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

Chromatin: a playground of drug interaction

by Dr. Erfaneh Firouzi Niaki

Supervisor: Prof. Dr. Gábor Szabó

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DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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by **Erfaneh Firouzi Niaki, Pharm.D**

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The Examination takes place at Room 1.406, Life Science Building, Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, 202109.09, 11 O'clock.

Head of the **Defense Committee**:	Prof. Dr. Tőzsér József, Ph.D., DSc
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	Dr. Penyige András, Ph.D.

The PhD Defense (online format) takes place via Zoom online meeting platform (Meeting ID: 917 2125 5912), at 14:00 p.m. on 6th of July 2021. Publicity is guaranteed during the online defense.

Part A. Interactions of Cisplatin and Daunorubicin

1. INTRODUCTION

The application of multiple anticancer drugs with a diverse mode of actions that target different cell components might lead to a desirable outcome so as to treat particular cancer types more effectively. A synergy of the drugs could lead to 1) increment in the effectiveness of therapy; 2) reduction in the dosage to decrease side effects while improving or maintaining efficacy; 3) mitigate or delay the development of resistance to the drugs. In this matter it is questionable what strategies and approaches are best for choosing candidates to be combined and tested even at the pre-clinical state of research. It also seems challenging to interpret and evaluate the results of pre-clinical tests, what is crucial to move forward to the clinical trials. Because numerous drug combinations only have additive or slight synergistic effects, it is the extent of synergy that becomes vital to be determined.

Anticancer drugs that target chromatin are some of the most effective agents in combating cancer. Anthracyclines and platinum-based drugs have been exploited in combination chemotherapy to treat a broad variety of different types of cancer. Although many of the cellular targets of these anticancer agents are known, their involvement in cytotoxicity is poorly understood. Therefore, how they interact with each other when used in combination is even more difficult to predict.

For both Cisplatin (Cis-Pt) and Daunorubicin (Dauno), the main drug target is chromatin and both influence DNA topology; thus, the possibility arises that they may mutually influence each other's binding to their chromatin targets. Therefore, we set out to determine how the Dauno evoked changes in chromatin structure and DNA topology affect the formation of Cis-Pt-DNA adducts, and vice versa how Cis-Pt influences Dauno binding.

Synergy between two drugs (when the observed effect is greater than the effects of the individual agent added), vs. antagonism (when the observed effect is smaller than what is expected when the effects add up), are not readily determined by comparison of the individual effects with those of the combinations, due mainly to the non-linearity of the dose-effect relationships. Therefore, we used the combination index (CI) method based on the median-

effect principle of the mass-action law performed according to Chou-Talalay to determine the mode of drug interaction. The CI reflects synergism, additivity or antagonism when CI < 1; CI = 1 or CI > 1, respectively.

1.1. Cisplatin

1.1.1. Chemistry of Cisplatin

Cisplatin (Cis-Pt), *Cis*-(Pt $(NH3)_2Cl_2$), consists of two ammine and two chloride ligands in the *cis* configuration, bound to a single atom of platinum in a square planar complex. Following its entry into the cell, due to the decrease of chloride concentration to ~2-10 mM, in the diammino*dichloro*platinum (II), chloro ligands are substituted with water molecules in two consecutive steps while preserving *cis* configuration, to become the chemically active *cis* diammine*di**aqua*platinum (II) species. This hydrolysis reaction is the rate-limiting step for DNA binding, and the half-life ($t_{1/2}$) for substitution of the first chloride ligand with water is about 2 h.

1.1.2. Cis-Pt and DNA

The cationic aquated Cis-Pt species behave as electrophilic reagents and interact with nucleophilic sites on the DNA. Intrastrand cross-links formed by the binding of Cis-Pt to two neighboring guanines (1,2-d(GpG)) comprises ~ 65% of the total Cis-Pt-DNA adducts, ~22% of the adducts formed on adenine and guanine in 1,2-d(ApG) sequence but not in pGpA, ~13% on two guanines separated by one or more nucleotides (1,3-d(GpNpG)), while Interstrand cross-links (ICLs) forming between the guanine bases at complementary strands were present at a frequency of about 1-5%.

1.1.3. Effect of adducts on DNA and nucleosome structure

The formation of Cis-Pt-DNA adducts causes distortions of the DNA conformation. Intrastrand cross-links tend to bend the DNA toward the major groove, while ICLs cause DNA bending toward the minor groove. Cis-Pt adducts also unwind the DNA helix: intrastrand adducts by 13˚- 23˚, whereas a single ICL unwinds DNA by approximately 79˚.

1.1.4. Cytotoxicity

Binding of Cis-Pt to the DNA is generally perceived as the leading cause of its toxicity. The contribution of the various types of cross-links to toxicity has not been clarified fully. On the one hand, as, intrastrand cross-links comprise the major form of adducts, it is suggested that this type of lesion may be responsible for the cytotoxic action of Cis-Pt. In line with this notion, transplatin (trans-isomer of Cis-Pt), is unable to form 1,2-intrastrand cross-links, therefore intrastrand cross-links are more often referred to as the main factors of cytotoxicity. ICLs are deemed not to be the primary cytotoxic lesions in conformity with the observation that transplatin, despite of the fact that is ~ 6 fold more apt to form ICLs than Cis-Pt, is clinically futile. Nonetheless, the hypersensitivity of Fanconi anemia cells (defective in the repair of ICLs), to ICL forming reagents, propose that their impact on cytotoxicity may be considerable. Cis-Pt binding to linker region is preferred over nucleosomal DNA and the ratio of the DNA damage caused by Cis-Pt is ~ 1.3 times higher at linker region than in the core region of nucleosome. While the above and most other similar studies examined the formation of all types of the Cis-Pt-DNA adducts together, in another publication specifically the formation of ICLs was measured in reconstituted nucleosomal and naked DNA. According to these latter results, Cis-Pt forms ICLs with naked and nucleosomal DNA at a similar efficiency.

1.1.5. Effect of DNA topology on adduct formation

Regarding the question whether DNA topology plays a role in the formation Cis-Pt-DNA adducts, indirect evidence was obtained in experiments where the influence of DNA supercoiling on the cytotoxicity of Cis-Pt in *E.Coli* K12 bacteria was studied. Bacteria carrying mutations in the Gyrase gene were treated with Cis-Pt. An ~1.5x increase in the amount of total Cis-Pt bound to DNA was observed in the mutant strains, implying an influence of DNA supercoiling on DNA platination. In *in vitro* experiments, the number of ICLs was compared in supercoiled and linear plasmid DNA. The results showed that at relatively low levels of total modification by Cis-Pt (< 1 Pt /500 bp), ICLs were formed in negatively supercoiled DNA at approximately 3x higher frequency than in linear or relaxed DNA. In these studies ICLs comprised 30%-40% of the total adducts. The kinetics of ICL formation were faster in supercoiled DNA (formation $t_{1/2}$ ~2h) than in linearized DNA ($t_{1/2}$~4h), suggesting that negative supercoiling indeed enhances ICL formation.

Anthracyclines, often used in cancer chemotherapy in combination with Cis-Pt, elicit changes in DNA conformation upon their intercalation between the adjacent base-pairs. Therefore, their co-administration with Cis-Pt may result in major consequences on Cis-Pt toxicity. Conversely, the Cis-Pt-elicited cross-links may fix genomic DNA in constrained conformational states

antagonizing intercalation. In view of these complexities, we set out to determine how the Dauno evoked changes in chromatin structure and DNA topology affect the formation of Cis-Pt-DNA adducts, and how Cis-Pt influences Dauno uptake and binding.

1.2 Superhelicity

The hierarchical packaging of the genomic DNA is eukaryotes involves formation of the nucleosomal structure (beads-on-a-string), then further packaging into a continuum of zigzag and ~30 nm fibers, formation of chromatin loops and further, higher order compaction; all these structural changes involve changes of DNA supercoiling. In bacterial systems, supercoiling appears to be essential for viability and is finely tuned by the antagonistic activities of DNA gyrase and topoisomerase I. Alterations in the level of supercoiling by more than 25% subsequent to mutation in corresponding genes are lethal. DNA supercoiling, affecting the conformation of larger DNA regions, as well as locally influencing DNA structure is an important player in the regulation of gene expression. For efficient transcription as well as replication, negative supercoiling facilitates the separation of DNA double strands. Such a topology is introduced into the bacterial systems in an ATP-dependent manner by gyrase enzymes; in eukaryotes, nucleosomal structure entails negative supercoiling. Toroidal writhes composed of 145-147 bp of DNA wrapped around the histone octamer, forms 1.65 turns. Upon unwrapping of DNA from nucleosomes, the constrained toroidal writhe is converted to unconstrained plectonemic writhe. When the constrained, negative supercoils form upon the assembly of the nucleosomes, compensatory positive supercoils are generated in the linker DNA that are assumed to be relaxed by topoisomerases. Upon the binding of large proteins and their subsequent translocation along the DNA (like DNA and RNA polymerases), the baseline supercoiled state is altered transiently: overwound and underwound supercoils are generated in front and behind the elongating polymerase, respectively. In other words, instead of one helical turn per 10.5 bp, there is one helical turn per < 10.5 bp ahead of RNA polymerase, whereas, in the rear, there is one helical turn per >10.5 bp. If the positive supercoils ahead of DNA- or RNA polymerases remain unrelaxed, it leads to replication and transcription blockade. Negative supercoils behind the polymerases favor formation of unconventional DNA conformations (non-B DNA), including Z-DNA, D-loops, and R-loops and G-quadruplexes. The strategy applied by both pro- and eukaryotes to resolve polymerase-induced supercoiling

is recruiting topoisomerases. Over- or underwound DNA behaves as a substrate for topoisomerases. Topoisomerases, upon forming “cleavage complexes” with the DNA, introduce transient single- or double-strand breaks thus removing the replication or transcription blockade. In prokaryotes, DNA is usually negatively supercoiled (excepting the thermophile archea). Writhe exists in both toroidal and plectonemic form. When in the toroidal form, DNA is wrapped around nucleoid-associated proteins including HU and FIS.

In eukaryotes, changes of supercoiling are effortful challenges for DNA replication and transcription. Also, the superhelical state of DNA in chromatin and the enzymes involved in the regulation of DNA topology are among the most effective targets in combating cancer. However, due to its complexity, supercoiling and its biological significance in human genomes is not yet well appreciated.

1.3 Anthracyclines

1.3.1. Chemistry of anthracyclines

The anthracyclines (including daunorubicin, doxorubicin, epirubicin, idarubicin and aclarubicin) belong to a class of chemotherapeutics with similar structures that are widely used for treatment of several types of cancer. Daunorubicin (Dauno) and Doxorubicin (Dox) consist of planar tetracyclic ring and sugar moieties. Although there is only a minuscule difference in the structure of Dauno and Dox, but apparently it has an impact on the spectrum of diseases where they are applied. Dox is used in the treatment of breast cancer, soft tissue sarcomas, lymphomas and solid tumors arising in endometrial tissue, whereas Dauno is applied in myeloblastic or acute lymphoblastic leukemia.

1.3.2. Daunorubicin and its mechanisms of action

Dauno, the first discovered anthracycline compound, in its uncharged form, enters the cell via passive diffusion. Under physiological pH, most of the Dauno molecules are positively charged, probably limiting uptake only to a small extent due to the equilibrium between the ionized and neutral form. Entry of the charged Dauno molecules appears also to be possible by a flip-flop mechanism. Dauno affects a broad range of biochemical processes, and a number of different mechanisms have been proposed to be responsible for Dauno-elicited cytotoxicity. These include inhibition of DNA and RNA synthesis (mainly due to binding of the drug to the DNA), topoisomerase II poisoning (by trapping the enzyme at cleavage sites), oxidative stress

(due to formation of reactive oxygen species), involving also lipid peroxidation (by chelating iron).

1.3.3. Interactions of Dauno with chromatin

In addition to topoisomerase II (Top2) inhibition, Dauno inhibits both DNA and RNA synthesis possibly due mainly to direct binding to and inhibition of the polymerases. Inhibition of DNA synthesis has been observed at concentrations of Dauno as low as ~0.25 μM. The fused tetracyclic structure intercalates between adjacent base pairs, while daunosamine extends into the minor groove. Dauno intercalation is slightly preferred at GC base pairs. Single-molecule measurements denote that the intercalation of one Dauno molecule untwists (unwinds) the double helix by 8°-12°. In negatively supercoiled DNA, such an alteration in *Tw* would generate compensatory positive torsional stress in the neighboring parts of the DNA, what makes the DNA resist further intercalation more-and-more as intercalator concentration is increased. Importantly, single-molecule magnetic tweezers studies showed that the assembly of a nucleosome on positively supercoiled DNA is inhibited. The torsional stress exerted by the compensatory positive supercoiling can weaken histone-histone and histone-DNA interactions in the nucleosome core particle and favor dissociation of the H2A/H2B dimers from the H3/H4 tetrasome (at low salt conditions). Published results by our lab as well as another lab showed that Dox (a congener derivative of Dauno) induces eviction of histones independently from Top2 inhibition. Furthermore, it was shown that Doxorubicinone (Dox lacking the sugar moiety) was unable to evict histones, suggesting the importance of sugar group and its extension into the minor groove. The postulated mechanism for histone eviction is a competition of the sugar moiety with histone residues critical for nucleosome stability like the arginine side chain of H4 (H4-R45). Dauno preferentially binds to linker DNA and nucleosome depleted regions like active promoters. Binding of Dauno to the linker DNA regions induces an unfolding followed by aggregation of chromatin fiber thereby influencing higher-order chromatin conformation. Dauno also binds to H1 and H5 linker histones with preference for H1 over H5.

2. AIM AND OBJECTIVES OF THE STUDY

The aim of the study is to elucidate if and how Dauno and Cis-Pt, chemotherapeutic agents primarily targeting the chromatin and frequently used in combination, influence each other's binding to chromatin components.

Objectives

1) To determine if Dauno and Cis-Pt synergize or antagonize each other's cytotoxicity in vitro.
2) To establish techniques for the measurement of the drugs bound in the different cell compartments.
3) To determine how the changes in chromatin induced by Dauno affect the amount of Cis-Pt-DNA adduct levels.
4) To determine how Cis-Pt affects the cellular uptake and chromatin binding of Dauno.

3. METHODS

3.1. Cells, treatments, cytotoxicity assay

HeLa cells expressing H2B-GFP or H3-GFP, control HeLa, HCT-116 and T47-D cells were cultured in DMEM, where Jurkat cells in RPMI1640, supplemented with FCS, L-glutamine, streptomycin and penicillin. Dauno was used in a concentration range of 0 –15 μM. Cis-Pt was applied at 0–80 μM. For median effect analyses, Cis-Pt was added to the cells at 40 μM final concentration together with a concentration series of Dauno, for 16 hours. Cytotoxicity was assessed using the Resazurin based assay. 100 μl aliquots of the drug treated cells were re-suspended in colorless media and added to 96-well flat-bottom microplates to 20,000 cells per well. 100 μl of freshly prepared Resazurin solution in colorless medium was added to each well to a final concentration of 18 μM. The plates were incubated at 37°C for 24 h and fluorescence signals were measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm using microplate reader. To determine synergism, additivity or antagonism, median effect analysis was performed using the method of Chou-Talalay, using CompuSyn ver. 1.0.

3.2. Urea-agarose gel electrophoresis

Jurkat cells were treated with 9 μM Dox and 10 or 25 μM Cis-Pt for 16 hrs. The DNA of the samples was analyzed by urea-agarose gel electrophoresis, the method of Materna et al., further developed in our lab. Briefly, the cells were embedded into LMP-agarose plugs, deproteinized and treated with the Nt.CviPII nickase enzyme. The samples of agarose plugs were equilibrated with 9 M urea-containing loading buffer, denatured at 90 ˚C for 5 min, allowed to cool to RT then loaded onto an agarose gel also containing urea. After conventional electrophoresis, the gel was stained with 5 μg/ml ethidium bromide (EBr).

3.3. Measurement of Dauno

3.3.1. *Determination of total intracellular Dauno fluorescence*

In order to assess Dauno fluorescence in live cells and in isolated nuclei by flow-cytometry, HeLa cells were treated with 1.5 μM of Dauno alone or in combination with 40 μM of Cis-Pt for 16 hours. After trypsinization and re-suspension of the cells, Dauno fluorescence intensity distributions were recorded in a flow-cytometer.

3.3.2. *Determination of nuclear Dauno fluorescence*

Aliquots of the drug treated cells above were suspended in 500 μl PBS and lysed in 500 μl ice-cold 0.2% (v/v) Triton X-100 dissolved in 1 × PBS/EDTA, for 20 minutes (min). After

lysis, nuclei were washed twice with 4 ml ice cold PBS/EDTA then re-suspended in 1 ml PBS/EDTA and Dauno fluorescence intensity distributions were recorded by flow-cytometry.

3.3.3. *Determination of DNA-bound Dauno via Hoechst-quenching*

HeLa cells were treated with 1.5 µM of Dauno alone or in combination with 40 µM of Cis-Pt for 16 h, then, without washing the samples, stained with 35 µM Hoechst 33342 for 30 min. After trypsinization, the cells were analyzed by flow-cytometry.

3.3.4. *Flow-cytometry*

A Becton Dickinson FACSAria III Cell Sorter was used. Dauno was excited using the 488 nm line of a solid state laser and the emitted light was detected using a 695/40 nm band-pass filter. Hoechst 33342 fluorescence was measured by excitation at 375 nm and emission detection using a 450/50 nm band-pass filter. Data were analyzed by the Reflex software.

3.4. Measurement of histone eviction

Histone eviction was measured by the QINESIn assay developed in our lab. Briefly, the agarose-embedded GFP-histone expresser cells at the bottom of ibidi wells were permeabilized with 500 µl ice cold 1% (v/v) Triton X-100 dissolved in 1x PBS/EDTA, washed with 500 µl ice cold 1x PBS/EDTA and these samples of nuclei were treated with different concentrations of Dauno. The histones remaining chromatin-bound were determined cell-by-cell using laser scanning cytometry (LSC) scans of the samples.

3.5. Measurement of ICLs and total Cis-Pt-DNA adduct levels by LSC

3.5.1. *Preparation of comets and halos*

Following exposure to drugs, the cells were centrifuged at 175 g for 5 min at 4 °C, then re-suspended to a final concentration of 1 × 106 cells/ml in PBS, kept at 37°C and mixed at a v/v ratio of 1: 3 with 1% Low melting point agarose dissolved in PBS. 100 µl of the cell-agarose suspension was pipetted onto Superfrost microscope slides and were covered with coverslips. The slides were pre-coated by submerging them in molten 1% agarose dissolved in water, and then allowed to dry overnight at room temperature. The cells were left to sediment on the surface of the slides for 4 min at 37°C, then kept on ice for 5 min. After polymerization of the agarose, the coverslips were gently removed, then the slides were submerged in glass staining tanks containing ice-cold PBS/EDTA for 5 min, then submerged in ice-cold TRIS/EDTA, 150 mM NaCl and 1% v/v Triton X-100 for 10 min, next in PBS/EDTA containing 1% Triton X-100 for another 10 min. After washing in PBS/EDTA for 10 min, the samples were equilibrated with nickase, then nickase treatment was performed covering the slides with 500 µl of nickase buffer containing the frequent cutter nicking endonuclease Nt.CviPII at a final concentration

of 0.001 U/ml and incubated in a wet chamber at 37 °C, for 20 min. After washing with ice-cold PBS/EDTA three times for 10 min, the slides were submerged in freshly made alkaline lysis buffer at room temperature, for 1h. After alkaline denaturation, the slides were placed in an electrophoresis tank filled with alkaline running buffer. After electrophoresis (for 20 min at 200 mA), the samples were neutralized with 0.4 M Tris (pH = 7.5) for 10 min before staining with SYBR Green I, 1: 5000 diluted in PBS/EDTA. Since no background fluorescence was detected following S1 digestion, simple intensity measurements instead of calculating tail-moments sufficed, so the steps related to comet formation (nickase digestion and electrophoresis) were omitted and the experiments were performed in ibidi 8-wells microslide. The samples were equilibrated with S1 for 30 min, then the samples were covered with 500 µl of S1 enzyme solution. The enzyme was diluted to 1000 U/ml concentration in S1 nuclease buffer. The samples were incubated on ice for 5 min with the enzyme for equilibration, then digestion was performed at 37 °C, for 20 min. After washing in PBS/EDTA, the samples were stained with SYBR Green I as described above, for 1 h.

3.5.2. *Determination of total amount of Cis-Pt-DNA adducts*

Nuclear halos were labeled by indirect immunofluorescence using anti-Cisplatin primary antibody as described below. Agarose-embedded nuclei on ibidi slides were exposed to the combinations of Cis-Pt and Dauno at 4 °C for 16 hours, washed with ice cold PBS/EDTA 3 times for 5 min each, treated with Proteinase K dissolved in 0.4 M EDTA, 1% Sodium lauroyl sarcosinate, 10mM Tris, pH = 8 at 0.5 mg/ml final concentration, at 42 °C, for 24h. The enzymatic reaction was stopped by incubating the samples with 1:10 dilution of 10mM PMSF) at room temperature, for 10 min. Then the samples were denatured and renatured as described above; electrophoresis was skipped. Immunofluorescence labeling was performed at 4 °C, overnight, using rat monoclonal anti-Cisplatin modified DNA antibody diluted at a titer of 1:800 in 150µl of PBS/EDTA/1% BSA. Samples were washed 6 times for 10 min each, followed by incubating the samples with 500µl 5% (m/v) Blotto Non-Fat Dry Milk in PBS/EDTA on ice for 30 min. Labeling with the secondary antibody was performed using Alexa Fluor 488 (A488) conjugated Donkey anti-rat IgG, at a titer of 1:800, diluted in PBS/EDTA/3% BSA, from 2 mg/ml stock solutions. After labeling with the secondary antibody, nuclei were washed with 500µl ice-cold PBS/EDTA 3 times for 10 min each. Then the samples were fixed using 1% formaldehyde at 4 °C, overnight. After fixation, the nuclei were washed with 500µl ice-cold PBS/EDTA 3 times for 5 min and fluorescence intensity distributions were recorded by LSC. For studying the effect of salt induced histone eviction on

total Cis-Pt-DNA adduct formation, the agarose embedded nuclei of HeLa cells were prepared on the slides as described above and were treated with 1.1 M or 1.6 M of NaCl in PBS/EDTA 6 times for 10 min each and subsequently washed with PBS/EDTA. These samples were exposed to 40 µM of Cis-Pt, for 16 hrs, then treated with proteinase K followed by PMSF and washing steps, then labelled by immunofluorescence for the LSC measurements, as described above. Then the same samples were dehydrated with an ascending series of ethanol for subsequent LA-ICP-MS analysis.

3.5.3. *Laser scanning cytometry (LSC)*

Automated microscopic imaging was performed using an iCys instrument. Green fluorescent protein (GFP), SYBR Green I, A488, and propidium iodide (PI) were excited using a 488nm Argon ion laser. A647 was excited with a 633nm HeNe laser. The fluorescence signals were collected via an UPlan FI 20x (NA 0.5) objective. GFP and A488 and SYBR Green fluorescence were detected through 510/21nm, 530/30nm and 550/30nm filters respectively, while A647 and PI were detected through a 650 nm/LP filter. Data evaluation was performed using the iCys 7.0 software. Gating of G1 phase cells was based on the fluorescence intensity distribution curves of the DNA stained with PI.

3.6. Determination of Pt in the nuclei using quantitative LA-ICP-MS

3.6.1. *LA-ICP-MS instrumentation*

The LA-ICP-MS measurements were conducted by our collaborators at the chemistry department of Ghent university, using an Analyte G2 193 nm ArF* excimer-based LA-unit, equipped with a low-dispersion Cobalt ablation cell, what was coupled to a quadrupole-based Agilent 7900 ICP-mass spectrometer *via* the ARIS, providing fast aerosol transport.

4. RESULTS

4.1. Cis-Pt and Dauno synergize or antagonize each other in a cell-type and dose dependent manner

To determine the mode of drug interaction I calculated the combination index (CI) based on the median-effect principle of the mass-action law, performed according to the Chou-Talalay method. Cytotoxicity was evaluated by performing a Resazurin cell viability assay. I chose the simultaneous addition of the two drugs because both form covalent linkages eventually, what would diminish the chances that any of the drugs could exert its effects solely on its own. Covalent (at least so tight as to resist the DNA isolation procedure) binding of Dauno to DNA was apparent in view of the quenching of EBr fluorescence in the Dauno treated DNA samples. The choice of timing of the drug treatment (16 hrs) was based on the required time for the formation of adducts and the kinetics of the repair of Cis-Pt-related DNA modifications. I made a comprehensive pre-titration of concentrations using the two drugs in several combinations and selected the conditions based on these data. The concentrations of both drugs were systematically varied in a matrix spanning 0.3 – 30 μM of Dauno and 1 – 100 μM of Cis-Pt, and the CI values were determined for each combination. Combination treatment of Jurkat cells confirmed the general synergism of the two drugs over a wide concentration ratio, what was expected in view of clinical experience and was exhibited best at 40 μM Cis-Pt for both low and high Dauno concentrations. Therefore, 40 μM of Cis-Pt was used in the further experiments that included other cell types as well. Unexpectedly, in sharp contrast with the Jurkat cells, in HeLa antagonism was observed at the subtoxic Dauno concentration range of 0.3–1.8 μM, and synergism at toxic Dauno concentrations ranging between 3–12 μM, using 40 μM Cis-Pt.

I performed experiments using Dox also. Jurkat and HeLa cells were treated with Dox or Cis-Pt at a concentration range of 0.3-32 μM and 5-160 μM, respectively, and using a combination of 40 μM of Cis-Pt with different Dox concentrations. The cells showed a varied response to the combination of Dox and Cis-Pt, which recapitulated the dose- and cell line-dependence of synergy vs. antagonism found in the case of Dauno and Cis-Pt. I also tested if Dox exhibits a concentration-dependent synergy/antagonism when combined with another drug that interferes with protein degradation pathways, which has come to the focus of our interest *via* another project. In those experiments, Jurkat cells were treated with Dox and PYR-41 alone at a concentration range of 2.25-36 μM and 6.25-100 μM, respectively, or in a combination of 50

μM of PYR-41 with various concentration of Dox. The combination of Dox and PYR-41 treatments on Jurkat cells is synergistic.

4.2. Co-treatment with Cis-Pt differentially alters the amount of total cellular, chromatin-bound and DNA-bound Dauno

I first evaluated whether the antagonism between the two drugs might be related to an effect of Cis-Pt on the amount of Dauno taken up into the cells, accumulating in the acidic endosomal compartment, by assessing Dauno fluorescence in live cells using flow-cytometry. In parallel, the total nuclear fluorescence of Dauno, accounting for the chromatin-bound drug, was also measured. Dauno fluorescence was significantly decreased in Cis-Pt-Dauno co-treated cells as compared to cells incubated with Dauno only, both measured in live cells and nuclei. Since Dauno also binds to histones in addition to DNA, I performed an assay to directly measure the effect of Cis-Pt co-treatment on DNA-bound Dauno in live cells via the quenching of Hoechst 33342 fluorescence. Hoechst 33342 enters live cells and becomes brightly fluorescent upon binding through the minor groove of the DNA to tandem AT base pairs. The green emission of Hoechst dye is absorbed by Dauno, thus quenching of Hoechst fluorescence can be utilized as a measure of DNA-bound anthracycline. The decrease of Hoechst fluorescence in the presence of Dauno was stronger when Cis-Pt was also added. This difference can be partly accounted for by the influence of Cis-Pt on Hoechst fluorescence. However, an increased level of DNA-bound anthracycline in the presence of Cis-Pt co-treatment was corroborated in experiments when the DNA of cells treated with Dox was isolated, run on agarose gels and visualized by EBr staining.

4.3. Cis-Pt facilitates Dauno-induced histone eviction

A newly discovered effect of anthracyclines, also implicated in their cytotoxicity, is induction of nucleosome eviction due to intercalation. I investigated whether this effect may be influenced by Cis-Pt. Live cells or isolated nuclei were co-treated with the intercalator and Cis-Pt and populations of cells were assessed on a cell-by-cell basis by flow cytometry. The intercalator-induced eviction of the histone dimers and tetrasomes were both substantially augmented. Thus, the amount of histone-bound Dauno is expected to decrease in the nucleus when cells are co-treated with Dauno and Cis-Pt, apparently in the entire concentration range of the intercalator.

4.4. Detection of ICLs using a modified alkaline comet assay

Another possible mode of interaction between the two classes of drugs is relaxation of supercoiling by the intercalator, mitigating ICL formation. In order to study this possibility, we

employed a modified version of the alkaline comet assay where the ICL-dependent increment in the renaturation of alkali-denatured samples of nuclei is measured. In view of the rather high background of renatured DNA in the absence of cross-linker in that procedure, we used S1 nuclease treatment to remove any non-renatured DNA, and the SYBR Green I dye to stain double-strand DNA. Using these modifications and employing LSC to measure the fluorescence of nuclear comets, ICL detection was made possible in a wide concentration range of Cis-Pt added to live cells, as low as ~5 μM.

4.5. Co-treatment with Dauno influences Cis-Pt-DNA adduct levels

In order to assess the effects of Dauno intercalation on the formation of Cis-Pt-DNA adducts, nuclei of HeLa cells were exposed to combinations of 40 μM of Cis-Pt and Dauno used at various concentrations. ICL levels were assessed employing the modified comet assay described above, while total Cis-Pt DNA adducts were measured via immunofluorescence using an antibody recognizing all Cis-Pt-DNA adducts. I observed no change in the level of total DNA-Pt adduct levels, while there was an apparent reduction in ICL formation. Notably, this effect was detected already in the low concentration range of the intercalator.

4.6. Effect of nucleosome eviction on Cis-Pt-DNA adduct levels

A model system was set up to learn how nucleosome eviction, in the absence of any other effects of Dauno, might influence Cis-Pt binding to the DNA. I measured total Cis-Pt adduct levels and ICLs in nuclei pre-treated with salt to remove the dimers (1.1 M NaCl), or all the histones (1.6 M NaCl). Total adduct levels were assessed by three independent methods: by measuring the fluorescence intensity of SYBR Green I staining dsDNA in the renatured samples when S1 digestion step was omitted so as to detect all the Cis-Pt adducts renaturing into ds DNA when (any) two strands are covalently linked by Cis-Pt, by immunofluorescence using the anti-Cis-Pt antibody, and also by LA-ICP-MS. ICLs were measured by the modified comet assay described above as well as by LA-ICP-MS following the treatments performed in the comet assay. The different approaches yielded results that were in agreement with each other. Total DNA platination was increased at intermediate salt levels only, while ICLs were decreased, moderately at 1.1 M, and to a greater extent at 1.6 M salt concentration.

5. DISCUSSION

We have observed a cell type- and dose-dependent interaction between Dauno and Cis-Pt, which warrants a detailed analyses of the mechanistic components of the interactions in the case of their combined application in cancer chemotherapy. Reminiscent of our findings, cell type and dose-dependent synergism vs. antagonism was described for the combination of Cis-Pt with a deoxycytidine analogue. In view of the fact that both Dauno and Cis-Pt appear to primarily target the chromatin, we set out to assess how they affect each other's binding to particular cellular components, focusing on the chromatin. There is little doubt that the mode of Cis-Pt action most relevant to cytotoxicity is its binding to the DNA. Although the effects of Dauno are more diverse, its interaction with the different components of the chromatin are generally considered of primary importance for its toxicity. Also in line with the hypothesis that Dauno exerts its cytotoxicity mainly in the nucleus, a shift in its cellular distribution was observed toward higher cytoplasmic/nuclear ratios in resistant cells. To investigate the possible scenarios of interactions that may lead to synergism or antagonism we employed a web-based resource that integrates multiple forms of pharmacological and genomic analyses, and also unifies the cancer cell line datasets. CellminerCDB analyses, revealed that despite the highly similar chemical structure and mechanisms of action of Dauno and Dox, there are differences even among cell lines of the same tissue type regarding their individual sensitivity to the two drugs. Hence the complexity of cell biological features influencing the toxicity of drugs must be enormous, which makes it difficult to pinpoint the target most relevant for cytotoxicity for any of the drugs studied herein. Thus, the differences observed in the case of combination of Dox and Cis-Pt may not be surprising.

Concerning the effect of Cis-Pt co-treatment on Dauno levels, total Dauno fluorescence representing mainly the acidic endosomal compartment where amphiphilic compounds spontaneously accumulate, decreases upon co-treatment. Therefore, if extra-nuclear effects are also important factors in Dauno toxicity, Cis-Pt could antagonize them by reducing the cytoplasmic Dauno levels. Cis-Pt also decreased total nuclear Dauno fluorescence. Dauno binds more avidly to DNA than to histones, but its fluorescence is quenched only by DNA. Therefore, the fluorescence intensities measured probably reflect the presence of both Dauno-DNA and Dauno-histone complexes, but in an unknown ratio. Therefore, we applied an assay that can directly measure DNA-bound Dauno, based on the quenching of Hoechst 33342 fluorescence by Dauno when they are in molecular proximity. Upon Cis-Pt co-treatment the levels of DNA-bound Dauno or Dox do not decrease, rather increase. Dimming of the EBr

fluorescence in the presence of the Dox was stronger when more Cis-Pt was added to the cells together with the intercalator. Thus, Cis-Pt indeed increases the amount of the DNA-bound anthracycline.

Dauno-induced eviction of histones was augmented in the nuclei co-treated with Cis-Pt. Two possible explanations for this augmentation by Cis-Pt were considered: 1) Increase of DNA-bound Dauno despite a decrease in its cellular uptake; 2) Cis-Pt unwinds DNA locally, thus inducing compensatory positive torsional stress around, what would also act to destabilize the nucleosomes enhancing Dauno –induced histone eviction. In other words, Cis-Pt causes distortions in the DNA structure which may be an important factor in its influence on Dauno-induced histone eviction. The Cis-Pt elicited increase in the eviction of H2B was detected already at low, while H3 eviction was augmented only at higher Dauno concentrations. Thus, interaction of the two drugs in terms of histone eviction appears to be synergistic in the whole concentration range of the anthracycline.

Regarding the effect of Dauno on Cis-Pt-DNA cross-links levels, total platination was not affected, while the level of ICLs, assessed using a modified version of the alkaline renaturation assay, was significantly decreased. The decreased ICL levels observed at low Dauno concentration were not further changed using higher concentrations of the anthracycline; thus, the antagonistic effect of Dauno on ICL formation appears to be similar in the entire Dauno concentration range tested. Therefore, if ICLs significantly contribute to Cis-Pt toxicity, Dauno co-treatment could mitigate these consequences in the low Dauno concentration range, while the synergistic other effects may overrule this at higher concentrations. The decrease of ICL formation is likely due to relaxation of negative supercoiling of the chromatin loops. Based on the above, the antagonism of low Dauno concentration with Cis-Pt in HeLa cells is suggestive of the importance of ICL formation in the cytotoxicity of Cis-Pt in certain experimental and treatment regimens.

We have set up a model system comprising isolated nuclei treated with moderate and high salt concentration, to evict nucleosomes and elicit topological changes of the DNA in a controlled manner and in the absence of topological changes induced directly by intercalators. The LA-ICP-MS approach let us obtain absolute numbers for the total adduct levels and for ICLs and these are comparable to the numbers determined in other studies. The changes in total platination levels (mainly intrastrand cross-links) and ICLs were comparable to the changes observed when nuclei were treated with Cis-Pt and Dauno. The eviction of nucleosomes by salt

or Dauno increases the target size for Cis-Pt binding. Furthermore, intrastrand crosslinks that involve bending of the DNA must be severely antagonized by any constrainment of its structure and augmented by the release of such constraints. On the other hand, the increase of free DNA regions upon nucleosome eviction is expected to favor formation of highly constrained, hyperplectonemic structures likely to occur in the case of long superhelical DNA molecules. These antagonistic effects can explain the changes observed in total Cis-Pt-DNA adduct formation in our model system. The increase of target size upon histone eviction may be dominant at moderate salt concentration, increasing adduct levels, while the hyperplectonemic constrainment in the naked DNA produced by high salt pre-treatment may prevent the bending of DNA required for intrastrand crosslinking. The superhelicity of the linker regions is generally assumed to be negative. About 30% of the overall linking number difference is estimated to partition as twist. The nucleosomal structure entails slightly overwound DNA (Tw =10.3 bp/turn compared to 10.5 bp/turn for B-DNA). The negative twist in the linker regions together with the slightly positive twist inside the nucleosomes can account for the linker-preference of ICL formation. At moderate salt treatment, upon eviction of H2A and H2B, the enlarged nucleosome-free regions are expected to adopt a topology with higher degree of freedom to redistribute twist (Tw) and writhe(Wr). As a consequence, Wr likely becomes more, and Tw less negative in the widening linker regions, in comparison with their state before eviction. Such changes would lead to a decrease in ICL numbers after treatment with 1.1 M salt. At high salt, upon complete eviction, the hyperplectoneme imposed topological constraints would antagonize proper apposition of the bases involved in ICL formation, further decreasing the chances of their generation. The data obtained in this model system are in line with the interpretation that, the Dauno evoked decrease of ICL formation observed in isolated nuclei is, at least partly, due to Dauno induced eviction of the histone dimers. To what extent Dauno-elicited Tw relaxation directly contributes to a decreased ICL formation could not be determined because nucleosome eviction necessarily accompanies intercalation. The scenario of complete eviction modeled by high salt concentration pre-treatment of isolated nuclei does not occur even at high Dauno concentrations. In summary, histone eviction by itself is expected to diminish ICL formation, while total adduct formation may not change due to the complexity of effects involving increased target size represented by a more relaxed naked DNA on the one hand, and constraints emerging in the wake of DNA-DNA interactions upon hyperplectoneme formation, on the other.

6. SUMMARY

Dauno and Cis-Pt antagonize one another through a decrease of ICLs in the presence of Dauno (at its low and high cc. alike), and also via the diminished Dauno uptake in the presence of Cis-Pt. They could synergize with each other through enhanced histone eviction by Dauno in the presence of co-treatment with Cis-Pt, accompanied by an increment of DNA bound anthracycline. These drug interactions have not been described before to the best of our knowledge and may impact cytotoxicity reached by combination treatment regimens *in vivo*.

In the presence of Cis-Pt, Dauno concentration decreases in the cytoplasm (due to decreased uptake into the cell and/or the endosomal compartment). In the nucleus, its total amount is not affected by Dauno co-treatment, but the number of ICLs is decreased.

Part B. The Relevance of Histone Levels for DNA-Targeted Therapy

I. INTRODUCTION

As mentioned in section 1.3 of part A, Dauno binds to the linker histones. Moreover, I showed that binding of Dauno alone, as well as its combination with Cis-Pt led to histone eviction. Since eviction mattered for anthracycline binding and for how the two drugs interact with each other, we systematically used CellminerCDB (https://discover.nci.nih.gov/cellminercdb/) to analyze how sensitivity to these drugs correlates with the expression level of the different histones. In contrast with the experimental observation of Braunestin. *et al*, we, by using CellminerCDB to explore the NCI-60 database, found no correlation between the sensitivity of Dauno and Dox with the expression level of H2A (HISTH2AC) and H2B (HISTH2BD) and H1.5 (HIST1H1B) histones,. However, we stumbled on an interesting phenomenon. We have observed that 10 out of 60 cell lines from 9 different tissue types have markedly decreased mRNA expression levels of the H1.5 variant linker histone gene, concomitant with mRNA overexpression of ODC1 (Ornithine Decarboxylase 1) and AMD1 (Adenosylmethionine Decarboxlase 1). I investigated two scenarios, trying to interpret this phenomenon: First, based on the assumption that the linker histone variants, unexpressed in the 10 cell lines and expressed in 50, may provide a selective advantage for the latter by shielding the linker DNA to drugs, I tested if cell lines with inducible H1.5 expression may exhibit any dependence of drug sensitivity on H1.5 expression levels. For this purpose drugs differentially toxic for the 50 and the 10 cell lines were selected based on Cellminer analyses. These studies have led to negative results, not demonstrated here. I also investigated another option: whether the elevated level of the enzymes involved in polyamine synthesis might have a nucleosome destabilizing effect, potentially influencing drug sensitivity. If it were so, then the overexpression of ODC1 in the 10 cell lines could make them more vulnerable to DNA-targeted drugs. This aspect, a fortuitous side-product in our attempt to interpret the strange dichotomies among the NCI60 cell lines, has led us to realize that at high enough concentration polyamines destabilize nucleosomes.

ODC1 and AMD1 are the genes of the key enzymes for polyamine (PA) synthesis. PAs (Putrescin, Spermidine and Spermine) are polycationic alkylamines. Putrescin is generated from ornithine catalyzed by the ODC1 enzyme, and it is a precursor for the production of Spermidine and Spermine. AMD1 is generated as a pro-enzyme (proAMD1), which, as a result

of self-cleavage and heterotetramerization, becomes an active enzyme. The active AMD1 enzyme catalyzes the conversion of *S*-adenosylemethionine to *S*-adenosyl-5′-(3-methylthiopropylamine) which is fully dedicated to polyamine biosynthesis. Investigations on the activity of ODC1 in brain cancer, as well as AMD1 activity in prostate cancer unveiled that their activity leads to the potentiation of these malignancies. Expression and activity of ODC1 and AMD1 are closely related to each-other (based on CellminerCDB) and to the cellular concentration of the polyamines. Furthermore, PAs are involved in the pathologies of several parasitic diseases such as Trypanosomiasis and Leishmaniasis. It is also worth noting that PAs are associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and certain age-related conditions, as the levels of polyamines are known to decrease with age. Since PA levels and the enzymes involved in their metabolism proved to play a role in cancer and several other diseases, inhibitors of PA synthesis or transport are in the focus of attention in view of the therapeutic prospects. Preclinical and clinical studies on polyamine antagonist regimens including Eflornithine and Sardomozide (ODC1 and AMD1 inhibitors, respectively), showed promising results in certain types of cancer. Combination of Eflornithine and some of the non-steroidal anti-inflammatory drugs such as Indomethacin, Sulindac and Celecoxib synergized with each other resulting in the suppression of polyamine-dependent cell proliferation and tumor growth. The transport system of PAs is another therapeutic target for decreasing the PAs level in cancer cells. Synthetic, lipophilic polyamine analogues restrict the cellular PA uptake system and when in combination with Eflornithine, they synergize each other strongly resulting in PA depletion. At physiological pH, positively charged (basic) PAs associate with negatively charged (acidic) sites on proteins, nucleic acids as well as phospholipid membranes. PAs are required for cells to proceed from G1 to S phase and they are directly involved in the DNA synthetic phase of the cell cycle. PAs bind to DNA and cause DNA aggregation. The stoichiometry of PA binding which leads to condensation of DNA is ~2 PA molecules per one helical turn of DNA. PAs appear to stabilize also nucleosomes and condense chromatin in the ~ 0.1-3 mM concentration range. The nucleosome destabilizing effect discussed below, an unexpected observation with possible ramification not contemplated earlier, appears at slightly higher, still biologically relevant PA concentrations.

II. AIM OF THE STUDY

The study aims to determine how PAs, when used at high, still biologically relevant concentrations, impact nucleosome stability?

III. METHODS

Preparation of the solutions:

Except for the permeabilizing solution (1% Triton/PBS/EDTA) that was prepared freshly, all the other solutions were prepared 24hrs before the day of the experimentation. The pH of the ice-cold PBS/EDTA (pH=7.8) solutions was alkalized to the corresponding pH of the solutions containing 2.5, 5, and 10 mM Spermidine (pH equals 9.6, 10.2, and 10.4, respectively) with NaOH. Moreover, the pH of the NaCl solutions containing 5 and 10 mM Spermidine were neutralized with HCl to achieve pH= 7.8. Worth mentioning that the addition of HCl was as minuscule that an excess salt concentration did not increase by more than 5 mM.

Preparation of nuclei/ permeabilization and histone eviction by salt

Embedding the cells in low melting agarose in Ibidi-wells, and permeabilization of cells is explained section 3.4 and work of Imre.et.al from our lab. After permeabilization, nuclei were washed with 400 µl ice cold 1×PBS/EDTA, 3 times for 3min. Then nuclei were treated with the solutions containing various NaCl concentration with or without Spermidine, for 60 min, on ice. After this step, the nuclei were washed with 400 µl ice cold 1×PBS/EDTA 3 times for 5 min. Since NaCl was dissolved in 1×PBS/EDTA, the salt concentrations indicated on the X axes of the graphs in Figures B.4 and B.5, show the additional NaCl concentrations regardless of NaCl present in the PBS buffer which is ~150 mM. EDTA was included in PBS so as to avoid complex effects involving divalent cations.

IV. RESULTS

The effect of PAs on the stability of nucleosomes was analyzed in permebilized nuclei of H2B-GFP expressing HeLa cells by utilizing our QINESIn method. Upon addition of Spermidine to the agarose-embedded nuclei at a concentration of 10 mM markedly lowered the concentration of salt required to destabilize the nucleosomes releasing H2B-GFP. However, the pH of the PBS/EDTA buffer was strongly alkalinized by Spermidine addition, so destabilization could be partly attributed to the shift of pH toward the isoelectric point of the histones (~10 for H2B, ~10.5 for H2A and ~11 for H3 and H4). Therefore, to determine if Spermidine itself exert a destabilizing effect, the salt elution curves were compared in the presence and in the absence of the Spermidine at the pH measured after adding the Spermidine to the PBS/EDTA + NaCl solution. Spermidine at a concentration as low as 2.5 mM, augmented NaCl-induced histone eviction. Comparison of NaCl solution with a pH adjusted to a similar pH as NaCl solutions containing the Spermidine indicates that the increment in histone eviction is not merely due to alkaline pH, but also Spermidine itself. Although 5 mM of Spermidine solution with no NaCl is unable to destabilize histone, but addition of 400 mM NaCl to the system suffice to boost eviction of histones. The same holds true for 10 mM of Spermidine solution supplemented with only 50 mM NaCl, in which histone eviction is augmented significantly in comparison to eviction ensued from alkaline NaCl solutions.

V. DISCUSSION

The millimolar concentration range of the nucleosome destabilizing effect observed in our experiments is attained by PAs in various physiological and pathological scenarios. For instance, PAs level can reach 10-12 mM in prostate cancer. Also, PAs are present in millimolar concentration in plants and have diverse functions, what can be modulated by their exogenous application in high concentration. Since expression of ODC1 and AMD1 correlate (Pearson correlation r =0.38) across the 60 lines, the high ODC1 and AMD1 expressor cells likely have higher intracellular PA levels in comparison with the other 50 cell lines.

Chromatin compaction in permeabilized nuclei treated with polyamines ensued at 0.2 and 0.5 mM of Spermine or Spermidine, which has not changed further above 0.8 and 1.5 mM concentration, respectively. The nucleosome destabilizing effect reported herein, an unexpected observation with possible ramification not contemplated earlier, appears at higher, still biologically relevant PA concentrations. Within the physiological range, up to pH=8.0, nucleosomes are expected to be stable. Alkalization to near the isoelectric point of the histones sensitized the nucleosomes to salt induced disruption, effectively decreasing the electrostatic binding forces. Alkaline conditions alone, at physiological ionic strength, did not dissociate H2B-GFP from the nucleosomes. Both alkaline pH and elevated salt concentration were necessary to demonstrate the destabilizing effect of PAs. The nucleosome destabilizing effect described herein is apparently superimposed on PAs' contribution to the condensed state of chromatin as well as on other, indirect effects on chromatin structure and function; these include the up-regulation of histone acetylation and activation of DNA methyltransferase, also ensuing at the elevated PA concentrations following ODC1 overexpression, or PA feeding, respectively. Therefore we suggest that elevated PA concentration in the nucleus leading to nucleosome destabilization may contribute to epigenetic diversification, possibly through alteration in the fine balance of factors regulating remodeling.

VI. SUMMARY

At high concentration of PA (what is present in certain cancer cells and in normal prostate tissue as well), nucleosomes are significantly destabilized. This is a novel, previously unrecognized effect of PAs considered primarily as DNA and nucleosome stabilizing agents.

Nucleosomal structure being repressive for most processes involving the chromatin, we speculate that their destabilization in cells exhibiting high metabolic flux of PAs may interfere with processes relying on chromatin remodeling.

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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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   Sci Rep. 7 (1), 1-15, 2017.
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