Characterizing the nucleosome site selectivity, adduct structures and impact on chromatin for a variety of metal-based anticancer compounds and structural studies of nucleosome-linker histone interactions

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**CHARACTERIZING THE NUCLEOSOME SITE
SELECTIVITY, ADDUCT STRUCTURES AND IMPACT
ON CHROMATIN FOR A VARIETY OF METAL-BASED
ANTICANCER COMPOUNDS AND STRUCTURAL
STUDIES OF NUCLEOSOME-LINKER HISTONE
INTERACTIONS**

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Abbreviations

3-HPA	3-hydroxypicolinic acid
AAS	Atomic absorption spectrophotometry
APS	Ammonium persulfate
BME	β -mercaptoethanol
CIA	Chloroform-isoamyl alcohol
CIAP	Calf intestine alkaline phosphatase
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EMSA	Electrophoretic mobility shift assay
H1.0	Full length linker histone
H1.0+His ₆	Full length linker histone with hexa-histidine tag
HER	High electronegativity region binding
HIS	Histidine binding
HPLC	High pressure liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
IPTG	Isopropyl- β -D-thiogalactopyranoside
gH1.0	Globular linker histone
gH1.0+His ₆	Globular linker histone with hexa-histidine tag
MALDI-MS	Matrix-assisted laser desorption ionization - mass spectrometry
MPD	2-methyl-2,4-pentanediol
NER	Nucleotide excision repair
NCP	Nucleosome core particle
NUC	Nucleosome
nucDNA	Nucleosomal DNA
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
PNK	Polynucleotide kinase
PTA	1,3,5-triaza-7-phosphaadamantane

QED	Quantitative estimate of drug-likeness
rATP	Riboadenosine triphosphate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SHL	Superhelix location
TEA	Triethanolamine
TEMED	Tetramethylethylenediamine

Abstract

The enormous length of DNA molecules is managed in the nucleus of eukaryotic cells by wrapping the double helix through histone protein association to form chromatin. The basic repeating unit of chromatin is the nucleosome. Due to this packaging, nucleosomal DNA and histones have different conformations and accessibility compared to that of naked DNA or chaperone-bound histones. The structure and dynamics of chromatin are important for many cellular functions. Different nuclear factors bind to nucleosomes to regulate chromatin dynamics and signal downstream cellular processes, like DNA replication and transcription. As a consequence, nucleosomes and their binding partners can be specifically targeted to modulate these functions. This is especially relevant to cancer cell function, whereby numerous epigenetic differences, such as alterations in nucleosome positioning, between transformed and healthy cells result from extensive changes in gene expression and the frequency of DNA replication (cell division). Distinguishing epigenetic features of cancer cells could be potentially exploited through the design of site-specific chromatin-targeting anticancer agents.

Because of the severe side effects and resistance problems associated with platinum anticancer drugs, many different compounds based on alternative heavy metals have been explored as potential therapeutic agents and many more are presently under investigation. However, the cellular targets and mechanisms of action for these newer agents are largely unknown. Based on studies with cisplatin and other platinum drugs, DNA is generally assumed to be the cellular target for reactive metal-based compounds. However, this thesis work and other recent evidence show that proteins are the main targets for at least some of these compounds. Since the nucleosome contains both DNA and protein components and is an important therapeutic target, it is useful for investigating the DNA- versus protein-binding preferences of different metal-based agents.

We investigated the site selectivity, adduct structures and impact on chromatin for many different metal-based compounds, including ruthenium, osmium, gold and rhodium agents. This involved a broad spectrum of biochemical, X-ray crystallographic and electron microscopic (EM) techniques in conjunction with collaborations on cellular, analytical chemistry and computational approaches. The

striking discovery is that, with the exception of one class of ruthenium agent, the compounds investigated show a preference for binding to histone protein sites as opposed to the DNA of the nucleosome. Moreover, by comparing two structurally similar ruthenium compounds, one a cytotoxic anti-primary tumor agent and the other an effectively non-cytotoxic antimetastasis agent, we could show the basis for their differential DNA versus histone protein targeting and consequently linkages to their distinct cellular impact. For the compounds that preferentially form adducts at histone protein sites, we also discovered that there are two distinct classes of binding site, which are distinguishable by virtue of electrostatic components, affinity for histidine, steric access and hydrophobic interactions. Beyond this, the results shed light on the basis for cleavage of ‘carrier’ ligands, the influence of different metal centers and the basis for a novel nucleosomal drug-drug synergy. Finally, we show that adducts formed by certain metalloagents can have a direct and dramatic influence on the structure and dynamics of the chromatin fiber. Together with the other findings, this suggests strong therapeutic potential surrounding many different features of chromatin, yielding new leads for the rational design of improved metal-based anticancer agents.

By compacting chromatin in a site specific manner, linker histone association with the nucleosome changes the structure and accessibility of defined genomic sites. However, the details of linker histone binding location on the nucleosome are still not clear, and in particular a high-resolution atomic structure for a nucleosome-linker histone assembly has not been solved. In addition to the biological significance, structural characterization of linker histone-nucleosome interactions could hold importance for drug development initiatives, for instance new targets for metal-based compounds. In this thesis, we investigated binding and crystallization of complexes of nucleosome with linker histones, focusing largely on the minimal sized assembly, the ‘chromatosome’. We found different affinities for nucleosome binding to the full length versus globular domain-only linker histone and also some degree of DNA length and sequence dependence in the interaction. Different crystals of the chromatosome were obtained, however the best so far diffract X-rays to only ~ 5.5 Å resolution. Nonetheless, the initial results of the study provide a platform for further optimization of chromatosome crystallizations and possible structural characterization.

1 Introduction

1.1 Chromatin

DNA in eukaryotic cells exists in complex with nuclear proteins to form chromatin^{1,2}. The basic repeating unit of chromatin is the nucleosome. The nucleosome core consists of 145-147 bp of DNA wrapped in 1.67 turns around a histone octamer in a left-handed superhelix. The histone octamer consists of two copies of each of the core histones H2A, H2B, H3 and H4³⁻⁵. Nucleosome core regions in the genome are connected to each other by linker DNA of 10 to 90 bp, giving the appearance of a beads-on-string structure with diameter of 11 nm⁶. This structure is then folded into a fiber of 30 nm diameter^{7,8}. Further compaction with twisting and coiling finally yield the metaphase chromosome (Figure 1.1). Linker histones such as H1 and H5 are involved in the folding process, even though their precise role and position on the nucleosome are not yet clear⁹.

When first discovered, the packaging of eukaryotic DNA into chromatin was thought to be purely for spatial reasons. Chromatin structure is utilized to pack long stretches of genetic material into a very small nucleus. It was later realized that chromatin is also involved in gene expression¹⁰. DNA regions arranged in compact higher order chromatin structures are generally transcriptionally less active while those in more loose arrangement are transcriptionally more active¹¹. At the molecular level, gene expression is regulated by the positioning of nucleosomes along genetic elements¹². Although this might give an impression that chromatin is a rigid structure, chromatin is actually very dynamic and constantly packed and unpacked to allow different genes to be activated or inactivated along with cellular dynamics and status changes. Histone variants and post-translational modifications, DNA methylation and chromatin remodeling factors are involved in this process¹³⁻¹⁵. This dynamic system also permits the regulation of other cellular processes like DNA replication and repair.

Figure 1.1. Eukaryotic cell's DNA is packed by nuclear proteins to form chromatin structure. Figure is adapted from ref. 10.

1.2 Nucleosome core structure

The highest resolution nucleosome core structure was solved to 1.94 Å using *in vitro* reconstituted nucleosome core particle (NCP) containing palindromic 147 bp DNA (Figure 1.2)⁴. A pseudo-two-fold symmetry axis runs through the central base pair of the DNA, separating the two symmetry-related halves of the NCP. This central base pair is numbered 0, while the two 73-bp halves of the DNA are numbered from 1 to 73 on one half, and -1 to -73 on the other half. Major and minor grooves of the DNA alternate between facing inward and outward with respect to the histone octamer. The superhelix location (SHL) denotes the number of turns of the DNA double helix, starting from the central base pair as SHL0.

Figure 1.2. Nucleosome core structure. a) A nucleosome core particle viewed down the dyad axis (black ellipse). DNA superhelix axis is shown as broken line. b) Half of the nucleosome core viewed down the DNA superhelix axis; numbers denote the superhelix axis locations where minor grooves face inward. DNA strands are colored orange and cyan; histones are yellow, red, blue, green for H2A, H2B, H3, and H4, respectively; histone fold extensions and N- and C-terminal regions are colored white and labeled. Figures are adapted from ref. 4.

The interactions of histone with DNA mainly involve the histone fold motifs, which bind to the phosphodiester backbone of the central 121 bp of the DNA. The

terminal 13 bp of each NCP half are bound to H3- α N' helices and H3 N-terminal tails. In addition, N-terminal tails of H2B go through minor groove channels between neighboring DNA gyres (SHL-2.7 and SHL4.7, and the symmetry-related elements). The direct interactions between DNA and histone proteins mostly involve elements from the proteins' main chains. Most notable interactions with the side chains are the insertion of twenty amino acid side chains (mostly arginine) into DNA minor grooves, forming interactions with DNA phosphodiester backbone and edges of the bases. Most of these function in facilitating bending into minor grooves, which are facing the histone octamer. Water molecules also mediated interactions between histone and DNA elements, further stabilizing the nucleosome structure. Furthermore, water also acts as bridges for interactions between groups of histone and DNA which are located too far from each other to form direct interactions. These interactions are non-specific in regards to DNA sequence, allowing the formation of nucleosomes over different DNA sequences in the genome.

Nucleosomal DNA adopts different conformations compared to free DNA or DNA bound to other nuclear proteins. The overall form of nucleosomal DNA is generally B-form or B-form-like. However, nucleosomal DNA is non-uniformly bent around histone octamer, and there are local conformational variations of nucleosomal DNA depending on the flexibility of the sequence and position along histone octamer¹⁶. For NCP147, there are three different bending modes characterized by different base-pair-step parameters (Figure 1.3). The different DNA base-pair-step parameters are listed in Figure 1.4. Roll and tilt are the main bending operators. Roll involves bending and unstacking of DNA base-pair-step along the base edges and is energetically more favorable than tilt, which encompasses unstacking of bases in one strand and compression in the other strand. Bending into major groove (positive rolling) is more favorable than rolling into minor groove (negative rolling). The occurrence of rolling is coupled to twisting and sliding, while tilting is coupled with shifting. In NCP147, bending into the major grooves is smooth with positive roll angles coupled with reduced twist and negative slide values. Bending into minor grooves can be smooth or kinked, depending on the DNA sequence. Smooth bending into minor grooves involves GC, GG=CC or AG=CT base-pair-steps and is characterized by negative roll angles and alternating shift values. Kinked bending into minor grooves happens at the more flexible CA=TG base-pair-step with extremely negative roll angle coupled with large slide and twist values.

Figure 1.3. DNA bending modes in NCP147. Figure is adapted from ref. 16.

Figure 1.4. DNA base-pair-step parameters. Figure is adapted from ref. 17.

Different DNA sequences can have varying flexibility, and the histone octamer tends to prefer DNA sequences with greater flexibility for bending. Hence, although nucleosome can be formed with different DNA sequences due to non-specificity of DNA-histone interactions, the DNA sequence still has an indirect influence on nucleosome positioning *in vivo*^{18,19}. The most notable example is the

preference for TA dinucleotide at locations where DNA needs to bend towards the minor grooves. Although it has been shown in NCP147 that bending into minor grooves can happen with different base-steps, DNA sequences with higher affinity to form nucleosome are often enriched with TA dinucleotides at their minor-groove-inward locations²⁰.

With every helical turn of the DNA, four phosphate groups come in close contact with histone elements and are constrained by the histones. Two of these four phosphate groups act as a hinge. Even though the DNA is constrained at these hinges, the region between them is free to adopt different conformations, albeit with some restriction based on their DNA sequence and surrounding histone environment. If this region is where the minor groove is facing the histone octamer, it would experience pressure to bend and narrow the minor groove. The 'pressure' to distort is maximal at the center of the minor groove and the presence of the most flexible TA base-step can facilitate this bending process. Hence DNA sequences with TA dinucleotides at these locations have higher propensity to form nucleosome²¹.

Besides bending, nucleosomal DNA also displays stretching and kinking properties at distinct locations in the nucleosome^{4,5}. In stretching, DNA is elongated and overtwisted, with minimal changes in histone-DNA contacts, which consequentially increases the effective DNA length by 1 bp. It can occur around $SHL \pm 2$ and ± 5 and has been observed for nucleosomes in crystal and solution states and over different DNA sequences and lengths^{22,23}. The elongation in stretched DNA is usually accompanied by DNA kinking around the location of stretching. Kinking is defined by extreme bending (rolling) into the major or minor groove, which results in unstacking between the bases within a base pair step. It is most clearly observed in NCP with 145 bp DNA, where extreme kinks towards major and minor grooves occur at $SHL -1$ and $+1.5$, respectively. These sites also correlate with the initiating point of DNA stretching regions in this construct. Exogenous factors like HIV-integrase and small molecules have been demonstrated to prefer superkinked DNA as their substrate^{23,24}. Similarly, cellular factors may also be able to recognize and exploit this unique feature.

Crystal contacts are formed by several different types of inter-nucleosomal interactions. The DNA termini of neighboring nucleosomes form major base stacking interactions. Divalent and monovalent cations are also involved in stabilizing the nucleosome arrangements. Besides binding to DNA²⁵, divalent cations like Mn^{2+} and

Mg²⁺ can mediate the most extensive inter-particle interaction in NCP crystals by a bridging histone-histone contact^{4,26}. Inter-nucleosomal interaction is also formed between the N-terminal of histone H4 of one nucleosome with an acidic patch formed by histone H2A and H2B on the surface of a neighboring nucleosome^{3,4}. This phenomenon has also been demonstrated in solution and seems to be governed by electrostatic attraction^{27,28}. In addition to contributing to the formation of higher-order chromatin structures, the acidic patch on the nucleosome surface is also implicated in the binding of cellular and exogenous factors²⁹⁻³³.

1.3 Linker histones

Binding of linker histone to the linker DNA in the nucleosome increase the length of DNA protected from micrococcal nuclease digestion by ~20 bp^{34,35}. The minimal sized (~167 bp) complex of nucleosome with linker histone is called the chromatosome. Upon binding, linker histone's presence changes the structure of linker DNA and stabilizes chromatin condensation³⁶⁻³⁸. Linker histones typically consist of a small globular domain of around ~70 amino acids, flanked by a short N-terminal tail of typically ~40 amino acids and a long C-terminal tail of ~100 amino acids. The structure of the globular domain of chicken H5, the homolog of linker histone H1 in human, shows a winged-helix motif common to DNA-binding proteins, with three α -helix bundles, a β -sheet at the C-terminus and several connecting loops (Figure 1.5)³⁹. In addition to the two β -strands at the C-terminal region, one short β -strand in the loop 1 region between helix 1 and helix 2 is added to the β -sheet. Loop 3 between the second and third β -strands together with the C-terminus of the globular domain look like the wing of a butterfly flanking helix 3; hence the term winged-helix motif. The globular domain sequence is conserved among different types of linker histones. It alone is capable of binding to the nucleosome and provides the 20 bp additional protection from micrococcal nuclease digestion⁴⁰. The N- and C-terminal domains of linker histone vary in sequence and length between different linker histone types, but in general contain a high density of basic amino acid residues. They are disordered in solution and became ordered upon binding to nucleosome⁴¹⁻⁴³. The function of the N-terminal domain is still unclear, although it has been proposed to

contribute to affinity and specificity of H1 binding to nucleosome⁴⁴⁻⁴⁶. The C-terminal domain is the main determinant of chromatin-condensing function of the linker histone^{38,45}. The first and third quarters of the C-terminus of histone H1.0 have been showed to be essential for this function⁴⁷.

1.4 Linker histone binding to nucleosome

Although linker histone was known to bind to the linker DNA of the nucleosome, details of its binding location and structure on the nucleosome have been the topic of controversy for many years. The two opposing models on this subject are the symmetric and asymmetric binding theories (Figure 1.6). In symmetric binding, linker histone binds to the dyad of the nucleosome core and linker DNA from both sides of the nucleosome^{40,48-50}. In asymmetric binding, linker histone interacts with the nucleosome core around half a turn away from the dyad and only linker DNA from one side of the nucleosome core is involved in the interaction⁵¹⁻⁵⁴. In both models, the globular domain of linker histone is the main binding partner of the nucleosome core, while the basic N- and C-terminal regions possibly interact with linker DNA regions, inducing the linker DNA to form a stem-like structure³⁷. Different regions of the globular domain of linker histone have been proposed to mediate interaction with nucleosome.

Figure 1.5. Structural model³⁹ and schematic representations of globular domain of linker histone H1/H5. The globular domain of linker histones is a member of the winged-helix protein class (topological representation in bottom figure), consisting of three α -helices (H1-3), three β -strands (S1-3) and several connecting loops (L1-3; top figure). Loop 3 and the C-terminal region of the protein form winged-like structures (W1 and W2) flanking helix 3. Bottom figure is adapted from ref. 55.

Figure 1.6. Different models of linker histone binding to nucleosome. Left) Symmetric binding. Right) Asymmetric binding. Histone octamer is depicted as a light blue circle, DNA as a dark blue line, and linker histone as red or orange circles. Figures are adapted from ref. 56.

In a symmetric binding model proposed by Fan and Roberts, linker histone H5 interacts with nucleosome dyad by insertion of Lys85 (at loop 3 of H5 globular domain) into the minor groove at the dyad. Meanwhile, residues from helix 3 interact with one of the linker DNA and residues from loop 1 and C-terminus interact with the second linker DNA⁵⁰. For the asymmetric binding mode, different location and binding sites have been proposed. In a model suggested by Zhou *et al.*, loop 1 of the globular domain interacts with nucleosomal DNA near the dyad and helix 3 interacts with one of the linker DNA⁵³. Brown *et al.* suggested the opposite, with helix 3 interacting with nucleosomal DNA off-centered from the dyad and loop 1 and C-terminus with one of the linker DNA from the opposite half of the nucleosome, possibly protecting longer linker DNA segments⁵². Based on the affinity of linker histone for AT-rich regions and the location of AT dinucleotides in the genome, Cui and Zhurkin proposed that linker histone binds to nucleosomal DNA near the dyad through helix 3 and to one of the linker DNA through loop 3, causing the linker DNA to bend towards the dyad⁵⁴.

In a recent cryo-EM study, linker DNA from both sides of the nucleosome seemed to be involved in linker histone binding, although the resulting structure was asymmetric in nature. This is due to one half of the nucleosome contacting a major part of the linker histone while the other half binds to a lesser portion of the linker histone⁵⁷. In an NMR study by Yawen Bai's group, the globular domain of *drosophila* linker histone H1 was also shown to bind asymmetrically and the C-terminal H1 tail

to interact with the C-terminal tail of histone H2A from the proximal half of the nucleosome⁵⁸. They proposed a model where helix 3 binds to nucleosomal DNA near the dyad, and lysine residues from helix 1 and β -strand 2 and 3 interact with the 10 bp linker DNA on one side of the nucleosome. Near the end of this thesis' writing, the same group published a 3.5 Å crystal structure of globular domain of chicken H5 in complex with nucleosome containing 167 bp 601 DNA sequence⁵⁶. Linker histone was shown to bind symmetrically to the nucleosome dyad, with loop 3 and the N-terminal region of helix 2 bound to the nucleosome dyad, and loop 1 and helix 3 interacting with linker DNA from the two ends of the nucleosome. The difference in binding mode in the NMR and crystal structures was attributed to differences in sequence between the two linker histones used (drosophila H1 and chicken H5). Although a crystal structure of linker histone in complex with nucleosome was already solved, the resolution needs to be improved to see more details. For example, electron density for some of the important linker histone side chains could not be seen in the current structure. In addition, all available structures were done with globular domains of different linker histones. Since the C- and N-terminal domains also seem to play role in nucleosome binding, a structure of full-length linker histone in complex with nucleosome still needs to be solved.

1.5 Higher order structures of chromatin

Since 30-nm fiber seems to be the predominant structure under physiological salt conditions^{8,59,60}, it is important to study its structure in detail in order to understand mechanisms for chromatin dynamics. Research in 30-nm fiber structure has been advanced by ability to reconstitute regularly-spaced nucleosome array *in vitro*. This reconstitution is based on the use of Widom's 601 DNA sequence¹⁸. The 601 sequence is a strongly positioning sequence, meaning it has high affinity for histone octamer. DNA with array of 601 sequences separated from each other by weak octamer binding sequences can be used to reconstitute regularly-spaced nucleosome array. Nucleosomes will be formed at 601 sequences, but not at the weak binding sequences. Hence the poor binding sequences will act as the linker DNA between any two nucleosomes formed at 601 sequences⁶¹.

The mechanism of folding in the 30-nm fiber is still controversial. There are two main models that are most widely accepted (Figure 1.7). The first is the two-start model, where two stacks of helically arranged nucleosomes are connected by straight linker DNA. This model is further subdivided into two types: two-start helical ribbon^{62,63} and two-start crossed-linker model⁶⁴. The second model is the one-start solenoid model, where arrays of nucleosomes follow each other in a helical path, connected by bent linker DNA^{65,66}. One possible explanation for the structure difference is the influence of linker DNA length. Electron microscopy of nucleosome array with 10-70 bp linker DNA supported the solenoid model with nucleosomes at different layers of the helix interdigitated with one another, although 50-70 bp linker DNA length seemed to create a different class of fiber with larger diameter than those with 10-40 bp linker DNA⁶⁷. Single-molecule analysis using magnetic tweezers revealed that nucleosome array with 50 bp linker DNA adopted solenoid structure, while that with 20 bp linker DNA assumed two-start structure⁶⁸, consistent with the crystal structure of tetranucleosome with 20 bp linker DNA⁶⁹. Disulfide cross-linking of nucleosome array with 30 bp linker DNA also demonstrated a preference for the two-start model with sequential nucleosomes arranged in zigzag conformation⁷. Recent cryo electron microscopy of nucleosome arrays with 30-40 bp linker DNA revealed a two-start helical structure composed of repeating tetranucleosome units⁵⁷. It has been suggested that nucleosome array with shorter linker DNA prefers to take on a two-start structure, while that with longer linker DNA prefers solenoid structures. The presence of linker histone has different extents of influence on the two-start and one-start structure. The two-start helix structure can be formed in the presence of divalent ion without linker histone, albeit in a looser conformation. Addition of linker histone caused further compaction of the fiber. The one-start solenoid, on the other hand, needs the presence of linker histone to be formed⁷⁰.

Figure 1.7. Model of one-start and two-start helices of chromatin 30-nm fiber structure. Each model contains 22 nucleosomes. Schematic representations are depicted in the insets. a) One-start helix with nucleosomes at different layers of the helix interdigitated with one another. Marine and magenta colors denote alternate helical gyres. b) Two-start helix with zigzag conformation. Marine and magenta colors denote sequential pairs of nucleosomes. Figures are adapted from ref. 67.

The traditional model of continuous folding of chromatin from 11-nm to 30-nm fibers to more folded conformations has been challenged by the discovery of a fractal organization of mitotic chromosome in the HeLa cancer cell line, which was formed from the 11-nm fiber without the pre-requisite of forming 30-nm structure⁷¹. Moreover, super resolution nanoscopy of chromatin organization *in vivo* demonstrated that nucleosome exists as groups of different sizes, termed “clutches”, which do not need to be formed in the orderly manner of 30-nm fiber⁷². However, 30-nm fiber structure was still found in chicken erythrocyte nuclei, and the 30-nm fiber clearly can exist *in vitro*. It is also possible that the 30-nm fiber can exist within the nucleosome clutches. Hence, it is still too early to rule out the existence of 30-nm fiber, although it seems that higher order chromatin condensation does not have to be achieved through this structure.

1.6 Chromatin as drug target

Cellular processes such as gene transcription and DNA replication require unpacking of chromatin structure. Interference with chromatin dynamics will therefore disrupt transcription and DNA replication. Tumor tissues are formed by actively dividing cells. Hence drugs that disrupt nucleosomal dynamics may have more prominent effects on tumor cells than healthy cells. Furthermore, tumor cells are likely to have different nucleosome positioning and dynamics than that of healthy cells due to differences in their gene expression patterns (Figure 1.8)^{73,74}. This makes the nucleosome an important target to consider in designing more tumor-specific drugs. Nucleosomal DNA, histones or both can be the target of such drugs.

As a consequence of the packaging into chromatin structure, DNA and histones organized into nucleosomes have reduced solvent-accessible surface area compared to free DNA or chaperone-bound histones. Moreover, nucleosomal DNA has different conformations compared to free DNA, depending on the sequence and position around histone octamer¹⁶. These factors influence the recognition of nucleosomal DNA and histones by nuclear proteins and small molecules^{24,29,75}. Although NCP and nucleosome array would be better models for examining genomic targeting, many studies of DNA-targeting drugs have been focused on naked DNA.

Figure 1.8. Epigenetic silencing of tumor suppressor gene in tumor cells. In healthy cells, the transcription start sites of MLH1 and nearby EPM2AIP1 genes are devoid of nucleosomes, allowing the genes to be expressed. In tumor cells, gene silencing is achieved by positioning nucleosomes at the transcription start sites of the genes. Figure is adapted from ref. 73.

1.7 Metals in medicine

Heavy metals, in particular transition metals, have been used in the medicinal world to serve as either structural scaffolds or a reactive center in drugs. The use of metals has significantly increased the range of biologically relevant compounds that can be used as drugs. This is owing to the wide variety of metal coordination geometries and accessible redox states which enable scientists to synthesize structures with stereochemistry and ligand orientations, which are not achievable with purely organic compounds. In addition, it is possible to adjust the thermodynamics and kinetics of ligand substitution, depending on the choice of metals and ligands⁷⁶⁻⁷⁸. Hence, it is possible to tune the reactivity of the drugs towards different targets *in vivo*.

As a structural scaffold, metals are usually connected to organic ligands. The metal atom would not react with the target molecule *in vivo*, but acts to maintain the structure of the drug in a specific orientation for optimum binding with the target. The organometallic bonds are kinetically inert, hence organometallic compounds act like organic compounds⁷⁹. Examples include the pioneering work of the Meggers group in developing ruthenium-based organometallic compounds that can act as protein kinase inhibitors⁸⁰. For example, Meggers lab has successfully synthesized the organometallic compound DW12 that mimics the natural kinase inhibitor staurosporine (Figure 1.9)⁸¹. While staurosporine is nonselective for different types of protein kinases, the R enantiomer of DW12 is selective and more potent than staurosporine towards GSK-3 and Pim kinases. Other types of ruthenium-based kinase inhibitors have also been synthesized^{82,83}.

Figure 1.9. Half sandwich ruthenium complex as kinase inhibitor. DW12 mimics staurosporine. Figure is adapted from ref. 81.

When metal is used as a reactive center in drugs, the metal atom itself would bind to the target molecule *in vivo*. The metal center might be connected to organic or inorganic ligands. The first of such drugs is cisplatin, which was found serendipitously by Rosenberg in the 1960s⁸⁴. Since then, cisplatin has been one of the most successful anticancer drugs^{85,86}. This led to the development of other platinum-based anticancer agents, like carboplatin, oxaliplatin and satraplatin (Figure 1.10)⁸⁷⁻⁸⁹. The mechanism of action of platinum drugs is thought to be based mostly on DNA binding. After a drug molecule enters a cell, the leaving group(s) is exchanged with water to form an aquated species. This aquated species is the active form of the drug and can form adducts on DNA^{90,91}. The bifunctional adducts cause distortion of the DNA structure, and nuclear proteins involved in DNA repair can recognize the distorted structure⁹². When repair fails, the cell cycle arrests and eventually apoptosis occurs⁹³. In other cases, the DNA adducts prevent transcription and replication of DNA^{94,95}.

Figure 1.10. Platinum-based drugs.

Binding of platinum compounds to DNA is mainly mediated through N7 atom of purine bases, especially guanines^{96,97}. Bifunctional platinum compounds form predominantly 1,2 intrastrand DNA crosslinks at GG and AG dinucleotides and some GNG 1,3 intrastrand crosslinks. In addition, a small fraction of interstrand DNA crosslinks are also formed (Figure 1.11). Packaging of DNA by histone octamer into nucleosome has been shown to modulate site selectivity of platinum compounds, mainly based on solvent accessibility governed by changes in different DNA base-step parameters^{98,99}.

Figure 1.11. Binding of cisplatin to DNA forms different types of DNA crosslinks. a) 1,2 intrastrand crosslink. b) 1,3 intrastrand crosslink. c) Interstrand crosslink. Figure is adapted from ref. 96.

Despite the success of these drugs, concerns have been raised regarding their toxicity. Platinum drugs are not specific to tumor cells. Hence, healthy cells are also affected by treatment using these drugs, and many side effects, such as nephrotoxicity, gastrointestinal tract toxicity and neurotoxicity, have been observed^{94,100}. Moreover, many tumor types are resistant to these platinum drugs. This resistance can be intrinsic or acquired after treatment with platinum drugs. The resistance can be based on reducing DNA binding or mediated after the drugs bind to DNA (Figure 1.12). To reduce the amount of platinum drugs binding to DNA, tumor cells may downregulate the expression of CTR1 (a receptor involved in platinum drugs influx into cells)^{101,102}, sequester the activated platinum drugs by sulfur-containing proteins^{103,104}, or actively efflux the drugs from cell¹⁰⁵. After the platinum drugs bind to DNA, they can be removed by upregulation of repair mechanisms, especially the nucleotide-excision repair (NER) pathway^{106,107}. Alternatively, tumor cells can increase their tolerance of platinum DNA adducts through loss of the DNA mismatch repair pathway (for which failed repair attempts lead to apoptosis)^{108,109}, bypassing the adducts by upregulating error-prone DNA polymerase during DNA replication^{110,111}, or reducing apoptotic signaling pathways¹¹².

Figure 1.12. Mechanisms of resistance towards platinum drugs. Top) Resistance can occur by preventing binding of platinum drugs to DNA. Platinum compounds enter cells through transporter proteins like copper transporter CTR1 or by passive diffusion. They can then undergo hydrolysis to form the active aquated species and enter cell nucleus to bind to DNA. Loss of CTR1 can reduce influx of platinum compounds. Meanwhile, the aquated species can be prevented from entering nucleus by binding to metallothionein or glutathione (facilitated by glutathione S-transferases, GSTs). In addition, the aquated species can be exported by copper exporters ATP7A and ATP7B or glutathione S-conjugate export pump GS-X/MRP2. Bottom) Resistance can also be achieved through increased removal of DNA adducts or increased tolerance towards DNA adducts. After platinum compounds enter the cell nucleus they can bind to DNA with preference towards the N7 atom of guanine bases,

forming intrastrand (majority) or interstrand (minority) crosslinks. These adducts can be removed by nucleotide-excision repair (NER). Increased NER can cause resistance to platinum compounds. Mismatch repair (MMR) pathway is not effective in removing platinum adducts. When cells engage in MMR to repair platinum adducts, apoptosis is usually triggered after several failed cycles. Loss of MMR can lead to tolerance of platinum adducts. In addition, tolerance can be achieved by bypassing of the adducts by polymerase and decreased apoptosis. Figures are adapted from ref. 94.

The toxicity and resistance related to platinum-based agents has led to efforts to develop better metal-based drugs with reduced toxicity and fewer resistance problems. Other transition metals are being investigated for this purpose. Ruthenium is one of the most promising alternatives to platinum^{77,78}. Two ruthenium-based compounds (NAMI-A and KP1019 – chemical structures shown in section 2.1.9) have successfully completed phase I clinical trials^{113,114}. Ruthenium drugs are deemed to be more specific to tumor cells, hence reducing side effects. The specificity is thought to be based on selective delivery of ruthenium drugs into tumor cells. Most Ru drugs in the blood are bound to plasma proteins, like albumin and transferrin. Tumor cells have a higher need for iron and hence more transferrin receptor. In this way, more Ru drugs are delivered to tumor cells than to healthy cells. Albumin is thought to serve as a reservoir for Ru drugs before they bind to transferrin¹¹⁴. In addition, Ru drugs are proposed to be more reactive towards biomolecules at lower pH. Ruthenium compounds, like RAPTA-C and KP1019, have been shown to be more reactive to DNA at pH 6.0 compared to pH 7.4¹¹⁵. Tumor tissues are usually hypoxic and rely on glycolysis for energy production. The accumulated lactic acid causes tumor tissues to have lower pH. Drugs with Ru^{III} center (such as KP1019, NAMI-A) are proposed to be inactive pro-drugs, which are activated by reduction to Ru^{II} in tumor cells^{116,117}. Resistance towards Ru drugs has been shown to be low or completely absent, indicating that such agents impact different cellular pathways compared to platinum drugs¹¹⁸.

The ruthenium agent KP1019 was found to cause apoptosis in colorectal tumor cell lines SW480 and HT29¹¹⁹. RAED-C also has been shown to be active against the human ovarian cancer cell line A2780¹²⁰. They are mainly effective against primary tumors, while NAMI-A and RAPTA-C have little effect on primary tumors, but are very active against metastases^{121,122}. For NAMI-A, the antimetastatic activity has been correlated with its antiangiogenic properties and selective targeting

of metastatic cells¹²³⁻¹²⁵. RAPTA-C has been shown to induce G2/M phase arrest and apoptosis in tumor cells¹²⁶, but it is unclear how this results in inhibition of metastasis with little effect on primary lesions. All of these agents are reactive towards both DNA and proteins *in vitro*^{115,127-134}. Their modes of action are still not well understood.

Other promising metals for anticancer agent development include osmium, rhodium and gold. Research on osmium-based antitumor agents was previously not very popular, largely because osmium complexes are usually regarded as toxic (OsO₄) or inert (Os(II) and Os(III) complexes)¹³⁵. However, the Sadler group has demonstrated that tuning of reactivity of osmium compounds is possible via variation of ligands¹³⁶. Moreover, some osmium complexes have been shown to be non-cross-resistant with cisplatin¹³⁷. Ruthenium and osmium share very similar chemical properties and many osmium analogues of ruthenium complexes have been synthesized. One such is OsAPTA-C, the osmium analogue of RAPTA-C. OsAPTA-C has been shown to bind to DNA oligonucleotides¹³⁸ and have similar cytotoxicity as RAPTA-C towards HT29 and A549 tumor cell lines¹³⁹.

Research on rhodium complexes was started when dirhodium carboxylate was found to exhibit anticancer properties, albeit with lower efficacy than that of cisplatin¹⁴⁰. However, toxic side effects limited the use of this compound. Indeed, rhodium was deemed not very promising initially because many rhodium analogues of platinum and ruthenium anticancer agents are too toxic. Fine-tuning the chemistry of rhodium complexes, for example by varying the length of alkyl group in dirhodium compounds or using Rh(I) with square planar geometry, has reduced the toxicity problem. Different types of rhodium complexes have since been studied and many are cytotoxic towards Erlich ascites tumor, oral carcinoma, melanoma, lung metastatic tumors and others^{141,142}.

Gold complexes have enjoyed increased interest as anticancer agent candidates since auranofin, an antiarthritic drug containing thiolate and phosphine ligands, was found to have anticancer activity similar to cisplatin. Analogues with modified phosphine or thiolate ligands, as well as substitution with different ligands have been studied since then. Proteins were believed to be the cellular targets of these compounds^{143,144}. The common mechanism was suggested to be inhibition of thioredoxin reductase and apoptosis through a mitochondrial pathway¹⁴⁵. In addition, cathepsin cysteine protease, deubiquitinases and cysteine-dependent protein tyrosine phosphatases have been shown to be inhibited by various gold compounds¹⁴⁶⁻¹⁴⁸.

1.8 Project aims

Despite increasing knowledge on antitumor effects of new metal-based drugs, the cellular targets and mechanisms of action are largely unclear. Even though metal-based drugs were initially assumed to target DNA, interactions with proteins are now being more seriously considered. DNA, proteins, or both may be the targets for metal-based drugs. To a certain extent, the preferred target may depend on the choice of metal center. Furthermore, for the same type of metal center, preferred targets may differ due to the chemistry of the ligands. In this study, we used NCP and nucleosome arrays as tools for characterizing the mechanism of action of ruthenium, osmium, rhodium and gold anticancer candidates. Taking advantage of the presence of both DNA and protein in nucleosome, we aimed to investigate the preferred targets of the compounds, the details of position and structure of the adducts and their effects on chromatin higher order structure. Knowledge on mechanistic details of these agents will assist in the rational design of more effective, tumor cell-specific, and low resistance drugs, especially when focusing on chromatin as the specific target.

The location and structure of linker histone binding on nucleosome are still under ongoing debate. We aimed to solve the atomic resolution structure of the chromatosome, utilizing X-ray diffraction methods. Atomic resolution details on how linker histone binds to nucleosomes will give insights on the role of linker histones in chromatin compaction and dynamics, including changes in DNA topology and how different types of higher order structure (solenoid, zigzag helix and others) may be formed. In addition, this knowledge will add to understanding how linker histones interact with nuclear factors and influence their access and interactions with nucleosomes and how the chromatosome structure may potentially be exploited for design of chromatin-targeting drugs.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemical	Source
2-methyl-2,4-pentadiol (MPD)	Sigma-Aldrich, USA
3-hydroxypicolinic acid (3-HPA)	Sigma-Aldrich, USA
β -mercaptoethanol (BME)	Sigma-Aldrich, USA
$\gamma^{32}\text{P}$ -ATP	PerkinElmer Inc., USA
Acetonitrile	Merck, USA
Acetic acid	Merck, USA
Acrylamide	Bio-Rad, USA
Acrylamide:Bis (29:1)	Bio-Rad, USA
Agarose	Affimetrix, USA
Ammonium persulfate (APS)	Acros Organics, Belgium
Ampicillin	Sigma-Aldrich, USA
Bacto agar	BD Biosciences, USA
Bacto tryptone	BD Biosciences, USA
Benzamidine	Sigma-Aldrich, USA
Bis-acrylamide	Bio-Rad, USA
Boric acid	Merck, USA
Bromophenol blue	Sigma-Aldrich, USA
Cacodylic acid	Sigma-Aldrich, USA
Chloramphenicol	Acros Organics, Belgium
Chloroform	Fischer Scientific UK Ltd., UK
D-(+)-trehalose	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck, USA
Dithiothreitol (DTT)	Gold Biotechnology, USA
DNA ladders and 6X loading dye	Promega, USA
Ethanol	Merck, USA
Ethylenediaminetetraacetic Acid (EDTA)	Bio-Rad, USA
Ethidium bromide	Bio-Rad, USA
Formamide	Sigma-Aldrich, USA
Formic acid	Sigma-Aldrich, USA
Glucose	Alfa Aesar, USA
Glycerol	USB Corporation, USA
Glycine	1 st Base Pte. Ltd., Singapore
Guanidine hydrochloride	Sigma-Aldrich, USA
Hydrochloric acid (HCl)	Merck, USA
Imidazole	Sigma-Aldrich, USA

Instant Blue	Expedeon, UK
Isoamyl alcohol	Acros Organics, Belgium
Isopropanol	Merck, USA
Isopropyl- β -D-thiogalactopyranoside (IPTG)	Gold Biotechnology, USA
Lithium chloride (LiCl)	Sigma-Aldrich, USA
Magnesium chloride (MgCl ₂)	Sigma-Aldrich, USA
Magnesium sulfate (MgSO ₄)	Sigma-Aldrich, USA
Manganese chloride (MnCl ₂)	Sigma-Aldrich, USA
Phenol	Sigma-Aldrich, USA
Phenylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich, USA
Piperidine	Sigma-Aldrich, USA
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Merck, USA
Potassium acetate	Alfa Aesar, USA
Potassium chloride (KCl)	Merck, USA
Potassium hydroxide (KOH)	Merck, USA
Polyethylene glycol (PEG) 6000	Sigma-Aldrich, USA
Protease inhibitor cocktail set III, EDTA-free	Calbiochem, USA
Protein ladder	Bio-Rad, USA
Riboadenosine triphosphate (rATP)	Sigma-Aldrich, USA
RNA, sodium salt	Merck, USA
Sodium acetate	Sigma-Aldrich, USA
Sodium chloride (NaCl)	Merck, USA
Sodium Dodecyl Sulfate (SDS)	Hoefer, Inc., USA
Sodium hydroxide (NaOH)	Merck, USA
Spermidine trihydrochloride	Sigma-Aldrich, USA
Spermine tetrahydrochloride	Sigma-Aldrich, USA
Sucrose	USB Corporation, USA
Tetramethylethylenediamine (TEMED)	Bio-Rad, USA
Thiourea	Sigma-Aldrich, USA
Triethanolamine (TEA)	Merck, USA
Triethylamine	Merck, USA
Triton X-100	Bio-Rad, USA
Tris	USB Corporation, USA
Urea	USB Corporation, USA
Xylene cyanol	Sigma-Aldrich, USA
Yeast Extract	USB Corporation, USA

2.1.2 Buffers and Solutions

Buffer/solution	Composition
Chloroform-isoamyl alcohol (CIA)	24:1 (v/v) chloroform : isoamyl alcohol
Exonuclease III stop buffer	0.9 M Na-Acetate 0.09 M EDTA
Formic acid stop buffer	0.3 M Na-Acetate

	0.1 M EDTA 25 µg/ml RNA
H1 Lysis Buffer	50 mM Tris-Cl pH 8.0 2% (v/v) Triton X-100 0.05% (v/v) protease inhibitor cocktail
Heparin Buffer A	50 mM Tris-Cl pH 8.8 150 mM NaCl
Heparin Buffer B	50 mM Tris-Cl pH 8.8 1 M NaCl
Histone Wash Buffer	50 mM Tris-Cl pH 7.5 100 mM NaCl 1 mM BME
Histone Sonication Buffer	50 mM Tris-Cl pH 8.0 500 mM NaCl 5% (v/v) glycerol 5 mM BME 0.2% (v/v) PMSF 2 mM benzamidine
Histone Unfolding Buffer 1	7 M guanidine hydrochloride 20 mM Na-Acetate pH 5.2 10 mM DTT
Histone Unfolding Buffer 2	7 M guanidine hydrochloride 50 mM Tris-Cl pH 8.0 5% (v/v) glycerol 5 mM BME
His Buffer A	50 mM Tris-Cl pH 8.0 500 mM NaCl 5% (v/v) glycerol 6 M urea 5 mM BME (for hH3 only)
His Buffer B	50 mM Tris-Cl pH 8.0 500 mM NaCl 5% (v/v) glycerol 6 M urea 1 M imidazole 5 mM BME (for hH3 only)
HisTrap Buffer A	50 mM Tris-Cl pH 8.8 500 mM NaCl 20 mM imidazole
HisTrap Buffer B	50 mM Tris-Cl pH 8.0 500 mM NaCl 500 mM imidazole
IMAC Buffer A	50 mM Tris-Cl pH 8.0 500 mM NaCl 20 mM imidazole
IMAC Buffer B	50 mM Tris-Cl pH 8.0 500 mM NaCl 500 mM imidazole
Lysis solution I	50 mM glucose

	10 mM EDTA 25 mM Tris-Cl pH 8.0
Lysis solution II	0.2 M NaOH 1% SDS
Lysis solution III	4 M K-Acetate 2 M acetic acid
Mono Q Buffer A	20 mM Tris-Cl pH 7.5 0.1 mM EDTA 0.3 M NaCl
Mono Q Buffer B	20 mM Tris-Cl pH 7.5 0.1 mM EDTA 1 M NaCl
Mono S Buffer A	50 mM Tris-Cl pH 8.0 5 mM BME 6 M urea
Mono S Buffer B	50 mM Tris-Cl pH 8.0 5 mM BME 6 M urea 1 M NaCl
NCP crystal Mg buffer	10 mM MgSO ₄ 20 mM K-Cacodylate pH 6.0 2% D-(+)-Trehalose 24% MPD
NCP crystal Mn buffer	37 mM MnCl ₂ 40 mM KCl 20 mM K-Cacodylate pH 6.0 2% D-(+)-Trehalose 24% MPD
Octamer Unfolding buffer	7 M guanidine hydrochloride 10 mM Tris-Cl pH 7.5 10 mM DTT
Octamer Refolding Buffer	2 M NaCl 10 mM Tris-Cl pH 7.5 1 mM EDTA 10 mM BME
SAUDE-0	7 M urea 20 mM Na-Acetate 1 mM EDTA 5 mM BME
SAUDE-1000	7 M urea 20 mM Na-Acetate 1 mM EDTA 5 mM BME 1 M NaCl
SPFF Buffer A	50 mM Tris-Cl pH 8.8 500 mM NaCl
SPFF Buffer B	50 mM Tris-Cl pH 8.8 1 M NaCl
TBE (1X)	89 mM Tris

	89 mM boric acid 2 mM EDTA
TE(10,50)	10 mM Tris-Cl pH 8.0 50 mM EDTA
TE(10,0.1)	10 mM Tris-Cl pH 8.0 0.1 mM EDTA
TE(10,10)	10 mM Tris-Cl pH 8.0 10 mM EDTA
TE(10,1)	10 mM Tris-Cl pH 8.0 1 mM EDTA
TNE	10 mM TEA-Cl pH 7.5 40 mM NaCl 1 mM EDTA
TCS-n	20 mM Tris-Cl pH 7.5 1 mM EDTA 1 mM DTT n M KCl
TGS	25 mM Tris 200 mM glycine 0.1% SDS

2.1.3 Competent Cells and Media

Competent Cell	Use	Source
BL21(DE3)pLysS	protein expression	Novagen, USA
HB101	plasmid amplification for DNA constructs preparation	Promega, USA
DH5 α	plasmid amplification for DNA constructs preparation	New England Biolabs, USA

Medium	Composition
2xTY	16 mg/ml bacto tryptone 10 mg/ml yeast extract 5 mg/ml NaCl 0.1% (w/v) glucose
TYE agar	10 mg/ml bacto tryptone 5 mg/ml yeast extract 8 mg/ml NaCl 15 mg/ml bacto agar
TB	12 mg/ml bacto tryptone 24 mg/ml yeast extract 72 mM K ₂ HPO ₄ 17 mM KH ₂ PO ₄ 0.4% (v/v) glycerol

2.1.4 Enzymes and Reaction Buffers

Enzyme	Source	Reaction Buffer
Aval	New England Biolabs, USA	NEB Buffer 4
Calf intestine alkaline phosphatase (CIAP)	New England Biolabs, USA	50 mM Tris-Cl pH 8.0 10 mM MgCl ₂ 0.1 mM EDTA
EcoRV	New England Biolabs, USA	50 mM Tris-Cl pH 7.6 100 mM NaCl 10 mM MgCl ₂ 1 mM DTT
Exonuclease III	New England Biolabs, USA	NEB Buffer 1
HRV-3C	Novagen, USA	50 mM Tris-Cl pH 7.5 150 mM NaCl
Lysozyme(from chicken egg white)	Sigma-Aldrich, USA	-
Hinfl	New England Biolabs, USA	50 mM Tris-Cl pH 8.0 50 mM NaCl 10 mM MgCl ₂ 1 mM DTT
RNaseA	Roche, USA	-
Scal	New England Biolabs, USA	NEB Buffer 3
T4 DNA ligase	New England Biolabs, USA	100 mM Tris-Cl pH 7.6 20 mM MgCl ₂ 10 mM DTT 2 mM rATP
T4 Polynucleotide kinase (PNK)	New England Biolabs, USA	NEB T4 PNK reaction buffer
Thrombin	GE Healthcare, USA	10 mM Tris-Cl pH 7.5 1 mM BME

2.1.5 Gels and Loading Buffers

Gel	Composition
6% PAGE	6% (v/v) acrylamide 0.15% (v/v) bis-acrylamide 0.25x TBE buffer 0.1% (w/v) APS 0.1% (v/v) TEMED
10% PAGE	10% (v/v) acrylamide 0.17% (v/v) bis-acrylamide 1x TBE buffer 0.1% (w/v) APS

	0.1% (v/v) TEMED
SDS-PAGE 15% resolving gel	15% (v/v) acrylamide 0.25% (v/v) bis-acrylamide 375 mM Tris-Cl pH 8.8 0.1% (w/v) SDS 0.1% (w/v) APS 0.1% (v/v) TEMED
SDS-PAGE 18% resolving gel	18% (v/v) acrylamide 0.3% (v/v) bis-acrylamide 375 mM Tris-Cl pH 8.8 0.1% (w/v) SDS 0.1% (w/v) APS 0.1% (v/v) TEMED
SDS-PAGE 5% stacking gel	5% (v/v) acrylamide 0.25% (v/v) bis-acrylamide 125 mM Tris-Cl pH 6.8 0.1% (w/v) SDS 0.1% (w/v) APS 0.1% (v/v) TEMED
Denaturing PAGE	7 M urea 10% (v/v) acrylamide 0.31% (v/v) bis-acrylamide 0.5x TBE Buffer 0.1% (w/v) APS 0.1% (v/v) TEMED

Loading Buffer	Composition
DNA (denaturing condition)	95% (v/v) formamide 0.025% (w/v) bromophenol blue 0.025% (w/v) xylene cyanol FF 20 mM EDTA
Protein	15 mM Tris-Cl pH 6.8 0.5% (w/v) SDS 5% (v/v) glycerol 0.00625% (w/v) bromophenol blue 2.63% (v/v) β -mercaptoethanol
Nucleosome	10% sucrose
Nucleosome array	20 mM Tris pH 7.4 1 mM EDTA 20% (v/v) glycerol 0.1% (w/v) bromophenol blue

2.1.6 Equipments

Instrument	Source
24-well crystallization plates	Hampton Research, USA
4800 MALDI TOF/TOF analyzer	Applied Biosystem, USA
AKTA FPLC	GE Healthcare, USA
Amicon concentrator device	Millipore, USA
DyeEx 2.0 Spin Kit	Qiagen, Germany
G:Box machine	Syngene, UK
Gel plates	Bio-Rad, USA
High vacuum grease	Dow Corning, USA
HiLoad 16/60 Superdex 200 column	GE Healthcare, USA
HiPrep 26/60 Sephacryl S-200 HR	GE Healthcare, USA
HiPrep SP FF 16/10	GE Healthcare, USA
HiTrap Heparin HP (5 ml) column	GE Healthcare, USA
HiTrap IMAC HP (5 ml) column	GE Healthcare, USA
HisTrap HP (5 ml) column	GE Healthcare, USA
Imaging Screen-K	Bio-Rad, USA
Jupiter C18 reverse phase (250x4.6mm, 300A, 5µm) column	Phenomenex, USA
Jupiter C18 reverse phase (250x10mm, 300A, 5µm) column	Phenomenex, USA
Micromax-007HF rotating anode X-ray generator	Rigaku, Japan
Minisart® syringe filters	Sartorius Stedim Biotech, France
Molecular Imager FX	Bio-Rad, USA
Mono Q 5/50 GL column	GE Healthcare, USA
Mono S 5/50 GL column	GE Healthcare, USA
Nanodrop 200 spectrophotometer	Thermo Fisher Scientific, USA
R-Axis IV++ image plate	Rigaku, Japan
Resource S (6 ml) column	GE Healthcare, USA
Single pump HPLC	Shimadzu, Japan
Siliconized glass cover slip	Hampton Research, USA
Slab Gel Drier Model GD2000	Hoefer Inc., USA
Sonics Vibra Cell Sonicator	Sonics & Materials, USA
Whatman chromatography paper, 3 mm	Sigma-Aldrich, USA
Z2000 polarized Zeeman atomic absorption spectrophotometer	Hitachi, Japan
T12 Icor transmission electron microscope	FEI, USA

2.1.7 DNA and Protein Constructs

Sequence of all DNA and protein constructs are listed in Appendix I and II.

Constructs	Remarks
DNA145	palindromic, derived from human α -satellite DNA sequence
DNA147	palindromic, derived from human α -satellite DNA sequence
DNA CMS155	non-palindromic, 10 bp linker on one site on the nucleosome, 601-based sequence
DNA CMS154	non-palindromic, 9 bp linker on one site on the nucleosome, 601-based sequence
DNA CMS156	non-palindromic, 11 bp linker on one site on the nucleosome, 601-based sequence
DNA PRP167	palindromic, 11 bp linker on both sites of nucleosome, 601-based sequence
DNA CMS167	palindromic, 11 bp linker on both sites of nucleosome, 601-based sequence
DNA CMS165	palindromic, 10 bp linker on both sites of nucleosome, 601-based sequence
DNA CMS169	palindromic, 12 bp linker on both sites of nucleosome, 601-based sequence
DNA601-36-177	array of thirty-six 177 bp repeats, 601-based sequence
xH2A	<i>Xenopus laevis</i> histone H2A
xH2B	<i>Xenopus laevis</i> histone H2B
xH3	<i>Xenopus laevis</i> histone H3
xH4	<i>Xenopus laevis</i> histone H4
hH2A	<i>Homo sapiens</i> histone H2A
hH2B	<i>Homo sapiens</i> histone H2B
hH3	<i>Homo sapiens</i> histone H3
hH4	<i>Homo sapiens</i> histone H3
H1.0	<i>Homo sapiens</i> full-length linker histone H1.0
gH1.0	<i>Homo sapiens</i> globular domain of linker histone H1.0

2.1.8 Crystallization Screening Kits

Screening Kit	Source
Natrix HR2-116	Hampton Research, USA
PEG Rx	Hampton Research, USA

2.1.9 Metal-based Compounds

All metal-based compounds below were provided by P.J. Dyson and C.G. Hartinger groups.

2.2 Methods

2.2.1 Palindromic DNA Production

DNA145 and DNA147 are palindromic 145 and 147 bp DNA constructs derived from human α -satellite sequences. They were produced from 84 or 85 bp DNA inserted in pUC19 vector^{4,5}. The palindromic DNA PRP167 sequence of 167 bp size was cloned into pUC57 vector as multiple repeats of 95 bp 601L-based²¹ half sites by EZBiolab Inc., USA. The DNA CMS165, 167 and 169 fragments (165, 167 and 169 bp) were cloned into pUC18 vector (CMS167) or pUC19 vector (CMS165 and 169) as multiple repeats of 94, 95 or 96 bp 601L-based half sites, respectively, by Dr. Bao Qiuye. The same protocol (modified from ref. 149) for overexpressing and reassembling palindromes from half-sites was used for the large scale preparation of these DNA constructs.

2.2.1.1 Transformation and cell culture

Plasmid was transformed into *E. coli* HB101 or DH5 α competent cells, by mixing 50 μ l cells with 100 ng of the plasmid. Following 10 minute incubation on ice, cells were heat-shocked at 42°C for 45 seconds and returned to ice for 2 minutes. Cold 2xTY media was added to a final volume of 500 μ l. After recovery by incubation at 180 rpm, 37°C for 1 hour, cells were plated onto TYE agar plates containing 100 μ g/ml Ampicillin and incubated at 37°C for 16 hours. Four single colonies were picked and each was used to inoculate 5 ml 2xTY media containing 100 μ g/ml Ampicillin. The culture was incubated in a shaker incubator at 180 rpm, 37°C until the media seemed turbid (3-5 hours). This starter culture was transferred into a flask containing 100 ml TB media with 100 μ g/ml Ampicillin and incubated at 180 rpm, 37°C until the media looked turbid (1-2 hours). The culture was divided into 15 flasks each containing 500 ml TB media with 100 μ g/ml Ampicillin and incubated at 150 rpm, 37°C for 16 hours.

2.2.1.2 Cell lysis and plasmid extraction

Bacterial cells were harvested by centrifugation at 7,000 rpm, 20°C for 7 minutes. Pelleted cells were resuspended in 300 ml Lysis Solution I. Following that, 600 ml Lysis Solution II was added and the solution was vigorously shaken for 2 minutes. After incubation on ice for 20 minutes, 1050 ml Lysis Solution III was added and mixed by inverting the bottles gently. After 20 minute incubation on ice, the mixture was centrifuged at 10,000 g, 4°C for 20 minutes. Remaining cell debris was removed from the supernatant by filtering the mixture through 4 layers of sterile gauze. To the supernatant, 0.52x volume of isopropanol was added and the mixture was incubated at room temperature for 30 minutes, followed by centrifugation at 16,000 g, 20°C for 30 minutes. The pellet was air-dried and dissolved in 40 ml TE(10,50). RNA contaminant was digested by adding 240 µl of 10 mg/ml RNase A and incubation at 180 rpm, 37°C overnight.

To remove aggregates, sample was centrifuged at 10,000 g, 20°C for 30 minutes. Protein contaminant was removed by adding 0.5x volume of buffered phenol and centrifugation at 27,000 g, 20°C for 20 minutes. This step was repeated until the interphase between aqueous and organic phase was clear. Following this, 10 ml of chloroform : isoamyl alcohol (CIA) was added and sample was centrifuged at 12,000 g, 20°C for 10 minutes. To the supernatant, 0.2x volume of 4 M NaCl and 0.4x volume of 40% polyethylene glycol (PEG) 6000 was added. Sample was incubated on ice for 30 minutes and centrifuged at 15,000 g 4°C for 15 minutes. Pellet containing plasmid was dissolved in TE(10,0.1) and PEG remnant was removed from sample by doing 2x CIA extraction as described above. Then 0.1x volume of 3 M sodium acetate pH 5.2 and 2.5x volume of cold 100% ethanol was added to the sample, followed by incubation on ice for 30 minutes and centrifugation at 15,000 g 4°C for 10 minutes. Pellet was dissolved in TE(10,0.1) and the DNA concentration measured with a Nanodrop 200 spectrophotometer.

2.2.1.3 Insert purification and palindromic DNA production

Insert purification and subsequent palindrome generation steps are depicted in Figure 2.1. To release insert half-sites, purified plasmid was digested with EcoRV (300 units/mg plasmid DNA) by shaker incubation at 150 rpm, 37°C for 24 hours.

Completion of EcoRV digestion was assessed by running 10% polyacrylamide gel electrophoresis (PAGE) in 1x TBE buffer. Upon completion, insert was separated from empty vector by adding 0.196x volume of 4 M NaCl and 0.374x volume of 40% PEG 6000 (final concentration was 9.5% PEG 6000). After 30 minute incubation on ice, sample was centrifuged at 15,000 g, 4°C for 10 minutes. The supernatant containing insert was ethanol-precipitated as described above. Pellet was dissolved in TE(10,0.1).

Dephosphorylation of the insert was carried out using Calf-Intestine Alkaline Phosphatase (CIAP) at a concentration of 38.4 units/mg DNA. After shaker incubation at 150 rpm, 37°C for 24 hours, DNA was extracted from the mixture by adding 0.5x volume of phenol:CIA (1:1 volume ratio) and centrifugation at 22,000 g, 20°C for 20 minutes. Ethanol precipitation was carried out and pellet dissolved in TE(10,0.1). Complete dephosphorylation was examined by setting up a ligation test and running 10% PAGE. Dephosphorylated DNA sample would not be ligated, while the control of non-dephosphorylated DNA would be ligated, forming a ladder of different DNA multimers.

Dephosphorylated insert was digested with HinfI restriction enzyme (276 units/mg DNA) to remove the extra 10 bp with 3 bases overhang (10bp+3 fragment); hence forming the half-sites for the palindromic DNA. To remove the smaller fragments, ion exchange chromatography using Mono Q column was carried out. Column was equilibrated with Mono Q Buffer A and DNA was eluted by applying a gradient of Mono Q Buffer B from 0% to 40% over 2 ml, to 70% over 30 ml, and a final jump to 100% for regeneration. For each run, 5-10 mg of DNA was injected. The fractions were assessed by 10% PAGE and ethanol precipitated. Pure DNA half-sites were ligated to each other to form the palindromic sequences. T4 DNA ligase (500 units/mg DNA) was added and sample was incubated at 18°C for 24 hours. Ligation completeness was examined by 10% PAGE. A second ion exchange chromatography was carried out as described above. However, only 1-3 mg of sample was injected for each run. Pure DNA was stored at -20°C.

2.2.2 Non-palindromic DNA Production

DNA CMS154, 155 and 156 are non-palindromic 154, 155 and 156 bp DNA fragments, respectively, which are based on the 601L sequence²¹ and were cloned into pUC57 vector as multiple direct repeats by EZBiolab Inc., USA. The protocol for plasmid production and digestion for these constructs is identical to that for palindromic DNA illustrated above. After EcoRV digestion of plasmid to release the final DNA fragment, insert was separated from empty vector by PEG precipitation with final concentration of 9% PEG 6000. The supernatant containing insert was purified with Mono Q column as described above.

Figure 2.1 Overview of palindromic DNA preparation.

2.2.3 DNA Array Production

DNA601-36-177 construct consist of 36 repeats of the 601 DNA nucleosome core sequence, flanked by 30 bp linkers (with the exception of the two termini), inserted into pUC18 vector. This construct was a gift from Prof. Daniela Rhodes¹⁵⁰. Plasmid was used to transform *E. coli* DH5 α cells, as described above. The DH5 α variant was used to suppress recombination activities on the plasmid. This is important to maintain plasmid integrity, especially for constructs with long inserts of high repeat number. One single colony was picked and used to inoculate 5 ml 2xTY media containing 100 μ g/ml Ampicillin. The culture was incubated in a shaker incubator at 180 rpm, 37°C until the media seemed turbid (3-5 hours) and transferred into 2 flasks each containing 500 ml TB media with 100 μ g/ml Ampicillin, which were subsequently incubated at 150 rpm, 37°C for 16 hours. Cells were harvested by centrifugation at 7,000 rpm, 20°C for 7 minutes and resuspended in 20 ml Lysis Solution I. Following addition of 50 mg lysozyme and incubation at room temperature for 15 minutes, 40 ml Lysis Solution II was added. After 5 minute incubation on ice, 70 ml Lysis Solution III was added. Precipitated cell debris was spun down at 9,000 g, 4°C for 20 minutes and supernatant was filtered through 4 layers of sterile gauze. Nucleic acid was precipitated by adding 0.6x volume of isopropanol, incubating on ice for 5 minutes and centrifuging at 12,000 g, 4°C for 20 minutes. Pellet was dissolved in 10 ml TE(10,10) and 10 ml 5 M LiCl was added to precipitate RNA and proteins. After spin down at 12,000 g, 20°C for 10 minutes, isopropanol precipitation was done as described above. Pellet was resuspended in 10 ml TE (10,1) and 50 μ l of 10 mg/ml RNase A was added. Following 15 minutes incubation at room temperature, 0.2x volume of 4 M NaCl and 0.4x volume of 40% PEG 6000 was added. The mixture was incubated on ice for 30 minutes and centrifuged at 15,000 g, 4°C for 30 minutes. Pellet was dissolved in 5 ml TE(10,1.0) and 3 ml of phenol:chloroform (1:1 volume ratio) was added to separate plasmid from remaining protein contaminants. Sample was centrifuged at 4,000 g, 20°C for 10 minutes. Aqueous layer was collected and ethanol-precipitated. The purified plasmid pellet was dissolved in TE(10,1).

EcoRV digestion of the purified array plasmid was performed to release insert. DraI and HaeII anzymes were used to cut the vector down into smaller fragments. PEG precipitation was performed to remove most of the vector fragments. 4 M NaCl and 40% PEG 6000 was added to the plasmid to make final concentrations of 1-2

mg/ml plasmid DNA, 0.5 M NaCl, and 7% PEG 6000. After incubation on ice for 30 minutes, the mixture was centrifuged at 10,000 g for 30 minutes at 4°C. The pellet was dissolved in TE(10,1) and assessed by 10% PAGE. The PEG precipitation was then repeated but with slightly less 40% PEG 6000 added to give a lower final concentration. After repeating this procedure 2-3 times, the sample would contain no or very little amount of digested vector fragments. Pure DNA601-36-177 was ethanol-precipitated and stored in TE(10,1) at -20°C.

2.2.4 *Xenopus* Core Histones Production

Xenopus laevis core histones were individually expressed and purified using a standard protocol from refs. 151,152, with minor modifications. The expression plasmids contain histone gene sequences in pET3a (xH2A, xH2B and xH4) and pET-3d (xH3) vectors.

2.2.4.1 Transformation and cell culture

Competent BL21(DE3)pLysS *E. coli* cells was transformed with the plasmid of interest by mixing 50 µl of cells with 100 ng plasmid in a volume of less than 10 µl. After incubation on ice for 10 minutes, cells were heat-shocked at 42°C for 45 seconds and again incubated on ice for 2 minutes. Cold 2xTY media was added to make a final volume of 500 µl and the culture was incubated at 37°C for 1 hour. Cells were spun down at 3,000 rpm 20°C for 3 minutes, resuspended in 50 µl 2xTY media, and plated onto agar plates containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and incubated at 37°C for 16 hours.

Four individual colonies were picked and each was used to inoculate 5 ml of 2xTY media containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol, which was incubated in a shaker incubator at 180 rpm at 37°C for 16 hours. This starter culture was used to inoculate a 100 ml 2xTY media containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and grown at 37°C until the OD₆₀₀ reached 0.4. The culture was distributed evenly into 15 flasks each containing 500 ml 2xTY media with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol, which were grown at 37°C until

the OD₆₀₀ reached 0.55. Cells were induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After 3 hours, cells were harvested by spinning down at 7,000 rpm, 20°C for 7 minutes. Pelleted cells were resuspended in 100 ml Histone Wash Buffer, flash frozen and stored at -20°C.

2.2.4.2 Preparation of inclusion bodies

Frozen cells were thawed at 37°C and sonicated at 35% amplitude, with 2 second pulses and 2 second pauses until there was no cell clump. The sample was centrifuged at 18,000 rpm, 4°C for 15 minutes. The pellet was dissolved in 150 ml Histone Wash Buffer with 1% Triton X-100, and centrifuged again. These steps were repeated once with Histone Wash Buffer containing Triton X-100 and once with Histone Wash Buffer without Triton X-100. Pellet containing inclusion bodies was then stored at -20°C.

2.2.4.3 Purification

The inclusion body pellet was dissolved in 1 ml dimethyl sulfoxide (DMSO) by stirring for 30 minutes. After that, 40 ml Histone Unfolding Buffer 1 was added and sample was stirred for another 1 hour. Centrifugation at 18,000 rpm 20°C for 30 minutes was done to remove insoluble material. Size exclusion chromatography was carried out using a HiPrep 26/60 Sephacryl S-200 HR column in SAUDE-1000. Typically, sample was divided into 7-8 runs. Fractions were analyzed with 18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing histone protein were dialyzed against 3 changes of water containing 5 mM β -mercaptoethanol (BME), for at least 4 hours each. Dialysis was carried out at 4°C. Sample was flash-frozen in liquid nitrogen, lyophilized and stored at -80°C.

Ion exchange chromatography was done using a Resource S column equilibrated in SAUDE-0. Lyophilized histone was dissolved in SAUDE-0 and injected into the column with a flow rate of 4 ml/minute. Proteins were eluted by applying a gradient of SAUDE-1000 from 0% to 50% over 100 ml. Up to 50 mg of sample was injected for each run. The fractions were assessed with 18% SDS-PAGE

and dialyzed as described above. Protein concentration was measured with a Nanodrop 200 spectrophotometer and aliquots were lyophilized and stored at -80°C.

2.2.5 Human Core Histones Production

Homo sapiens core histones were individually expressed and purified using an established protocol. The methods for recombinant human histones preparation were developed by Tanaka *et al.*¹⁵³ and Dr. T.S. Kumaravel of the RIKEN Harima Institute at SPring8, Japan. The plasmids contain histone gene sequences (with additional hexa-histidine N-terminal tag) in pHCE (hH2A, hH2B and hH3) and pET-15b (hH4) vectors. The plasmids were kindly provided by Prof. Hitoshi Kurumizaka (RIKEN Genomic Sciences Center, Japan).

2.2.5.1 Transformation and cell culture

Transformation was carried out as in the *Xenopus laevis* histone production protocol. BL21(DE3) cells were used for hH2A, hH2B and hH3, while JM109 cells were used for hH4. One-tenth of the colonies were scraped and used to inoculate 500 ml 2xTY medium with 100 µg/ml ampicillin (and additional 25 µg/ml chloramphenicol for hH4). Culture was incubated in a shaker incubator at 180 rpm at 37°C for 4-6 hours until growth was visible and then distributed into 12 flasks each containing 500 ml 2xTY media with 100 µg/ml ampicillin (and additional 25 µg/ml chloramphenicol for hH4). This culture was grown at 37°C overnight, whereby for hH4, additional induction with 1 mM IPTG had to be carried out when the OD₆₀₀ reached 0.8. Cells were harvested by spinning down at 7,000 rpm, 20°C for 7 minutes.

2.2.5.2 Preparation of inclusion bodies

Pelleted cells were resuspended in 100 ml Histone Sonication Buffer with 1 mM ethylenediaminetetraacetic acid (EDTA) and sonicated at 35% amplitude, with 20 second pulses and 30 second pauses for 3 minutes. This was repeated 3-4 times

and sample was centrifuged at 20,000 rpm, 4°C for 15 minutes. The pellet was dissolved in 100 ml Histone Sonication Buffer, sonicated and centrifuged again. Pellet containing inclusion bodies was dissolved in 100 ml Histone Unfolding Buffer 2, sonicated as before and stirred at room temperature for 1 hour. Sample was spun down at 50,000 g, 20°C for 20 minutes and supernatant was collected.

2.2.5.3 Purification

HiTrap IMAC HP (5 ml) column was charged with nickel ions and equilibrated in Histone Unfolding Buffer 2, sample was loaded and the column was run with His Buffer A and B. Protein was eluted by step-wise gradient of 5, 25, 50, 250 and 500 mM imidazole concentration. Fractions were examined with 15% SDS-PAGE, pooled and dialyzed against three changes of water at 4°C. Protein concentration was estimated and histidine tag was cleaved by adding thrombin (1-1.5 units/mg protein) and stirring at room temperature for 2 hours. Digestion was confirmed by 15% SDS-PAGE and stopped by adding 200 µl of saturated PMSF and 200 µl of 1 M benzamidine.

Urea was added to the sample to make a 2.5-3.0 M final concentration (for hH3 and hH4, additional NaCl was added to give 100 mM final concentration) and sample was loaded onto Resource S or Mono S columns. Purification was conducted with Mono S Buffer A and B at flow rate of 4 and 1 ml/minute for Resource S and Mono S columns, respectively. Protein was eluted by applying gradients of Mono S Buffer B from 0% to 50% over 30 ml and then to 100% over 10 ml. Up to 50 mg of sample was injected per run for hH2A and hH2B, while only 15-20 mg was injected for hH3 and hH4. OD260/260 was measured for the fractions to avoid pooling those with DNA contamination. Fractions were examined with 15% SDS-PAGE and dialyzed against three changes of water at 4°C. Aliquots were lyophilized and stored at -20°C.

2.2.6 Linker Histone Production

2.2.6.1 Full length H1.0

The protocol for full length linker histone H1.0 production was modified from ref. 61. Competent BL21(DE3)pLysS *E. coli* cells were transformed with plasmid containing the gene of full length *Homo sapiens* linker histone H1.0 (with additional hexa-histidine N-terminal tag) in pET-15b vector (EZBiolab Inc., USA) using the same protocol as for the core histones. Four individual colonies were picked and each was used to inoculate 5 ml of 2xTY media containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol, which was incubated in a shaker incubator at 180 rpm at 37°C for 16 hours. This starter culture was used to inoculate a 100 ml 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and grown at 37°C until the OD₆₀₀ reached 0.6. The culture was distributed into 8 flasks (5 ml each) of 500 ml 2xTY media with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and grown at 37°C until the OD₆₀₀ reached 0.6. Cells were induced by adding IPTG to a final concentration of 0.4 mM. After 3 hours, cells were harvested by spinning down at 7,000 rpm, 20°C for 7 minutes. Pelleted cells were flash-frozen and stored at -20°C. Frozen cells were thawed and resuspended in 100 ml H1 Lysis Buffer with 4 mg/ml lysozyme, stirred at 4°C for 1 hour and centrifuged at 22,000 g, 4°C for 30 minutes. Pellet was resuspended in 40 ml of H1 Lysis Buffer with 1 M NaCl. Sonication was done at 35% amplitude with 20 second pulses and 30 second pauses for 3 minutes and repeated 1-2 times. Sample was stirred at 4°C for 24 hours. Insoluble material was removed by spinning down at 22,000 g, 4°C for 30 minutes.

Supernatant containing H1.0 protein was injected into a HiTrap IMAC HP (5 ml) column charged with nickel ions and equilibrated with IMAC Buffer A. DNA contamination was removed by a high salt wash with IMAC Buffer A containing 2 M NaCl. Elution was done by applying a gradient of IMAC Buffer B from 0% to 50% over 50 ml and then to 100% to regenerate. Fractions were assessed with 15% SDS-PAGE and those containing H1.0 were dialysed against 50 mM Tris-Cl pH 7.5 and 150 mM NaCl. His-tag was cleaved with HRV-3C protease 8 units/mg protein and removed by injecting the sample into a HiTrap IMAC HP column and collecting the flow-through. This digestion step was omitted when H1.0 with his-tag was needed.

Sample was then injected into a Heparin column equilibrated with Heparin Buffer A. Sample was eluted with a gradient of Heparin Buffer B from 0% to 50% to wash, followed by 50% to 100% over 100 ml. Fractions were assessed by 15% SDS-PAGE and pure H1.0 was stored in -80°C.

2.2.6.2 Globular H1.0 (gH1.0)

Competent BL21(DE3)pLysS *E. coli* cells were transformed with plasmid containing the globular domain of *Homo sapiens* linker histone H1.0 gene (with an additional hexa-histidine N-terminal tag) in pET-15b vector (EZBiolab Inc., USA) using the same protocol as for the core histones. The method for globular linker histone (gH1.0) production is identical to that for the *Xenopus laevis* core histones up to inclusion body preparation. Inclusion body from 4 L culture was dissolved in 25 ml of H1 Lysis Buffer with 50 mM EDTA and 1 M NaCl by mixing at 4°C overnight. Sample was centrifuged at 22,000 g 4°C for 30 minutes to remove insoluble material. Following the protocol for H1 purification in ref. 61, sample was diluted 1:1 in SPFF Buffer A and injected into a HiPrep SP FF 16/10 column equilibrated in SPFF Buffer A. Elution was conducted with SPFF Buffer B and fractions were examined by 15% SDS-PAGE. Since no protein was bound to the column, flow-through was concentrated and dialyzed into SPFF Buffer A.

Sample was subsequently loaded into a HisTrap HP (5 ml) column charged with nickel ions and equilibrated with HisTrap Buffer A. Elution was done by applying a gradient of HisTrap Buffer B from 0% to 100% over 60 ml. Fractions were assessed with 15% SDS-PAGE and those containing gH1.0 were pooled and dialyzed against 50 mM Tris-Cl pH 8.8, 500 mM NaCl. The next step of purification was done with a HiLoad 16/60 Superdex 200 column. Sample in 50 mM Tris-Cl pH 8.8, 500 mM NaCl was injected. Fractions containing pure gH1.0 were pooled and stored at -80°C. The his-tag was cleaved with HRV-3C protease (10 units/mg protein) and removed by injecting the sample into a HisTrap HP (5 ml) column and collecting the flow-through. Sample was dialyzed into 20 mM K-Cacodylate pH 6.0 and stored at -80°C.

2.2.7 Histone Octamer Production

The protocol used for histone octamer production was a modified version of that from refs. 151,152.

2.2.7.1 Refolding

Lyophilized H2A, H2B, H3 and H4 proteins (from *Homo sapiens* or *Xenopus laevis*) were dissolved in Octamer Unfolding Buffer and mixed in equimolar stoichiometry. Octamer Unfolding Buffer was added to make a final histone concentration of 1 mg/ml and the mixture was dialyzed against Octamer Refolding Buffer at room temperature (*Xenopus laevis*) or 4°C (*Homo sapiens*). After two changes of Octamer Refolding Buffer every 4 hours, the dialysis was allowed to run overnight. Precipitate was removed by centrifuging the sample twice at 18,000 g, 20°C for 10 minutes each.

2.2.7.2 Purification

Histone octamer was purified by size exclusion chromatography using a HiLoad 16/60 Superdex 200 column. Sample was concentrated with a 10 kDa Amicon concentrator to 1 ml before loading onto the column. The column was run in Octamer Refolding Buffer and fractions were assessed by 18% SDS-PAGE and the pure histone octamer was concentrated and stored at -20°C in 50% glycerol.

2.2.8 Reconstitution of Nucleosome and Nucleosome Core Particle

The protocol for nucleosome (NUC) or nucleosome core particle (NCP) *in vitro* reconstitution was modified from refs. 151,152. DNA and histone octamer were mixed in high salt (2 M KCl) and 1 mM DTT. The presence of glycerol in the histone octamer stock causes inaccuracy in histone octamer concentration measurements. Hence, screening of different histone octamer to DNA molar ratio should be done for

every batch of histone octamer. DNA concentration was fixed at 6 μ M while histone octamer concentration was varied depending on the targeted ratio. The mixture was dialyzed against TCS Buffer containing 0.85 M KCl. Every 2.5 hours, the salt concentration was decreased by 0.2 M until it reached 0.45 M. The sample was then dialyzed against buffer without salt overnight. Precipitate was spun down at 14,500 rpm, 4°C for 15 minutes, and the concentration of reconstituted NUC or NCP was calculated based on DNA concentration.

The quality of reconstituted NUC or NCP was examined by 6% PAGE. Typically, 1-2 μ g of sample was loaded in 10% sucrose. Pure NUC or NCP should contain a single band with no free DNA or high molecular weight species. If off-centred species was present, the sample was heated at 37-50°C followed by centrifuging at 14,500 rpm, 4°C for 15 minutes. Buffer exchange to 20 mM K-Cacodylate pH 6.0 was done with a 50 kD Amicon concentrator and sample was concentrated to 5-10 mg/ml. NUC or NCP can then be stored at 4°C. After a favorable histone octamer to DNA molar ratio was established in initial small scale screening, this ratio would be used for a larger scale reconstitution.

2.2.9 Reconstitution of Nucleosome Array NUC601-36-177

The protocol for NUC601-36-177 *in vitro* reconstitution was modified from ref. 70. DNA601-36-177 was mixed with histone octamer in 10 mM TEA-Cl pH 7.5, 1 mM EDTA and 2 M NaCl. Different histone octamer to DNA molar ratios were screened to determine a ratio that gave optimally saturated nucleosome array. The DNA concentration was fixed at 0.1 mg/ml. Competitor DNA, of 84 bp (DNA145) or 85 bp (DNA147) half-site fragment, was added at a half mass-ratio of the DNA array. A double bag dialysis system was used by dialyzing the sample in one dialysis bag against 10 mM TEA-Cl pH 7.5, 1 mM EDTA and 2 M NaCl in a second dialysis bag, which was at the same time being dialyzed against 10 mM TEA-Cl pH 7.5, 1 mM EDTA. In this way, the dialysis and reconstitution processes occurred slowly and the quality of nucleosome array was better. Dialysis was carried out overnight at 4°C, precipitate was removed by spinning down at 14,500 rpm, 4°C for 15 minutes and the concentration of reconstituted nucleosome array was calculated based on the DNA

concentration. The quality of the reconstituted nucleosome array was determined by gel electrophoresis using 0.8% agarose 0.2x TBE buffer. The saturation of the reconstituted nucleosome array can be visualized in 6% PAGE after digesting the sample with *Ava*I enzyme (5000 units/mg DNA) at 37°C for 1 hour. If needed, buffer exchange to 20 mM K-Cacodylate pH 6.0 was done using a 50 kD Amicon concentrator.

2.2.10 Electrophoretic Mobility Shift Assay (EMSA)

EMSA was conducted to detect reaction between DNA, NCP or nucleosome array with metal compounds. Free DNA, NCP or nucleosome array was incubated with different molar ratios of metal compounds in 20 mM K-Cacodylate pH 6.0 for 1-2 days at room temperature. Nucleosome concentration was fixed at 1 μ M. Reaction was stopped by passing the sample through DyeEx 2.0 spin column to remove any unreacted metal compounds.

After reaction with NCP, nucleosomal DNA (nucDNA) was extracted by adding 1x volume of 2 M KCl and heating at 50°C for 30 minutes. After that, 1x volume of phenol:ClA (1:1) was added and the mixture was centrifuged at 14,500 rpm, 20°C for 10 minutes. The aqueous phase containing nucleosomal DNA was collected, ethanol precipitation was performed and pellet was dissolved in TE 10/0.1. Upon binding of metal compounds, DNA, NCP or nucleosome array bands were expected to migrate more slowly due to additional mass added by bound metal compounds. 10% PAGE, 6% PAGE or 0.8% agarose was run to visualize the shifts for DNA, NCP and nucleosome array, respectively.

EMSA was also used to detect binding of linker histone with nucleosome. Nucleosome concentration was fixed at 1 μ M. Linker histone was added to nucleosome at different molar ratios. After incubation on ice for 15 minutes, 6% PAGE was run to visualize band shifting resulting from binding of linker histone to nucleosome.

2.2.11 Atomic Absorption Spectrophotometry (AAS)

The concentration of metals in the gas phase can be measured by passing light through the sample and measuring the amount of absorption. Different metals in atomic form absorb light at different wavelengths. Hence, using light of specific wavelength, we can measure the concentration of a specific metal in our sample. A Z-2000 Polarized Zeeman Atomic Absorption Spectrophotometer was used in graphite furnace mode. A default program was used for measurement, which involved 40 second drying at 80-110°C, where solvent was evaporated from the sample, 20 second ashing at 800°C, where sample was vaporized into gas, and a 10 second atomization at 2700°C, where the sample was broken down to its free atoms. A standard curve was made by measuring the absorbance of solutions of known concentration of the metal element of interest. The concentration of that specific metal in sample was then calculated based on the standard curve.

Free DNA or NCP was incubated with different metal compounds in different molar ratios for 2 days at room temperature. Reaction was stopped by passing the sample through a DyeEx 2.0 spin column. Nucleosomal DNA was extracted from NCP as described in section 2.2.11. Concentration of metal in samples was measured by AAS. By comparing the metal concentration with concentration of DNA or NCP, the number of metal adducts per molecule of DNA or NCP was calculated.

2.2.12 Exonuclease III Footprinting

Free DNA or NCP was incubated with different metal compounds in different molar ratios for 1-2 days at room temperature. Sample was passed through a DyeEx 2.0 spin column to remove unbound metal compound. Nucleosomal DNA was extracted from the NCP. Exonuclease III footprinting⁹⁹ was carried out to deduce the position of metal adducts on free or nucleosomal DNA. A schematic representation of the method is shown in Figure 2.2.

DNA was labeled at its 5' end with ³²P by mixing 3 µg DNA, 1x T4 polynucleotide kinase (PNK) Buffer, 10 units of T4 PNK and 10 µCi of γ³²P-ATP in a total of 20 µl volume. After 1 hour incubation at 37°C, the reaction was stopped by

passing the sample through a DyeEx 2.0 spin column to sequester unbound $\gamma^{32}\text{P}$ -ATP. DNA was then digested with Exonuclease III enzyme by mixing 3 μg ^{32}P -labeled DNA, 1x Exonuclease III Buffer and 250 units of Exonuclease III in a total of 50 μl volume.

Exonuclease III digests one strand of double-stranded DNA in the 3' to 5' direction. The presence of metal compound adduct halts Exonuclease III digestion generally one nucleotide before the adduct position. The digestion would result in DNA fragments with different lengths, depending on the location of metal adducts. After 1 hour incubation at 37°C, reaction was stopped by adding 25 μl Exonuclease Stop Buffer and 190 μl cold 100% ethanol. After incubation on ice for 10 minutes, the sample was centrifuged at 12,000 rpm for 3 minutes. The pellet was washed with 200 μl cold 70% ethanol and centrifuged at 12,000 rpm for 3 minutes. The pellet was dissolved in 200 μl of 1 M thiourea and incubated at 58°C for 16 hours to remove the metal adducts from DNA. Positions of the metal adducts were derived by comparing the DNA fragments from the sample with a purine sequencing marker in denaturing PAGE. Since the presence of metal adducts would add to the molecular weight of the DNA fragments, metal adducts need to be removed for the sample bands to align with the purine marker bands. The thiourea reaction was stopped by adding 20 μl 3 M Na-Acetate and 540 μl cold 100% ethanol. After incubation on ice for 10 minutes, the mixture was centrifuged at 12,000 rpm for 3 minutes. Following 70% ethanol wash, the pellet was dissolved in 20 μl of 1x Denaturing PAGE Loading Dye.

To prepare purine sequencing marker, 25 μl of 85% formic acid was added to 1.5 μg of ^{32}P -labeled DNA in a 10 μl volume. The mixture was incubated at room temperature for 8 minutes and quenched by adding 200 μl Formic Acid Stop Buffer and 750 μl cold 100% ethanol. Following 10 minutes of incubation on ice and centrifugation at 12,000 rpm for 3 minutes, the pellet was dissolved in 200 μl of 10% (v/v) piperidine and incubated at 95°C for 45 minutes. Reaction was stopped by ethanol precipitation. After 70% ethanol wash, the pellet was dissolved in TE (10, 0.1). To dephosphorylate the DNA fragments, the dissolved pellet was mixed with 1x T4 PNK Buffer and 250 units of T4 PNK in a total of 50 μl volume. The reaction was incubated overnight at 37°C and stopped by ethanol precipitation. After 70% ethanol wash, the pellet was dissolved in 20 μl of 1x Denaturing PAGE Loading Dye. Denaturing PAGE was run to examine the size of different DNA fragments resulting from Exonuclease III digestion of metal agent-treated DNA. Sample was compared

with purine marker to deduce the positions of metal adducts on the DNA at approximately single base pair resolution.

Figure 2.2. Schematic representation of Exonuclease III footprinting.

2.2.13 Reversibility assay

To examine the stability of ruthenium agent adducts on DNA, we designed a reversibility assay, where DNA145 was treated with different ruthenium anticancer agents at different molar ratios and incubated at room temperature in the dark for 24 hours. Unreacted metal agents were removed by passing the sample through a DyeEx 2.0 spin column. The sample was left to stand at room temperature in the dark for another 24 hours, before passing it through DyeEx 2.0 spin column for the second time. AAS and 10% PAGE were utilized to examine the samples.

2.2.14 Co-crystallization of Oligonucleotides with Metal-based Compounds

To examine the structure of adducts of metal-based compounds on DNA, co-crystallization methods were employed. Unlike soaking experiments, where metal-based agents were added to NCP145 crystals, here the oligonucleotide was treated with the agents before setting up for crystallization. Single-stranded oligonucleotides in powder form were dissolved in autoclaved Milli-Q water to make 10 mM stocks. Annealing was done by heating single-stranded oligonucleotides in 50 mM Na-Cacodylate pH 6.0 for 10 minutes at 50°C, then slowly cooling down to room temperature. Annealed double-stranded oligonucleotide DNA was treated with RAPTA-C at 1:1 molar ratio. Due to lower reactivity, reactions with 5:1 KP1019 to double-stranded oligonucleotide molar ratio were set up. All reactions were conducted in 20 mM Na-Cacodylate pH 6.0 buffer. Samples were incubated at room temperature in the dark for 48 hours.

To purify the ruthenated oligonucleotides from unreacted oligonucleotides and free ruthenium agents, high pressure liquid chromatography (HPLC) was done on a single pump HPLC instrument. The analytical separation was done using a Jupiter C18 reverse phase column (250 x 4.6 mm, 300 Å, 5 µm), while the preparative separation was done with a Jupiter C18 reverse phase column (250 x 10 mm, 300 Å, 5 µm). Column was equilibrated with 1.4% triethylamine acetate pH 7.5. After sample was injected, a gradient using 100% acetonitrile was generated by increase from 5% to 40% over 50 minutes. For analytical HPLC runs, the flow rate was 1 ml/minute,

while for preparative runs it was 2 ml/minute. Temperature was maintained at 48°C. For RAPTA-C runs, absorbance at 220 nm (RAPTA-C absorbance peak) and 260 nm (DNA absorbance peak) were recorded. For KP1019 runs, absorbance at 260 nm and 280 nm (KP1019 absorbance peak) were recorded. Fractions were collected and lyophilized. Annealing, reaction and HPLC were carried out by Soon Cin Huang.

To confirm the identity of the peaks, AAS and matrix-assisted laser desorption ionization - mass spectrometry (MALDI-MS) were performed. For MALDI-MS, a 4800 MALDI TOF/TOF analyzer was used in linear mode; 3-hydroxypicolinic acid matrix (0.3 µl) was applied onto the MALDI plate and allowed to dry. Lyophilized samples were dissolved in Milli-Q water and 0.3 µl was applied on top of the matrix.

Due to the low yield of ruthenated oligonucleotide preparations, initial crystallization screens were done using unreacted oligonucleotides. Conditions that gave crystals were used to screen ruthenated oligonucleotides. The initial screen was done with a Natrix HR2-116 kit. Oligonucleotide (1 mM) in 0.5x crystallization buffer (2 µl) was equilibrated against a reservoir of 500 µl of 1x crystallization buffer in hanging drop vapor diffusion set ups. Crystallization plates were incubated at 18°C. Diffraction test images were collected using an in-house collection port of the MicroMax-007HF rotating anode generator with a Rigaku Raxis IV+ image plate detector.

2.2.15 Soaking of NCP Crystals with Metal-based Compounds

NCP145 crystals were grown and harvested according to the established protocol⁵. Chloride and manganese ions can interfere with drug binding and data analysis, respectively, and had to be removed from the buffer. Mn Buffer was replaced with Mg Buffer in a stepwise manner. Metal-based compounds were dissolved in Mg Buffer and used to soak NCP145 crystals. In cases where metal compounds have low solubility in Mg Buffer, as much powder as possible was dissolved. Although the exact concentration was unknown, it would have been as high as achievable. The soaked crystals were incubated at room temperature for different lengths of time. The soaking for RAPTA-C was done by Dr. Wu Bin and Dr. Michelle S. Ong.

2.2.16 Crystallization of Nucleosome in Complex with Linker Histone

Linker histone H1.0 or gH1.0 was mixed with nucleosome at ratios determined from EMSA results. Salting out and salting in equilibration methods were attempted. In salting out, sample in 0.5x crystallization buffer was equilibrated against a reservoir of 1x crystallization buffer. In salting in, sample in 2x crystallization buffer was equilibrated against a reservoir of 1x crystallization buffer. Crystallizations were set up in hanging drop vapor diffusion mode with sample concentrations of 4 mg/ml in 2-10 μ l volume, and a reservoir of 500 μ l. Crystallization plates were incubated at 18°C. Crystals were harvested in buffers with a half concentration of components of the crystallization reservoir, containing up to 2% trehalose and 40% 2-methyl-2,4-pentadiol (MPD) or PEG6000, and directly mounted in the cryo-stream.

2.2.17 X-ray Diffraction Data Collection

Preliminary X-ray diffraction test for nucleosome crystals was done using the in-house collection port of the Micromax-007HF rotating anode X-ray generator (1.54 Å) with R-Axis IV++ image plate detector. Collection of X-ray diffraction data was done at beam lines X06DA or X06SA of the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) with MarCCD or Pilatus detectors. X-ray wavelength of 1.50 Å was used for native nucleosome crystals and those soaked with ruthenium and rhodium compounds. Wavelength was tuned to 1.14 Å and 1.04 Å for crystals soaked with osmium and gold compounds, respectively. Full data sets consisted of 360 images collected with 0.5° oscillation range and 1 second exposures.

2.2.18 Data Processing and Model Building

Data integration was done by MOSFLM¹⁵⁴ and data reduction by SCALA from the CCP4 package¹⁵⁵. Otherwise, data were processed with the “go.com” suite at the Swiss Light Source. Rigid body and restrained refinements were done using

Refmac5 from CCP4 package and the native NCP model (pdb code: 3REH). Model building was carried out with Coot¹⁵⁶. Figures were made with PyMOL¹⁵⁷.

2.2.19 Electron Microscopy (EM)

NUC601-36-177 was incubated with different molar ratios of metal compounds in 20 mM K-Cacodylate pH 6.0 for 1 day at room temperature. Nucleosome array concentration was fixed at 0.5 μ M. Reaction was stopped by passing the sample through DyeEx 2.0 spin columns to remove any unreacted drug molecule. Sample was diluted to make 30 μ g/ml nucleosome array that was dialyzed against TNE buffer (with or without MgCl_2) at 4 °C overnight. To fix the sample, 2 μ l of 25% glutaraldehyde was added to 98 μ l sample buffer to make 0.5% stock. Two microliters of this solution was then added to 8 μ l of sample, so that the final glutaraldehyde concentration was 0.1%. Sample was incubated on ice for 10 minutes.

Carbon-coated grids (home-made by NTU Cryo EM lab) were glow discharged for 30 seconds. Four microliters of sample were pipetted onto the grid, incubated for 1 minute and blotted with Whatman paper. Four microliters of stain solution of 2% uranyl acetate was placed on the grid, incubated for 1 minute and blotted. The grid was air-dried for 10 minutes. Electron microscopy was done with Icor T12 microscope operated at 120 kV. Negative stained grids were imaged at calibrated magnification of 49,000X with -1.0 to -2.0 μ m defocus applied. The EM studies were done in collaboration with Prof. Sara Sandin's lab.

3 Results and Discussion Part 1 – Preparation of DNA, Nucleosomes and Linker Histones

3.1 DNA Production

In order to prepare DNA with different sequences and lengths needed for the assembly of nucleosome core particle (NCP), nucleosome (NUC) and nucleosome array, different DNA constructs were produced as described in the Materials and Methods section. Single repeat DNA constructs were used for reconstituting NCP (145 or 147 bp DNA) and NUC (154 to 169 bp DNA). Meanwhile, a DNA array construct with 36 repeats of 177 bp sequence was used for reconstitution of nucleosome array.

3.1.1 Single repeat DNA

The single repeat DNA constructs can be divided into palindromic and non-palindromic classes. It is difficult to prepare long sequences of palindromic DNA directly in bacterial cells due to recombination problems. Hence, for the palindromic DNA constructs, the final products need to be ligated from two nearly identical halves of the sequences. The resultant DNA materials from different steps of DNA145 production are shown in Figure 3.1a. After releasing 84 bp insert from pUC18 vector by EcoRV digestion (lane 1), PEG precipitation was carried out to separate the two species. Empty vector was pelleted while most of the 84 bp insert stayed in the supernatant (lane 2 and 3). After dephosphorylation, the 84 bp DNA was subjected to ligation test. The non-dephosphorylated control was ligated to form a ladder of DNA with different lengths, while the dephosphorylated DNA was not ligated (lane 4 and 5). After HinfI digestion, the 84 bp insert was cut into 71bp+3 (71 bp DNA with 3 bases overhang) and 10bp+3 (10 bp DNA with 3 bases overhang; lane 6). This sample was put through anion exchange column to separate the 71bp+3 from 10bp+3 (Figure 3.1b, 3.1a lanes 7-9). The pure 71bp+3 DNA was ligated to each other to form

palindromic DNA145 (Figure 3.1a lane 10). A second round of anion exchange was done to purify the 145 bp DNA (Figure 3.1c, 3.1a lanes 11-13). The process of DNA147, DNA PRP167, DNA CMS167, 165 and 169 production is the same as DNA145 and purified materials are shown in Figure 3.1a lane 14 and Figure 3.2. Production of DNA PRP167 was done by Dr. Bao Qiuye (data not shown). The final products of these DNA constructs migrated in between 100 and 200 bp bands of the DNA ladder, while their half sequences migrated just below the 100 bp band.

Figure 3.1. DNA145 production. a) The resultant materials from the steps of DNA145 production. Lanes: L – DNA ladder, 1 – after EcoRV digestion, 2 – pellet after PEG precipitation, 3 – supernatant after PEG precipitation, 4 – ligation test control (84 bp before dephosphorylation), 5 – ligation test sample (84 bp after dephosphorylation), 6 – after HinfI digestion, 7 – flow-through from first anion exchange, 8 – peak eluted at 53 mS/cm during the first anion exchange, 9 – peak eluted at 61 mS/cm

during the first anion exchange, 10 – after ligation, 11 – flow-through from second anion exchange, 12 – peak eluted at 69 mS/cm during the second anion exchange, 13 – peak eluted at 71 mS/cm during the second anion exchange, 14 – DNA147. b, c) Anion exchange FPLC profiles. For these and all subsequent FPLC profiles: dashed pink line – injection, blue line – UV absorbance at 280 nm, green line – Buffer B concentration, brown line – conductivity reading, X axis – volume in ml, Y axis – UV absorbance at 280 nm in mAU. b) First anion exchange. c) Second anion exchange.

Figure 3.2. Final products of DNA CMS167, 165 and 169. L – DNA ladder.

For non-palindromic DNA constructs, the full-length fragment sequences (not the half sequences) were directly amplified in bacterial cells. Figure 3.3 shows the resultant materials from different steps of DNA CMS155 production. Digestion by EcoRV was not 100% complete and generated additional species of two or more repeats of DNA CMS155 (Figure 3.3a lane 1), which cannot be removed by PEG fractionation. Purification in Mono-Q column (Figure 3.3b and c) yielded pure DNA CMS155 (Figure 3.3a lane 2). The preparation of DNA CMS154 and 156 was done in the same way as DNA CMS155. DNA CMS156 is shown in Figure 3.3a lane 3. Production of DNA CMS154 was done by Dr. Bao Qiuye (data not shown). The final products of these DNA constructs migrated in between 100 bp and 200 bp bands of DNA ladder.

Figure 3.3. DNA CMS155 production. a) Resultant material after EcoRV digestion (lane 1) and final products of DNA CMS155 and 156 (lanes 2 and 3). b) Anion exchange FPLC profile of DNA CMS155. c) Fractions from anion exchange purification in (b).

3.1.2 DNA array

The DNA array construct used for nucleosome array reconstitution is non-palindromic. The production process is similar to single repeat non-palindromic DNA constructs, involving digestion of the plasmid containing the construct and purification from empty vector. The resultant materials from different steps of DNA601-36-177 production are shown in Figure 3.4. After EcoRV digestion, the insert was released from the vector (lane 1). The insert DNA (~6.4 kbp) is larger than the empty vector (~2.7 kbp) but the difference in size is not enough to be separated by PEG fractionation. To increase the size difference, the empty vector was digested further with DraI and HaeII to convert it into smaller fragments (lane 2). After several rounds of PEG fractionation, only small amount of vector fragments remained (lane 3).

Figure 3.4. DNA601-36-177 production. Lanes: L – DNA ladder, 1 – after EcoRV digestion, 2 – after DraI and HaeII digestion, 3 – final product after PEG fractionation.

3.2 Core Histones Production

Core histones for NCP, NUC and nucleosome array assembly were prepared following protocols described in Materials and Methods section. NCP constructs used in the metal compound studies were reconstituted with *X. laevis* core histones due to their availability and our established method of crystal preparation using these constructs. To get a better model for the human system, we then moved to using human core histones for the study of metal compound effects on nucleosome arrays and crystallization attempts of nucleosome in complex with linker histone. In addition, a hexa-histidine tag was added to the human core histones for ease of purification.

3.2.1 *Xenopus laevis* core histones

In bacterial cells, excessively produced histone proteins accumulate in inclusion bodies. To purify histones, inclusion bodies were resuspended and the soluble material was passed through a HiPrep 26/60 Sephacryl S-200 HR size exclusion column. The size exclusion chromatography profile of *X. laevis* histone H2B (xH2B) is shown in Figure 3.5a. Total volume of the column is 318 ml with a

void volume of 105 ml. The first peak eluted at 86-128 ml and contained high molecular weight proteins and DNA contaminants. The peak eluting at 128-160 ml (total elution volume of ~32 ml) contained histone xH2B (13.9 kD; protein migrated a bit slower than its size in gel). Considering the separation capacity of the column of 5-250 kD, xH2B should elute near the end of purification. However, we found that xH2B eluted just after the void volume, possibly due to the presence of DNA contaminants, as evidenced by high OD_{280/260} value (>1). This DNA contaminant will be removed further during the next step of purification. A small amount of other proteins with higher molecular weight than xH2B were also present in the fractions containing xH2B. Additionally, one protein band with lower molecular weight than xH2B could be observed. This might come from another protein or it might be a truncated form of xH2B. Resource S column was used to further purify the histone (Figure 3.5b). Initial fractions contained some higher molecular weight contaminants while later fractions were of high purity.

3.2.2 *Homo sapiens* core histones

Unlike *X. laevis* histones, the *H. sapiens* core histone constructs are tagged with hexa-histidine at their N-termini. This allows us to purify these constructs by affinity chromatography using columns charged with nickel ions. Elution was done by increasing concentration of imidazole and all fractions were examined with SDS-PAGE. For *H. sapiens* histone H4 (hH4), most of the histone (13.4 kD with hexa-histidine tag) eluted at 250-500 mM imidazole concentration (Figure 3.6a). Hexa-histidine tag was then cleaved by thrombin (Figure 3.6b) yielding hH4 of 11.6 kD size and the final step of purification was done by cation exchange chromatography (Figure 3.6c). Human histone H4 with and without hexa-histidine tag migrated a bit slower than their theoretical sizes on gels. Protein molecular weight was confirmed by mass spectrometry.

Figure 3.5. *Xenopus laevis* core histone preparation. a) Size exclusion chromatography profile and corresponding 18% SDS-PAGE of the fractions. b) Cation exchange chromatography profile and corresponding 18% SDS-PAGE of the fractions.

Figure 3.6. *Homo sapiens* core histone preparation. a) 15% SDS-PAGE of fractions from affinity chromatography purification. b) Thrombin digestion. c) Cation exchange chromatography profile and corresponding 15% SDS-PAGE of the fractions.

3.3 Linker Histone Production

Two linker histone constructs (full length and globular domain) were prepared for the crystallization of nucleosome in complex with linker histone. Both of these constructs were prepared with hexa-histidine tags to make purification easier.

3.3.1 Full length H1

Full length *H. sapiens* linker histone H1.0 with hexa-histidine tag (22.8 kD) was purified by three steps of affinity chromatography. Sample was first passed through a HiTrap IMAC HP (5 ml) column charged with nickel ions (Figure 3.7a; protein migrated slower than its size in gel). Before elution, the column was washed with high salt buffer to remove DNA contaminant from protein of interest. After dialysis, hexa-histidine tag was cleaved off by HRV3C protease to yield 20.9 kD sized protein (Figure 3.7b; protein migrated slower than its size in gel). Uncleaved protein was removed by passing the sample through the same column once again, this time collecting the flow-through. When H1.0 with hexa-histidine tag was desired, these digestion and purification steps were omitted. The last purification step was done in HiTrap Heparin HP (5 ml) column (Figure 3.7c). Protein molecular weight was confirmed by mass spectrometry.

Figure 3.7. Full length H1.0 purification. a) FPLC profile from first affinity chromatography and 15% SDS-PAGE of the fractions. b) FPLC profile from second affinity chromatography and 15% SDS-PAGE of the fractions. c) FPLC profile from heparin and 15% SDS-PAGE of the fractions.

3.3.2 Globular H1

Globular domain of *H. sapiens* linker histone gH1.0 with hexa-histidine tag (11.5 kD) was first purified from inclusion body by cation exchange chromatography in HiPrep SP FF 16/10 column (Figure 3.8a). However, no gH1.0 protein was bound to the column. This may be due to the high concentration of salt used. Affinity chromatography with HiTrap IMAC HP (5 ml) column (charged with nickel ions) was then tried. The protein sample was about 90% pure after affinity chromatography as judged by the SDS-PAGE and Coomassie staining, although some faint high-molecular weight bands were still present (Figure 3.8b). A last step of size exclusion chromatography yielded pure gH1.0, which still had the hexa-histidine tag (Figure 3.8c). After removal of hexa-histidine tag which yielded protein of 9.5 kD size, the flow-through of affinity chromatography was collected (Figure 3.9). Protein molecular weight was confirmed by mass spectrometry.

Figure 3.8. Globular H1.0 purification. a) FPLC profile from cation exchange chromatography and 15% SDS-PAGE of the fractions. b) FPLC profile from affinity chromatography and 15% SDS-PAGE of the fractions. c) FPLC profile from size exclusion chromatography and 15% SDS-PAGE of the fractions.

Figure 3.9. FPLC profile of gH1.0 from affinity chromatography after hexa-histidine tag removal and corresponding 15% SDS-PAGE.

3.5 Histone Octamer Refolding

Core histones need to be refolded together to form histone octamer, which would be used in assembly of NCP, NUC and nucleosome array. (Figure 3.10). Core histones were mixed at equimolar ratio and dialyzed to allow refolding, then histone octamer was purified by size exclusion chromatography. The purification profile of *X. laevis* histone octamer purification is shown in Figure 3.11. The profile for *H. sapiens* histone octamer purification is very similar to this. Peak eluting at 40-55 ml contained aggregated material. The histone octamer peak eluted at 63-78 ml, followed by H2A-H2B dimer peak at 80-90 ml, in agreement with published protocol in ref. 152. The identity of the histone octamer was confirmed using 18% SDS-PAGE. In *X. laevis* histone octamer, the lowest band represented xH4 (11.2 kD) and the highest band represented xH3 (15.3 kD). The middle band was a mixture of xH2A (13.9 kD) and xH2B (13.8 kD) which cannot be separated in 18% SDS-PAGE. For *H. sapiens* histone octamer, the fastest-running band represented hH4 (11.6 kD) and the middle band represented hH2A (14.4 kD). hH2B (14.2 kD) and hH3 (15.7 kD) were both present in the slowest-running band. All of the core histones migrated a bit slower than their theoretical sizes in gel, especially hH2B which migrated together with hH3.

Figure 3.10. Reconstitution of nucleosome. Core histones were mixed and refolded together to make histone octamer, which with DNA are reconstituted into nucleosome.

Figure 3.11. Histone octamer refolding. a) Size exclusion profiles of *Xenopus laevis* histone octamer. b) 18% SDS-PAGE of histone octamer. Lanes: 1 – *Xenopus laevis* histone octamer, 2 – *Homo sapiens* histone octamer.

3.6. Reconstitution of nucleosome core particle, nucleosome and nucleosome array

NCP, NUC and nucleosome array were reconstituted from a mixture of DNA fragment and histone octamer. The naming convention follows the DNA constructs they were reconstituted from. For example, NCP reconstituted with DNA145 will be referred to as NCP145.

3.6.1 Nucleosome and nucleosome core particle reconstitution

NCP145 and 147 were reconstituted from DNA sequences derived from human α -satellite DNA which have moderate nucleosome-positioning ability. Because of that, sometimes two conformations of NCP were produced: centered and off-centered NCP. They differ in the translational position of histone octamer with respect to DNA. In centered NCP, histone octamer is symmetrically positioned along DNA, while in off-centered NCP, histone octamer is asymmetrically positioned with

one of the DNA ends overhanging outside the core region (Figure 3.12a, ref. 158). The two conformations can be identified by their different migration in native polyacrylamide gels: off-centered NCP migrated slower than centered NCP (Figure 3.12a lane 1)¹⁵⁹. The off-centered conformation is energetically less favorable and could be repositioned to the centered state upon heat treatment (Figure 3.12a lane 2). The final products of NCP145 and 147 are shown in Figure 3.12a lane 2 and 3. *X. laevis* histone octamer was used for reconstituting NCP145 and 147. Reconstitution of NUC CMS154, 155, 156, 165, 167 and 169 did not give off-centered species because these constructs are based on the Widom strong positioning 601 sequence. The final products of NUC CMS155, 156, 165, 167 and 169 are shown in Figure 3.12b. Reconstitution of NUC CMS154 and PRP167 was done by Dr. Bao Qiuye (data not shown). These constructs were reconstituted with *H. sapiens* histone octamer.

Figure 3.12. Nucleosome production. a) The resultant materials from NCP reconstitution. Lanes: L – DNA ladder, 1 – NCP145 before heat-treatment (off-centered and centered NCP145 positions are indicated by pink and blue arrows, respectively), 2 – NCP145 after heat-treatment, 3 – NCP147 after heat-treatment. Cartoon is adapted from ref. 158. b) Final products of NUC CMS155, 156, 167, 165 and 169.

3.6.2 Nucleosome array reconstitution

DNA601-36-177 and *H. sapiens* histone octamer (hHO) were used to reconstitute nucleosome array; NUC601-36-177 produced after reconstitution is illustrated in Figure 3.13. With increasing amount of hHO loaded onto the DNA array, the nucleosome array band was shifted upwards until it reached a plateau (Figure 3.13a). Competitor DNA and remaining vector fragments then started to take on histone octamer, and nucleosome-like species originating from competitor DNA and vector fragments were observed. Although the competitor DNA added was only 84 bp long, it has been shown that a complete histone octamer can bind to ~80 bp DNA¹⁶⁰. NUC601-36-177 was not purified from contaminating competitor DNA and the resulting nucleosome-like species because purification steps may cause histone loss from the nucleosome array (personal communication with Prof. Daniela Rhodes). The saturation of the resulting nucleosome array was examined by digestion with *Ava*I restriction enzyme. Upon *Ava*I digestion (Figure 3.13b), optimum saturation is indicated by the presence of a mononucleosome NUC177 band (Figure 3.13b, green triangle) and disappearance of DNA177 species (Figure 3.13b lane 4). When the amount of histone octamer was too little, free DNA177 was still present in the sample. NUC177 with one H2A-H2B dimer dissociated (NUC177-hexasome; Figure 3.13b, blue triangle) was also observed in addition to full NUC177. With more histone octamer added, the band representing NUC177-hexasome became faint (Figure 3.13b lane 4). However, at 1.50 histone octamer to DNA ratio, another band started to appear below the NUC177 band (Figure 3.13b, red triangle). Although this band migrated very similarly to that of the NUC177-hexasome, it seemed to migrate still slightly slower and its intensity increased with increasing amount of histone octamer added. Hence this appears to relate to a different species. Although it is still unclear, a likely explanation is that it is a nucleosome-like species formed on the competitor DNA.

Figure 3.13. Nucleosome array NUC601-36-177 production. a) Nucleosome array reconstituted with different amount of histone octamer. Numbers indicate histone octamer:DNA molar ratios. b) *Ava*I digestion of the materials from (a).

Compaction assays were conducted by incubating nucleosome array with different amounts of divalent metal, followed by glutaraldehyde cross-linking and then band shift analysis. In the presence of $MgCl_2$, NUC601-36-177 migrates faster due to more compact conformation (Figure 3.14). The increase in migration speed is

elevated with increasing concentration of MgCl_2 , until a fully folded conformation is attained at approximately 1.6 mM MgCl_2 . Increasing the MgCl_2 concentration further causes aggregation and precipitation of the nucleosome array.

Figure 3.14. Nucleosome array folding induced with different concentrations of MgCl_2 .

4 Results and Discussion Part II – Nucleosome Site Selectivity of Novel Metal-based Anticancer Agents

4.1 Binding of RAPTA-C and KP1019 to DNA, NCP and nucDNA

The project was initiated by studying two ruthenium compounds, RAPTA-C and KP1019. Initial biochemical tests were mainly done using these two agents. DNA147 (and hence NCP147) was chosen for the initial tests as it is the standard construct in our lab with an established protocol for preparation of DNA, NCP and crystals. DNA147 or NCP147 were treated with RAPTA-C or KP1019, and reactivity was assessed by EMSA on native polyacrylamide gel (Figure 4.1). Due to lower solubility of KP1019, only up to 50:1 KP1019 to DNA147 or NCP147 molar ratio was achievable. The nucDNA samples correspond to DNA that was extracted from treated NCP.

Figure 4.1. Reactivity of RAPTA-C and KP1019 to DNA147, NCP147 and nucDNA147. Numbers indicate compound to NCP147 or DNA147 molar ratio used in the reaction.

When naked DNA147 was treated with RAPTA-C, the DNA147 band was shifted upwards, indicating binding of RAPTA-C on DNA147. At higher ratio the DNA147 band became smeary, probably because nonspecific binding started to take place. In addition, the presence of excess amount of ruthenium agents caused precipitation of DNA147. For KP1019, band shifting became evident at 50:1 molar ratio. At 10:1 and 50:1 molar ratio, precipitation of DNA147 was observed. RAPTA-C was also reactive towards NCP147, as shown by slight band shift, which was more apparent at 50:1 ratio. The high molecular weight band likely indicated intermolecular association as a result of RAPTA-C cross-linking. At 50:1 ratio, the sample started to precipitate due to excessive intermolecular association. For KP1019, it was difficult to see if there was any shift of NCP147 band, but the formation of high molecular weight species suggested reactions had taken place. To examine whether adducts formed by RAPTA-C and KP1019 on NCP147 were on the DNA component of NCP, DNA was extracted from NCP147 after reaction with RAPTA-C or KP1019. This extracted DNA is called nucleosomal DNA147 (nucDNA147.) It was apparent that RAPTA-C formed adducts on nucDNA147. Meanwhile, no noticeable band shift was observed for KP1019.

The results from EMSA suggested that RAPTA-C was reactive towards DNA147 and NCP147. The RAPTA-C adducts on NCP147 were formed on its DNA component, although possibility of adduct formation on histones could not be ruled out at this point. KP1019 was reactive towards DNA147 and NCP147. It is not clear whether the adducts on NCP147 were formed on the DNA component. Both ruthenium agents were more reactive towards naked DNA147 than nucDNA147, indicating the presence of protection from the compounds when DNA was wrapped around histone octamer in nucleosome.

4.2 Quantification of RAPTA-C and KP1019 adducts on DNA, NCP and nucDNA

To understand whether RAPTA-C and KP1019 favor adduct formation on DNA or protein, the number of adducts formed by RAPTA-C and KP1019 on DNA, NCP and nucDNA was measured by AAS. In addition to the 147 bp construct, we

also used a 145 bp construct, which was hypothesized to be more favorable for metal adduct formation due to the presence of stretching and kinking in the DNA element of NCP145. When the number of ruthenium adducts on whole NCP and nucleosomal DNA is known, the number of adducts on nucleosomal histone can be deduced and used to determine whether the compounds prefer to bind to DNA or histone.

Figure 4.2. Standard curve made from absorbance measurement of RAPTA-C with known concentration.

A standard curve was made from RAPTA-C solution of known concentration (Figure 4.2), and used to quantify the concentration of ruthenium in the samples (Figure 4.3). The association of RAPTA-C was higher for naked DNA than for NCP, again indicating protection phenomenon in NCP. Meanwhile, naked DNA and NCP have similar affinity for KP1019. The number of ruthenium adducts on nucleosomal DNA was very low for all samples. The direct interpretation of these results was that most of ruthenium adducts on the NCP were histone-associated. However, the extraction of nucDNA from compound-treated NCP gave a low yield (this was not the case for non-treated NCP), and this caused a suspicion of a biased result due to selective loss of ruthenated nucDNA (potentially cross-linked) during the extraction process. From the AAS data alone, the number of adducts on nucleosomal DNA and histone and therefore the preferred targets of RAPTA-C and KP1019 cannot be determined.

Figure 4.3. Graphs showing results of AAS experiments. a) RAPTA-C reacted with DNA147 or NCP147. b) RAPTA-C reacted with DNA145 or NCP145. c) KP1019 reacted with DNA147 or NCP147. d) KP1019 reacted with DNA145 or NCP145. a-d) nucDNA was extracted from drug-treated NCP. Error bar is standard deviation from two independent experiments.

4.3 Co-crystallization attempts of oligonucleotides with RAPTA-C and KP1019

4.3.1 Derivatization of ruthenated oligonucleotides

Co-crystallizations of oligonucleotides with RAPTA-C or KP1019 were conducted in an attempt to characterize the structures of RAPTA-C and KP1019 adducts on naked DNA. This was not based on an assumption that DNA is the target of action for these drugs, but instead to understand the structure of the adducts if these drugs are to bind to DNA at all. However, a note of caution was kept in our mind that any result might not represent the structure of adducts for nucleosomal DNA. Seven types of oligonucleotides were tested for RAPTA-C. Due to limitations in KP1019 availability, only three were tested for KP1019, based on the reactivity of these oligonucleotides towards RAPTA-C. The sequences of oligonucleotides used in these experiments are listed in Table 4.1. Palindromic oligonucleotide sequences with only one guanine nucleotide per strand were chosen, allowing only one adduct to form per strand.

After reaction with RAPTA-C, oligonucleotide 3c14 generally gave larger peaks in the HPLC profiles corresponding to ruthenated oligonucleotide as compared to other oligonucleotide types (Figure 4.4). Hence it was chosen for scaling-up and subsequent experiments. AAS allowed identification of peak A as unruthenated 3c14, while peaks B to F were 3c14 with roughly two RAPTA-C molecules per double strand 3c14 (one RAPTA-C per strand; Table 4.2).

Table 4.1. Oligonucleotides used in ruthenation attempt.

Oligonucleotide	Sequence (5' to 3')
3	5' A A C A A A T T T G T T
3b	5' A A A C A A T T G T T T
3d	5' A A A A A C G T T T T T
6d	5' A A A A A G C T T T T T
3b14	5' A A A A A A C G T T T T T T
3c14	5' A A A A A C A T G T T T T T
3d14	5' A A A A C A A T T G T T T T

Figure 4.4. HPLC profiles of different oligonucleotides treated with RAPTA-C at 2:1 compound to double stranded oligonucleotide molar ratio. 3c14 control (a) corresponds to HPLC run of unruthenated oligonucleotide 3c14. Letter A denotes the peak of unruthenated oligonucleotides, while B to F at (g) correspond to peaks of ruthenated oligonucleotides. Boxes at (b), (c), (d), (e), (f) and (h) correspond to region of peaks B to F. For these and all subsequent HPLC profiles, pink line corresponds to UV absorbance at 220 nm for reaction with RAPTA-C and 280 nm for reaction with KP1019, blue line at 260 nm. X axis is time in minutes, Y axis is peak intensity in mAU.

Table 4.2. AAS results of 2:1 RAPTA-C to double stranded 3c14 molar ratio.

Peaks	RAPTA-C:double strand
A	0.20:1
B	1.84:1
C	1.70:1
D	2.12:1
E	1.92:1
F	2.60:1

Figure 4.5. Scaled-up RAPTA-C – 3c14 reaction with 2:1 molar ratio. a) HPLC profile. b) MALDI-MS profile of peak C in (a).

Table 4.3. AAS results of scaled-up reaction of 2:1 RAPTA-C to double strand 3c14 molar ratio.

Peaks	RAPTA-C:DNA
A	0.12:1
C	1.28:1
E	1.24:1

Scaling-up resulted in a different HPLC profile compared to the small scale HPLC run (Figure 4.5a). Peak B and F disappeared while peak C and E became more prominent. The AAS results were also different (Table 4.3). When peak C was sent for MALDI-MS analysis (Figure 4.5b), the most prominent peak corresponded to the molecular weight of unruthenated 3c14 (4267 g/mol). A small peak of 3c14 with one molecule of RAPTA-C with two chloride ligands absent (total 4659 g/mol) was also

observed. This puzzled us because peak C was supposed to be already purified ruthenated 3c14. Another round of HPLC for peak C was carried out (Figure 4.6a). In agreement with MALDI-MS results, both peak A and C were detected instead of peak C alone. It seems that in the time between the first HPLC and the MALDI-MS and the second HPLC, some of RAPTA-C adducts were detached from the 3c14. Peak C from this second HPLC run was collected and sent for MALDI-MS (Figure 4.6b). Intriguingly, only material corresponding to unruthenated 3c14 was present, with all of RAPTA-C adducts removed from the 3c14. We thus hypothesized that the adducts of RAPTA-C on DNA were unstable and may be cleaved over time. These adducts are also likely to be nonspecific.

Figure 4.6. HPLC re-run of peak C from Figure 4.5a. a) HPLC profile. b) MALDI-MS profile of peak C from (a).

After treatment with KP1019, oligonucleotide 3b14 showed a better reactivity compared to other oligonucleotides (Figure 4.7) and was chosen for scaling-up. In small scale, peak A was confirmed by AAS to be unruthenated 3b14 (KP1019 to double-stranded 3b14 molar ratio of 0.3:1). Peak B was 3b14 with roughly two KP1019 adducts per double-stranded 3b14 (KP1019 to double-stranded 3b14 molar ratio of 2.18:1). Scaling-up gave a similar HPLC profile as a small scale HPLC run with an additional appearance of peak C (Figure 4.8a). Peak A and B were sent for MALDI-MS (Figure 4.8b and c). Peak A corresponded to unruthenated 3b14 with

molecular weight of 4263 g/mol. A peak at 4301 g/mol was probably unruthenated 3b14 with one potassium ion (MW = 39 g/mol) bound to it. Trace of potassium ion might be present in the buffers used since samples were not desalted. Peak B corresponded to 3b14 with one KP1019 per strand. Many different peaks were present in the region, likely due to different states of aquation of KP1019 and the presence of potassium ions. Peak C was present on the HPLC run of unreacted KP1019 but not that for unreacted oligonucleotide (data not shown); hence it was assigned as free KP1019 molecules.

Figure 4.7. HPLC profiles of different oligonucleotides treated with KP1019 at 5:1 KP1019 to double-stranded oligonucleotide molar ratio. A denotes the peak of unruthenated oligonucleotides, while B corresponds to peaks of ruthenated oligonucleotides.

Figure 4.8. Scaled-up KP1019 – 3b14 reaction with 5:1 molar ratio. a) HPLC profile. b) MALDI-MS profile of peak A. c) MALDI-MS profile of peak B.

4.3.2 Co-crystallizations

A homogenous population of oligonucleotide with one molecule of RAPTA-C per strand could not be purified. On the other hand, oligonucleotide 3b14 with one KP1019 per strand could be isolated, albeit with different aquation states of KP1019. Therefore, only co-crystallization of KP1019-3b14 was attempted. Due to limitation in the amount of purified KP1019-3b14, a full Natrix HR2-116 screening was done with unruthenated 3b14. Small crystals appeared with condition 27 (0.2 M ammonium acetate, 0.01 M magnesium acetate, 0.05 M Na-Cacodylate pH 6.5, 30% w/v PEG 8000) and 48 (0.2 M ammonium chloride, 0.01 M calcium chloride, 0.05 M Tris-Cl pH 8.5, 30% w/v PEG 4000) after 24 hours (Figure 4.9a and b). Large irregular crystals appeared with condition 16 (0.04 M magnesium acetate, 0.05 M Na-Cacodylate pH 6.0, 30% v/v MPD) after 12 days (Figure 4.9c). X-ray diffraction data

were collected and a diffraction pattern with characteristic of DNA double helix was observed (Figure 4.9d) although resolution was very low (~ 8 Å). These three conditions were pursued further by changing the concentration of each ingredient (Table 4.4). This extended screening did not yield crystals with better quality than the original conditions. Therefore, crystallization of KP1019-3b14 with conditions 16, 27 and 48 were set up. Unfortunately, no crystals grew under these conditions.

Figure 4.9. Crystal from Natrix HR2-116 screening using unruthenated 3b14. a) Crystals from condition 27. b) Crystals from condition 48. c) Large irregular crystal from condition 16. d) Diffraction pattern of crystal from (c).

Table 4.4. Conditions used for further screening of condition 16, 27 and 48.

No.	Difference from original condition
16a	0.06 M Mg(CH ₃ COOH) ₂
16b	0.02M Mg(CH ₃ COOH) ₂
16c	0.075M Na-Cacodylate pH 6.0
16d	0.025M Na-Cacodylate pH 6.0
16e	35% v/v MPD
16f	25%v/v MPD
27a	0.3M CH ₃ COONH ₄
27b	0.1M CH ₃ COONH ₄
27c	0.015M Mg(CH ₃ COO) ₂
27d	0.005M Mg(CH ₃ COO) ₂
27e	0.075M Na-Cacodylate pH 6.5
27f	0.025M Na-Cacodylate pH 6.5
27g	35% w/v PEG 8,000
27h	25% w/v PEG 8,000
27i	40% w/v PEG 8,000
27j	20% w/v PEG 8,000
27k	0.9x original condition
27l	0.8x original condition
27m	0.7x original condition
27n	0.6x original condition
27o	0.5x original condition
48a	0.3M NH ₄ Cl
48b	0.1M NH ₄ Cl
48c	0.015M CaCl ₂
48d	0.005M CaCl ₂
48e	0.075M Tris-Cl pH 8.5
48f	0.025M Tris-Cl pH 8.5
48g	35% w/v PEG 4,000
48h	25% w/v PEG 4,000
48i	40% w/v PEG 4,000
48j	20% w/v PEG 4,000
48k	0.9x original condition
48l	0.8x original condition
48m	0.7x original condition
48n	0.6x original condition
48o	0.5x original condition

4.4 Stability of RAPTA-C and KP1019 adducts on DNA

Even though EMSA and AAS showed reactivity of RAPTA-C towards DNA, the difficulty to isolate oligonucleotide derivatized with RAPTA-C made us think that RAPTA-C adducts on DNA are unstable. We designed a reversibility assay where ruthenium agent-treated DNA145 was separated from unreacted ruthenium agent molecules by passing the samples through a spin column, leaving at room temperature for 24 hours, and passing through a spin column for the second time. Hence, any ruthenium agent which was released from DNA145 after the first clean-up would be removed during the second clean-up. Besides RAPTA-C, KP1019 was also investigated in this assay as it is also reactive towards DNA.

Figure 4.10. Reversibility assay of RAPTA-C and KP1019 binding to DNA. a) AAS results of reversibility assay for reaction of RAPTA-C (RC) or KP1019 with DNA145. Efficiency corresponds to the % of ruthenium, relative to treatment, that remains DNA-associated. b) The same samples as (a) run in 10% PAGE (lanes: L - 1 kb DNA ladder; 1 – DNA145; 2 to 5 - RAPTA-C : DNA145 2/10/50/200:1; 6 to 8 - KP1019 : DNA145 2/10/50:1).

After the second clean-up, most of the RAPTA-C adducts were removed from DNA145, as shown by the large decrease in binding efficiency (Figure 4.10a). At 50:1 compound to DNA145 ratio, binding efficiency decreases from around 50% to around 10% for RAPTA-C, as opposed to only about 10% decrease for KP1019. Removal of RAPTA-C adducts from DNA145 can also be observed by comparing lane 5 in both gels in Figure 4.10b, where the sample migrated faster after the second clean-up, suggesting less amount of compound bound to DNA145 compared to after the first clean-up. These observations suggest that there were two types of DNA adducts: stable adducts (which were probably specific, covalent adducts) and unstable adducts (likely non-specific and non-covalent). RAPTA-C seemed to form more of the unstable adducts, which were detached from DNA over time, leaving only a small amount of stable adducts. Meanwhile, KP1019 seemed to form more of the stable adducts.

4.5 Binding sites of metal-based compounds on nucleosome core

The binding sites and structures of metal-based compound adducts on the nucleosome core were studied by soaking NCP145 crystals with metal agent and collecting X-ray diffraction data. For NCP145 crystals soaked with heavy metals, the optimal data collection wavelength would be at the absorption edge of that particular heavy metal (Figure 4.11). For NCP crystals soaked with osmium or gold compounds, data were collected at their absorption edges of 1.14 Å and 1.04 Å, respectively, which is achievable at most synchrotrons. The absorption edges of ruthenium and rhodium are at 0.56 Å and 0.53 Å, which are difficult to achieve at most synchrotron sources. We chose 1.50 Å for X-ray diffraction for ruthenium and rhodium compound-treated NCP145 crystals as these metals still absorb X-rays with observable signals at this wavelength. The harvest buffer for NCP145 crystals contained manganese which has a similar X-ray absorption capacity as ruthenium and rhodium at 1.50 Å. In the presence of manganese and ruthenium or rhodium, it is not possible to distinguish whether anomalous signal comes from manganese or ruthenium or rhodium. Before soaking, manganese in the buffer was replaced with magnesium, which does not absorb X-rays at 1.50 Å. At the same time, chloride ions,

which can prevent aquation and binding of metal compounds, were also removed. This strategy allowed confident assignment of metal agents' binding sites and structures on the nucleosome core. The complete removal of manganese is evident from the absence of any anomalous difference electron density signal at the most ordered (initially) manganese site in the crystal (Figure 4.12b).

Figure 4.11. X-ray absorption profiles (f'') of Ru and Os (top) and Rh and Au (bottom) compared to Mn and Mg. Figures were adapted from http://skuld.bmsc.washington.edu/scatter/AS_periodic.html.

When metal agents react with the NCP crystals, it would interfere with the ordering of molecules in the lattice, thus disturbing crystal integrity and quality. When

the quality of the crystals was significantly reduced, we would only get lower resolution data, from which accurate information on the structure of the adducts cannot be obtained. With higher concentrations of metal agents and longer soaking times, crystals would get progressively more damaged. Consequently, we needed to make a compromise between getting high binding site occupancy and good resolution data. The concentration of metal compounds and the duration of soaking were adjusted for different types of agents, in an effort to get the best data with good resolution and detectable adducts. The soaking conditions are summarized in Table 4.5. An example of the diffraction profile of NCP145 crystal treated with ruthenium agent is shown in Figure 4.12a.

Figure 4.12. Diffraction image of and manganese removal from an NCP145 crystal. a) Example of diffraction profile of NCP145 crystal treated with ruthenium agent. b) The most ordered divalent metal site (light blue sphere) is located between two NCP145 molecules (yellow and green carbon atoms). This divalent metal site was devoid of anomalous difference electron density, indicating the absence of manganese at this site (full replacement by Mg^{2+}). 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (red, contoured at 3σ) are superimposed on the model.

Table 4.5. Soaking conditions for different metal-based agents.

Compound	Concentration (mM)	Duration (hours)
RAPTA-C	0.750	48
RAPTA-B	1.000	5 / 19 / 19
RAPTA-T	1.000	3 / 3
RAPTA-3Me	1.000	3 / 3
RAPTA-H	0.250	42
	1.000	4 / 4
RAPTA-I	1.000	5 / 21 / 21
RAED-C	1.000	21 / 24 / 41
OsAPTA-C	1.000	22 / 22 / 27 / 68 / 93 / 120 / 166
	4.000	21 / 68 / 68 / 68 / 68 / 68
NAMI-A	1.000	24 / 25 / 31 / 69 / 97
KP1019	1.000	40 / 90 / 90
Alna058	<0.250	18 / 43
	1.000	25
Alna094	< 0.250	23 / 41 / 44 / 69
Alna159	0.250	43
Alna071	<1.000	21 / 68
Alna122	<1.000	20 / 68
KP1581	0.125	20
	0.250	19 / 19
KP1582	1.000	23 / 46
ELJI085	<1.000	22 / 48
ELJI079	<1.000	22 / 47
ELJI065	<1.000	44 / 44 / 96
SAME136	<1.000	46 / 96
KP1535	0.250	21 / 27
KP1648	<1.000	25 / 26
KP1653	<1.000	27 / 71 / 97
KP1548	1.000	26 / 68 / 93
KP1745	< 0.250	24 / 28
KP1834	< 0.250	23
Rh-MAL	0.250	19
Auranofin	1.000	24 / 68 / 92
Auranofin + RAPTA-T	2.000+0.500	16 / 43 (Ru edge) 17 / 43 (Au edge)
	2.000+0.500 (after 22h RAPTA-T was topped up to 1.000)	40 (Ru edge) 40 / 40 (Au edge)

From the data of 23 ruthenium, 4 osmium, 1 rhodium and 1 gold compounds tested so far, 9 different sites on histone proteins were observed. These are depicted as sites 1, 2, 3, 4/4', 5, 5', 6', 7 and 7' in Figure 4.13. A prime symbol denotes a symmetry-equivalent site in the other half of the NCP. In addition, there were two

DNA adduction sites observed at the one and one-half turn positions from the dyad, on both halves of the nucleosome. The adduction sites of each agent are listed in Table 4.6. Closer inspection of the sites are illustrated for different metal-based compounds adducts in sections 4.6 to 4.11.

For some of the sites, only those on one half of the NCP were occupied by adducts. For sites 2 and 3, this is easily understandable because site 2' and 3' are sterically blocked in NCP145 crystals (Figure 4.14). Around this region, histones H2A and H2B form an electronegative cleft in which the positively-charged tail of histone H4 from a neighboring nucleosome is inserted. Even though metal-hydrates can bind to these sites¹⁶¹, bulkier ruthenium or osmium agents would not be able to access them. For site 1, it seems that the opposite scenario is the case. Site 1 is the location of the major interparticle interaction in NCP145 crystals, which involves a divalent cation bridge. The presence of adduct at this site and not at the more sterically accessible site 1' implies that extensive interactions with neighboring NCP assist the formation of adducts. Site 4 and 4' are located at the center of the nucleosome core. Histone H2A Glu41 residues from the two nucleosome halves are located very close to each other. The anomalous signal seen at this location seemed to be resulting from a mixture of binding to either one of the two available Glu41 side chains. Sites 5, 5', 7 and 7' are accessible and adducts were found at all of these sites. Site 6 is the location of the single Mg^{2+} ion bound to NCP145 and not available for metal agent binding, hence only site 6' is available for binding. Site 6' showed positive Fo-Fc electron density but no strong anomalous difference electron density, meaning this site is a potential, but not definite, binding site for some of the metal agents. An aspartate residue from H3' might act as the potential binding partner if a metal agent is bound there (Figure 4.15).

Figure 4.13. Major sites of adduct formation for ruthenium, osmium, rhodium and gold anticancer candidates. DNA strands are colored orange and cyan. Histone proteins are blue for H3, green for H4, yellow for H2A, and red for H2B. Top: view is down the dyad axis with DNA superhelix axis oriented vertically. Bottom: view is after 180° rotation of the top view. Prime symbol denotes symmetry-equivalent site in the other half of NCP145.

Figure 4.14. Crystal packing of NCP145. Color scheme is the same as in Figure 4.13. Dashed pink arrows show possible route of entry for small molecules to different sites of the NCP. Sites 2 and 3 of the middle NCP molecule are accessible, while site 2' and 3' are sterically blocked due to interaction with neighboring NCP. Site 1, where interparticle interactions occur, was occupied. Meanwhile, site 1' was not occupied even though it is sterically accessible.

Table 4.6. Binding sites of different metal-based compounds on the NCP145.

Metal agent	Binding Sites										
	1	2	3	4/4'	5	5'	6'	7	7'	SHL +1.5	SHL -1.5
RAPTA-C	X	X	X								
RAPTA-BT3HI		X	X								
RAED-C		X		X						X	X
OsAPTA-C											
NAMI-A		X		X			X				
KP1019		X									
Alna058		X	X								
Alna094		X	X								
Alna159		X	X								
Alna071		X									
Alna122		X									
KP1581			X		X	X					
KP1582											
ELJI085					X	X					
ELJI079			X		X	X					
ELJI065			X		X	X					
SAME136					X	X					
KP1535			X		X	X					
KP1648			X		X	X					
KP1653						X					
KP1548			X		X	X					
KP1745			X		X						
KP1834			X		X	X					
Rh-Maltol			X								
Auranofin											
Auranofin +RAPTA-T		X	X					X	X

Figure 4.15. Site 6'. NCP145 model is shown with 2Fo-Fc electron density map (gray, contoured at 1σ), positive Fo-Fc electron density map (green, contoured at 3σ) and anomalous difference electron density map (red, contoured at 3σ) superimposed on the model.

Anomalous difference electron density was also observed near the bases of DNA terminal nucleotides for some of the agents (Figure 4.16). The ruthenium center seemed to be coordinated to N7 of the terminal adenine on both I and J chains of DNA. This is the most sterically accessible purine base in the NCP⁹⁸. Since there are effectively no DNA termini *in vivo*, the adduct formation at these sites seems unlikely to be relevant to the mechanism of action *in vivo*. Hence, we regard these adducts as DNA end artefacts. Interestingly, many experiments in the field of metal anticancer agents used single nucleotides or short oligonucleotides as the substrates^{115,162,163}. Single nucleotides and short oligonucleotides are more susceptible to the DNA end artifacts and hence can yield misleading results in considering the preferred binding partner of metal-based agents.

Figure 4.16. DNA terminus binding artefact. a) and b) NCP145 model is shown with 2Fo-Fc electron density maps (gray, contoured at 1σ), positive Fo-Fc electron density maps (green, contoured at 3σ) and anomalous difference electron density maps (red, contoured at 3σ) superimposed on the model. a) The NCP145 crystal was treated with NAMI-A. Positive Fo-Fc electron density and anomalous difference electron density are present near Adenine -72 of DNA chain I from one NCP molecule (carbon shown in yellow). b) NCP145 crystal had been soaked with KP1834. Positive Fo-Fc electron density and anomalous difference electron density are present near Adenine -72 of DNA chain J from one NCP molecule (carbon shown in yellow) and Adenine -72 of chain I from neighboring NCP molecule (carbon shown in green).

Atomic model building has been done for RAPTA-C, RAED-C, Alna094, KP1834, Alna071, Alna122, ELJI065, ELJI079, ELJI085, SAME136 and RAPTA-T/auranofin. Data collection and refinement statistics are listed in Table 4.7. For the remaining metal agents, adducts were not built into the models, but adduction sites were identified based on 2Fo-Fc, Fo-Fc and anomalous difference electron density maps.

Table 4.7. Data collection and refinement statistics of NCP145 crystals soaked with different metal-based agents. Soaking with RAPTA-C was done by B. Wu and M. Ong.

Anticancer agent	RAPTA-C	RAED-C	KP1834	Alna094	Alna071
Soaking time (hours)	56	21	23	23	68
Soaking concentration (mM)	0.75	1.00	0.25	0.25	1.00
Data Collection					
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions a (Å)	106.35	106.62	106.43	106.80	106.59
b (Å)	109.86	109.71	107.75	109.69	109.76
c (Å)	182.59	181.94	181.28	182.67	182.06
Resolution (Å) (highest shell)	2.50-94.1 (2.50-2.64)	2.69-60.6 (2.69-2.84)	3.18-93.9 (3.18-3.36)	2.66-48.8 (2.66-2.81)	2.56-54.9 (2.56-2.70)
<i>R</i> _{merge} % (highest shell)	6.1 (48.4)	5.4 (48.7)	6.4 (49.8)	5.4 (49.7)	6.0 (49.3)
<i>I</i> / <i>σI</i> (highest shell)	9.7 (1.7)	18.5 (2.6)	16.5 (3.4)	18.2 (3.5)	15.7 (2.1)
Completeness % (highest shell)	99.7 (98.2)	93.1 (66.4)	98.2 (89.7)	98.4 (96.8)	93.3 (67.7)
Redundancy (highest shell)	4.4 (3.5)	6.8 (4.8)	6.5 (5.2)	6.8 (6.7)	6.3 (3.8)
Refinement					
Resolution (Å)	2.50-94.1	2.69-60.6	3.18-93.9	2.66-48.8	2.56-54.9
No. reflections	72837	54577	33808	59632	63261
<i>R</i> _{work} / <i>R</i> _{free} (%)	24.7/27.7	24.8/28.2	26.3/29.9	24.6/28.8	25.2/26.9
No. atoms	12126	12093	12075	12079	12052
Protein	6086	6078	6086	6086	6086
DNA	5939	5939	5939	5939	5939
Solvent	38	16	16	16	16
Adduct	63	60	34	56	11
<i>B</i> -factors (Å ²)	86	84	105	79	75
Protein	59	57	78	51	48
DNA	113	112	133	108	102
Solvent	74	88	119	99	74
Adduct	130	163	152	172	116
R.m.s. deviations					
Bond lengths (Å)	0.008	0.009	0.006	0.009	0.010
Bond angles (°)	1.48	1.43	1.35	1.45	1.43

Alna122	ELJI085	ELJI079	ELJI065	SAME136	Auranofin + RAPTA-T
68	48	47	44	46	40
1.00	1.00	1.00	1.00	1.00	2.00+1.00
P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
106.58	106.80	106.48	106.71	106.78	106.73
109.87	109.81	109.82	109.93	109.73	109.95
182.00	182.38	181.39	181.84	181.91	182.04
2.40-54.9 (2.40-2.53)	2.58-60.8 (2.58-2.72)	2.87-60.5 (2.87-3.03)	2.38-60.6 (2.38-2.51)	2.41-58.6 (2.41-2.54)	2.80-48.9 (2.80-2.95)
5.0 (45.0)	6.8 (47.8)	11.7 (32.9)	4.1 (45.9)	7.5 (40.4)	3.7 (43.3)
18.1 (2.0)	15.8 (2.3)	8.2 (2.0)	21.3 (2.1)	12.8 (2.0)	23.2 (1.6)
90.4 (59.6)	84.2 (46.5)	99.7 (98.5)	82.6 (39.3)	96.5 (81.6)	95.8 (76.6)
6.4 (3.6)	6.4 (4.2)	6.4 (3.5)	6.1 (3.0)	6.1 (2.9)	5.0 (2.0)
2.40-54.9	2.58-60.8	2.87-60.5	2.38-60.6	2.41-58.6	2.80-48.9
74491	55962	48147	69745	78440	48472
25.7/27.1	25.2 / 27.3	23.7 / 28.0	25.2 / 28.0	26.5 / 27.5	21.5/23.6
12052	12063	12063	12119	12122	12093
6086	6086	6086	6086	6086	6086
5939	5939	5939	5939	5939	5939
16	16	16	16	16	16
11	22	22	78	81	52
76	77	72	76	75	99
49	51	45	47	48	70
104	105	99	104	102	132
78	74	85	78	75	121
145	123	109	119	117	145
0.009	0.008	0.010	0.009	0.009	0.007
1.33	1.30	1.50	1.34	1.28	1.46

4.6 Protein versus DNA targeting and anticancer activity

RAPTA-C and RAED-C are two related compounds from the ruthenium-arene class containing chloride leaving groups. The only difference between the two lies in the 1,3,5-triaza-7-phosphaadamantane (PTA) and one chloride group of RAPTA-C being replaced by ethylenediamine group in RAED-C. This means that RAPTA-C has 2 chloride leaving groups while RAED-C has only one. Our results based on X-ray diffraction of NCP145 crystals and DNA footprinting show that this modest distinction resulted in a difference in their preferred binding target; RAPTA-C binds mainly to protein while RAED-C favors binding to DNA (see below). A summary of the nucleosome core binding sites of these two compounds is shown in Figure 4.17.

When NCP crystals were soaked with RAPTA-C, we found 3 binding sites on the histones on the surface of the NCP and no DNA adducts. The structure of RAPTA-C adducts at sites 1, 2 and 3 are depicted in Figure 4.18. RAPTA-C was bound to Lys59 and Glu63 of histone H4 at site 1. As mentioned above, binding to this site also involved extensive hydrophobic interactions with residues from histone H3 and H4, including those which coordinate the Mg^{2+} ion, and even involves histone H2B of a neighboring NCP molecule (Figure 4.18b). In fact, from all the compounds examined in this study, only RAPTA-C binds to this site. RAPTA-C seems to have just the appropriate size and shape to bind within the crowded environment at this site. Compounds that are too large cannot be accommodated here, while those that are too small cannot make sufficient hydrophobic interactions needed for binding. This also implies that hydrophobic interactions may account for the initial attractive forces for association at site 1.

RAPTA-C bound to Glu61 and Glu64 of histone H2A at site 2 and His106 of histone H2B at site 3. Binding to site 3 also involved possible weak or partial interaction with Glu102 of histone H2B. Besides hydrophobic interactions with nearby histone elements, binding to sites 2 and 3 also encompassed van der Waals interactions between the arene group of RAPTA-C molecule at site 2 and the PTA group from that at site 3 (Figure 4.18b), suggesting cooperativity of binding to these two sites. Data from soaking with different durations and/or concentrations of RAPTA-C and related compounds revealed that site 2 was derivatized first. Compounds that did not bind to site 3 are probably unable to form the necessary non-polar contacts with the first bonded molecule of site 2.

Figure 4.17. Binding sites of RAPTA-C and RAED-C on the nucleosome. a) Chemical structure and adduction sites in the NCP crystal. NCPs are shown looking down the superhelix axis. Dyad positions are marked by arrows. Colour scheme of NCP is the same as in Figure 4.13. Adducts are shown in space-filling representation (grey). b) Adduction sites on naked and nucleosomal DNA in solution (Figure 4.20) are shown as arrowheads (filled – strong signal, open – moderate signal). Adduction sites on nucleosomal DNA in NCP crystal are shown as green dots. DNA sequence in orange denotes regions where minor grooves face the histone octamer. Dyad positions are marked by black arrows. Stretched regions are depicted with magenta arrows. Figure is adapted from ref. 164.

Figure 4.18. RAPTA-C adducts on histones. a) Structure of the RAPTA-C adducts with 2Fo-Fc density map (rust, contoured at 1σ) and anomalous difference electron density map (cyan, contoured at 4.0σ (top) and 4.5σ (bottom)) are superimposed on the model. b) Hydrophobic interactions formed at the adduction sites. Arrow (bottom) denotes interaction between the adducts at sites 2 and 3. Ru and Mg atoms and water molecules are shown as magenta, black and red spheres, respectively. Neighboring NCP is shown in grey. Coordinate (-covalent) bonds are represented by magenta and black lines. Ru-coordinating and nearby histone protein side chains are shown in (a) while those forming hydrophobic interactions with the adducts are shown in (b). Figures are adapted from ref. 165.

RAED-C formed adducts at 2 histone sites: site 2, as in the case of RAPTA-C, and also site 4/4' (Figure 4.19, bottom panels). RAED-C did not form adduct at site 3, likely due to the ethylenediamine group's inability to form adequate hydrophobic contact with the arene group from site 2. The same reason might account for the absence of binding to site 1. On the other hand, RAPTA-C did not form adduct at 4/4' likely due to the bulkiness of PTA group which prevented access to this site. RAED-C also formed adducts at two symmetry-related DNA sites at the SHL ± 1.5 of the NCP (Figure 4.19, middle panels). In fact, it is the only compound among those tested in the NCP crystal soaking study, which showed substantial DNA binding. The structure of the DNA adduct sites are essentially the same in both halves of the NCP. The ruthenium atom of RAED-C was coordinated to the N7 atom of the middle

guanine base of an AGG element. One of the amine groups of the ethylenediamine ligand of RAED-C also formed a hydrogen bond with the O6 atom of the guanine base. In addition, hydrophobic interactions were formed with nearby bases and sugar-phosphate backbone. SHL ± 1.5 are the locations of stretching and kinking in the NCP145 structure. Consequentially, the guanine bases at these locations are more accessible for binding by RAED-C. Although both SHL ± 1.5 locations encompass stretching and kinking of the DNA, their structures in native NCP are very different from each other. After RAED-C binding, however, the structure of the derivatized state of these two locations became almost identical. This suggests that the RAED-C binding caused these structural changes, and there is an intrinsic preference for the derivatized conformation to accommodate RAED-C binding. Comparing the native and derivatized states, RAED-C coordination to the guanine base would cause steric clashes between the ethylenediamine group of RAED-C and the adenine base at the 5' location (Figure 4.19, top panels). Conformation rearrangement upon RAED-C binding involves some unstacking of the 5' adenine away from the coordinating guanine. This also coincides with the DNA stretching relocating from the SHL ± 1.5 to the SHL ± 5 locations (magenta arrows in Figures 4.17b and 4.20).

To study the DNA binding site preference of RAPTA-C and RAED-C to NCP145 in solution, we employed exonuclease-III DNA footprinting. Naked DNA and NCP145 were both derivatized with the ruthenium compounds and nucleosomal DNA was then extracted from the derivatized NCP145. Labeling of both DNA 5'-ends did not pose a problem since the construct used was palindromic in nature. We found a significant difference in binding site preference between naked and nucleosomal DNA for both compounds (Figures 4.17b and 4.20). An extensive amount of sites derivatized in naked DNA were protected in nucleosomal DNA due to changes in conformation/dynamics induced by histone octamer packaging. Both RAPTA-C and RAED-C showed selectivity towards GG dinucleotides, with reactivity apparently governed by initial electrostatic attraction. RAPTA-C had low reactivity towards both naked and nucleosomal DNA, except at the DNA termini (a DNA end artefact; see section 4.5) and SHL ± 1.5 (also the preferred location of RAED-C attack). In NCP145, RAED-C formed DNA adducts at SHL ± 1.5 and ± 2.5 and the last double helical turn of the termini. The adducts at SHL ± 1.5 and ± 2.5 were more prevalent than in naked DNA. Moreover, the adduct location seemed to be shifted by one nucleotide relative to the adducts formed on naked DNA. This means packaging

of DNA into nucleosome can increase reactivity for certain structurally preferred sites and also shift inherent reactivity by a single nucleotide.

Figure 4.19. Crystal structures of RAED-C adducts on nucleosome core. Top – Space filling representation of DNA conformational change from native NCP (cyan) to RAED-C derivatized (magenta). Middle – Structure of RAED-C adducts on SHL ± 1.5 . Bottom - Structure of RAED-C adducts on histones. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines and hydrogen bonds black dashed lines. Ru-coordinating and proximal residues are shown. Figure is adapted from ref. 164.

Figure 4.20. Exonuclease footprinting of naked (D) and nucleosomal (N) DNA derivatized with RAPTA-C or RAED-C. Purine markers are shown as “m”. Green asterisks denote location of guanines. Stretched regions are shown as magenta arrows (solid – native NCP, dashed – RAED-C derivatized NCP). Figure is adapted from ref. 164.

The results from X-ray diffraction and DNA footprinting experiments showed that while RAED-C is reactive towards both DNA and protein components of nucleosome core, RAPTA-C favors histone binding and has low reactivity for DNA. These are in agreement with *in vitro* and *in vivo* works done by our collaborators, summarized below (data will not be shown, see ref. 164).

Inductively coupled plasma mass spectrometry (ICP-MS) was employed to quantify the reactivity of RAPTA-C and RAED-C towards NCP in solution and chromatin in ovarian cancer cells. In ICP-MS, sample is ionized by passing it through high temperature argon plasma formed by electromagnetic induction. The ionized sample is then streamed to a mass spectrometer. In solution, 85% of RAPTA-C adducts and 61% in cells were associated with the histone component, while 71% of RAED-C adducts in solution and 77% in cells were associated with the DNA component. Hybrid quantum mechanical/molecular mechanical computational calculations also revealed their difference in site selectivity, with RAPTA-C having a 2-fold higher reaction energy barrier compared to RAED-C in forming DNA adduct. Moreover, RAPTA-C-DNA adducts are also energetically less stable than those of RAED-C. This is a result of the bulkier PTA ligand of RAPTA-C, which yields steric obstacles in accommodating adducts on the double helix. Meanwhile, the activation energy for histone site binding is similar for both compounds.

Treatment of ovarian cancer cells with RAPTA-C and RAED-C resulted in different patterns of cellular impact, for example, RAPTA-C induced more G2/M-phase arrest than RAED-C, although their overall effects in inducing apoptosis and cell cycle arrest were less severe than that of cisplatin. Ovarian cancer cells recovered faster from the effects of ruthenium compound treatment compared to that with cisplatin. These compounds also showed little or no cross-resistance with cisplatin, suggesting different mechanisms of action, even though both cisplatin and RAED-C target DNA.

The DNA binding preference of RAED-C (~6.4% of total cellular uptake) is switched to protein-favoring of RAPTA-C by exchanging the ethylenediamine to a PTA ligand, suggesting the potential of controlling drug targeting preference by modulating ligands of metal-based compounds. The difference in site selectivity between DNA and protein binding may account for different effects of the highly cytotoxic RAED-C and the relatively non-cytotoxic RAPTA-C, with RAED-C mainly active against primary tumors, whereas RAPTA-C is highly active against metastasis.

Histone proteins may be involved in the RAPTA-C cellular mechanism of action, since ~2.5% of total cellular uptake of RAPTA-C was bound to histones, as compared to ~4.1% cisplatin binding to DNA¹⁶⁶. However, binding to other nuclear, cytoplasmic and even extracellular matrix proteins may also play role in its therapeutic effects. In addition, the bifunctional nature of RAPTA-C binding potential may also play role in its binding and activity. For example, RAPTA-C may form cross-links between DNA and protein or between different sites in DNA or protein.

For this interdisciplinary study, the ICP-MS works were done by M. Groessl, C. Clavel, C.H.F. Yeo, and W.H. Ang, the computational calculations were carried out by P. Campomanes and U. Röthlisberger and all cell-related experiments were done by G.E. Davey (with contributions from H. Yu and P. Dröge).

4.7 Combining protein and DNA binding

Alna094 and Alna159 are composed of RAPTA components with nitrogen mustard chlorambucil alkylating groups linked to the arene part. It was hoped that these agents will bind to protein through their ruthenium center and alkylate DNA through the chlorambucil component, possibly forming protein-DNA crosslinks. Two histone binding sites were observed for both compounds, but we did not observe DNA binding with these agents.

The structure of Alna094 adducts at sites 2 and 3 are depicted in Figure 4.21. We did not observe any electron density for the alkylating group of Alna094, likely because of its disordered nature. The RAPTA part of Alna094 behaved like RAPTA-C and bound to the same residues as RAPTA-C at sites 2 and 3. The PTA group from the Alna094 in site 3 also formed hydrophobic interactions with the arene from site 2. The alkylating group did not seem to interfere with binding at these sites. Alna094 did not occupy site 1. Alna094 is larger than RAPTA-C and the alkylating group may render the molecule too big to enter site 1, which is sterically crowded.

Figure 4.21. Alna094 adducts at sites 2 and 3. NCP145 model (yellow carbon) and Alna094 adducts (green carbon) is shown with 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (teal, contoured at 3σ) superimposed on the model. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines.

Experiments in solution demonstrated that Alna094 was able to bind to ethyl-guanine both through its ruthenium center and the alkylating group¹⁶⁷. Only a minor fraction of Alna094 was found to crosslink amino acid and DNA base, binding to amino acid through the ruthenium center and alkylating ethyl-guanine through the arene-chlorambucil part. Despite this, Alna094 was found to be more cytotoxic than chlorambucil or RAPTA-T (closest analogue among RAPTA compounds) alone, or combination of both, in A2780r and MCF7 cell lines.

Coordination through the ruthenium center was found to be faster than alkylation. Moreover, compounds in the RAPTA series were found to prefer protein binding. Hence in the crystal structure, we found adducts only through the ruthenium center at protein sites. In the environment of the preferred nucleosome binding sites (sites 2 and 3), the nearest DNA site available for binding is about 20 Å away. Hence, alkylation with protein-DNA crosslinking are not possible with Alna094. Furthermore, the arene group in Alna094 was found to be labile and can be cleaved off in solution. Longer linker and more stable arene coordination to the ruthenium center may be needed for crosslinking to occur in the nucleosomal context.

4.8 Effect of carrier ligand cleavage

KP1834 is a complex of ruthenium arene and thiomorpholine-substituted hydroxypyridone ligand. Hydroxypyridone compounds have been used as MRI contrasting media, metal chelators, antiviral and antibacterial agents and for other medicinal purposes¹⁶⁸⁻¹⁷². The structure of KP1834 adducts at sites 3, 5 and 5' are depicted in Figure 4.22. The carboxylate ligand was not observed in the electron density. Instead, there was density for water molecule at site 3 (red sphere in Figure 4.22a). This is in agreement with ESI-MS results of binding of KP1834 to amino acids, histidine and methionine, and also proteins, ubiquitin and transferrin, where only the ruthenium and cymene group was observed to bind to the proteins, while the pyridine ligand has been cleaved off (C.G. Hartinger lab, unpublished). The ruthenium atom is bound to the same group as RAPTA-C at site 3. At sites 5 and 5', the ruthenium center of KP1834 was bound only to one nitrogen atom of His79 of histone H2B.

The carrier groups of metal-based agents are usually assumed to stay with the metal center, while the leaving groups would be exchanged with water before reaction with cellular molecules. However, we observed that the carrier ligands of at least three agents (KP1834, ELJI079 and ELJI085; see below) investigated in this study were cleaved from the metal center. Despite the carrier group being cleaved off, KP1834 showed high antiproliferative effects, with IC₅₀ in the low micromolar range for different cancer cell types (CH1, SW480 and A549 cells), which was lower than that for an analogue with a more stable carrier ligand (C.G. Hartinger lab, unpublished). These results were unexpected and suggest that carrier group cleavage does not equate with inactivation of the compounds. Instead, these compounds may act as pro-drugs which became active after being detached from their carrier ligands. Otherwise, cleavage may happen after binding, with transition states still containing the intact carrier groups. This factor needs to be considered in designing and fine-tuning metal-based compounds. Stability of compounds always needs to be tested since compounds may behave differently in more complex, more physiologically relevant, contexts.

Figure 4.22. KP1834 adducts at site 3, 5 and 5'. NCP145 model (yellow carbon) and KP1834 adducts (green carbon) is shown with 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (teal, contoured at 3σ) superimposed on the model. Ru-coordinating side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines. a) Site 3. b) Sites 5 and 5'.

4.9 Tri-ruthenium clusters compounds

Alna071 and 122 are unique compared to other multinuclear metal compounds studied here in that they contain direct metal-metal bonds. The structure of Alna071 and Alna122 adducts at site 2 are depicted in Figure 4.23. Only one of the three ruthenium atoms in each compound formed covalent bonds with histone components at site 2. There seems to be no nucleosome core component that is close enough for the other two ruthenium atoms to bind. Site 3 is too far for binding by other ruthenium

atoms from one molecule. The bulkiness of these compounds may prevent binding of two molecules to sites 2 and 3, and since site 2 seems to be the more reactive site, only this site was observed to be occupied by these compounds. Only density for one ruthenium atom and one PTA group was observed for either compound. The remaining parts of the compounds may be disordered because they were unbound.

Related compounds of tri-ruthenium clusters with bicyclopophosphite instead of PTA group have been shown to exhibit antiproliferative and antiangiogenic activities¹⁷³. It is not clear whether these effects came from covalent binding of the ruthenium center to cellular targets or carbon monoxide release, since carbon monoxide-releasing compounds have also been shown to induce antiproliferative effects¹⁷⁴.

Figure 4.23. Adducts of Alna071 (a) and Alna122 (b) at site 2. NCP145 model (yellow carbon) and Alna068 and Alna122 adducts (green carbon) is shown with 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (teal, contoured at 3σ) superimposed on the model. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines.

4.10 Different binding properties of osmium and ruthenium compounds

Compounds in the ELJI series (ELJI079, ELJI085, ELJI065 and SAME136) contain carbothioamide ligands which by themselves were shown to have gastric mucosal protection effects, but very low acute toxicity ($>1\text{g/kg}$)¹⁷⁵. Placing carbothioamide ligands in metallodrug complexes with ruthenium or osmium centers

increased the cytotoxicity. These compounds represent the first reported metal-based drug candidates with S,N-bidentate ligand systems¹⁷⁶. Metal-based drugs are usually present in a pro-drug state and activated by hydrolysis which leads to aquation and binding to biomolecules. For the ELJI compounds, hydrolysis did not lead to aquation but to ligand cleavage and dimerization. Dimerized molecules seemed to be inactive but an equilibrium between monomer and dimer states provided monomeric molecules for binding to cellular targets. In fact, dimerization may act to prevent deactivation by binding to sequestering molecules in the blood stream or cellular environments.

ELJI079 and ELJI085, having ruthenium centers, bound to His79 from histone H2B at sites 5 and 5'. In addition, the carbothioamide ligands were cleaved off the metal center. It was not clear whether cleavage happened before, during or after binding to histidines. Ligand cleavage usually correlated with reduced cytotoxicity; however, both compounds still have high antiproliferative activity. ELJI065 and SAME136, having osmium centers, bound also to His79 at sites 5 and 5', and additionally to His106 from histone H3 at site 3. The carbothioamide ligands remained intact and seemed to be involved in hydrophobic contacts with nearby histone elements. Sites 5 and 5' are located at histone dimer-dimer and dimer-tetramer interfaces, and adducts formed at these sites may interfere with chromatin dynamics. Compounds with larger carbothioamides may be sterically hindered from binding to these sites.

Figure 4.24. Adducts of ELJI085 and ELJI079 on NCP145. NCP145 model (yellow carbon) and ELJI085 and ELJI079 adducts (green carbon) is shown with 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (teal, contoured at 3σ) superimposed on the model. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines.

Figure 4.25. Adducts of ELJI065 and SAME136 on NCP145. NCP145 model (yellow carbon) and Alna094 ELJI065 and SAME136 adducts (green carbon) is shown with 2Fo-Fc electron density map (gray, contoured at 1σ) and anomalous difference electron density map (teal, contoured at 5σ) superimposed on the model. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines. Ru-coordinating histone protein side chains are shown. Ru atoms are shown as magenta spheres. Coordinate (-covalent) bonds are represented by magenta lines.

Although ruthenium and osmium compounds can be considered as bioisosteres with similar pseudo-octahedral geometries, osmium compounds have

slower hydrolysis rates. This may account for the carbothioamide ligands remaining attached when bound to biomolecules. As shown for KP1834, removal of the carrier ligand did not render the compounds inactive. In fact, the ruthenium compounds showed higher cytotoxicity compared to their respective osmium analogues. The most interesting trait of these compounds is their potential oral activity, a feature currently exclusive to Pt(IV) compounds, such as satraplatin. ELJ085 and ELJI065 were shown to be stable in 60 mM HCl pH 1.2 for at least 7 days, suggesting that these compounds would be stable in the stomach environment, and hydrolysis would be delayed until they reached the blood stream or cytoplasm. Furthermore, these compounds had quite high lipophilicity, a property related to increased cellular uptake, and also good permeability and a quantitative estimate of drug-likeness (QED) similar to approved anticancer drugs, like imatinib and tamoxifen. Combined with high cytotoxicity (even in cisplatin-resistant SW480 and multi drug-resistant A549 cancer cell lines), these properties suggested that compounds in the ELJI series have potential to be developed as antitumor drugs which are orally active¹⁷⁶. *In vivo* tests still need to be done to confirm oral bioavailability.

4.11 Combination of different metal-based compounds

The use of drug combinations for treating cancer is becoming more prevalent due to problems of toxicity and resistance to single drug therapy. Even though cancer cells can also gain multi-drug resistance, it would be less likely to develop. Different cellular components and pathways may be targeted simultaneously, attacking multiple weaknesses of cancer cells all at once. Combination of drugs may have effects, which are more than the sum of the individual drugs. Furthermore, using several drugs at the same time may be more effective than sequential administration¹⁷⁷.

Auranofin is an inorganometallic compound used to treat rheumatoid arthritis. It contains a gold center with thioglucose and phosphine ligands. *In vivo*, it is hydrolysed rapidly and the tetraacetylthioglucose group is cleaved off the gold center¹⁷⁸. The activated cationic Au-phosphine component can then bind to cysteine and histidine residues of cellular proteins¹⁷⁹. Even since the early days, when it started to be used as an antiinflammatory agent, auranofin has also been shown to exhibit

antitumor properties¹⁸⁰. It inhibits redox enzymes, like thioredoxin reductase and thioredoxin glutathione reductase, which are essential for maintaining intracellular levels of reactive oxygen species¹⁸¹. Inhibition of these enzymes leads to cellular oxidative stress and apoptosis.

RAPTA-T is an analogue of RAPTA-C, with toluene instead of cymene for the arene group. It binds to site 2 and 3 in NCP145 with essentially the same structure of adducts as RAPTA-C in these sites. RAPTA-T did not form adduct at site 1, probably due to insufficient hydrophobic interactions.

When NCP145 crystals were soaked with auranofin, no binding was observed despite even extensive soaking with 1 mM of compound for up to 4 days. When the combination of auranofin and RAPTA-T was used to soak NCP145 crystals, we found that, in addition to the two RAPTA-T sites, there are 2 auranofin molecules bound to symmetry-related His113 sites of histone H3 (Figure 4.26). The thioglucose ligand of auranofin was cleaved from the gold center and only the phosphine group remained. These results are in agreement with results from other experiments. In cell studies, where A2780 tumor cells were treated with RAPTA-T, auranofin or combination of both, the level of auranofin binding to chromatin was increased ~3-fold in the presence of RAPTA-T. Cell survival analysis also showed that the presence of RAPTA-T reduced tumor cell survival substantially compared to treatment with auranofin only. These findings on the synergistic impact and adduct accumulation of auranofin and RAPTA-T on tumor cells are from P.J. Dyson lab (unpublished), and are the basis for our original motivation to study these two compounds together.

Although the auranofin binding sites are ~35 Å away from RAPTA-T sites, there are alterations in the dynamics of the nucleosome core structure caused by RAPTA-T binding that influence the reactivity towards auranofin. This is revealed by molecular dynamic simulations of the NCP, NCP with only RAPTA-T or auranofin adducts and NCP with both RAPTA-T and auranofin adducts (G. Palermo and U. Röthlisberger, unpublished). In the presence of RAPTA-T, a slight kink in the histone H2A α 2 helix and systemic intra-helical conformational changes are triggered, leading to a conformational switch of histone H3 Ala135, which renders the histidine sites susceptible to auranofin binding.

For this RAPTA-T/auranofin study, NCP crystals were prepared and soaking was done by Ma Zhujun.

Figure 4.26. Adducts of auranofin and RAPTA-T on NCPI45. a) Auranofin binding sites relative to RAPTA-T. b) NCPI45 model (green carbon) and auranofin and RAPTA-T adducts (gray carbon) are shown with a 2Fo-Fc electron density map (gray, contoured at 1σ) and an anomalous difference electron density map (teal, contoured at 5σ for auranofin and 3σ for RAPTA-T) superimposed on the model. Au- and Ru-coordinating histone protein side chains are shown. Au and Ru atoms are shown as yellow and magenta spheres, respectively. Coordinate (-covalent) bonds are represented by gold or magenta lines.

4.12 Histone site-selectivity of the metal-based compounds

The presence of defined sites of adduction implies that reaction of metal-based compounds with NCP145 is not random. Glutamate and histidine are the main amino acids involved in adduction. One lysine residue is involved at site 1, while aspartate is involved at site 6'. Ruthenium agents are also known to be highly reactive towards histidine residues in proteins^{132,182}. The binding usually involves a nitrogen atom of the imidazole ring in histidine¹⁸³. All solvent accessible histidines in the NCP145 crystal are implicated in binding with metal-based agents in this study.

Based on the 29 compounds studied so far, 2 compounds (OsAPTA-C and KP1582) did not show any binding site on nucleosome core crystal. For the 27 compounds which showed binding to nucleosome core in soaking experiments, there are two general types of binding sites at play. The first type involves binding to high electronegativity region (HER) sites, such as sites 1, 2, 3, 4/4' and 6' and appears to be governed by electrostatic attraction. The RAPTA series, RAED-C, KP1019, NAMI-A and Alna series compounds all bind NCP in this manner. The second type involves binding to accessible histidine (HIS) sites (sites 3, 5, 5', 7 and 7'). The KP series (except for KP1019 and KP1582), ELJI series, SAME136, Rh-Maltol and auranofin utilize this mode in binding NCP. Since it contains both a histidine and a high electronegative potential, site 3 is considered both a HIS and HER type, which is why it is a common binding site for many of the compounds. The mechanisms governing the preference of each compound to react at one or the other site type are still not clear, and a combination of different factors may be involved in directing this selectivity. We are presently investigating this further with our computational chemist collaborators (G. Palermo and U. Röthlisberger).

Hydrophobic interactions and shape complementarity are certainly key players in determining the binding site selectivity of the compounds we studied here. As mentioned above, the fact that only RAPTA-C binds to site 1 seems to stem from the exact complementarity of this compound to this site. Even small changes in the ligands, including slightly different arene groups within the RAPTA series, prevent other compounds from forming adducts here. Also, simultaneous binding to sites 2 and 3 requires van der Waals contact to be established between these two sites. When this condition is not met, only site 2 (for HER binders) or site 3 (for HIS binders) is occupied. In agreement with this hypothesis, adduct formation at HIS sites also

appears to involve extensive hydrophobic interactions with neighboring amino acids. In fact, ELJI079 and ELJI085, which have lost their carbothioamide ligands required for van der Waals contact formation, unlike ELJI065 and SAME136, could not form adduct at site 3.

Steric access appears to also be involved in directing histone binding preference. Most of the HER compounds seems to be too bulky to access the interior of the nucleosome and bind only to the surface residues. The HIS compounds, on the other hand, seem to be small enough to access the more buried histidine residues at site 5, 5', 7 and 7'. Exceptions exist for RAED-C and NAMI-A which are not too large to access the nucleosome interior. Both compounds bind to glutamate residues at site 4/4'. They did not bind to site 5, 5', 7 and 7' possibly because the intrinsic preference for more electronegative residues and/or the absence of shape complementarity needed for adduction at these sites.

All osmium compounds studied here are in the HIS category and have intrinsic preferences for histidine residues. OsAPTA-C, the osmium analogue of RAPTA-C was found not to form adduct at all in the soaking experiments, even after high concentration (up to 4 mM) for extended period (3 days) of soaking was done. Its osmium center may confer preference to bind to histidine and the bulky PTA group may prevent binding to interior histidine sites. Hence the only available site for binding could be site 3. However, compounds with the piano-stool configuration like OsAPTA-C apparently require binding to site 2 first in order to provide shape complementarity for subsequent binding to site 3. These factors may explain the inability of this compound to bind at all to NCP.

4.13 Effects of metal compound adducts on chromatin dynamics

The implications of binding of metal-based agents to nucleosomes can already be predicted from the location of the adducts. Sites 2 and 3 are involved in the binding of RCC1, Sir3, CENP-C, PRC1 and LANA proteins with nucleosome²⁹⁻³³. The presence of adducts at this site may influence binding of these proteins and other nuclear and exogenous factors with chromatin. Sites 1, 2 and 3 are also involved in inter-nucleosome interactions in chromatin. We believed it would be interesting to see

if the adduct formation would influence chromatin compaction into higher order structures. Nucleosome array with 36 repeats of 177 bp DNA (NUC601-36-177) was utilized to investigate this hypothesis. NUC601-36-177 was treated with metal compounds at different molar ratios, and EMSA and EM were conducted to study the effect on chromatin folding.

Binding of metal compounds would add to the molecular weight of nucleosome array and hence slower migration on agarose gels. On the other hand, nucleosome arrays with more folded conformations would run faster than their unfolded counterparts due to their compact structure. We found that the presence of adducts of metal-based compounds in nucleosome arrays caused folding of the arrays (Figure 4.27). The derivatized nucleosome arrays ran faster than the untreated nucleosome array control despite additional mass from the coordinated compounds. This was more apparent when compared to competitor DNA and nucleosome-like species bands, which were running slower than their untreated counterparts due to added molecular weight. RAPTA-C was the first compound tested, due to its ability to bind to sites 1, 2 and 3 on the nucleosome core. RAED-C and cisplatin were examined because of their capability and preference to bind to the nucleosomal DNA. In addition, 3 binuclear ruthenium compounds (BM C2CL4, BM C10CL4 and BM PEGCL4) were studied due to their potential in forming internucleosomal interactions. From agarose gel results, we found that DNA binders like cisplatin and RAED-C caused only small extents of folding as shown by slight shifting of the nucleosome array bands. RAPTA-C caused more extensive folding, but the binuclear ruthenium compounds seemed to yield by far the highest degree of folding (Figure 4.27, see comparison between RAPTA-C and BM C2CL4 on bottom left gel).

Results from EMSA were then confirmed with EM. In addition to the negative control of native nucleosome array with an open conformation, we also examined the structure of NUC601-36-177 in the presence of 1.6 mM MgCl₂ which yielded the fully compacted state even in the absence of linker histone (Figure 4.28a)⁷⁰. In the absence of MgCl₂, the nucleosome arrays appeared extended with observable nucleosome core and linker DNA. In 1.6 mM MgCl₂, the nucleosome arrays were folded into a two-start helix structure with zig-zag conformation. When this is compared to the cryo-EM map of 12 x 187 bp and model of 48 x 187 bp chromatin fibers (Figure 4.28b, ref. 57), the two stacks of nucleosome cores (the two “strands” of the two-start helix) could be seen clearly. In addition, we also observed regions

where the two stacks of nucleosome cores seemed as if they cross each other when viewed as 2D image. In the model with 48 nucleosomes, this cross-like structure is observed every 12 nucleosomes.

When treated with cisplatin, some of the nucleosome arrays remained unfolded with observable nucleosome core and linker DNA regions (Figure 4.29a, left). Some molecules showed a small degree of folding, as implied by a less extended conformation with closer nucleosome-nucleosome distances and no observable linker DNA (Figure 4.29a, right). RAED-C treatment resulted in most arrays having a small degree of folding (Figure 4.29b, left). Despite neither causing much folding, RAED-C seemed to be more effective than cisplatin, probably because RAED-C also binds to site 2 on histone proteins. RAPTA-C treatment gave rise to some folded molecules and aggregated chromatin fibers, unlike what we have seen in agarose gels (Figure 4.29b, right). The binuclear ruthenium compounds, on the other hand, yielded what appears to be fully compacted nucleosome arrays (Figure 4.30).

The compaction caused by these agents looked different from that caused by divalent ion alone. Also, compaction induced by the metal compounds seemed to be unaffected by the presence/absence of divalent ion (Figure 4.30, bottom panels). In addition, small compounds with similar charge did not give similar folding type/extent (cisplatin and RAPTA-C both have 2+ charges). This suggests that the effect of these compounds did not stem from electrostatic influences alone. Binding to specific sites on the nucleosome is most likely linked to their compacting effect. The compacted structures induced by the binuclear metal compounds are highly distinct and seemed to involve more twisting than that caused by Mg^{2+} alone. The binuclear agent-compacted arrays look shorter and thicker, suggesting a more compressed folded state, with more nucleosomes in each layer (Figure 4.30). They are also more heterogeneous, with some folded nucleosome arrays that looked like solenoid, two start-helix, clutches of nucleosomes or other shapes.

This heterogeneity might have been caused by binding of the metal compounds to different sites on the nucleosome arrays. The irreversible nature of drug coordination might also play a role; once binding happened at one or several sites, the nucleosome array might become more constrained and forced to take on shapes according to where these initial adducts were formed.

The strong chromatin compacting affect caused by the binuclear metal compounds may disturb chromatin dynamics inside the cell. Regions of chromatin,

which are required to be unfolded may be prevented from doing so by the presence of covalent adducts formed by these agents. In addition, proper compaction and folding into higher order structures may also be interrupted due to aberrant compaction configurations induced by the compounds.

Figure 4.27. Chromatin compaction induced by binding of metal-based compounds.

Figure 4.28. Conformations of nucleosome array. a) Electron microscope images of nucleosome array without and with MgCl₂ b) Cryo-EM map of 12 x 187 bp and model of 48 x 187 bp chromatin fiber (adapted from ref. 57). Red stars denote the cross-like structure between the two stacks of nucleosome cores.

a)

Cisplatin : NUC601-36-177 100:1 in 40 mM NaCl

Figure 4.29. Electron microscope images of nucleosome array treated with different mononuclear metal compounds. a) Cisplatin treatment. b) RAED-C and RAPTA-C treatments.

a)

BM C2CL4 : NUC601-36-177 80:1 in 40 mM NaCl
and 0.5 mM MgCl₂

BM C10CL4 : NUC601-36-177 80:1 in 40 mM NaCl
and 0.5 mM MgCl₂

b)

BM PEGCL4 : NUC601-36-177 100:1 in 40 mM NaCl
and 0.5 mM MgCl₂

BM PEGCL4 : NUC601-36-177 100:1 in 40 mM NaCl

Figure 4.30. Electron microscope images of nucleosome array treated with different binuclear ruthenium compounds. a) BM C2Cl4 and BM C10Cl4 treatments. b) BM PEGCl4 treatment.

5 Results and Discussion Part III – Crystallization of Nucleosome in Complex with Linker Histone

5.1 Crystallization of asymmetric nucleosome with linker histone

The binding of full length and the globular domain of human histone H1.0 to nucleosome was first studied by EMSA before setting up crystallizations for X-ray diffraction studies. When nucleosome was loaded with linker histone, the nucleosome band would migrate slower due to the additional molecular weight from the bound linker histone. Different linker histone to nucleosome ratios were tested to find a ratio where all nucleosome was bound to linker histone. Under-saturation would result in the presence of free nucleosome in the sample. Over-saturation would yield non-specific association and precipitation. Both deviations would result in heterogeneous sample, which is not suited for crystallization. An optimal ratio that yields the most homogenous sample of linker histone-nucleosome complex was chosen for crystallization set ups.

Linker histone was shown to bind to the linker DNA around the DNA entry/exit point of the nucleosome core¹⁸⁴. Based on studies, which have shown binding of linker histone to be asymmetric and preferring AT-rich regions, the nucleosome CMS155 (NUC CMS155) was designed. This construct contains a 10 bp linker DNA with a poly-A sequence on only one side of the nucleosome, to form an asymmetric nucleosome. NUC CMS156 and 154 were then designed with +/- 1 bp in the linker DNA length compared to NUC CMS155.

Since standard conditions for NCP crystallization use K-Cacodylate buffer pH 6.0, binding reactions done in this buffer were used to determine optimal ratios for crystallization set ups. For full length H1.0 with hexa-histidine tag (H1.0+His₆), around half of the nucleosome band was shifted at 1:1 linker histone to nucleosome ratio, the sample started to precipitate at 2:1 and all samples precipitated at 4:1 ratio (Figure 5.1a left), for either NUC CMS155 or NUC CMS156. No ratio gave a clean full saturation, without having any precipitation. Further screens with finer ratios (Figure 5.1a right) suggested 1.5:1 to be the best ratio for binding, maximizing the

fraction of nucleosome bound to linker histone while minimizing precipitation. A ratio of 1.5:1 was initially chosen for crystallization with NUC CMS155 but further optimization showed that the lower ratio of 1:1 was better for crystallization (see below). Hence, for NUC CMS156, the ratio of 1:1 was used for crystallization set ups. For full length H1.0 without the hexa-histidine tag (will be referred to as just “H1.0”), the binding affinity towards NUC CMS155 and NUC CMS156 was similar to that with the his-tag (Figure 5.1b). Ratios from 1:1 to 1.5:1 were used for crystallizations.

Figure 5.1. Binding of linker histone H1.0+His₆ (a) and H1.0 (b) to NUC CMS155 and NUC CMS156.

For globular H1.0 (gH1.0), binding to NUC CMS155 would not reach saturation, even after 4 equivalents of linker gH1.0 was added, suggesting lower affinity of the globular H1.0 to nucleosome compared to its full length counterpart

(Figure 5.2a). This phenomenon was observed clearly for gH1.0 with histidine tag (gH1.0+His₆). For gH1.0 without the tag (“gH1.0”), this also seemed to be the case, although the extent of band shifting was very small and difficult to be observed on the gel. For NUC CMS156, gH1.0+His₆ binding was still not complete at 4:1 ratio, and sample precipitated at 8:1 ratio (Figure 5.2b). For gH1.0, binding seemed to be complete at 2:1 ratio, but it was not clear from the gel. Since no significant improvement of binding was observed above the ratio of 1.5:1, and in accordance to EMSA result from full length linker histone, the ratio from 1.0:1 to 1.5:1 was used for crystallization set ups.

Since linker histone, particularly the full length, precipitated nucleosome even at low ratios (some precipitation was already observed at 1:1 ratio) in pH 6.0, the higher pH of 7.5 and different types of buffer were tested. However, no significant difference in binding was observed in these conditions. EMSA for NUC CMS154 was done by Dr. Bao Qiuye (data not shown).

Figure 5.2. Binding of gH1.0+His₆ and gH1.0 to NUC CMS155 (a) and NUC CMS156 (b).

Crystallizations were conducted with the hanging droplet vapor diffusion method. In the salting-out approach, the salt concentration is initially lower and becomes higher as equilibration occurs. We found that complex containing full length H1.0 in the sample precipitated at the low salt concentration used in salting-out method. Almost all drops types precipitated with the salting-out method. On the other

hand, the salting-in method was found to be better, because the initial salt concentration is higher, preventing precipitate formation at the start.

A screen of conditions similar to the standard NCP crystallization condition was initially set up to determine the precipitation point for the H1.0+His₆ and NUC CMS155 complexes. Since no precipitation was observed for this set up, lower MnCl₂ and KCl concentrations were screened. The precipitation point was determined to be around 5.0-12.5 mM MnCl₂ with 5 mM KCl. Only small, thin, and irregularly-shaped crystalline materials were observed at 25 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0 (Figure 5.3a). Screening was extended by substituting MnCl₂ with MgCl₂, CaCl₂, spermidine or spermine. Thin and bent crystals were observed in MgCl₂ conditions (Figure 5.3b). Small, rectangular prism-shaped crystals were observed for CaCl₂ conditions (Figure 5.3c). In spermidine conditions, bent twinned crystals were observed (Figure 5.3d). Bent, fused crystals were also observed for spermine conditions (Figure 5.3e). Screening was focused on CaCl₂ conditions since crystal morphology was the best compared to other multivalents. Finer screen around CaCl₂ concentrations did not yield better crystals. A screen of H1.0+His₆ to NUC CMS155 ratio was done and the lower ratio (1:1) seemed to yield better crystals (small rods) than the ratio used initially (1.5:1). Larger drop volume (10 µl instead of 2 µl) gave bigger crystals (Figure 5.3f), and when the concentration of complex was decreased or increased, no crystals were observed.

As a control to suggest if the crystals obtained contained linker histone, drops containing only NUC CMS155 or only linker histone in crystallization buffer were set up. Drops without linker histone resulted in NUC CMS155 crystals, which differed in appearance compared to drops with linker histone present. Crystals of NUC CMS155 alone looked thin and plate-shaped (Figure 5.4a). Drops with linker histone only did not give any crystals.

Salting-in with CaCl₂, MnCl₂, MgCl₂, spermidine and spermine were also set up for complexes of H1.0 with NUC CMS155. These conditions became our standard set for initial screening (Table 5.1). Only spermine gave crystalline material, and it was only small, irregularly-shaped crystals. Co-crystallization of H1.0+His₆ and H1.0 with NUC CMS156 also resulted in similar looking crystalline material, which also grew under conditions containing spermine (Figure 5.5).

Figure 5.3. Co-crystallization of H1.0+His₆ with NUC CMS155. a) 25 mM MnCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) 37.5 mM MgCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) 37.5 mM CaCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. d) 25 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. e) 25 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. f) 37.5 mM CaCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0; 1:1 ratio in 10 μ l drop.

Figure 5.4. Crystals of NUC CMS155. a) 37.5 mM CaCl_2 , 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) 37.5 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

Figure 5.5. Co-crystallizations of full length linker histones with NUC CMS155 and NUC CMS156. a) H1.0 and NUC CMS155 complex in 12.5 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) H1.0+His₆ and NUC CMS156 complex in 25 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) H1.0 and NUC CMS156 complex in 12.5 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

Table 5.1. Standardized conditions for initial crystallization screening of linker histone-nucleosome assemblies.

12.5 mM MnCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	12.5 mM MgCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	12.5 mM CaCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	12.5 mM spermidine 5 mM KCl 10 mM K-Cacodylate pH 6.0	12.5 mM spermine 5 mM KCl 10 mM K-Cacodylate pH 6.0
25 mM MnCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	25 mM MgCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	25 mM CaCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	25 mM spermidine 5 mM KCl 10 mM K-Cacodylate pH 6.0	25 mM spermine 5 mM KCl 10 mM K-Cacodylate pH 6.0
37.5 mM MnCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	37.5 mM MgCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	37.5 mM CaCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	37.5 mM spermidine 5 mM KCl 10 mM K-Cacodylate pH 6.0	37.5mM spermine 5 mM KCl 10 mM K-Cacodylate pH 6.0
50 mM MnCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	50 mM MgCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	50 mM CaCl ₂ 5 mM KCl 10 mM K-Cacodylate pH 6.0	50 mM spermidine 5 mM KCl 10 mM K-Cacodylate pH 6.0	50 mM spermine 5 mM KCl 10 mM K-Cacodylate pH 6.0

For globular linker histone, initially the salting-out method was again tested because incubation of gH1.0+His₆ with NUC CMS155 did not give as much precipitation as with the full length linker histone. Different precipitants were added to the drops, but this again yielded mainly precipitation. Hampton PEGRx screens were also tested, but no crystals grew from these conditions; most drops precipitated although some drops remained clear.

The salting-in method was then tested with the standard screening conditions (Table 5.1). MnCl₂ and MgCl₂ conditions gave only small crystals (Figure 5.6a and b). CaCl₂ gave clustered, irregularly-shaped crystals (Figure 5.6c). Spermidine gave long, clustered rods (Figure 5.6d). Spermine gave bent crystals similar to those with full length H1.0+His₆ (Figure 5.6e). The spermidine condition was further screened by varying the concentration of buffer ingredients and gH1.0+His₆ to NUC CMS155 ratio. Ratios of 1:1 and 1.5:1 gave nice crystals, but higher ratios yielded smaller and worse-looking crystals. Larger drop size (10 µl) yielded larger individual crystals (Figure 5.6f).

As a control to suggest if the crystals obtained contained linker histone, drops containing only NUC CMS155 or only gH1.0+His₆ in crystallization buffers were set up. Drops with only NUC CMS155 resulted in crystals, which differed in appearance compared to drops containing gH1.0+His₆. Crystals of NUC CMS155 alone looked like clusters of plates (Figure 5.4b). Drops with gH1.0+His₆ only did not give any crystals.

Salting-in with MnCl₂, MgCl₂, CaCl₂, spermidine and spermine were set up for complexes of gH1.0 with NUC CMS155. MnCl₂ only gave crusts (Figure 5.7a). MgCl₂ gave very small needle/rod clusters (Figure 5.7b). CaCl₂ gave small plate clusters (Figure 5.7c). Spermidine gave small hexagonal crystals (Figure 5.7d). Spermine yielded bent, twinned crystals (Figure 5.7e).

Figure 5.6. Co-crystallizations of gH1.0+His₆ with NUC CMS155. a) 50 mM MnCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) 37.5 mM MgCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) 37.5 mM CaCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. d) 37.5 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0 e) 25 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. f) 37.5 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0, 1:1 ratio in 10 μ l drop.

Figure 5.7. Co-crystallizations of gH1.0 with NUC CMS155. a) 50 mM MnCl_2 , 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) 50 mM MgCl_2 , 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) 25 mM CaCl_2 , 5 mM KCl, 10 mM K-Cacodylate pH 6.0. d) 25 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0 e) 25 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

Crystallization of NUC CMS156 with gH1.0+His6 only gave birefringent crystalline material under conditions containing spermine (Figure 5.8a). Meanwhile, those with gH1.0 yielded small birefringent needles or sticks under CaCl_2 conditions,

thick plates in spermidine and small crystalline material in spermine (Figure 5.8b-d). Crystallization screening for NUC CMS154 was done by Dr. Bao Qiuye. Complexes with both full length and globular domain gave clusters of small needles (data not shown).

Figure 5.8. Co-crystallizations of NUC CMS156 with globular linker histone. a) gH1.0+His₆ in 25 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. b) gH1.0 in 50 mM CaCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) gH1.0 in 50 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. d) gH1.0 in 37.5 mM spermine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

Crystals were initially harvested in their respective reservoir buffers, but most of the crystals dissolved in these conditions. Optimized harvest buffer contained a half-concentration of components of the reservoir buffer, with up to 2% trehalose and

40% MPD or PEG6000 included. X-ray diffraction tests for all of these crystals yield mostly no or very low resolution diffraction (lower than 40 Å), although one crystal of gH1.0+His₆ with NUC CMS155 diffracted with reflections up to ~8 Å (Figure 5.9).

Figure 5.9. Diffraction image of a crystal from a co-crystallization of gH1.0+His₆ with NUC CMS155. Crystals were grown in 37.5 mM spermidine, 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

5.2 Crystallization of palindromic chromatosome

Binding of linker histone to symmetric nucleosomes with linker DNA on both sides of the nucleosome core were also investigated. NUC PRP167 is composed of a palindromic 167 bp DNA fragment, having an 11 bp mixed-sequence linker DNA on each end of the nucleosome core, and was used first due to its availability in the lab. Based on the results from asymmetric nucleosomes, only near-stoichiometric linker histone to nucleosome ratios were tested. EMSA was done by Dr. Bao Qiuye (data not shown) and a ratio of 1.2:1 was chosen for crystallization of full length linker histone with NUC PRP167. Globular H1.0 was not included in crystallization screening due to very weak binding to NUC PRP167. Crystallization screening was done together with Dr. Bao Qiuye. Crystals of H1.0+His₆ with NUC PRP167 grew as thick plates in conditions containing MnCl₂ (Figure 5.10), and the presence of linker histone was confirmed by SDS-PAGE (Dr. Bao Qiuye, data not shown). However, these crystals did not diffract.

Figure 5.10. Co-crystallizations of H1.0+His₆ with NUC PRP167 in 30 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0.

The NUC CMS167 construct, with 11 bp linker DNA at each end of the nucleosome core, was then designed based on the NUC CMS155 sequence. EMSA of H1.0+His₆ and H1.0 with NUC CMS167 was done by Dr. Bao Qiuye (data not shown). Ratios of 1.2:1 and 0.9:1 were chosen for crystallization set ups for H1.0 with and without histidine tag, respectively. Binding to globular linker histone was again weaker compared to the full-length protein (Figure 5.11). Binding of gH1.0+His₆ seemed to be even weaker than gH1.0, since at 2:1 ratio less than 50% of the nucleosome band was shifted by linker histone binding, while for gH1.0 around half of the nucleosome was bound to the linker histone. The ratio chosen for crystallization set up was 2:1 for globular H1.0 both with and without tag.

Figure 5.11. Binding of gH1.0+His₆ (a) and gH1.0 (b) to NUC CMS167.

Crystallizations for NUC CMS167 were done together with Dr. Bao Qiuye. Crystals of H1.0+His₆ or H1.0 with NUC CMS167 grew as fused sticks, although those with H1.0 looked a bit better with some sticks appearing to be single crystals (Figure 5.12a-b). These crystals gave diffraction patterns with streaks instead of individual spots, possibly due to their twinned nature (Figure 5.13a-b). As evident from the crystal morphology, H1.0-NUC CMS167 crystals diffracted better, with streaks reaching ~ 9 Å and some spots observed within the streaks. Crystals of H1.0-NUC CMS167 complex also grew as thick plates which diffracted to ~ 5.5 Å (Figure 5.14). Crystals of gH1.0 with NUC CMS167 yielded mostly twinned sticks as for the full length constructs, but there were some plates albeit thinner than those with H1.0 (Figure 5.12c-d). The sticks diffracted with a characteristically streaky pattern. Some spots were also observed, which seemed to come from different lattices (Figure 5.13c). The plates diffracted quite well with regular spots reaching ~ 8 Å (Figure 5.13d). Globular H1.0 with histidine tag gave stick crystals with similar characteristics and a diffraction quality as those without histidine tag, but did not give the plate type crystal. We concluded that crystals with plate morphology diffract better than the sticks. However, only a few drops gave the plate-shaped crystals and it was difficult to reproduce them. Salting-out, seeding and microbatch methods were also tested for

symmetric nucleosome NUC PRP167 and/or NUC CMS167, but did not yield better crystals than the salting-in vapor diffusion method.

Figure 5.12. Co-crystallizations of linker histones with NUC CMS167. a) H1.0+His₆ in 50 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0. b) H1.0 in 50 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0. c) gH1.0 in 45 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0. d) gH1.0 in 30 mM MnCl₂, 5 mM KCl and 10 mM K-Cacodylate pH 6.0.

Figure 5.13. Diffraction patterns of crystals of linker histones in complex with NUC CMS167. a) H1.0+His₆ in 50 mM MnCl₂, 10 mM KCl, 10 mM K-Cacodylate pH 6.0. b) H1.0 in 50 mM MnCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0. c) gH1.0 in 50 mM MnCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0 (twinned sticks crystal). d) gH1.0 in 30 mM MnCl₂, 5 mM KCl, 10 mM K-Cacodylate pH 6.0 (plate-shaped crystal).

Figure 5.14. Co-crystallizations of H1.0 with NUC CMS167 and the corresponding diffraction pattern. Crystals were grown in 50 mM MnCl_2 , 5 mM KCl, 10 mM K-Cacodylate pH 6.0.

The presence of linker histone in the crystals of full-length H1.0 with NUC CMS167 was confirmed by SDS-PAGE (Figure 5.15). Crystals of H1.0-NUC CMS167 (lane 5) contain all 4 core histones and linker histone H1.0. Crystals of H1.0+His₆-NUC CMS167 (lane 6) contain all 4 core histones but it was difficult to see if linker histone was in the crystals, due to low amount of material loaded on the gel.

Figure 5.15. Linker histone's presence in the crystals.

Variations of NUC CMS167 were then designed where 1 bp was added or removed to the length of the linker DNA at each side of the nucleosome core. These were the NUC CMS165 and 169. Figure 5.16a shows the EMSA for binding of H1.0 to NUC CMS169. Most of the nucleosome was occupied with linker histone when the linker histone to nucleosome ratio was 1:1, and there seemed to be no significant improvement with more linker histone added. Hence, this ratio was used for crystallization set ups. Crystallization of H1.0 with NUC CMS169 resulted in crystals with a peculiar screw morphology with some plates at the end of the screws (Figure 5.16b). EMSA and crystallization of NUC CMS165 were done by Dr. Bao Qiuye (data not shown). Crystal shapes and conditions used were very similar to those for NUC CMS169. These crystals have not been tested for X-ray diffraction yet.

NUC CMS167 gave twinned stick crystals, but adding or subtracting 1 bp from each of the linker DNA sections resulted in a completely different shape of crystals. The peculiar thing was that changes in both directions gave crystals which are similar to each other. This is unexpected since there seemed to be no trend observed from longer to shorter linker DNA. Instead, 167 bp seems to be the non-optimal length for individual crystal formation and resulted in mostly twinned crystals. Individual crystals were observed for NUC CMS165 and 169 but they were of screw shape, which suggests very unique packing configuration of macromolecules in the crystals. Reducing or increasing the linker DNA length beyond 165 and 169 bp has not been done yet.

Many types of crystals of the chromatosome were obtained and many different combinations of different linker histone H1.0 and nucleosome constructs yielded crystals. However, most of these crystals were small and irregularly shaped. Due to limitations in time and resources, the screening was focused mainly on the conditions that gave crystals with better morphology. Different methods were tested to get bigger versions of these small-but-morphologically-good-crystals. Among the bigger crystals obtained, some of them were twinned and gave streak-shaped diffraction patterns. There were also some big and healthy-looking crystals which did not diffract well. Only a few of the bigger and individual crystals diffracted to better than 10 Å. Difficulties in further optimizing the conditions arose due to problems in reproducing the bigger crystals. With different batches of nucleosome and linker histone, sometimes no big crystals grew, even using conditions which previously gave nice big crystals. Moreover a batch of nucleosome and linker histone might also give different-

looking crystals as the material was stored and aged. These factors also limited the possibility to screen different harvest conditions, which may have a substantial impact on the final crystal diffraction quality.

Figure 5.16. Binding and co-crystallization of H1.0 with NUC CMS169. a) Binding over different molar ratios. b) Crystals from 37.5 mM spermidine, 5 mM KCl and 10 mM K-Cacodylate pH 6.0.

Conclusion and Future Direction

Most of the metal-based agents examined in this study are reactive towards the nucleosome core. From the NCP crystallographic experiments, only one compound (RAED-C) formed substantial adducts on the DNA. The other compounds only formed adducts at histone protein sites. Although DNA adduct formation by these agents *in vivo* cannot be presently ruled out, at least for one compound, RAPTA-C, protein is certainly the preferred binding partner within the cell. In agreement with its binding profile in NCP crystals, RAPTA-C was also shown to favor protein binding to the NCP in the solution state and also in cancer cells. In addition, exonuclease footprinting revealed that most RAPTA-C adducts on DNA were formed at the double helix termini which contain the most accessible bases, suggesting severe steric access requirements for adduction, which we confirmed to be the case by high level molecular dynamics simulations. Furthermore, in our biochemical experiments, most of the initially DNA-associated RAPTA-C seems to be unstable, and this is again consistent with the computational results that show relatively low stability (compared to RAED-C) of the DNA adducts for this compound. All of this evidence, in combination with its very low cytotoxicity, indicates that proteins are the primary cellular target and thus strongly suggests that proteins are at least partly involved in the therapeutic mechanism of action, in particular the antimetastatic activity, of RAPTA-C.

In contrast to the chemically very similar RAPTA-C, RAED-C favors targeting of the DNA, as is clear from all of our experimental and computational studies. Collectively, our results suggest that ruthenium compounds will have the potential to form DNA adducts, as long as there is sufficient steric access to the metal center. Although both cisplatin and RAED-C prefer DNA binding, their mechanisms of action are apparently dissimilar since their cellular effects are different and RAED-C displays only a basal level of cross-resistance (common to DNA damaging agents completely unrelated to cisplatin¹⁸⁵) with cisplatin towards cisplatin-resistant A2780 cells. Binding of RAED-C to internal DNA sites within the nucleosome core can trigger substantial local conformational changes and effectively remodel the DNA at a distance by inducing transfer of DNA stretching towards terminal locations (from around SHL ± 2 to SHL ± 5). This could also enter into the impact of RAED-C adducts on cellular function.

The histone protein binding sites of the many metal compounds we investigated can be divided into two groups, high electronegativity region (HER) and histidine (HIS). In addition, in dictating why certain sites within a HIS or HER category are differentially reactive towards different compounds, hydrophobic complementarity and steric access clearly play an important role in determining precise site preference. Adduct formation is also influenced by the chemical properties of the compounds, in particular the metal identity and stability of carrier ligands. However, this may be reducible to a more simplified attribute, since we found that osmium has an absolute preference for HIS over HER sites, and the identity of the metal center itself is the strongest determinant of ligand cleavage kinetics.

Beyond our HIS/HER and steric model system for potentially predicting the nucleosome site selectivity of metal-based agents, we have discovered several new principles for chromatin targeting. Notably, by working with our collaborators to employ an atom-to-cell approach, like we utilized for the RAPTA-C/RAED-C study, we can explain the basis for the observed RAPTA-T/auranofin antitumor cell activity synergy. This involves a novel allosteric mechanism that plays out through cross-talk at a distance between the two types of adducts on the nucleosome. Such a new mechanism suggests there may be much more potential for drug-drug synergies operating within chromatin. In addition, we found new possibilities for exerting therapeutic effects through histone binding at sites on the H2A-H2B acidic patch that are known to be important in the nucleosome association of cellular and exogenous factors²⁹⁻³³ and in the formation of higher order chromatin structure^{3,4}. In particular, the new binuclear ruthenium agents induce spontaneous nucleosome array folding, which results in chromatin fiber conformations dramatically different than that induced by divalent ions alone. Some of these compounds are known to be highly cytotoxic¹⁸⁶, and if such an effect occurs within the cell, chromatin dynamics and corresponding cellular functions may be substantially altered.

The cellular DNA/protein targeting of most of the other metal compounds studied in this thesis has yet to be established. However, the ‘comprehensive’ atom-to-cell methodology we used for the RAPTA-C/RAED-C and RAPTA-T/auranofin works can also be applied to investigate the cellular targeting and impact of any other metal-based anticancer candidates. In addition, further insight is needed for understanding the details of histone site preference of the different metal-based

compounds, although we are still actively working this out with our collaborators. This information can then be utilized for fine-tuning compounds for specific cellular targeting and activities. One of the key targets to consider is the nucleosome core, either the DNA or histone components, but perhaps in the future, the linker histone-bound nucleosome may also become a potential therapeutic target. Moreover, besides designing compounds to selectively target either the DNA or histones, we are also investigating possibilities for cross-linking between different DNA and/or histone sites, and this is a result of the site selectivity and adduct structures discovered from this work.

Full length and the globular domain of linker histone H1.0 are able to bind to nucleosomes having linker DNA on just one or both termini. However, the binding affinity of the globular domain is obviously lower than that of the full length protein, and having a minimal nucleosome size of ~167 bp, to generate a full chromatosome, may be a more viable approach. Complexes of different nucleosome and linker histone constructs yielded many types of crystals. Most of these crystals are small, and the presence of linker histone seems to influence nucleosome packing in the crystals to yield some peculiar crystal morphologies. Some bigger crystals were also obtained, but they still did not diffract to higher resolution. Difficulties in crystallization reproducibility also hindered further optimizations. Problems with reproducibility need to be resolved in order to be able to optimize the crystallization and harvesting conditions further. In addition, different linker histone subtypes and different DNA constructs could be screened for crystallization. We are presently testing some new strategies that may bear fruit.

References

1. van Holde, K.E. *Chromatin, Springer Series in Molecular Biology*, (Springer-Verlag, New York, 1988).
2. Kornberg, R.D. Chromatin structure: a repeating unit of histones and DNA. *Science* **184**, 868-71 (1974).
3. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251-60 (1997).
4. Davey, C.A., Sargent, D.F., Luger, K., Maeder, A.W. & Richmond, T.J. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 Å resolution. *J Mol Biol* **319**, 1097-113 (2002).
5. Ong, M.S., Richmond, T.J. & Davey, C.A. DNA stretching and extreme kinking in the nucleosome core. *J Mol Biol* **368**, 1067-74 (2007).
6. Oudet, P., Gross-Bellard, M. & Chambon, P. Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. *Cell* **4**, 281-300 (1975).
7. Dorigo, B., Schalch, T., Kulangara, A., Duda, S., Schroeder, R.R. & Richmond, T.J. Nucleosome arrays reveal the two-start organization of the chromatin fiber. *Science* **306**, 1571-3 (2004).
8. Hansen, J.C. Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu Rev Biophys Biomol Struct* **31**, 361-92 (2002).
9. Bednar, J., Horowitz, R.A., Grigoryev, S.A., Carruthers, L.M., Hansen, J.C., Koster, A.J. & Woodcock, C.L. Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. *Proc Natl Acad Sci U S A* **95**, 14173-8 (1998).
10. Felsenfeld, G. & Groudine, M. Controlling the double helix. *Nature* **421**, 448-53 (2003).
11. Weintraub, H. & Groudine, M. Chromosomal subunits in active genes have an altered conformation. *Science* **193**, 848-56 (1976).
12. Jiang, C. & Pugh, B.F. Nucleosome positioning and gene regulation: advances through genomics. *Nat Rev Genet* **10**, 161-72 (2009).
13. Henikoff, S. & Smith, M.M. Histone variants and epigenetics. *Cold Spring Harb Perspect Biol* **7**, a019364 (2015).
14. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693-705 (2007).
15. Clapier, C.R. & Cairns, B.R. The biology of chromatin remodeling complexes. *Annu Rev Biochem* **78**, 273-304 (2009).
16. Richmond, T.J. & Davey, C.A. The structure of DNA in the nucleosome core. *Nature* **423**, 145-50 (2003).
17. Dickerson, R.E. Definitions and nomenclature of nucleic acid structure components. *Nucleic Acids Res* **17**, 1797-803 (1989).
18. Lowary, P.T. & Widom, J. New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. *J Mol Biol* **276**, 19-42 (1998).
19. Travers, A., Hiriart, E., Churcher, M., Caserta, M. & Di Mauro, E. The DNA sequence-dependence of nucleosome positioning in vivo and in vitro. *J Biomol Struct Dyn* **27**, 713-24 (2010).
20. Widom, J. Role of DNA sequence in nucleosome stability and dynamics. *Q Rev Biophys* **34**, 269-324 (2001).

21. Chua, E.Y., Vasudevan, D., Davey, G.E., Wu, B. & Davey, C.A. The mechanics behind DNA sequence-dependent properties of the nucleosome. *Nucleic Acids Res* **40**, 6338-52 (2012).
22. Vasudevan, D., Chua, E.Y. & Davey, C.A. Crystal structures of nucleosome core particles containing the '601' strong positioning sequence. *J Mol Biol* **403**, 1-10 (2010).
23. Davey, G.E., Wu, B., Dong, Y., Surana, U. & Davey, C.A. DNA stretching in the nucleosome facilitates alkylation by an intercalating antitumour agent. *Nucleic Acids Res* **38**, 2081-8 (2010).
24. Pryciak, P.M. & Varmus, H.E. Nucleosomes, DNA-binding proteins, and DNA sequence modulate retroviral integration target site selection. *Cell* **69**, 769-80 (1992).
25. Davey, C.A. & Richmond, T.J. DNA-dependent divalent cation binding in the nucleosome core particle. *Proc Natl Acad Sci U S A* **99**, 11169-74 (2002).
26. Wu, B. & Davey, C.A. Using soft X-rays for a detailed picture of divalent metal binding in the nucleosome. *J Mol Biol* **398**, 633-40 (2010).
27. Dorigo, B., Schalch, T., Bystricky, K. & Richmond, T.J. Chromatin fiber folding: requirement for the histone H4 N-terminal tail. *J Mol Biol* **327**, 85-96 (2003).
28. Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R. & Peterson, C.L. Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* **311**, 844-7 (2006).
29. Makde, R.D., England, J.R., Yennawar, H.P. & Tan, S. Structure of RCC1 chromatin factor bound to the nucleosome core particle. *Nature* **467**, 562-6 (2010).
30. Armache, K.J., Garlick, J.D., Canzio, D., Narlikar, G.J. & Kingston, R.E. Structural basis of silencing: Sir3 BAH domain in complex with a nucleosome at 3.0 Å resolution. *Science* **334**, 977-82 (2011).
31. Kato, H., Jiang, J., Zhou, B.R., Rozendaal, M., Feng, H., Ghirlando, R., Xiao, T.S., Straight, A.F. & Bai, Y. A conserved mechanism for centromeric nucleosome recognition by centromere protein CENP-C. *Science* **340**, 1110-3 (2013).
32. McGinty, R.K., Henrici, R.C. & Tan, S. Crystal structure of the PRC1 ubiquitylation module bound to the nucleosome. *Nature* **514**, 591-6 (2014).
33. Barbera, A.J., Chodaparambil, J.V., Kelley-Clarke, B., Joukov, V., Walter, J.C., Luger, K. & Kaye, K.M. The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* **311**, 856-61 (2006).
34. Noll, M. & Kornberg, R.D. Action of micrococcal nuclease on chromatin and the location of histone H1. *J Mol Biol* **109**, 393-404 (1977).
35. Simpson, R.T. Structure of the chromatosome, a chromatin particle containing 160 base pairs of DNA and all the histones. *Biochemistry* **17**, 5524-31 (1978).
36. Furrer, P., Bednar, J., Dubochet, J., Hamiche, A. & Prunell, A. DNA at the entry-exit of the nucleosome observed by cryoelectron microscopy. *J Struct Biol* **114**, 177-83 (1995).
37. Hamiche, A., Schultz, P., Ramakrishnan, V., Oudet, P. & Prunell, A. Linker histone-dependent DNA structure in linear mononucleosomes. *J Mol Biol* **257**, 30-42 (1996).
38. Thomas, J.O. Histone H1: location and role. *Curr Opin Cell Biol* **11**, 312-7 (1999).
39. Ramakrishnan, V., Finch, J.T., Graziano, V., Lee, P.L. & Sweet, R.M. Crystal structure of globular domain of histone H5 and its implications for nucleosome binding. *Nature* **362**, 219-23 (1993).
40. Allan, J., Hartman, P.G., Crane-Robinson, C. & Aviles, F.X. The structure of histone H1 and its location in chromatin. *Nature* **288**, 675-9 (1980).
41. Clark, D.J., Hill, C.S., Martin, S.R. & Thomas, J.O. Alpha-helix in the carboxy-terminal domains of histones H1 and H5. *EMBO J* **7**, 69-75 (1988).

42. Vila, R., Ponte, I., Collado, M., Arrondo, J.L., Jimenez, M.A., Rico, M. & Suau, P. DNA-induced alpha-helical structure in the NH2-terminal domain of histone H1. *J Biol Chem* **276**, 46429-35 (2001).
43. Roque, A., Iloro, I., Ponte, I., Arrondo, J.L. & Suau, P. DNA-induced secondary structure of the carboxyl-terminal domain of histone H1. *J Biol Chem* **280**, 32141-7 (2005).
44. Oberg, C. & Belikov, S. The N-terminal domain determines the affinity and specificity of H1 binding to chromatin. *Biochem Biophys Res Commun* **420**, 321-4 (2012).
45. Allan, J., Mitchell, T., Harborne, N., Bohm, L. & Crane-Robinson, C. Roles of H1 domains in determining higher order chromatin structure and H1 location. *J Mol Biol* **187**, 591-601 (1986).
46. Vyas, P. & Brown, D.T. N- and C-terminal domains determine differential nucleosomal binding geometry and affinity of linker histone isoforms H1(0) and H1c. *J Biol Chem* **287**, 11778-87 (2012).
47. Lu, X. & Hansen, J.C. Identification of specific functional subdomains within the linker histone H10 C-terminal domain. *J Biol Chem* **279**, 8701-7 (2004).
48. Meyer, S., Becker, N.B., Syed, S.H., Goutte-Gattat, D., Shukla, M.S., Hayes, J.J., Angelov, D., Bednar, J., Dimitrov, S. & Everaers, R. From crystal and NMR structures, footprints and cryo-electron-micrographs to large and soft structures: nanoscale modeling of the nucleosomal stem. *Nucleic Acids Res* **39**, 9139-54 (2011).
49. Syed, S.H., Goutte-Gattat, D., Becker, N., Meyer, S., Shukla, M.S., Hayes, J.J., Everaers, R., Angelov, D., Bednar, J. & Dimitrov, S. Single-base resolution mapping of H1-nucleosome interactions and 3D organization of the nucleosome. *Proc Natl Acad Sci U S A* **107**, 9620-5 (2010).
50. Fan, L. & Roberts, V.A. Complex of linker histone H5 with the nucleosome and its implications for chromatin packing. *Proc Natl Acad Sci U S A* **103**, 8384-9 (2006).
51. Goytisolo, F.A., Gerchman, S.E., Yu, X., Rees, C., Graziano, V., Ramakrishnan, V. & Thomas, J.O. Identification of two DNA-binding sites on the globular domain of histone H5. *EMBO J* **15**, 3421-9 (1996).
52. Brown, D.T., Izard, T. & Misteli, T. Mapping the interaction surface of linker histone H1(0) with the nucleosome of native chromatin in vivo. *Nat Struct Mol Biol* **13**, 250-5 (2006).
53. Zhou, Y.B., Gerchman, S.E., Ramakrishnan, V., Travers, A. & Muyldermans, S. Position and orientation of the globular domain of linker histone H5 on the nucleosome. *Nature* **395**, 402-5 (1998).
54. Cui, F. & Zhurkin, V.B. Distinctive sequence patterns in metazoan and yeast nucleosomes: implications for linker histone binding to AT-rich and methylated DNA. *Nucleic Acids Res* **37**, 2818-29 (2009).
55. Gajiwala, K.S. & Burley, S.K. Winged helix proteins. *Curr Opin Struct Biol* **10**, 110-6 (2000).
56. Zhou, B.R., Jiang, J., Feng, H., Ghirlando, R., Xiao, T.S. & Bai, Y. Structural Mechanisms of Nucleosome Recognition by Linker Histones. *Mol Cell* **59**, 628-38 (2015).
57. Song, F., Chen, P., Sun, D., Wang, M., Dong, L., Liang, D., Xu, R.M., Zhu, P. & Li, G. Cryo-EM study of the chromatin fiber reveals a double helix twisted by tetranucleosomal units. *Science* **344**, 376-80 (2014).
58. Zhou, B.R., Feng, H., Kato, H., Dai, L., Yang, Y., Zhou, Y. & Bai, Y. Structural insights into the histone H1-nucleosome complex. *Proc Natl Acad Sci U S A* **110**, 19390-5 (2013).
59. Woodcock, C.L. & Dimitrov, S. Higher-order structure of chromatin and chromosomes. *Curr Opin Genet Dev* **11**, 130-5 (2001).

60. Horowitz-Scherer, R.A. & Woodcock, C.L. Organization of interphase chromatin. *Chromosoma* **115**, 1-14 (2006).
61. Schalch, T. Swiss Federal Institute Of Technology Zurich (2004).
62. Woodcock, C.L., Frado, L.L. & Rattner, J.B. The higher-order structure of chromatin: evidence for a helical ribbon arrangement. *J Cell Biol* **99**, 42-52 (1984).
63. Worcel, A., Strogatz, S. & Riley, D. Structure of chromatin and the linking number of DNA. *Proc Natl Acad Sci U S A* **78**, 1461-5 (1981).
64. Williams, S.P., Athey, B.D., Muglia, L.J., Schappe, R.S., Gough, A.H. & Langmore, J.P. Chromatin fibers are left-handed double helices with diameter and mass per unit length that depend on linker length. *Biophys J* **49**, 233-48 (1986).
65. Widom, J. & Klug, A. Structure of the 300A chromatin filament: X-ray diffraction from oriented samples. *Cell* **43**, 207-13 (1985).
66. Finch, J.T. & Klug, A. Solenoidal model for superstructure in chromatin. *Proc Natl Acad Sci U S A* **73**, 1897-901 (1976).
67. Robinson, P.J., Fairall, L., Huynh, V.A. & Rhodes, D. EM measurements define the dimensions of the "30-nm" chromatin fiber: evidence for a compact, interdigitated structure. *Proc Natl Acad Sci U S A* **103**, 6506-11 (2006).
68. Kruithof, M., Chien, F.T., Routh, A., Logie, C., Rhodes, D. & van Noort, J. Single-molecule force spectroscopy reveals a highly compliant helical folding for the 30-nm chromatin fiber. *Nat Struct Mol Biol* **16**, 534-40 (2009).
69. Schalch, T., Duda, S., Sargent, D.F. & Richmond, T.J. X-ray structure of a tetranucleosome and its implications for the chromatin fibre. *Nature* **436**, 138-41 (2005).
70. Routh, A., Sandin, S. & Rhodes, D. Nucleosome repeat length and linker histone stoichiometry determine chromatin fiber structure. *Proc Natl Acad Sci U S A* **105**, 8872-7 (2008).
71. Nishino, Y., Eltsov, M., Joti, Y., Ito, K., Takata, H., Takahashi, Y., Hihara, S., Frangakis, A.S., Imamoto, N., Ishikawa, T. & Maeshima, K. Human mitotic chromosomes consist predominantly of irregularly folded nucleosome fibres without a 30-nm chromatin structure. *EMBO J* **31**, 1644-53 (2012).
72. Ricci, M.A., Manzo, C., Garcia-Parajo, M.F., Lakadamyali, M. & Cosma, M.P. Chromatin fibers are formed by heterogeneous groups of nucleosomes in vivo. *Cell* **160**, 1145-58 (2015).
73. Lin, J.C., Jeong, S., Liang, G., Takai, D., Fatemi, M., Tsai, Y.C., Egger, G., Gal-Yam, E.N. & Jones, P.A. Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. *Cancer Cell* **12**, 432-44 (2007).
74. Gaspar-Maia, A., Alajem, A., Meshorer, E. & Ramalho-Santos, M. Open chromatin in pluripotency and reprogramming (vol 12, pg 36, 2011). *Nature Reviews Molecular Cell Biology* **12**(2011).
75. Yamada, K., Frouws, T.D., Angst, B., Fitzgerald, D.J., DeLuca, C., Schimmele, K., Sargent, D.F. & Richmond, T.J. Structure and mechanism of the chromatin remodelling factor ISW1a. *Nature* **472**, 448-53 (2011).
76. Sadler, P.J. & Peacock, A.F.A. Medicinal Organometallic Chemistry: Designing Metal Arene Complexes as Anticancer Agents. *Chemistry-an Asian Journal* **3**, 1890-1899 (2008).
77. van Rijt, S.H. & Sadler, P.J. Current applications and future potential for bioinorganic chemistry in the development of anticancer drugs. *Drug Discov Today* **14**, 1089-97 (2009).
78. Gasser, G., Ott, I. & Metzler-Nolte, N. Organometallic Anticancer Compounds. *J Med Chem* (2010).

79. Sadler, P.J. & Bruijninx, P.C.A. New trends for metal complexes with anticancer activity. *Current Opinion in Chemical Biology* **12**, 197-206 (2008).
80. Meggers, E., Atilla-Gokcumen, G.E., Bregman, H., Maksimoska, J., Mulcahy, S.P., Pagano, N. & Williams, D.S. Exploring chemical space with organometallics: Ruthenium complexes as protein kinase inhibitors. *Synlett*, 1177-1189 (2007).
81. Atilla-Gokcumen, G.E., Di Costanzo, L. & Meggers, E. Structure of anticancer ruthenium half-sandwich complex bound to glycogen synthase kinase 3beta. *J Biol Inorg Chem* **16**, 45-50 (2011).
82. Bregman, H. & Meggers, E. Ruthenium half-sandwich complexes as protein kinase inhibitors: an N-succinimidyl ester for rapid derivatizations of the cyclopentadienyl moiety. *Org Lett* **8**, 5465-8 (2006).
83. Smalley, K.S., Contractor, R., Haass, N.K., Kulp, A.N., Atilla-Gokcumen, G.E., Williams, D.S., Bregman, H., Flaherty, K.T., Soengas, M.S., Meggers, E. & Herlyn, M. An organometallic protein kinase inhibitor pharmacologically activates p53 and induces apoptosis in human melanoma cells. *Cancer Res* **67**, 209-17 (2007).
84. Rosenberg, B., Vancamp, L. & Krigas, T. Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode. *Nature* **205**, 698-9 (1965).
85. Galanski, M., Jakupec, M.A. & Keppler, B.K. Update of the preclinical situation of anticancer platinum complexes: novel design strategies and innovative analytical approaches. *Curr Med Chem* **12**, 2075-94 (2005).
86. Dyson, P.J. & Sava, G. Metal-based antitumour drugs in the post genomic era. *Dalton Trans*, 1929-33 (2006).
87. Harrap, K.R. Preclinical studies identifying carboplatin as a viable cisplatin alternative. *Cancer Treat Rev* **12 Suppl A**, 21-33 (1985).
88. Kidani, Y., Inagaki, K., Iigo, M., Hoshi, A. & Kureitani, K. Antitumor activity of 1,2-diaminocyclohexane--platinum complexes against sarcoma-180 ascites form. *J Med Chem* **21**, 1315-8 (1978).
89. Kelland, L.R., Abel, G., McKeage, M.J., Jones, M., Goddard, P.M., Valenti, M., Murrer, B.A. & Harrap, K.R. Preclinical antitumor evaluation of bis-acetato-amine-dichloro-cyclohexylamine platinum(IV): an orally active platinum drug. *Cancer Res* **53**, 2581-6 (1993).
90. Davies, M.S., Berners-Price, S.J. & Hambley, T.W. Rates of platination of -AG- and -GA- containing double-stranded oligonucleotides: effect of chloride concentration. *J Inorg Biochem* **79**, 167-72 (2000).
91. Fichtinger-Schepman, A.M., van der Veer, J.L., den Hartog, J.H., Lohman, P.H. & Reedijk, J. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry* **24**, 707-13 (1985).
92. Chaney, S.G., Campbell, S.L., Temple, B., Bassett, E., Wu, Y. & Faldu, M. Protein interactions with platinum-DNA adducts: from structure to function. *J Inorg Biochem* **98**, 1551-9 (2004).
93. Eastman, A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* **2**, 275-80 (1990).
94. Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7**, 573-84 (2007).
95. Siddik, Z.H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**, 7265-79 (2003).
96. Jung, Y. & Lippard, S.J. Direct cellular responses to platinum-induced DNA damage. *Chem Rev* **107**, 1387-407 (2007).
97. Baik, M.H., Friesner, R.A. & Lippard, S.J. Theoretical study of cisplatin binding to purine bases: why does cisplatin prefer guanine over adenine? *J Am Chem Soc* **125**, 14082-92 (2003).

98. Wu, B., Davey, G.E., Nazarov, A.A., Dyson, P.J. & Davey, C.A. Specific DNA structural attributes modulate platinum anticancer drug site selection and cross-link generation. *Nucleic Acids Res* (2011).
99. Wu, B., Droge, P. & Davey, C.A. Site selectivity of platinum anticancer therapeutics. *Nat Chem Biol* **4**, 110-2 (2008).
100. Knox, R.J., Friedlos, F., Lydall, D.A. & Roberts, J.J. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* **46**, 1972-9 (1986).
101. Holzer, A.K., Manorek, G.H. & Howell, S.B. Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin. *Mol Pharmacol* **70**, 1390-4 (2006).
102. Holzer, A.K. & Howell, S.B. The internalization and degradation of human copper transporter 1 following cisplatin exposure. *Cancer Res* **66**, 10944-52 (2006).
103. Mistry, P., Kelland, L.R., Abel, G., Sidhar, S. & Harrap, K.R. The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br J Cancer* **64**, 215-20 (1991).
104. Yang, P., Ebbert, J.O., Sun, Z. & Weinshilboum, R.M. Role of the glutathione metabolic pathway in lung cancer treatment and prognosis: a review. *J Clin Oncol* **24**, 1761-9 (2006).
105. Safaei, R., Holzer, A.K., Katano, K., Samimi, G. & Howell, S.B. The role of copper transporters in the development of resistance to Pt drugs. *J Inorg Biochem* **98**, 1607-13 (2004).
106. Ferry, K.V., Hamilton, T.C. & Johnson, S.W. Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF. *Biochem Pharmacol* **60**, 1305-13 (2000).
107. Dabholkar, M., Bostick-Bruton, F., Weber, C., Bohr, V.A., Egwuagu, C. & Reed, E. ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients. *J Natl Cancer Inst* **84**, 1512-7 (1992).
108. Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R.D. & Howell, S.B. The role of DNA mismatch repair in platinum drug resistance. *Cancer Res* **56**, 4881-6 (1996).
109. Zdraveski, Z.Z., Mello, J.A., Farinelli, C.K., Essigmann, J.M. & Marinus, M.G. MutS preferentially recognizes cisplatin- over oxaliplatin-modified DNA. *J Biol Chem* **277**, 1255-60 (2002).
110. Bassett, E., Vaisman, A., Tropea, K.A., McCall, C.M., Masutani, C., Hanaoka, F. & Chaney, S.G. Frameshifts and deletions during in vitro translesion synthesis past Pt-DNA adducts by DNA polymerases beta and eta. *DNA Repair (Amst)* **1**, 1003-16 (2002).
111. Albertella, M.R., Green, C.M., Lehmann, A.R. & O'Connor, M.J. A role for polymerase eta in the cellular tolerance to cisplatin-induced damage. *Cancer Res* **65**, 9799-806 (2005).
112. Gadducci, A., Cosio, S., Muraca, S. & Genazzani, A.R. Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. *Eur J Gynaecol Oncol* **23**, 390-6 (2002).
113. Rademaker-Lakhai, J.M., van den Bongard, D., Pluim, D., Beijnen, J.H. & Schellens, J.H. A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. *Clin Cancer Res* **10**, 3717-27 (2004).
114. Hartinger, C.G., Jakupec, M.A., Zorbas-Seifried, S., Groessl, M., Egger, A., Berger, W., Zorbas, H., Dyson, P.J. & Keppler, B.K. KP1019, a new redox-active anticancer agent--

- preclinical development and results of a clinical phase I study in tumor patients. *Chem Biodivers* **5**, 2140-55 (2008).
115. Groessl, M., Hartinger, C.G., Dyson, P.J. & Keppler, B.K. CZE-ICP-MS as a tool for studying the hydrolysis of ruthenium anticancer drug candidates and their reactivity towards the DNA model compound dGMP. *J Inorg Biochem* **102**, 1060-5 (2008).
 116. Clarke, M.J., Zhu, F. & Frasca, D.R. Non-platinum chemotherapeutic metallopharmaceuticals. *Chemical Reviews* **99**, 2511-34 (1999).
 117. Reisner, E., Arion, V.B., Guedes da Silva, M.F., Lichtenecker, R., Eichinger, A., Keppler, B.K., Kukushkin, V.Y. & Pombeiro, A.J. Tuning of redox potentials for the design of ruthenium anticancer drugs -- an electrochemical study of [trans-RuCl(4)L(DMSO)](-) and [trans-RuCl(4)L(2)](-) complexes, where L = imidazole, 1,2,4-triazole, indazole. *Inorg Chem* **43**, 7083-93 (2004).
 118. Heffeter, P., Pongratz, M., Steiner, E., Chiba, P., Jakupec, M.A., Elbling, L., Marian, B., Korner, W., Sevela, F., Micksche, M., Keppler, B.K. & Berger, W. Intrinsic and acquired forms of resistance against the anticancer ruthenium compound KP1019 [indazolium trans-[tetrachlorobis(1H-indazole)ruthenate (III)] (FFC14A)]. *J Pharmacol Exp Ther* **312**, 281-9 (2005).
 119. Jakupec, M.A., Arion, V.B., Kapitza, S., Reisner, E., Eichinger, A., Pongratz, M., Marian, B., Graf von Keyserlingk, N. & Keppler, B.K. KP1019 (FFC14A) from bench to bedside: preclinical and early clinical development--an overview. *Int J Clin Pharmacol Ther* **43**, 595-6 (2005).
 120. Morris, R.E., Aird, R.E., Murdoch Pdel, S., Chen, H., Cummings, J., Hughes, N.D., Parsons, S., Parkin, A., Boyd, G., Jodrell, D.I. & Sadler, P.J. Inhibition of cancer cell growth by ruthenium(II) arene complexes. *J Med Chem* **44**, 3616-21 (2001).
 121. Bergamo, A., Gagliardi, R., Scarcia, V., Furlani, A., Alessio, E., Mestroni, G. & Sava, G. In vitro cell cycle arrest, in vivo action on solid metastasizing tumors, and host toxicity of the antimetastatic drug NAMI-A and cisplatin. *J Pharmacol Exp Ther* **289**, 559-64 (1999).
 122. Scolaro, C., Bergamo, A., Brescacin, L., Delfino, R., Cocchietto, M., Laurenczy, G., Geldbach, T.J., Sava, G. & Dyson, P.J. In vitro and in vivo evaluation of ruthenium(II)-arene PTA complexes. *J Med Chem* **48**, 4161-71 (2005).
 123. Pintus, G., Tadolini, B., Posadino, A.M., Sanna, B., Debidda, M., Bennardini, F., Sava, G. & Ventura, C. Inhibition of the MEK/ERK signaling pathway by the novel antimetastatic agent NAMI-A down regulates c-myc gene expression and endothelial cell proliferation. *Eur J Biochem* **269**, 5861-70 (2002).
 124. Kostova, I. Ruthenium complexes as anticancer agents. *Curr Med Chem* **13**, 1085-107 (2006).
 125. Bergamo, A. & Sava, G. Ruthenium complexes can target determinants of tumour malignancy. *Dalton Trans*, 1267-72 (2007).
 126. Chatterjee, S., Kundu, S., Bhattacharyya, A., Hartinger, C.G. & Dyson, P.J. The ruthenium(II)-arene compound RAPTA-C induces apoptosis in EAC cells through mitochondrial and p53-JNK pathways. *J Biol Inorg Chem* **13**, 1149-55 (2008).
 127. Sava, G., Zorzet, S., Turrin, C., Vita, F., Soranzo, M., Zabucchi, G., Cocchietto, M., Bergamo, A., DiGiovine, S., Pezzoni, G., Sartor, L. & Garbisa, S. Dual Action of NAMI-A in inhibition of solid tumor metastasis: selective targeting of metastatic cells and binding to collagen. *Clin Cancer Res* **9**, 1898-905 (2003).
 128. Pluim, D., van Waardenburg, R.C., Beijnen, J.H. & Schellens, J.H. Cytotoxicity of the organic ruthenium anticancer drug Nami-A is correlated with DNA binding in four different human tumor cell lines. *Cancer Chemother Pharmacol* **54**, 71-8 (2004).

129. Malina, J., Novakova, O., Keppler, B.K., Alessio, E. & Brabec, V. Biophysical analysis of natural, double-helical DNA modified by anticancer heterocyclic complexes of ruthenium(III) in cell-free media. *J Biol Inorg Chem* **6**, 435-45 (2001).
130. Heffeter, P., Bock, K., Atil, B., Reza Hoda, M.A., Korner, W., Bartel, C., Jungwirth, U., Keppler, B.K., Micksche, M., Berger, W. & Koellensperger, G. Intracellular protein binding patterns of the anticancer ruthenium drugs KP1019 and KP1339. *J Biol Inorg Chem* **15**, 737-48 (2010).
131. Groessel, M., Reisner, E., Hartinger, C.G., Eichinger, R., Semenova, O., Timerbaev, A.R., Jakupec, M.A., Arion, V.B. & Keppler, B.K. Structure-activity relationships for NAMI-A-type complexes (HL)[trans-RuCl₄(S-dmso)ruthenate(III)] (L = imidazole, indazole, 1,2,4-triazole, 4-amino-1,2,4-triazole, and 1-methyl-1,2,4-triazole): aquation, redox properties, protein binding, and antiproliferative activity. *J Med Chem* **50**, 2185-93 (2007).
132. Casini, A., Gabbiani, C., Sorrentino, F., Rigobello, M.P., Bindoli, A., Geldbach, T.J., Marrone, A., Re, N., Hartinger, C.G., Dyson, P.J. & Messori, L. Emerging protein targets for anticancer metallodrugs: inhibition of thioredoxin reductase and cathepsin B by antitumor ruthenium(II)-arene compounds. *J Med Chem* **51**, 6773-81 (2008).
133. Han, Y., Luo, Q., Hao, X., Li, X., Wang, F., Hu, W., Wu, K., Lu, S. & Sadler, P.J. Reactions of an organoruthenium anticancer complex with 2-mercaptobenzanilide--a model for the active-site cysteine of protein tyrosine phosphatase 1B. *Dalton Trans* **40**, 11519-29 (2011).
134. Liu, H.K., Wang, F., Parkinson, J.A., Bella, J. & Sadler, P.J. Ruthenation of duplex and single-stranded d(CGGCCG) by organometallic anticancer complexes. *Chemistry* **12**, 6151-65 (2006).
135. van Rijt, S.H., Mukherjee, A., Pizarro, A.M. & Sadler, P.J. Cytotoxicity, hydrophobicity, uptake, and distribution of osmium(II) anticancer complexes in ovarian cancer cells. *J Med Chem* **53**, 840-9 (2010).
136. Peacock, A.F., Habtemariam, A., Fernandez, R., Walland, V., Fabbiani, F.P., Parsons, S., Aird, R.E., Jodrell, D.I. & Sadler, P.J. Tuning the reactivity of osmium(II) and ruthenium(II) arene complexes under physiological conditions. *J Am Chem Soc* **128**, 1739-48 (2006).
137. van Rijt, S.H., Hebden, A.J., Amaresekera, T., Deeth, R.J., Clarkson, G.J., Parsons, S., McGowan, P.C. & Sadler, P.J. Amide linkage isomerism as an activity switch for organometallic osmium and ruthenium anticancer complexes. *J Med Chem* **52**, 7753-64 (2009).
138. Dorcier, A., Dyson, P.J., Gossens, C., Rothlisberger, U., Scopelliti, R. & Tavernelli, I. Binding of organometallic ruthenium(II) and osmium(II) complexes to an oligonucleotide: A combined mass spectrometric and theoretical study. *Organometallics* **24**, 2114-2123 (2005).
139. Peruzzini, M., Dorcier, A., Ang, W.H., Bolano, S., Gonsalvi, L., Juillerat-Jeannerat, L., Laurenczy, G., Phillips, A.D., Zanobini, F. & Dyson, P.J. In vitro evaluation of rhodium and osmium RAPTA analogues: The case for organometallic anticancer drugs not based on ruthenium. *Organometallics* **25**, 4090-4096 (2006).
140. Erck, A., Rainen, L., Whileyman, J., Chang, I.M., Kimball, A.P. & Bear, J. Studies of rhodium(II) carboxylates as potential antitumor agents. *Proc Soc Exp Biol Med* **145**, 1278-83 (1974).
141. Katsaros, N. & Anagnostopoulou, A. Rhodium and its compounds as potential agents in cancer treatment. *Crit Rev Oncol Hematol* **42**, 297-308 (2002).

142. Medici, S., Peana, M., Nurchi, V.M., Lachowicz, J.I., Crisponi, G. & Zoroddu, M.A. Noble metals in medicine: Latest advances. *Coordination Chemistry Reviews* **284**, 329-350 (2015).
143. Berners-Price, S.J. & Filipovska, A. Gold compounds as therapeutic agents for human diseases. *Metallomics* **3**, 863-73 (2011).
144. Casini, A. & Messori, L. Molecular mechanisms and proposed targets for selected anticancer gold compounds. *Curr Top Med Chem* **11**, 2647-60 (2011).
145. Bindoli, A., Rigobello, M.P., Scutari, G., Gabbiani, C., Casini, A. & Messori, L. Thioredoxin reductase: a target for gold compounds acting as potential anticancer drugs. *Coordination Chemistry Reviews* **253**, 1692-1707 (2009).
146. Fricker, S.P. Cysteine proteases as targets for metal-based drugs. *Metallomics* **2**, 366-77 (2010).
147. Krishnamurthy, D., Karver, M.R., Fiorillo, E., Orru, V., Stanford, S.M., Bottini, N. & Barrios, A.M. Gold(I)-mediated inhibition of protein tyrosine phosphatases: a detailed in vitro and cellular study. *J Med Chem* **51**, 4790-5 (2008).
148. Zhang, J.J., Ng, K.M., Lok, C.N., Sun, R.W. & Che, C.M. Deubiquitinases as potential anti-cancer targets for gold(III) complexes. *Chem Commun (Camb)* **49**, 5153-5 (2013).
149. Dyer, P.N., Edayathumangalam, R.S., White, C.L., Bao, Y., Chakravarthy, S., Muthurajan, U.M. & Luger, K. Reconstitution of nucleosome core particles from recombinant histones and DNA. *Methods Enzymol* **375**, 23-44 (2004).
150. Robinson, P.J., An, W., Routh, A., Martino, F., Chapman, L., Roeder, R.G. & Rhodes, D. 30 nm chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction. *J Mol Biol* **381**, 816-25 (2008).
151. Luger, K., Rechsteiner, T.J., Flaus, A.J., Waye, M.M. & Richmond, T.J. Characterization of nucleosome core particles containing histone proteins made in bacteria. *J Mol Biol* **272**, 301-11 (1997).
152. Luger, K., Rechsteiner, T.J. & Richmond, T.J. Preparation of nucleosome core particle from recombinant histones. *Methods Enzymol* **304**, 3-19 (1999).
153. Tanaka, Y., Tawaramoto-Sasanuma, M., Kawaguchi, S., Ohta, T., Yoda, K., Kurumizaka, H. & Yokoyama, S. Expression and purification of recombinant human histones. *Methods* **33**, 3-11 (2004).
154. Leslie, A.G. The integration of macromolecular diffraction data. *Acta Crystallogr D Biol Crystallogr* **62**, 48-57 (2006).
155. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **50**, 760-3 (1994).
156. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**, 2126-32 (2004).
157. DeLano, W.L. The PyMOL Molecular Graphics System. (2002).
158. Wu, B. & Davey, C.A. Platinum drug adduct formation in the nucleosome core alters nucleosome mobility but not positioning. *Chem Biol* **15**, 1023-8 (2008).
159. Luger, K. Structure and dynamic behavior of nucleosomes. *Curr Opin Genet Dev* **13**, 127-35 (2003).
160. Fei, J., Torigoe, S.E., Brown, C.R., Khuong, M.T., Kassavetis, G.A., Boeger, H. & Kadonaga, J.T. The prenucleosome, a stable conformational isomer of the nucleosome. *Genes Dev* **29**, 2563-75 (2015).
161. Mohideen, K., Muhammad, R. & Davey, C.A. Perturbations in nucleosome structure from heavy metal association. *Nucleic Acids Res* **38**, 6301-11 (2010).
162. Pizarro, A.M. & Sadler, P.J. Unusual DNA binding modes for metal anticancer complexes. *Biochimie* **91**, 1198-211 (2009).

163. Dorcier, A., Hartinger, C.G., Scopelliti, R., Fish, R.H., Keppler, B.K. & Dyson, P.J. Studies on the reactivity of organometallic Ru-, Rh- and Os-ptp complexes with DNA model compounds. *J Inorg Biochem* **102**, 1066-76 (2008).
164. Adhireksan, Z., Davey, G.E., Campomanes, P., Groessler, M., Clavel, C.M., Yu, H., Nazarov, A.A., Yeo, C.H., Ang, W.H., Droge, P., Rothlisberger, U., Dyson, P.J. & Davey, C.A. Ligand substitutions between ruthenium-cymene compounds can control protein versus DNA targeting and anticancer activity. *Nat Commun* **5**(2014).
165. Wu, B., Ong, M.S., Groessler, M., Adhireksan, Z., Hartinger, C.G., Dyson, P.J. & Davey, C.A. A ruthenium antimetastasis agent forms specific histone protein adducts in the nucleosome core. *Chemistry* **17**, 3562-6 (2011).
166. Wolters, D.A., Stefanopoulou, M., Dyson, P.J. & Groessler, M. Combination of metallomics and proteomics to study the effects of the metallodrug RAPTA-T on human cancer cells. *Metallomics* **4**, 1185-96 (2012).
167. Nazarov, A.A., Meier, S.M., Zava, O., Nosova, Y.N., Milaeva, E.R., Hartinger, C.G. & Dyson, P.J. Protein ruthenation and DNA alkylation: chlorambucil-functionalized RAPTA complexes and their anticancer activity. *Dalton Trans* **44**, 3614-23 (2015).
168. Jocher, C.J., Moore, E.G., Xu, J., Avedano, S., Botta, M., Aime, S. & Raymond, K.N. 1,2-hydroxypyridonates as contrast agents for magnetic resonance imaging: TREN-1,2-HOPO. *Inorg Chem* **46**, 9182-91 (2007).
169. Grazina, R., Gano, L., Sebestik, J. & Amelia Santos, M. New tripodal hydroxypyridinone based chelating agents for Fe(III), Al(III) and Ga(III): Synthesis, physico-chemical properties and bioevaluation. *J Inorg Biochem* **103**, 262-73 (2009).
170. Agrawal, A., de Oliveira, C.A., Cheng, Y., Jacobsen, J.A., McCammon, J.A. & Cohen, S.M. Thioamide hydroxypyrothiones supersede amide hydroxypyrothiones in potency against anthrax lethal factor. *J Med Chem* **52**, 1063-74 (2009).
171. Lewis, J.A., Mongan, J., McCammon, J.A. & Cohen, S.M. Evaluation and binding-mode prediction of thiopyrone-based inhibitors of anthrax lethal factor. *ChemMedChem* **1**, 694-7 (2006).
172. Feng, M.H., van der Does, L. & Bantjes, A. Iron (III)-chelating resins. 3. Synthesis, iron (III)-chelating properties, and in vitro antibacterial activity of compounds containing 3-hydroxy-2-methyl-4(1H)-pyridinone ligands. *J Med Chem* **36**, 2822-7 (1993).
173. Nazarov, A.A., Baquie, M., Nowak-Sliwinska, P., Zava, O., van Beijnum, J.R., Groessler, M., Chisholm, D.M., Ahmadi, Z., McIndoe, J.S., Griffioen, A.W., van den Bergh, H. & Dyson, P.J. Synthesis and characterization of a new class of anti-angiogenic agents based on ruthenium clusters. *Sci Rep* **3**, 1485 (2013).
174. Niesel, J., Pinto, A., Peindy N'Dongo, H.W., Merz, K., Ott, I., Gust, R. & Schatzschneider, U. Photoinduced CO release, cellular uptake and cytotoxicity of a tris(pyrazolyl)methane (tpm) manganese tricarbonyl complex. *Chem Commun (Camb)*, 1798-800 (2008).
175. Kinney, W.A., Lee, N.E., Blank, R.M., Demerson, C.A., Sarnella, C.S., Scherer, N.T., Mir, G.N., Borella, L.E., DiJoseph, J.F. & Wells, C. N-phenyl-2-pyridinecarbothioamides as gastric mucosal protectants. *J Med Chem* **33**, 327-36 (1990).
176. Meier, S.M., Hanif, M., Adhireksan, Z., Pichler, V., Novak, M., Jirkovsky, E., Jakupec, M.A., Arion, V.B., Davey, C.A., Keppler, B.K. & Hartinger, C.G. Novel metal(ii) arene 2-pyridinecarbothioamides: a rationale to orally active organometallic anticancer agents. *Chemical Science* **4**, 1837 (2013).
177. Bozic, I., Reiter, J.G., Allen, B., Antal, T., Chatterjee, K., Shah, P., Moon, Y.S., Yaqubie, A., Kelly, N., Le, D.T., Lipson, E.J., Chapman, P.B., Diaz, L.A., Jr., Vogelstein, B. & Nowak, M.A. Evolutionary dynamics of cancer in response to targeted combination therapy. *Elife* **2**, e00747 (2013).

178. Bhabak, K.P., Bhuyan, B.J. & Mugesh, G. Bioinorganic and medicinal chemistry: aspects of gold(I)-protein complexes. *Dalton Trans* **40**, 2099-111 (2011).
179. Zou, J., Taylor, P., Dornan, J., Robinson, S.P., Walkinshaw, M.D. & Sadler, P.J. First Crystal Structure of a Medicinally Relevant Gold Protein Complex: Unexpected Binding of. *Angew Chem Int Ed Engl* **39**, 2931-2934 (2000).
180. Mirabelli, C.K., Johnson, R.K., Sung, C.M., Faucette, L., Muirhead, K. & Crooke, S.T. Evaluation of the in vivo antitumor activity and in vitro cytotoxic properties of auranofin, a coordinated gold compound, in murine tumor models. *Cancer Res* **45**, 32-9 (1985).
181. Rubbiani, R., Kitanovic, I., Alborzinia, H., Can, S., Kitanovic, A., Onambele, L.A., Stefanopoulou, M., Geldmacher, Y., Sheldrick, W.S., Wolber, G., Prokop, A., Wolf, S. & Ott, I. Benzimidazol-2-ylidene gold(I) complexes are thioredoxin reductase inhibitors with multiple antitumor properties. *J Med Chem* **53**, 8608-18 (2010).
182. Casini, A., Mastrobuoni, G., Terenghi, M., Gabbiani, C., Monzani, E., Moneti, G., Casella, L. & Messori, L. Ruthenium anticancer drugs and proteins: a study of the interactions of the ruthenium(III) complex imidazolium trans-[tetrachloro(dimethyl sulfoxide)(imidazole)ruthenate(III)] with hen egg white lysozyme and horse heart cytochrome c. *J Biol Inorg Chem* **12**, 1107-17 (2007).
183. Wang, F., Bella, J., Parkinson, J.A. & Sadler, P.J. Competitive reactions of a ruthenium arene anticancer complex with histidine, cytochrome c and an oligonucleotide. *J Biol Inorg Chem* **10**, 147-55 (2005).
184. McBryant, S.J. & Hansen, J.C. Dynamic fuzziness during linker histone action. *Adv Exp Med Biol* **725**, 15-26 (2012).
185. Behrens, B.C., Hamilton, T.C., Masuda, H., Grotzinger, K.R., Whang-Peng, J., Louie, K.G., Knutsen, T., McKoy, W.M., Young, R.C. & Ozols, R.F. Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47**, 414-8 (1987).
186. Murray, B.S., Menin, L., Scopelliti, R. & Dyson, P.J. Conformational control of anticancer activity: the application of arene-linked dinuclear ruthenium(II) organometallics. *Chemical Science* **5**, 2536-2545 (2014).

Appendix I – DNA sequences

DNA 147 and 145 *red color denotes the locations where minor grooves are facing the histone octamer

```
70      60      50      40      30      20      10      0      10      20      30      40      50      60      70
--+-+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
ATCAATATCCACCTGCAGATACTACCAAAAGTGTATTTGGAAACTGCTCCATCAAAAGGCATGTTCAGCTGGAATCCAGCTGAACATGCCTTTTGATGGAGCAGTTTCCAAATACACTTTGGTAGTATCTGCAGGTGGATATTTGAT
ATCAATATCCACCTGCAGATACTACCAAAAGTGTATTTGGAAACTGCTCCATCAAAAGGCATGTTCAGCTG AATC AGCTGAACATGCCTTTTGATGGAGCAGTTTCCAAATACACTTTGGTAGTATCTGCAGGTGGATATTTGAT
```

DNA CMS155, 154 and 156

```
70      60      50      40      30      20      10      0      10      20      30      40      50      60      70
--+-+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
ATCACAATCCCCGTGCCGAGGCCCT CAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTAGGGAATGTTTGTTCTTATTTAAGCGCACCTAGAGCTCGCTACTCGCATTCT ACGATCCGCAAGGGATATTTGGAGAAAAAAAACGAT
ATCACAATCCCCGTGCCGAGGCCCT CAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTAGGGAATGTTTGTTCTTATTTAAGCGCACCTAGAGCTCGCTACTCGCATTCT ACGATCCGCAAGGGATATTTGGAGAAAAAAAAGAT
ATCACAATCCCCGTGCCGAGGCCCT CAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTAGGGAATGTTTGTTCTTATTTAAGCGCACCTAGAGCTCGCTACTCGCATTCT ACGATCCGCAAGGGATATTTGGAGAAAAAAAACCGAT
```

DNA PRP-167

```
70      60      50      40      30      20      10      0      10      20      30      40      50      60      70
--+-+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
ATCTACCGGTTCGCACAATCCCGTGCCGAGGCCCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGGAATCCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACCGGGATTGTGCGAACCGGTAGAT
```

DNA CMS167, 165 and 169

```
70      60      50      40      30      20      10      0      10      20      30      40      50      60      70
--+-+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
ATCTTTTTTTTTTCACAATCCCGTGCCGAGGCCCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGGAATCCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACCGGGATTGTGAAAAAAAAAAGAT
ATCTTTTTTTTTTCACAATCCCGTGCCGAGGCCCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGGAATCCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACCGGGATTGTGAAAAAAAAAAGAT
ATCGTTTTTTTTTCACAATCCCGTGCCGAGGCCCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGGAATCCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACCGGGATTGTGAAAAAAAAAACGAT
```

DNA601-36-177

ATC- (CCGAGTCAATACATGCACAGGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCTTGGCGGTTAAACGCGGGGACAGCGCTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCA
CCGGGATTCTCCAGGGCGGCCGCTATC) x36 - CCGAGAT

Appendix II – Protein sequences

xH2A SGRGKQGGKTRAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLAHVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDEELNKLGRVTIAQGGVLPNIQSVLLPKKTESSKSKSK
xH2B PEPAKSAPAPKKGSKKAVTKTQKKDGKKRRKTRKESYAIYVYVKLQVHPDTGISSKAMSIMNSFVNDVFERIAGEASRLAHYNKRSTITSREIQTAVRLLPGELAKHAVSEGTKAVTKYTSK
xH3 ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVLREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA
xH4 SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDAVITYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG

hH2A GSSHHHHHHSSGLVPRGSH
 MSGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYSERVGAGAPVYLAHVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLGRVTIAQGGVLPNIQAVLLPKKTESHKAKGK
hH2B GSSHHHHHHSSGLVPRGSH
 MPEPAKSAPAPKKGSKKAVTKAQQKDGKKRRKRSRKESYSIYVYVKLQVHPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRLLPGELAKHAVSEGTKAVTKYTSK
hH3 GSSHHHHHHSSGLVPRGSH
 MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVLREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA
hH4 GSSHHHHHHSSGLVPRGSH MSGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIRDAVITYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG

H1.0+His₆ HHHHHHSSGLEVLFGQPTENSTSAPAAKPKRAKASKKSTDHPKYS DMIVAAIQAEKNRAGSSRSIQKYIKSHYKVG ENADSIKLSIKRLVTTGVLKQTKGVGASGSFRLAKSDEPKKSVAFKKTKEIKKV
 ATPKKASKPKKAASKAPTCKPKATPVKKAKKKLAATPKKAKKPKTVKAKPVKASKPKKAKPVKPAKSSAKRAGKKK
H1.0 GPTENSTSAPAAKPKRAKASKKSTDHPKYS DMIVAAIQAEKNRAGSSRSIQKYIKSHYKVG ENADSIKLSIKRLVTTGVLKQTKGVGASGSFRLAKSDEPKKSVAFKKTKEIKKVATPKKASKPKKAASKAP
 TKKPKATPVKKAKKKLAATPKKAKKPKTVKAKPVKASKPKKAKPVKPAKSSAKRAGKKK
gH1.0+His₆ HHHHHHSSGLEVLFGQPSKKSTDHPKYS DMIVAAIQAEKNRAGSSRSIQKYIKSHYKVG ENADSIKLSIKRLVTTGVLKQTKGVGASGSFRLAKSDEPKKS
gH1.0 QGPSKKSTDHPKYS DMIVAAIQAEKNRAGSSRSIQKYIKSHYKVG ENADSIKLSIKRLVTTGVLKQTKGVGASGSFRLAKSDEPKKS

Publications of candidate

1. Wu, B., Ong, M.S., Groessl, M., Adhireksan, Z., Hartinger, C.G., Dyson, P.J. & Davey, C.A. A ruthenium antimetastasis agent forms specific histone protein adducts in the nucleosome core. *Chemistry* **17**, 3562-6 (2011).
2. Meier, S.M., Hanif, M., Adhireksan, Z., Pichler, V., Novak, M., Jirkovsky, E., Jakupec, M.A., Arion, V.B., Davey, C.A., Keppler, B.K. & Hartinger, C.G. Novel metal(ii) arene 2-pyridinecarbothioamides: a rationale to orally active organometallic anticancer agents. *Chemical Science* **4**, 1837 (2013).
3. Adhireksan, Z., Davey, G.E., Campomanes, P., Groessl, M., Clavel, C.M., Yu, H., Nazarov, A.A., Yeo, C.H., Ang, W.H., Droge, P., Rothlisberger, U., Dyson, P.J. & Davey, C.A. Ligand substitutions between ruthenium-cymene compounds can control protein versus DNA targeting and anticancer activity. *Nat Commun* **5**(2014).