SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Doxorubicin-induced global changes and topological heterogeneity of chromatin

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The Examination takes place at the 1.405/B room, 1st floor, Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, at 9 a.m. on 10th of June, 2021.

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The PhD Defense (online format) takes place at 11 a.m., on 10th of June, 2021.

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Introduction

DNA topology is a complex and in many ways still enigmatic aspect of DNA and consequently chromatin structure. It is strongly affected by drugs of chemotherapeutic relevance, e.g. anthracyclines. My general goal was to study and better understand the effect of a prototypic anthracycline, Doxorubicin (Dox) on chromatin in settings resembling live cell conditions. I noticed previously unrecognized phenomena which are the focus of my thesis (part I). In its second part I will summarize the results of yet another line of research I pursued during my PhD studies, connected to the Dox-related project but of more general interest for global nuclear organization and potentially for transcriptional regulation (part II).

1. The chemotherapeutic significance of anthracyclines and their side effects

Anthracyclines are a significant group of chemotherapeutic agents and are currently widely used. Among the most commonly used members of this group, it is important to mention daunomycin (Dau), doxorubicin (Dox), aclarubicin and idarubicin. Dox is used either alone or in combination with other chemotherapeutic agents, thus reducing toxicity and increasing therapeutic efficacy. Among other solid tumors, Dox is currently present in the therapeutic protocol of breast, ovarian, bladder cancer, Kaposi's sarcoma, small cell lung cancer and multiple myeloma, furthermore, Dox is also used to treat various lymphomas and leukemias. Anthracyclines, like other cytostatics, have many side effects. Non-specific side effects typical of cytostatics are fatigue, headache, nausea, vomiting and hair loss due to the inhibition of cell division in the hair follicles are common. Inhibition of bone marrow hematopoietic stem cell division causes myelosuppression. Anthracyclines cause secondary malignancies in many cases and their most important and dangerous specific side effects are on myocardial cells, causing cardiomyopathy. Understanding the exact mechanism of action of Dox may help to mitigate the side effects that severely limit therapy.

2. Mechanism of action of anthracyclines and superhelicity of DNA

Anthracyclines are pleiotropic drugs that exert their effects at several points. Their best described mechanism of action is the inhibition of Topoisomerase II (TopII) enzymes involved in the regulation of DNA superhelicity. The superhelicity of DNA is altered by various nuclear processes such as DNA replication, transcription, recombination, DNA repair and chromatin remodeling. Topological changes arising due to nuclear processes are relaxed by topoisomerases. By creating strand breaks, DNA can rotate around its own axis, thus relaxing under- or overwinding of DNA. The target of Dox is the TopII enzyme. In the first step of its catalytic cycle TopII binds to DNA, followed by the cleavage of one or both strands of the DNA molecule, independently by the two TopII subunits, so that they become covalently bound to the 5' phosphates via the strategic tyrosin residues of the enzyme. The next step is the passage of the DNA strands in the case of decatenation, or simple relaxation in the absence of another DNA molecule, followed by the ligation of the strand breaks. The whole process is ATP-dependent. Although TopII enzymes are capable of creating transient double-strand breaks, they are also known to cause transient single-strand breaks. Human cells express two isotypes, alfa and beta, involved dominantly in decatenation activity at the end of S phase and in transcriptional regulation, respectively. As we will see below, Dox can modulate superhelicity of DNA not only through inhibition of the TopII enzyme, but also via direct effects on chromatin structure.

3. Binding of anthracyclines to chromatin: intercalation, DNA adduct formation and histone binding

Dox and the other anthracyclines intercalate between DNA bases. During intercalation the anthracycline forms a hydrogen bond with one of the guanine bases of DNA, thus stabilizing itself. Using the reactive groups of formaldehyde present intracellularly, it forms a covalent bond with one of the guanine bases of the DNA, thus cross-linking the strands and forming a DNA adduct. Intercalation of Dox and cross-linking of DNA strands inhibit strand separation, thus hampering DNA replication and transcription. Binding to intracellular histones also contributes to the pleiotropic effect of anthracyclines. Dau (Daunomycin) binds to both free linker and core histones in solution, however, as for chromatin-bound histones, only binding to the linker histone seems significant, with little binding to chromatin-bound core histones.

4. Relaxation due to intercalation, consequent nucleosome destabilization and its relation to DNA topology

DNA intercalation of anthracyclines has topological consequences. During intercalation, the negative superhelicity of DNA becomes less negative, causing structural changes in the nucleosome structure by reducing first the binding strength of H2A / H2B dimers, thus increasing the likelihood of dimer dissociation, which causes the destabilization of the nucleosome. Due to the synthetic processes and the organization of chromatin, superhelicity is not homogeneously distributed in the genome, and since the process of intercalation depends on the superhelicity of DNA, the destabilizing effect of intercalation on nucleosomes may be locally different. We have previously described that nucleosome destabilization due to intercalation is a consequence of DNA relaxation. We have previously shown that promoter-proximal nucleosomes (with H3K4me3 and H3K27ac posttranslational modifications) dissociate from chromatin at lower Dox concentrations. Thus, the global phenomena we detected (see Results) may occur locally at lower Dox concentrations in the Dox-preferred regions of chromatin. To investigate the posibility that the effect of intercalators on chromatin may be uneven along the chromatin, we mapped the chromatin regions that contain endogenous strand discontinuities and consequently formed topological domains containing relaxed DNA, potentially accomodating Dox more readily. This line of research has got further, more general implications on its own right, in addition to its relevance to the anthracycline effects.

5. Mapping of endogenous DNA breaks

Anthracyclines, like etoposide, inhibit the religation step of topoisomerase II, increasing the number of TopII enzymes bound to the 5' end of DNA (cleavage complexes), thus leading to the accumulation of DNA strand discontinuities containing a free 3' OH group. Since anthracyclines poison TopII in a DNA-bound state and prevent the ligation of the DNA strand discontinuities, by mapping the accumulated DNA breaks we get data regarding the interaction of the genome with TopII. Single-strand discontinuities containing a free 3' OH group can be enzymatically labeled with E. coli DNA polymerase, which incorporates biotin-labeled nucleotides in a template-dependent manner at the free 3' OH groups at the end of the DNA breaks, replacing a stretch of old DNA with newly incorporated nucleotides in a process called nick-translation, or

by the template-independent terminal transferase enzmye. Labeled DNA fragments can be visualized and validated by molecular combing of DNA isolated from both yeast and human cells. Using these labeling methods, I examined the distribution of single-stranded discontinuities containing free 3 'OH groups at the sequence level using DNA immunoprecipitation in combination with new generation sequencing (in collaboration descibed in Materials and Methods) and validated the data by quantitative PCR.

6. Anthracycline-induced chromatin aggregation

In addition to intercalating into DNA, destabilizing nucleosomes and binding to histones, anthracyclines also cause chromatin aggregation, which occurs at therapeutic concentrations. Based on earlier experiments published by other groups, the mechanism of chromatin aggregation was explained as follows: anthracyclines bind to chromatin-bound H1 histones, which promotes their dissociation. Furthermore, anthracyclines intercalate into internucleosomal linker DNA, resulting in more rigid linker DNA, thus the supranucleosomal folding of chromatin is changed, which gradually spreads to the nucleosomal DNA, changing the structure of chromatin which eventually leads to the formation of new contacts between histones and other chromatin fibers. As the amount of DNA wound on the nucleosome decreases, the histone octamer is partially destabilized, resulting in aggregated chromatin.

7. Nucleo-cytoplasmic transport of histones H1, H2A and H2B

According to my observations, Dox treatment results in the nucleo-cytoplasmic translocation of histone H2B, so it is important to include a brief overview of the major known nucleo-cytoplasmic transport processes involving histones. Most histones are synthesized in the cytoplasm in a cell cycle-dependent manner. The nuclear import process of histones H2A and H2B, similarly to other proteins, occurs through the nuclear pore complex. An N-terminal amino acid sequence of the histone proteins H2A and H2B contains the nuclear localization sequence (NLS) that is responsible for their entry into the nucleus. Export of major structural nuclear proteins from the nucleus also occurs in certain conditions. The mechanistic aspects of such export processes are little known Exportin 1 (XPO1, chromosomal maintenance 1, CRM1) is the

most common karyopherin involved in nuclear protein export, characterized by its inhibition by leptomycin B. This karyopherin is also responsible for the export of rRNA, snRNA, and mRNA, although other specialized transport proteins that export mature mRNAs are also known. CRM1-independent nuclear export of certain proteins has also been described. The export of TDP-43 and FUS from the nucleus e.g. are exportin-1 independent. These two proteins are involved in mRNA processing; they also play a role in the pathogenesis of diseases called amyotrophic lateral sclerosis and fronto-temporal dementia. During the development of the diseases, aggregation of these two proteins were detected in the cytoplasm, while their nuclear levels decreased in both neurons and glial cells.

Aims

Dox is a pleiotropic drug and its diverse mechanism of action has not been fully elucidated. I aimed to gain a more accurate understanding of the effect of the drug on chromatin, which is important for the understanding of chemotherapeutic effects and also for the prevention of the side effects of anthracyclines, and can provide insight into basic features of the chromatin structure.

A1. I wanted to investigate the mechanism of chromatin aggregation induced by Dox within the nucleus *in situ* using a laser scanning microscopic approach.

A2. I wanted to investigate what biochemical processes may play a role in the nucleo-cytoplasmic translocation of H2B, a phenomenon I observed first.

A3. I wanted to study by mass spectrometry which nuclear proteins show nucleo-cytoplasmic translocation related to protein degradation mechanisms apart from the H2B histone.

Since intercalators preferentially bind to relaxed chromatin regions marked by endogenous DNA breaks, the anthracyclines may target these at lower concentrations. In the second part of my thesis I present the results of my experiments aimed to map these breaks in the genome. This part of my thesis includes also unpublished data.

I had the following aims:

B1. My goal was to set up and validate an enzymatic labeling method that can specifically and efficiently label single-stranded DNA breaks.

B2. Using a molecular combing-based technique, I wanted to visualize the labeled single-strand discontinuities in the DNA of both yeast and mammalian cells in order to establish the relationship between strand breaks and TopII enzyme activity.

B3. My goals included determination of the distribution of DNA breaks containing free 3’ OH groups in the human genome using DIP-seq.

B4. I also wanted to determine the relationship between DNA strand breaks and transcription using superresolution microscopy.

B5. My aim was to study the spatial localization of DNA strand breaks within the nucleus by superresolution microscopy on nuclear halo samples.

Results I.

1. Marked aggregation of H2A but not of H2B induced by Dox treatment

We detected intranuclear aggregation of H2A, but not of H2B histones, in Jurkat cells after treatment with Dox, using a LSC-based procedure. The agarose-embedded live Jurkat cells were treated with 0-36 μM Dox for 2 hrs, then either fixed (pre-fixation) and subsequently permeabilized by the Triton X-100-containing lysing solution, or treated with this solution without previous fixation. Free histones, including those evicted by Dox, could diffuse out of the nuclei and were subsequently washed out, while aggregated and/or DNA-bound histones remained in the nuclei and were labeled by indirect immunofluorescence for subsequent analyses by LSC. The level of H2A in the nuclei of control cells was much lower than that of the pre-fixed samples, because the latter contain a pool of free histones readily diffusing out from the nuclei of the Dox-untreated cells. After treatment of the cells with Dox, increased amounts of H2A remained in the nuclei obtained by Triton X-100/PBS-EDTA lysis. Retention was Dox concentration-dependent, starting below somewhat 9 μM and reaching the H2A levels of the pre-fixed control nuclei at 36 μM drug concentration. In sharp contrast with H2A, no retention of H2B was detected in the assay when H2A and H2B were simultaneously measured. The aggregated histones appeared to localize mostly to the space outside the trabeculate compartment of genomic DNA stained with propidium iodide (PI) corresponding to aggregated chromatin.

2. Massive nucleo-cytoplasmic translocation of endogenous H2B, but not of H2A, after Dox treatment

As compared to H2A, histone H2B behaved in a sharply different manner upon Dox treatment not only in terms of aggregation tendency, but also in its intracellular localization. H2B accumulated in the cytoplasm in a Dox concentration and time-dependent manner, forming a gradient with ~3x higher amount of the histone in the cytoplasmic compartment relative to the nucleus. H2B nucleo-cytoplasmic translocation, likely an active process in view of the gradient formed, started at a Dox concentration as low as ~2 μM. Depletion of H2B from the center of the nucleus occurred already after 15-30 mins of treatment and propagated to the periphery

concomitantly with a gradual elevation of H2B detected in the cytoplasm. After 2 hrs of Dox treatment, the majority of H2B was depleted from the nuclei and accumulated in the cytoplasm.

To investigate the possible mechanisms of cytoplasmic H2B accumulation, treatment of the Jurkat cells with inhibitors of biochemical processes that could perhaps account for the cytoplasmic accumulation of H2B were performed. The possible role of an increased *de novo* protein synthesis in the accumulation of cytoplasmic H2B was excluded by blocking the synthetic process with puromycin or cycloheximide. Inhibition of CRM1 mediated nuclear export by leptomycin B failed to diminish the Dox-induced H2B translocation. The anthracycline-induced histone aggregates may be recognized by the cell as denatured proteins destined for elimination by degradation and/or export out of the nuclei. Therefore, inhibitors affecting such pathways were also tested. Indeed, H2B export could be strongly diminished by PYR-41, an inhibitor of diverse processes including E1-mediated ubiquitination. After co-treatment with Dox and PYR-41, H2B showed a characteristic nuclear localization pattern with spatial separation of the histone and the DNA-containing chromatin, reminiscent of the segregation of H2A from chromatin after Dox treatment alone. However, Dox-induced H2B export could not be reduced by another ubiquitination inhibitor MLN7243, the NEDDylation inhibitor MLN4924 or the SUMOylation inhibitor 2-D08. On the other hand, co-treatment with 2-D08 partially attenuated the effect of PYR-41 on Dox-induced H2B translocation, leading to a moderate cytoplasmic accumulation of H2B upon Dox treatment. Neither inhibitors of transcription (α-amanitin or actinomycin D), nor an inhibitor of Hsp90 (17-AAG) were able to revert the Dox-induced H2B export. H2B cytoplasmic accumulation was detected using two different (monoclonal vs. polyclonal) anti-H2B antibodies. Both commercially available antibodies are extensively used to visualize H2B with high specificity and sensitivity. Nonspecific binding of the secondary antibody was ruled out by incubating the cells with the secondary antibody only. We investigated cytoplasmic H2B relocation also in primary human lymphoid cells. Dox treatment elicited cytoplasmic relocation of H2B in human peripheral blood mononuclear cells (hPBMCs). In further similarity with the effects demonstrated in Jurkat cells, Dox caused no translocation of H2A in hPBMC nuclei but induced its segregation from chromatin that exhibited a trabeculate staining pattern. H2B translocation was overruled by PYR-41 treatment also in these cells, when the histone accumulated between the DNA-containing chromatin domains. In view of the involvement of extranuclear H2B in innate

immunity, we tested the effect of Dox on the distribution of this histone in monocyte-derived human DCs. Similarly to the other cells studied, Dox triggered a near-complete eviction of H2B from the nuclei, what was reverted by PYR-41. Remarkably, H2B was present in the cytoplasm of these cells at a surprisingly high level even in the absence of any treatment. The Dox-induced nucleo-cytoplasmic H2B export was confirmed using mass spectrometric (MS) analyzes of supernatants of the cytoplasmic cell lysates, performed in parallel with the microscopic assessment of H2B remaining in the nuclei. MS analysis showed an elevated H2B content in the supernatant of Dox-treated lyzed cells, simultaneously with the vanishing of the histone from the nuclei, as compared to the untreated control. The MS analyses also showed that a number of other histones and nuclear proteins were released from the nuclei by Dox. Under these experimental conditions, *i.e.* upon lysis of the cytoplasmic content by Triton X-100 and following centrifugation, increased amounts of other histones, prominently H1.2, was also detected in the supernatant of Dox-treated cells. However, only the appearance of H2B in the supernatant was PYR-41-sensitive. A series of non-histone proteins also exhibited increased elution from the nuclei upon Dox treatment under these conditions.

Results II.

1. Detection of DNA breaks using molecular combing

I optimized an experimental system based on "molecular combing", where individual DNA fibers are deposited on coverslips and can be visualized separately by fluorescence. I examined the distribution of DNA breaks containing enzymatically labeled 3′ OH groups on yeast XII. chromosomes, after labeling by E. coli DNA Polymerase I (Pol I). This enzyme incorporates biotin-labeled nucleotides to the free 3′ OH groups of strand breaks in a template-dependent manner. Data obtained from genomic and rDNA experiments have shown that the 3′ OH labeling method indeed detects DNA breaks and not R-loops, therefore it can be used in DIP-seq experiments on human cells.

2. DNA strand breaks accumulate on the promoters of active genes and co-localize with the initiating and elongating RNA polymerases in human cells

As revealed in the past 15 years, TopII activation and the appearance of transient DNA breaks accompany gene activation in the case of certain genes. Strand breaks present in the DNA of hPBMC samples were enzymatically labeled and mapped using DNA-immunoprecipitation sequencing (DIP-seq) (The experiment was performed according to the protocol established in our lab by Lóránt Székvölgyi. Bioinformatic evaluation was the work of László Halász and Erik Czipa. Validation of the results at particular nick-positive and negative promoters was performed by myself.) Approximately 10% of the mapped DNA breaks could be localized to genes and accumulation of the reads was detected on the promoters of active genes. The number of reads on the promoters of inactive genes was much lower. To confirm this, I examined the extent of co-localization of DNA breaks with RNA polymerase using superresolution microscopy. It is known that the phosphorylation of Ser 5 in the C-terminal domain (CTD) of RNA polymerase is characteristic of the enzyme present at transcription initiation, while the phosphorylation of Ser 2 is characteristic of RNA polymerase in the transcription elongation and termination phases. Using STED microscopy and immunofluorescence we have shown that the initiating RNA polymerases and the labeled strand breaks showed significant co-localization. The RNA

polymerases conducting elongation and termination co-localized with the breaks to a smaller extent.

Materials and methods

1. Cell culture

Jurkat cells were grown in T150 tissue culture flasks (Corning Glass Works, Corning, NY) using RPMI-1640 (Gibco, Grand Island, NY) and 10% fetal bovine serum. The cultures contained penicillin (100 µg/ml), streptomycin (0.25 µg/ml), and glutamine to a final concentration of 2 mM.

2. hPBMCs and human monocyte-derived DC cultures

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service of the University of Debrecen, Faculty of Medicine (Hungary) and from the Regional and Institutional Research Ethical Committee of the University of Debrecen. Written, informed consent was obtained from the blood donors prior to blood donation, their data were processed and stored according to the directives of the European Union. hPBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from hPBMCs by positive selection using immunomagnetic cell separation and anti-CD14 microbeads, according to the manufacturer's instruction (MiltenyiBiotec, Bergisch Gladbach, Germany).

3. Embedding live cells into low melting point agarose

Embedding was carried out according to Imre et al. Briefly, the wells of 8-well chambers (Ibidi, Martinsried, Germany) were coated with 1% (m/v) low melting point (LMP) Agarose. The cell suspension containing 6 x 10^6 cells/ml of PBS was mixed with 1% LMP agarose diluted in 1 x PBS at 37 ^oC, and the cell-agarose suspension was dispensed in the middle of the wells. After polymerization of the agarose on ice, complete culture medium was added to each well.

4. Sample preparation for mass spectrometry

Lysis of the 36 μM Dox-treated and control cells was by mixing 0.8 ml of cell suspension containing 2 x 10^7 cells/ml with 0.2 ml ice cold PBS containing 1% Triton X-100 on ice. After incubation on ice for 10 minutes, 14 ml of PBS was added in order to terminate lysis, then the nuclei were centrifuged at 550 g for 5 minutes. The supernatant was concentrated using Amicon Ultra 0.5 ml Centrifugal Filters (EMD Millipore, Darmstadt, Germany), then sent for Mass Spectrometry. The nuclei were fixed by incubating the pellet in freshly prepared 4% formaldehyde dissolved in PBS/EDTA, for 10 minutes, then embedded into agarose in 8-well chambers (Ibidi, Martinsried, Germany) as described above.

5. Drug treatments

Doxorubicin, puromycin, cycloheximide, leptomycin B, PYR-41, α-amanitin, actinomycin D, 2-D08 (all Sigma, Budapest, Hungary), 17-AAG (Reagents Direct, Encinitas, USA), MLN4924 (EMD Millipore, Darmstadt, Germany) and MLN7243 (Chemgood, Glen Allen, USA) were diluted to the final concentrations and added to live cells in complete DMEM medium for the time indicated, prior to fixing and lysis.

6. Preparation of nuclei

The agarose-embedded cells at the bottom of the wells were washed with 500 μl ice cold 1 x PBS, three times for three minutes, then lysis/permeabilization was carried out: (I) Samples were pre-fixed with 400 μl ice cold 4% (m/m) formaldehyde dissolved in 1 x PBS/EDTA on ice for 15 minutes and subsequently permeabilized by replacing the fixative with 500 μl ice cold 1% (v/v) Triton X-100 dissolved in 1 x PBS/EDTA (5 mM EDTA in PBS), for 10 minutes; (II) Samples were lyzed with ice cold 1% (v/v) Triton X-100 dissolved in 1 x PBS/EDTA; (III) Samples were lyzed with 500 μl ice cold 1% (v/v) Triton X-100 dissolved in 300 mM sucrose, 5 mM EDTA.
The lysis/permeabilization step was repeated once more, then nuclei were washed with 500 μl ice cold 1 x PBS/EDTA 3x, for three min each.

7. Immunofluorescence labeling

After lysis/permeabilization the samples were incubated with 500 μl 5 % (m/v) Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) in 1 x PBS/EDTA for

30 minutes on ice, to decrease non-specific binding of the antibodies. The blocking solution was washed out with 500 µl ice cold 1 x PBS/EDTA three times for three minutes and indirect immunofluorescence labeling was performed using mouse monoclonal anti-H2B (ab52484, Abcam, Cambridge, UK; 1 mg/ml), rabbit polyclonal anti-H2B (Sigma-Aldrich; 1 mg/ml) or rabbit polyclonal anti-H2A (ab18255, Abcam, Cambridge, UK; 1 mg/ml). Primary antibodies, all diluted in 150 µl of 1 x PBS/EDTA/1% BSA (1 x PBS/ EDTA supplemented with 1% w/v bovine serum albumin), at 4 °C, overnight. All the above antibodies were applied to the wells at a titer of 1:800. After labeling with the primary antibodies, the nuclei were washed with 500 µl ice cold 1 x PBS/EDTA three times for 10 minutes. Labeling with the secondary antibodies was performed in 150 µl 1 x PBS/EDTA for 2 hrs on ice, using Alexa fluor 488 (A488) or Alexa fluor 647 (A647) conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with identical results. H2A was detected by A647 (red channel) and H2B by A488 (green channel). The secondary antibodies were also used at a titer of 1:800, diluted in 1 x PBS/EDTA from 2 mg/ml stock solutions. After labeling with the secondary antibodies, the agarose-embedded nuclei were washed with 500 µl ice cold 1 x PBS/EDTA three times for 10 minutes. Then the samples were stained with 200 µl 12.5 µg/ml PI (dissolved in 1 x PBS/EDTA) for 60 minutes, on ice. The stained nuclei were washed 3 times with 500 µl ice cold 1 x PBS/EDTA for 3 minutes. Fluorescence intensity distributions were recorded using an iCys LSC, as described below.

8. Confocal microscopy

Imaging was carried out with an Olympus FluoView 1000 CLSM equipped with 488 and 633 nm lasers, using a 60x oil immersion oil objective. Composite images were constructed and evaluated using ImageJ software. Instrument settings (laser power, photomultiplier tube voltage, gains, pixel dwell time) and image analyses parameters (brightness, contrast, gamma factor of ImageJ) were identical in the case of all the samples compared in a particular experiment. Nuclear H2B content was calculated by selecting the nuclei on stack images and determining their total H2B fluorescence integral. Cytoplasmic H2B was calculated by subtracting nuclear from the total intracellular H2B fluorescence integral (8-10 representative cells /sample).

9. Automated microscopy (LSC)

Automated microscopic imaging was performed using an iCys instrument (iCys® Research Imaging Cytometer; CompuCyte, Westwood, Massachusetts, USA). A488 and PI were excited using a 488 nm Argon ion laser, A647 was excited with a 633 nm HeNe laser. The fluorescence signals were collected via an UPlan FI 20 x (NA 0.5) objective. A488 was detected through 510/21 nm and 530/30 nm filters, respectively, while A647 and PI were detected through a 650/LP nm filter. Each field (comprising 1000 x 768 pixels) was scanned with a step size of 1.5 µm. Gating of G_1 phase nuclei was according to the fluorescence intensity distribution of the DNA labeled with PI. The integral fluorescence intensity values, representing the summed fluorescence intensity of all the pixels representing the nuclei, were measured and averaged by LSC.

10. Enzymatic labeling of DNA breaks

E. coli DNA Polymerase I can be used to label the 3' OH groups of DNA breaks using biotin-tagged nucleotides (biotin-dUTP) in agarose-embedded nuclei. 1 µM dNTP mix (1 µM biotin-dUTP, 1 µM dATP, 1 µM dCTP, 1 µM dGTP) was diluted in DNA Polymerase I buffer together with 5 µM ddNTP mix (5 µM ddATP, 5 µM ddTTP, 5 µM ddCTP, 5 µM ddGTP). The reaction mix was added to each agarose-embedded sample. Labeling was performed at 37 °C for 20 min. For standard (non-limiting) labeling, the terminating ddNTP was omitted from the reaction mixture. For some samples, the labeling reaction was performed with Terminal Transferase enzyme in TdT buffer using 1 µM biotin-dUTP. Other reaction conditions were the same as described for Pol I. In the case of TdT labeling, any labeled RNA ends could be removed using a combined RNase treatment. The TdT-labeled samples were subsequentially digested with RNase HI (12.5 U / block) at 37 °C overnight, then, 5 µl of 10 mg / ml RNase A was added and incubated for 1 hour at room temperature. Finally, 5 µl of human RNase H2 enzyme was added to the samples incubated overnight at 37 °C. Prior to each digestion step, the blocks were equilibrated with the appropriate enzyme buffers 3x for 20 mins.

11. DNA combing

The DNA in the agarose blocks was solubilized at 70 °C, then Agarase enzyme was added to the samples and incubated at 42 °C overnight. DNA combing was performed according to the manufacturer's instructions on a vinylsilane-coated coverslip using a Genomic Vision instrument. Non-specific labeling of antibodies was reduced by incubation with 30 µl of 5% BSA / 1x PBS / 0.1% Triton X-100 for 20 min. The incorporated biotin molecules were detected by indirect immunofluorescence using an anti-biotin antibody. As a secondary antibody, Alexa Fluor 647-conjugated anti-mouse IgG antibody was used. Labeling of DNA was with YOYO-1 dye diluted to 30 µl 1: 5000 (0.1 M in MES pH 6.5).

Discussion I.

Dox treatment induced a marked nuclear aggregation of H2A, as opposed to H2B, at therapeutically relevant concentrations. Chromatin aggregation upon anthracycline treatment has been described earlier in *in vitro* and *in vivo* systems. This is the first report to our knowledge describing such phenomena involving histones alone. Histones are known to be evicted from the chromatin by Dox in an intercalator concentration dependent manner. Through the spectacles of confocal microscopy the majority of H2A molecules detected by immunofluorescence becomes topologically separated from the DNA-containing regions upon treatment with >9 μM Dox and get trapped inside the nuclei as measured in our LSC-based assay. Thus, the nuclear consequences of Dox treatment are the composite result of eviction and aggregation of histones. H2B also appeared to be segregated from the DNA-containing chromatin compartment when its exodus to the cytoplasm was prevented by PYR-41 co-treatment. Since aggregation was not detectable in our LSC-assay, the composition, size and/or stability of the aggregates (formed in the presence of PYR-41) may be different from that of the H2A aggregates. The fact that H2A and H2B can be separated by HPLC, implying a difference in the overall hydrophobicity of the two histones, is in line with their differential aggregation tendencies upon Dox treatment, what may be due to different amounts of the drug bound to the two histones. In the absence of PYR-41 H2B probably escapes aggregation because of its disappearance from the nuclei. Dox caused a massive increase of cytoplasmic H2B, but not of H2A levels. The cytoplasmic accumulation of H2B upon Dox treatment is a phenomenon not described before for any of the histones to our knowledge. This is to be taken into account among the responses of cells to Dox treatment in view of the small concentration of Dox required for the effect, what was detected in Jurkat cells, hPBMCs as well as DCs. The fact that H2B accumulated in a trabecular pattern outside the DNA-containing compartment in the samples co-treated with PYR-41 shows that the dissociation of the histone from chromatin induced by Dox was not affected, i.e. just the export of H2B was inhibited by PYR-41. The concomitant accumulation of H2B in the cytoplasm was detected by immunofluorescence, using either a monoclonal or a polyclonal antibody, and it was observed in the MS experiment as well, where it also exhibited PYR-41 sensitivity. The fact that H2B could be detected by the same antibodies in the nucleus in PYR-41 co-treated cells is also taken as evidence for the specificity of immunofluorescence detection.

Highly reduced intranuclear H2B content along with highly increased cytoplasmic H2B levels upon Dox treatment can be explained by the presence of an active export mechanism and intact nuclear membranes, the latter serving as a barrier impeding H2B nuclear re-entry. In contrast with its complete reversal effected by PYR-41, cytoplasmic accumulation of H2B was not significantly altered by inhibiting CRM1-dependent nuclear export, or applying inhibitors of various other biochemical processes. PYR-41 acts mainly as an inhibitor of the ubiquitin-activating enzyme (E1), with little or no effect on the ubiquitin-conjugating enzyme (E2). However, the agent was also shown to enhance total SUMOylation in cells. Induction of non-specific protein cross-linking may also contribute to its toxicity. Furthermore, it is also a deubiquitinase (DUB) inhibitor. The other inhibitor of E1-mediated ubiquitination tested herein did not reproduce the effect of PYR-41, and the combined treatment with 2-D08 SUMOylation inhibitor mitigated the PYR-41-elicited reversal of cytoplasmic H2B accumulation following Dox treatment (when H2B was present at high levels both in the cytoplasm and in the nucleus). Thus we assume that H2B export may be connected to the biochemical circuitry controlling protein degradation via the ubiquitin-like posttranslational modification, SUMO. The main nuclear export pathway is CRM1 dependent, which can be inhibited by leptomycin B that alkylates and inhibits the CRM1 protein. The insensitivity of H2B translocation to even high concentrations of this agent argues against the involvement of this mechanism. Several CRM1 independent nuclear export pathways have been described, including the transport of mature mRNAs, of poly(A)$^+$ RNAs and RNA helicases. Biochemical processes involving SUMOylation are important determinants of nuclear export of mRNAs and proteins. H2B translocation from the nucleus to the cytoplasm further increases the plethora of molecular events that are to be considered as components of Dox toxicity.

The likely involvement of protein degradation-related pathways in Dox-induced H2B nucleo-cytoplasmic export raises the possibility that the phenomenon may have relevance in connection with the following published observations: Enhanced protein degradation may play an important role in the acute cardiotoxicity of Dox therapy; down-regulation of UBC9, an E2-conjugating enzyme that is required for SUMOylation, increases the sensitivity of hepatocellular carcinoma to Dox; the SUMO pathway is a major determinant of Dox cytotoxicity in yeast; down-regulation of histone H2A and H2B pathways is associated with anthracycline sensitivity in breast cancer. It will be interesting to elucidate the exact relationship between the protein

degradation-related pathways and H2B cytoplasmic accumulation to investigate the above possible links.

The nucleo-cytoplasmic translocation of H2B induced by Dox may be connected to H2B export occurring in certain physiological and pathological circumstances. Instances of extra-nuclear location of histones have been documented. Extracellular histones can function as microbicidal proteins, on the other hand histones released in sepsis contribute to endothelial dysfunction. Apoptosis unrelated nucleo-cytoplasmic translocation of H1 histone was detected in HeLa cells, and extrachromosomal H2B is known to mediate innate antiviral immune responses. Remarkably, H2B in complex with gamma-interferon-inducible protein 16 was shown to be present in the cytoplasm during Epstein-Barr Virus and Herpes Simplex Virus-1 infection in non-apoptotic cells. Dox treatment evoked a PYR-41 sensitive nucleo-cytoplasmic translocation of H2B also in DCs, similarly to Jurkat and hPBMCs, resulting in significantly decreased intranuclear H2B levels. Intriguingly, DCs exhibit high cytoplasmic levels of H2B in the absence of any treatment, especially in plasma membrane proximal areas. Since macrophages and DCs are the frontline cells of innate immunity, it is possible that the elevated cytoplasmic H2B levels in DCs are required for pattern recognition. The fact that the cytoplasmic levels of H2B can be readily increased by treatment with >2 μM Dox raises the intriguing possibility that the H2B-related pathways of antiviral and antimicrobial immune response may be boosted by Dox treatment.

Dox treatment facilitated the release of a wide variety of nuclear proteins. The proteins exhibiting enhanced release from the nucleus upon Dox treatment are very diverse, suggesting that the entire nuclear structure is affected by the drug. This Dox-induced effect is apparently superimposed on a background of non-specific release of nuclear proteins likely explained by the conditions required in the MS experiment. The Dox-induced perturbations of intermolecular interactions in the nucleus may all contribute to the effects and side-effects of anthracyclines observed in the clinical practice.

The reported range of peak plasma concentrations of Dox is rather wide: values between 0.5 and 9 μM have been reported. Therefore, the Dox concentration range used in our experiments and the peak plasma concentrations in clinical settings could overlap. Our observations expand the list of molecular changes supporting such a scenario, raising the possibility that the severe

perturbations in H2A and H2B intracellular and intranuclear localization observed may be of interest also from the medical point-of-view, regarding both the effects and side-effects of anthracycline treatment. It will be intriguing to test if the novel phenomenon of Dox-induced H2B nucleo-cytoplasmic export may be exploited to boost or modulate immune response involving extrachromosomal H2B. The LSC-based assay of H2A aggregation described herein may find its application also in studies addressing various other cell biological conditions involving protein aggregation in the nucleus.

Discussion II.

The nucleosome-evicting effect of intercalators is well known from the literature and also from research experience of our lab. Using a quantitative nucleosome stability assay developed in our laboratory, we have found that the relaxation of genomic DNA causes a prominent nucleosome destabilization, which itself favors the binding of intercalators to DNA. Thus, any local relaxation of DNA coupled with the concomitant increase of intercalator binding is expected to locally destabilize the resident nucleosomes. This implies that the relaxed chromatin domains along the genome are more prone to Dox binding and these regions are likely to contribute eminently to the histone aggregation and export phenomena described. Since localized DNA breaks are generally the result of TopII activity, we aimed to detect the free 3′ OH ends characteristic of this enzyme activity. Labeled breaks designating relaxed chromatin regions, have been identified on active promoters. These observations also fit into our efforts to address the biological role of physiological DNA strand breaks and are planned to be published in the latter context.

1. Setting up the labeling of DNA breaks using a molecular combing system

The rDNA cluster of *S. cerevisiae* is located on chromosome XII. and consists of 100-200 identical, naturally amplified DNA segments. Yeast XII. chromosome has proven to be a good model system for optimizing and validating the enzymatic labeling of DNA strand breaks containing 3′ OH groups. The labeled transcription units of the rDNA cluster could also be recognized in the form of adjacent spots at the ends of the fibers, which implies that the DNA remains continuous at these sites, showing that single strand breaks were present here. I have also demonstrated that since single-strand breaks tend to be converted into double-strand breaks upon combing, so we concluded that the labeled free 3' OH groups mostly probably correspond to single-strand breaks. Although this finding contradicts the canonical enzymatic function of TopII, it is in line with other data from literature also suggesting that TopII can create single-strand breaks as well. Single-strand breaks were detected in yeast XII. chromosomes and also in genomic DNA in molecular combing experiments. Since I detected a similar number of signals in cells synchronized in G_1 phase with α-factor, most of the strand breaks must have been

generated in a DNA replication-independent manner. Possible labeling of transcription associated RNA ends was ruled out by treating the samples with RNases after TdT labeling. RNase A cleaves misincorporated ribonucleotides scattered in the genome also generating free 3' OH groups, so I used the combined RNase treatment following the TdT reaction. This allowed me to conclude that 3'OH groups of DNA origin are detected in our assays. This aspect has not been previously considered in the earlier reports on the transient, promoter-proximal DNA breaks.

2. TopII-associated DNA breaks on promoters of active genes and their co-localization with Ser5 / Ser2-phosphorylated RNA polymerase II

Our DIP-seq experiments were performed on the chromatin of G_0 phase cells. These results suggest that DNA strand breaks accumulate on the promoters of active genes in hPBMC cells. Confirming the results of DIP-seq experiments, I detected co-localization of DNA strand breaks and RNA polymerases characteristic of transcription initiation and elongation by STED microscopy in formaldehyde-fixed preparations. The presence of promoter-proximal single-strand breaks detected in my assays is consistent with literature data on activity dependent transient DNA breaks reported for several genes. Our data extends these observations to the majority of active genes. Furthermore, our data suggest that DNA breaks typically correspond to single-strand breaks, what might be a safer scenario for the cell than the double-strand breaks assumed to be present on the grounds that the enzyme is detected at promoters and that is its canonical function.

Summary

Effects of Dox on chromatin was examined and the following results were obtained:

1. I set up a scanning laser microscopic experimental system to show that Dox induces intranuclear aggregation of H2A histone that is not accompanied by aggregation of H2B.

2. Dox causes nucleo-cytoplasmic translocation of endogenous H2B histone in a concentration- and time-dependent manner in Jurkat, hPBMC, and DC cells.

3. Nucleo-cytoplasmic translocation of Dox-induced H2B can be inhibited by PYR-41, which affects protein degradation processes, and cannot be abolished by inhibition of CRM1-mediated nuclear export. Inhibition of SUMO-ylation partially antagonized the inhibitory effect of PYR-41 on Dox-induced H2B translocation.

4. I have shown by mass spectrometry that Dox treatment induces nucleo-cytoplasmic translocation of additional nuclear proteins in addition to H2B in a PYR-41 sensitive manner.

My observations can be interpreted by spontaneous aggregation of histones evicted during intercalation, taking into account the degradation of aggregates and the nucleo-cytoplasmic export. The Dox-induced changes in global chromatin structure described in my work may have biomedical significance for both the effects and side effects of anthracyclines.

My observations on the topological heterogeneity of chromatin:

Because the intercalation of Dox and other anthracyclines into chromatin depends on the topological state of DNA, I mapped the relaxed chromatin regions characterized by endogenous DNA single-strand breaks that may be predilection points for DNA binding of DNA intercalator drugs.

1. I developed and validated on *S. cerevisiae* genomic DNA an enzymatic labeling method suitable for the specific labeling of DNA strand breaks, furthermore I set up a molecular combing-based experimental system suitable for the fluorescence visualization of labeled individual DNA strands.

2. Using the former labeling strategy, we mapped endogenous DNA single-strand breaks on the DNA of hPBMC cells in a DNA immunoprecipitation and sequencing experiment. We found that they accumulate on the promoters of active genes.

3. With super-resolution microscopy we detected co-localization between DNA single-strand breaks and Ser5 / Ser2-phosphorylated RNA polymerase II.

4. I have shown by molecular combing experiments that DNA single-strand breaks co-localize with TDP2-labeled TopII on hPBMC cells.

5. On nuclear "halo" preparations, I demonstrated that the protruding part of the halo through the nuclear lamina was superhelical, while I detected the accumulation of DNA single-strand breaks in the lamina-proximal areas.

The topological duality of the chromatin architecture described above appears to be the basic organizing mechanism of the higher order chromatin structure. Relaxed domains, as preferred sites of interaction for intercalating agents, may primarily contribute to inducing the effects of such drugs.

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Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Nánási, P. P. i.**, Imre, L., Firouzi Niaki, E., Bosire, R., Mocsár, G., Türk-Mázló, A., Ausio, J., Szabó, G.: Doxorubicin induces large-scale and differential H2A and H2B redistribution in live cells.
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