
Advancing anti-tumor agents is of significant importance, as effective chemotherapy of malignant tumors is one of the great challenges of modern medicine^[1]. Conventional agents have been applied to many cancer therapies, and new anti-tumor agents having therapeutic potential are being discovered. Duocarmycin, first reported in 1988^{[2][3]} and their analogues have been beneficial in treating many types of tumor disease^{[1][4]} via covalent interaction with DNA minor groove adenine N3 alkylation site^{[5][6]}. However, in eukaryotic cells, the assembly of DNA and histone proteins into nucleosomes (the basic repeating subunits of chromatin) packs over two meters of DNA into the confines of the nucleus^[7]. In such a highly compact structure, whether antitumor agents exhibit similar or distinct accessibility to duplex DNA as in the naked condition is yet to be clarified.

A report about the accessible regions of nucleosome core particles (NCPs) to minor-groove-binding pyrrole-imidazole polyamides (PIPs) showed that PIPs fail to bind NCPs only at sites that are blocked by interactions with the histone octamer^[8]. NCPs were also shown to be fully accessible to some minor-groove-alkylating agents, and both the selectivity and efficiency of DNA alkylation were suggested to be unaffected by NCP packaging^[9]. Furthermore, the base pair unstacking arising from DNA stretch induce extreme minor groove kinking near the nucleosome center, which in turn generate a hot spot for intercalation facilitating the alkylation by a novel anticancer compound^[10].

However, in the actual chromatin condition, each nucleosome

encompass not only nucleosome 'core', but also linker DNA, and in most instances, a linker histone^[11]. As the essential composition of nucleosome, linker DNA provide a binding region for linker histone and then regulate epigenetic transcription^[12]. Cisplatin and analogues were demonstrated that the preferred site of DNA binding was in the linker region of the nucleosome^[13]. Some researchers adopted DNA fragment from the *Xenopus* Zaeuis 5 S rRNA gene for nucleosome reconstitution. The results exhibited that marked inhibition of the DNA cleavage induced by bleomycin (5 fold) and neocarzinostatin (2.4 fold) in the central region of nucleosomal DNA. Also in melphalan, about 2 fold inhibition in alkylation at adenine N-3 throughout the nucleosome was shown^{[14][15]}.

Taken together, the previous reports suggest the existence of undiscovered nucleosome features that can be harnessed in advancing the cancer drug discovery. Accordingly, 382 bp DNA containing Widom 601 nucleosome positioning sequence and duocarmycin B₂ were adopted for the nucleosome reconstitution



[a] T. Zou, S. Kizaki, Prof. H. Sugiyama
Department of Science, Graduate School of Science
Kyoto University
sakyo, Kyoto 606-8501, Japan
hs@kuchem.kyoto-u.ac.jp

[b] Dr. N. G. Pandian, Prof. H. Sugiyama
Institute for Integrated Cell-Material Sciences (iCeMS)
Kyoto University
Yoshida Ushinomiya-cho,
Sakyo, Kyoto 606-8502, Japan

Figure 1. (A) Preparation of mono-nucleosome; (B) Mechanism of duocarmycin B₂ alkylating DNA

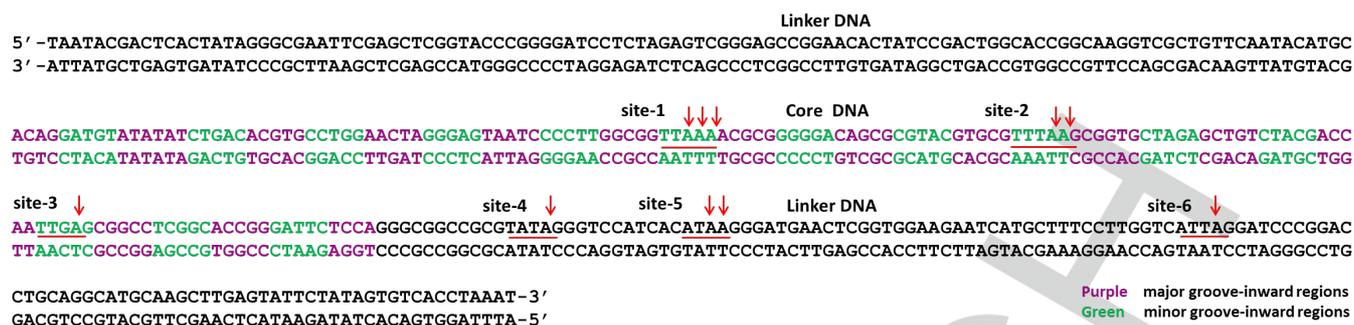


Figure 2. The sequence of 382 bp DNA and alkylating sites in core and linker DNA fragment.

and evaluation, to verify the influence of nucleosome assembly with linker part on the accessibility of small molecular agents into duplex DNA.

To construct the nucleosome structure, we used Texas red labeled 382 bp DNA containing 146 bp nucleosome-positioning 601 sequence^[16] flanked by two DNA arms of different lengths, 125 and 111 bp, respectively (Figure 1A, Figure 2). Subsequently, we amplified DNA through PCR from pGEM3Z-601 using the following primers: forward, 5'-Texas Red TAATACGACTCACTATAGG-3', and reverse, 5'-ATTAGGTGACACTATAGAATAC-3'^[17]. After the reaction, the amplified DNA was purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA). The purified DNA was then used for nucleosome reconstitution with histone octamer (Epicpypher, NC, USA) by standard salt gradient method^{[18][19]}. The reconstitution efficiency was confirmed by gel mobility shift assay (6% Native PAGE, 100V, 1h).

Duocarmycin B₂ at various concentrations with 6.25 mM sodium phosphate buffer (pH 7.0) alkylated the 5'-Texas Red labeled 382 bp free-DNA or nucleosomes at room temperature for 18 h. After incubation, the reaction mixture was quenched by addition of calf thymus DNA followed by heat treatment at 95°C for 10 min. The alkylating mechanism of duocarmycin B₂ was showed in Figure 1B. The solution was concentrated using vacuum centrifugation. The pellet was re-dissolved in 7 μ L of loading dye, heated at 95°C for 25 min, and then immediately cooled down to 0°C. A 0.8 μ L aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel by using a Hitachi DNA sequencer^[20]. The results were analyzed and shown with FRAGLYS version 2 in SSCP calculation mode.

The reconstituted nucleosomes confirmed by gel mobility shift assay (Figure S1) were directly used for alkylating reaction. After incubation at room temperature for 18 h, gel shift was also used for confirming the nucleosome condition, in consistent with previous report^[9], the result (Figure S2) indicated that nucleosome dissociation was not caused by the treatment with agents, nucleosomes incubated at room temperature showed similar stability as incubated at 4°C for 18 hours. Because prolonging the cleaving time from 10 min to 50 min did not yield significant difference (Figure S3), heating at 95°C for 10 min was adopted in the subsequent investigations.

Varying concentrations of duocarmycin B₂ were tested in the presence of free-DNA or nucleosome in 20 nM (Figure 3). In accordance with a previous report^[9], no unexpected alkylating sites were observed in either free DNA or nucleosomes. Both DNA and nucleosomes were alkylated to a higher degree with increasing concentrations of duocarmycin B₂. Figure 2 indicates that, in the core DNA, all three alkylating sites located in the minor groove inward region, which is consistent with previous

research. While evaluating the alkylating efficiency, while we observed notable alkylating efficiency differences between nucleosome and free DNA samples. In the core DNA region, three alkylating sites were found which all showed an obvious decreasing alkylating efficiency for nucleosomes compared with free DNA, site 2 even exhibited a decrease in efficiency more than 3 fold (from 21.5% to 6.3%) in the presence of 0.5 μ M duocarmycin B₂ (Figures 3 and 4). However, the first site, which includes three possible alkylating adenines (Figure 2), sometimes shows fluctuating efficiency changes for predictable better agent accessibility.

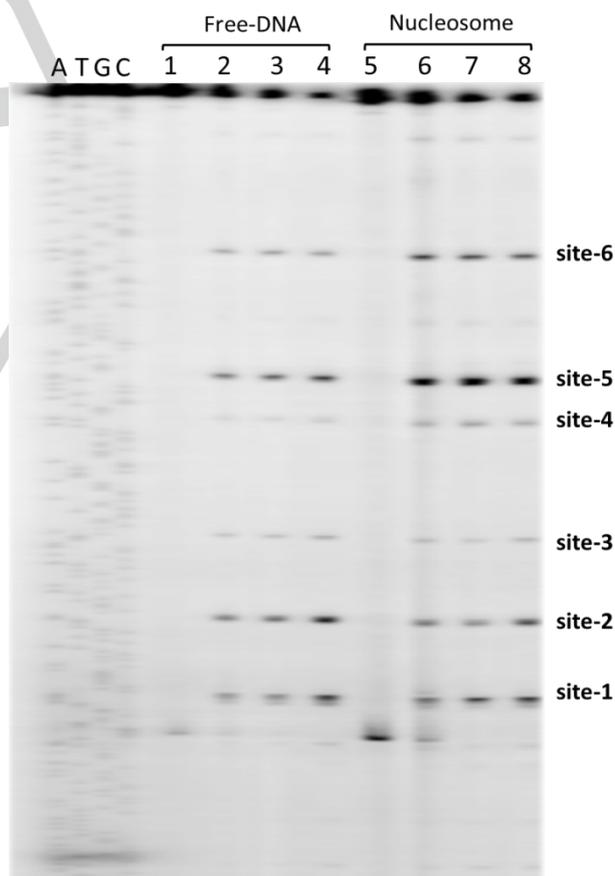


Figure 3. Thermally induced strand cleavage (10 min, 95°C) of 382 bp DNA and nucleosome with different concentration of duocarmycin B₂. lanes 1-4, free DNA (20 nM) + duocarmycin B₂ (0.1 μ M, 0.25 μ M, 0.5 μ M, respectively); lanes 5-8, nucleosome (20 nM) + duocarmycin B₂ (0.1 μ M, 0.25 μ M, 0.5 μ M, respectively).

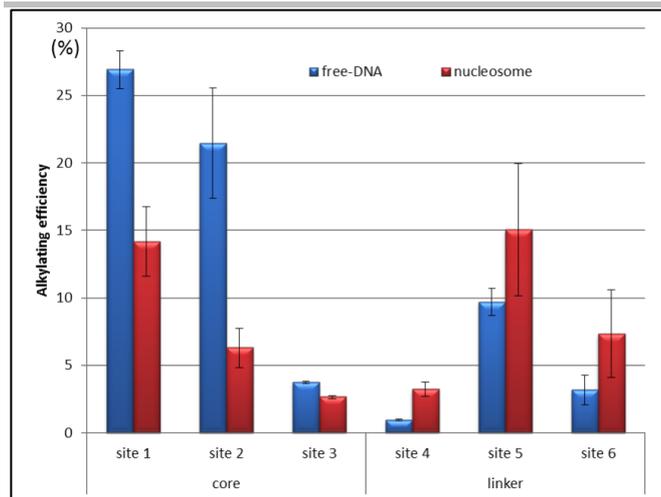


Figure 4. Alkylating efficacy of duocarmycin B₂ in 0.5 μM, with free DNA or nucleosome in 20 nM. The data are mean ± SD of repeated experiments (n=3).

In contrast with the decreasing alkylating efficacy observed at core sites, the efficiency of alkylating sites in the nucleosome linker DNA region was significantly increased when compared with that observed in the free DNA (Figure 3, Figure 4). Alkylating sites 5 on linker DNA region, which possesses a relatively higher reaction activity for two potential alkylating adenines, showed the most obvious alkylating band and efficiency increased from 9.7% to 15.0% in nucleosomal DNA. These results also suggest that, in the nucleosome structure, the linker DNA region shows similar or higher reactivity than the core DNA region. While in the naked DNA condition, lower reactivity in DNA linker region could be observed.

We also attempted to confirm these results using different concentrations of free DNA and nucleosome in the presence of 0.5 μM duocarmycin B₂, and similar tendencies were observed (Figure S4). These results indicated that the accessibility of duocarmycin B₂ to duplex DNA is altered in the presence of nucleosome assembly and the agent preferentially alkylates the naked DNA part (linker region) rather than the DNA that is packaged with histone proteins.

In conclusion, this present *in vitro* study was conducted to elucidate the mechanistic information regarding the accessibility of duocarmycin B₂ to duplex DNA in nucleosomes containing a linker DNA region. Compared with free DNA, in the presence of nucleosome structure, duocarmycin B₂ showed a significantly decreased efficiency in accessing the nucleosome core DNA, while exhibiting an increased efficiency in accessing the linker DNA region. This finding also suggested that although the efficiency of alkylating agents enters into nucleosome core region showed decreased binding, the targeting capability is still retained, and even higher in linker DNA region, while might be considered when designing drugs for targeting key promoter region.

Keywords: Nucleosome • Duocarmycin B₂ • Alkylation • Accessibility

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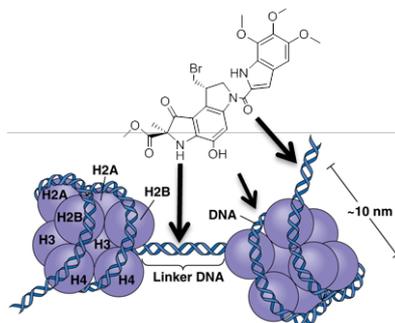
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Layout 1:

COMMUNICATION

Nucleosome assembly influence:

In vitro studies aimed at evaluating the alkylation level of duocarmycin B₂ on nucleosomes with core and linker DNA were conducted using sequencing gel electrophoresis. Our results suggested that in nucleosome structure, the alkylating efficiencies of duocarmycin B₂ were significantly decreased in core DNA and increased at the histone-free linker DNA sites compared with naked DNA condition.



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Namasivayam Ganesh Pandian^b,
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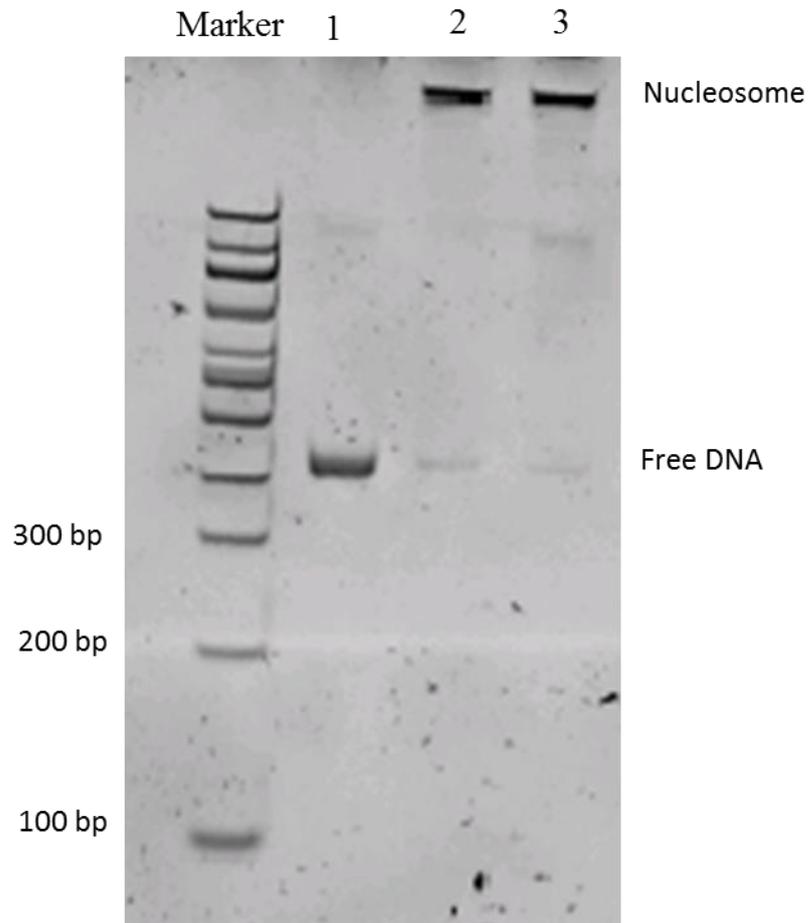


Figure S1. Nucleosome reconstitution efficiency confirmation, native gel electrophoresis of Texas red labeled 382 bp DNA and reconstituted nucleosomes (DNA: histone octamer in 2:3 molar ratio). Lane 1, control free DNA; lane 2, reconstituted nucleosome; lane 3, reconstituted nucleosome with unlabeled 382 bp DNA.

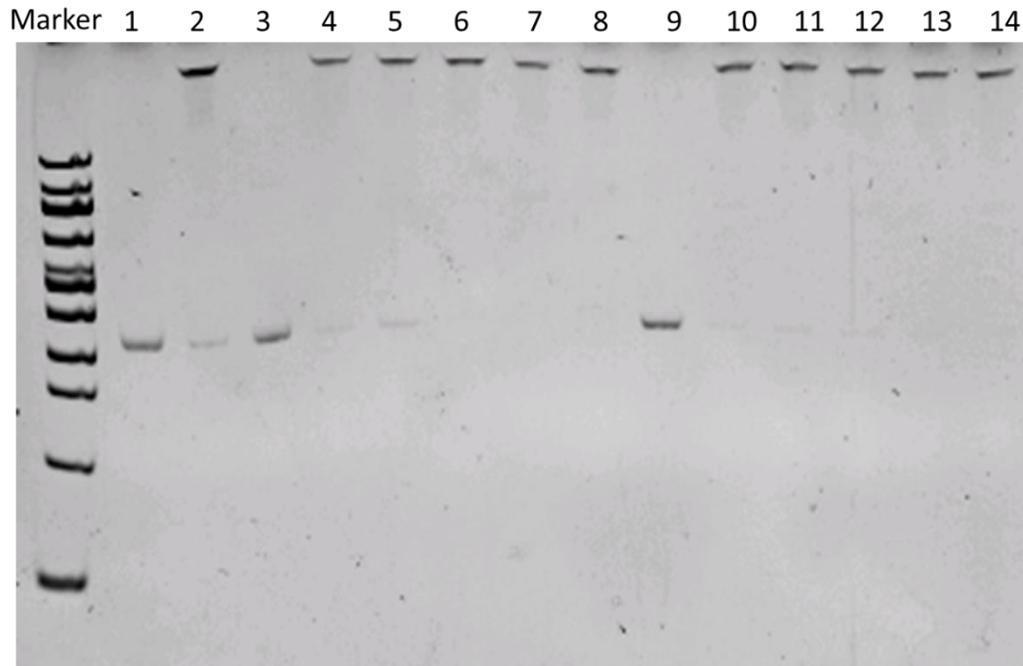


Figure S2. Nucleosome stability confirmation, non-denaturing gel electrophoresis of 5' end-Texas red labeled 601 DNA and reconstituted nucleosomes (DNA : histone octamer in 2:3). Lane 1, control free DNA; lane 2, reconstituted 601 nucleosome; lanes 3-8, room temperature, 18 h, DNA or nucleosome in 20 nM, 3: DNA with duocarmycin (0.1 μ M), 4: nucleosome control, 5-6: nucleosome with duocarmycin (0.1 μ M), 7-8: nucleosome with duocarmycin (0.5 μ M); lanes 9-14, 4°C, 18 h, 9: DNA with duocarmycin (0.1 μ M), 10: nucleosome control, 11-12: nucleosome with duocarmycin (0.1 μ M), 13-14: nucleosome with duocarmycin (0.5 μ M).

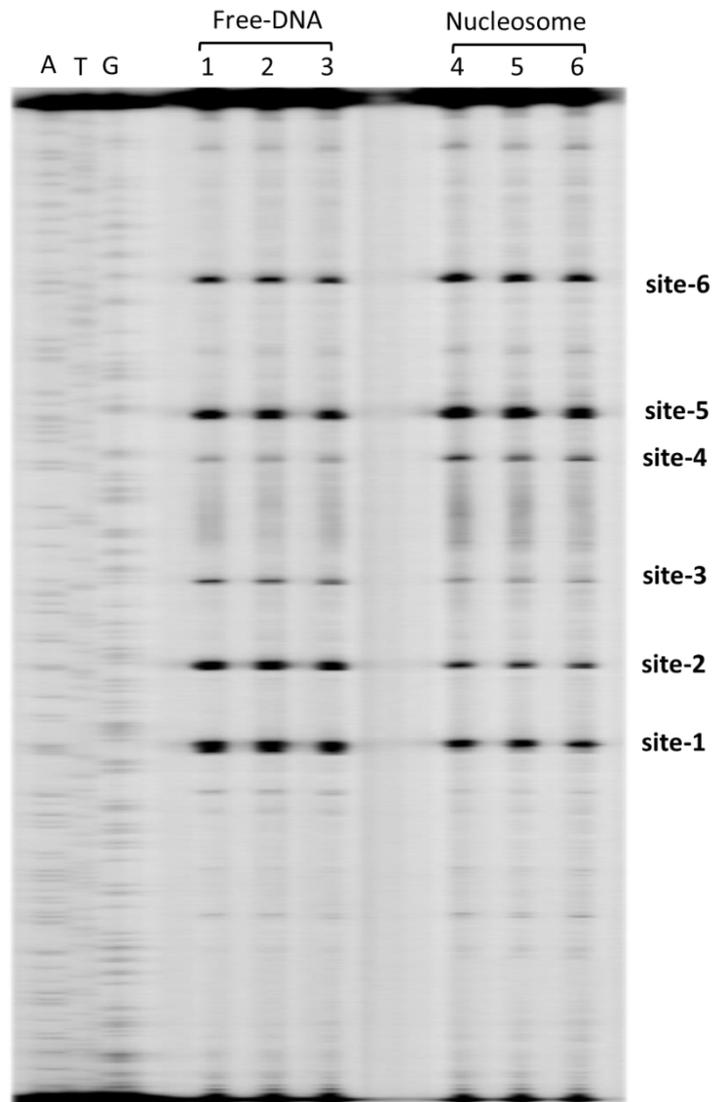


Figure S3. Thermally induced strand cleavage of 382 bp DNA and nucleosome with different heating time. Lanes 1-3, free DNA (30 nM) + duocarmycin B₂ (0.5 μM), heating in 95°C (10 min, 25 min, 50 min, respectively) ; lane 4-6, nucleosome (30 nM) + duocarmycin B₂ (0.5 μM), heating in 95°C (10 min, 25 min, 50 min, respectively).

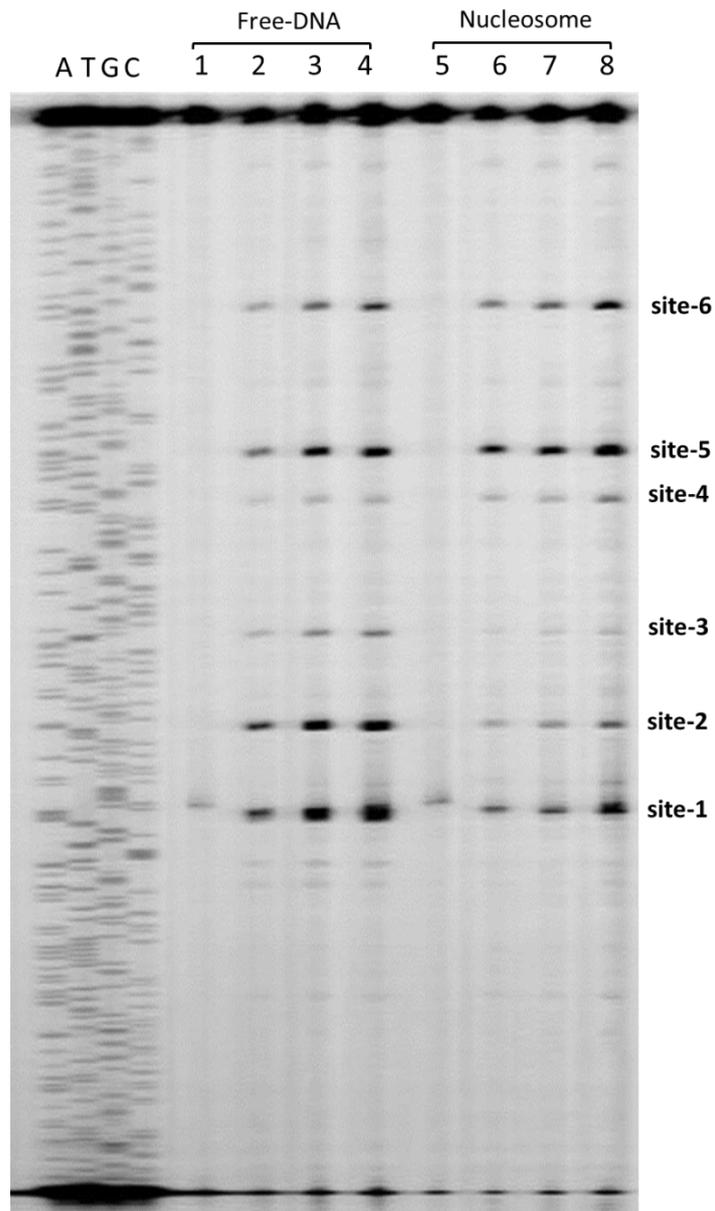


Figure S4. Thermally induced strand cleavage (10 min, 95°C). lane 1, control free DNA; lanes 2-4, duocarmycin B₂ (0.5 μM) + free DNA (10 nM, 20 nM and 30 nM respectively); lane 5, control nucleosome; lanes 6-8, duocarmycin B₂ (0.5 μM) + nucleosome (10 nM, 20 nM and 30 nM, respectively).