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Exposure to Metals Can Be Therapeutic

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Abstract: Because of the widespread epigenetic changes ensuing from carcinogenesis, structural and chemical features of chromatin provide unique targets for developing safer and more effective anticancer drugs. Metal-based agents have a potential advantage over other small molecular species in that characteristics of coordination geometry, redox state and ligand exchange allow one to fine-tune reactivity and affinity properties in a distinct fashion. This intersection of chromatin biology and bioinorganic medicinal chemistry is the subject of multiple collaborations in and between Switzerland and Singapore.

Keywords: Anticancer agent · Chromatin · DNA structure · Metal-based drug · Nucleosome

I have been a faculty member at the Nanyang Technological University for over ten years, but before moving to Singapore I was a post-doctoral fellow at the ETH Zurich. It was there that I started to have ideas about ways to target specific sites in the genome that have the potential to selectively disrupt cancer cell proliferation. This emerged from my work on DNA structure in nucleosomes – the basic repeating units of our histone protein-packaged genome, called chromatin.

Nucleosome Structure in Switzerland

My chromatin work in the lab of Timothy Richmond at the ETH was mainly focused on getting the highest possible resolution crystal structure of the nucleosome core particle (NCP), with the electron density sufficiently well-defined to build an accurate atomic model for the DNA double helix.[1,2] The nucleosome core is the primary element of DNA packaging, in which 145 to 147 base pairs are in direct association with a histone octamer typically composed of two copies each of histone proteins H2A, H2B, H3 and H4 (Fig. 1a,b).[7] Nucleosome core regions in the genome are connected by linker DNA, generally around 10 to 90 bp, which can be protein-free or associated with linker histone proteins to yield highly compact chromatin states.[8]

For many years since the discovery that DNA is wrapped into nucleosomes, it was evident that the double helix would have to be substantially deformed to achieve the level of compaction observed in chromatin. Since DNA has sequence-dependent structure and flexibility,[9–11] pronounced distortion in the nucleosomal state is consistent with early work showing that the sequence influences where the histone octamers prefer to localize.[12,13] This so-called nucleosome positioning is now well understood to be a foundation for genomic regulation by modulating the context and accessibility of target DNA sites.[14] However, prior to elucidating an accurate model for the nucleosomal double helix, it was unclear how the structure may be related to or different from other DNA forms and thus how the DNA sequence may influence conformation and affinity for the histone octamer. Clarifying this would shed light on the molecular recognition of DNA in nucleosomes and rationalize how the DNA sequence contributes to chromatin function.

The X-ray diffraction quality of NCP crystals is strongly dependent on the length and sequence of the DNA fragment, but using a 147 bp DNA construct we obtained high resolution data with exceptionally well defined electron density for the double helix.[1] Extensive analysis of the resulting NCP model (Fig. 1b) yielded an illuminating perspective of DNA structure and histone-DNA interactions in the nucleosome,[1,2] which would provide a foundation for later work my group in Singapore carried out that culminated in a mechanical model for understanding sequence-dependent properties of nucleosomes (Fig. 1c,d).[3,6,15–18] In addition, however, the detailed view of NCP structure also revealed some unexpected insight into selective recognition of nucleosomal DNA by small molecules.

The best diffracting NCP crystals are grown in buffers containing the divalent metal manganese(II). While Mn^{2+} is a transition metal, its macromolecule-binding properties are similar to the ubiquitous biological cation, Mg^{2+}. However, in contrast to magnesium, manganese evokes a strong anomalous component of X-ray scattering, which allows one to accurately pinpoint sites of Mn^{2+} binding in the crystals – including those with only partial occupancy. This provided a view of Mn^{2+} association with the DNA in the NCP, and although metal binding is only observed at highly electronegative regions as expected, what was surprising was that many such candidate sites were not occupied with Mn^{2+} (Fig. 2).[20] In the DNA major groove, cations preferentially associate with guanine N7 and/or O6 atoms of GG and GC dinucleotides. However, some of these potential sites were not occupied with metal, and we could show that this was a consequence of the histone-imposed DNA conformation being incompatible with coordination of Mn^{2+}. I gathered that this stringent DNA structure-based discrimination for the binding of just simple metal hydrates was an indication of the potential for highly site selective nucleosomal double helix recognition by small molecules.

No Escape from Metals

When I started my research group in Singapore at the end of 2004, I wanted to contribute to the development of improved anticancer agents, and because of my sustained (academic) exposure to metals, I already had some appreciation

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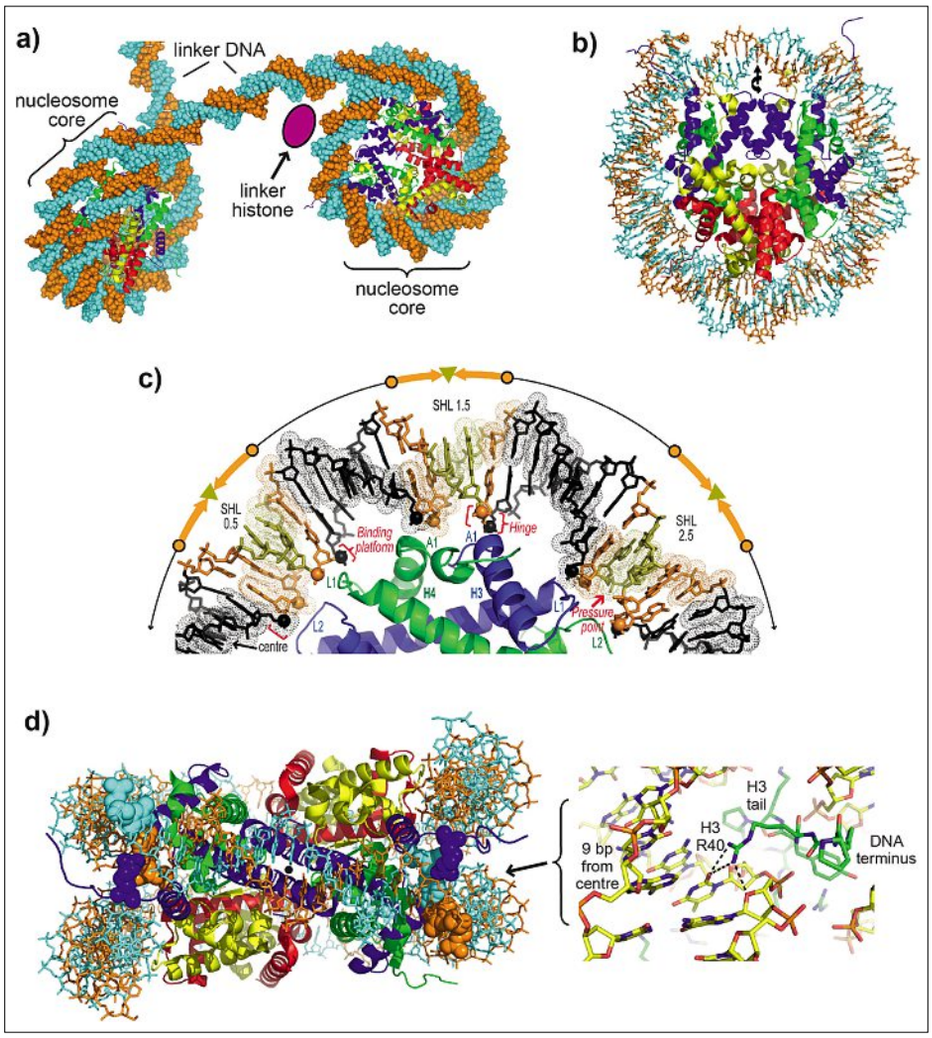

Fig. 1. Nucleosome structure and DNA sequence dependent attributes. (a) A two-nucleosome section of chromatin. (b) The 1.9 Å resolution crystal structure of the NCP, composed of a 147 bp DNA fragment.[1] The view is along the DNA superhelical axis, with the pseudo two-fold axis of the nucleosome running vertically through the central DNA base pair (straight arrow). (c) A mechanical model for explaining the general DNA sequence-dependent properties of the nucleosome.[3] Shown is an ~31 bp section starting from the nucleosome centre (SHL, double helical turns from centre). Systematic compression into and narrowing of minor groove inward (orange/gold nucleotides) versus major groove inward (black nucleotides) sections of the DNA yield a preference for A|T versus G|C sequences, respectively. Locations where the double helix is under the greatest 'pressure' to deform in the most energetically challenging mode (gold nucleotides) yield a special preference for the most flexible dinucleotide type, TA.[4,5] (d) The model in (c) portrays an 'indirect readout' mode of the DNA sequence, which is consistent with the premise that the histone proteins have evolved to largely minimize sequence dependency.[6]
However, there appears to be at least one example, illustrated here, where a histone element makes direct contacts with DNA base substituents to select for an A|T base pair (groups highlighted with space filling representation for the left panel). Such a 'direct readout' interaction could be used for modulating nucleosome unfolding and factor access in a DNA sequence-dependent fashion. (a-d) The two strands of the DNA (a, b, left panel of d) and the H3 (blue), H4 (green), H2A (yellow) and H2B (red) core histone proteins (a-c, left panel of d) are coloured individually.

for the unique potential of inorganic and bioinorganic compounds. In fact, medicinal agents based on a metal reactive center have the possible advantage that distinct characteristics of coordination geometry, redox state and ligand exchange allow one to fine-tune reactivity and affinity properties of the compound in a unique fashion.[23–25] For instance, the therapeutic potential of platinum, osmium or ruthenium compounds relies in part on favorably slow ligand exchange rates, which prevent rapid equilibration reactions that could cause inactivation, while allowing formation of long-lived bonds with key biological ligands.

As it turned out, my fascination with metals was sparked by my earliest research experiences. I began as an undergraduate at the University of Colorado in Boulder at a time when RNA biochemistry was just coming to the forefront because of the recently discovered ribozyme. Midway through my studies, Thomas Cech was awarded the Nobel prize for his discovery of catalytic RNA, at a time when I could not decide between two opposite ends of the scientific spectrum for my major. In the same department as Tom Cech was a graduate student in the lab of Marvin Caruthers, Doug Dellinger, who had convinced me during the course of many weightlifting sessions (here the metal of interest was iron) that I should give biochemistry a try. I figured this domain lies perhaps midway in the spectrum, so I adopted it as my second major.

After starting courses in biochemistry, I needed to experience lab work, and I managed to convince Olke Uhlenbeck to take me into his lab for a research project after stalking him at several RNA Club meetings. My project was to study the influence of tRNA base identity on ribosome binding, and for this I had to produce 'hot' tRNA by *in vitro* transcription that required massive quantities of ^{32}P-nucleotide label. Although I was primarily focused on avoiding Chernobyl-like incidences, it was at this early stage impressed upon me that divalent metal cations (Mg^{2+}/Ca^{2+} *in vivo*) are required for RNA folding and activity.

For my subsequent PhD work, I conducted X-ray crystallographic studies of myeloperoxidase in the lab of Roger Fenna at the University of Miami.[26–29] Myeloperoxidase is a heme-enzyme that plays a prominent role in our immune defense system by destroying phagocytized microorganisms and tumor cells through catalyzing the production of hypochlorous acid (HOCl). To do this, the enzyme is able to efficiently harness the energy inherent in the reduction of a peroxide molecule to oxidize chloride anion. This makes myeloperoxidases one of the most powerful oxidizing systems in the human body, and here again, metals were at the center of attention, as there is not only a unique (ferric, Fe^{3+}) heme prosthetic group, but also a Ca^{2+}-binding site essential for catalytic activity. I became particularly captivated by how the protein environment can intricately modulate redox properties of metal functional groups, especially after we discovered that the 'green' myeloperoxidase heme has an unprecedented three covalent bonds with protein side chains.[26]

I followed this doctorate work with a short stint in computational chemistry at the other University of Miami campus while my wife was completing her own PhD studies. I had become so fascinated with heme-protein chemistry, I decided to work on quantum mechanical calculations of heme-enzyme active site model systems, including the high symmetry, and thus computationally cheaper, iron-porphines.[30] When I was not vacillating between suicidal and homicidal thoughts over designing viable Z-matrices for the

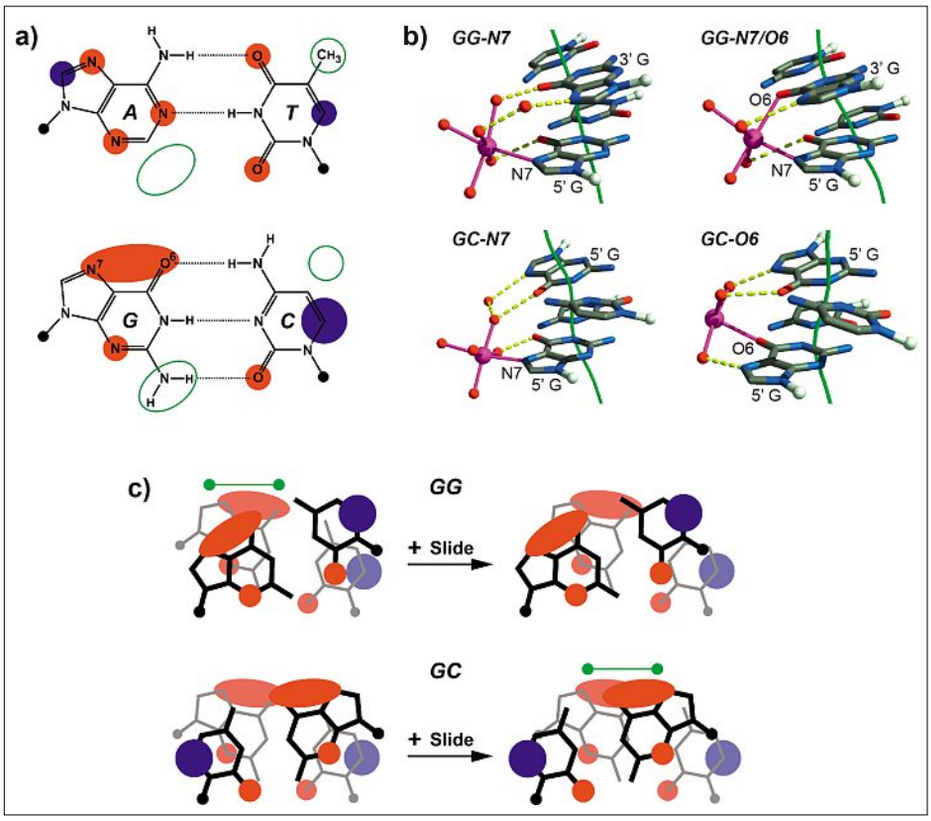

Fig. 2. DNA base chemical structure and metal cation binding. (a) Differences in electrostatic profiles (red, negative; blue, positive)[19] and steric factors (key distinctions between the two purines and the two pyrimidines highlighted in green) between the bases give rise to DNA sequence dependent structure/form and flexibility by influencing the stacking forces between base pairs. The extensive electronegative zone associated with the guanine N7 and O6 atoms provides the primary site for metal cation association with DNA base groups. (b) Mn^{2+} binding in the nucleosome core.[20] Two distinct modes of coordination are observed for both GG and GC dinucleotide sites. Coordinate bonds and Mn^{2+} ions are shown in magenta and hydrogen bonds as dashed yellow lines. The green tube corresponds to the double helix axis. (c) An example of how DNA structure can modulate metal binding.[20–22] Overlap of guanine bases (green dumbbells) electrostatically promotes cation association. Double helix bending into the minor groove is conformationally coupled to positive slide, but the effect of this base pair translation within a dinucleotide is opposite for GG versus GC, decreasing overlap for the former and increasing overlap in the latter.

optimizations, I did obtain some insight into bioinorganic chemistry that would hold my interest to this domain and come in handy later. Since I had already acquired an infatuation for Switzerland from spending a year as a high school (Gymnasium) exchange student in Basel, the next move to Zürich for post-doctoral work at the ETH, so that I could "better understand DNA", was not surprising.

Linking Nucleosomes with Bioinorganic Medicinal Chemistry in Singapore

Off of the heels of chromatin studies at the ETH, I thought that a good project to start up my new research group in Singapore would be to look at how platinum anticancer drugs form adducts on nucleosomes. Up until then, most of our knowledge on DNA-adduct formation processes was based on studies with naked (protein-free) DNA, or even short oligonucleotide fragments where end effects can dominate. However, the cellular substrate for DNA attacking agents is chromatin, and moreover most of the 6 billion bases pairs in each cell are directly associated with histones as part of one of the ~30 million nucleosome cores.

Platinum anticancer drugs act therapeutically by forming DNA lesions – generally cross-linking two adjacent guanine bases within a single strand – which interfere with genomic activities like transcription and ultimately induce apoptosis (Fig. 3a,b).[34,35] The prototypic platinum drug, cisplatin, was discovered to have antitumor activity already more than 45 years ago, and it is still today one of the most commonly used chemotherapeutic agents. However, cisplatin and congener drugs frequently yield substantial problems with toxicity and intrinsic or acquired resistance in patients.[36,37] This limits their utility, and yet improved agents are still at large in spite of a great many derivatives having been tested. One argument here is that, unlike for protein targets, specific genomic sites have generally not been considered in the drug development process.

If one considers that platinum drugs are exploiting some genomic weak points of tumor cells and that there are certain genes critical to cancer cell proliferation and survival that are relatively dispensable to healthy tissue,[38,39] then it is possible that specific DNA targets in the genome are more responsible for the therapeutic effects while others are largely counterproductive.[40] As such, one avenue to find better compounds could be to improve site selectivity properties. We started by first trying to understand where exactly platinum drugs prefer to form adducts in nucleosomes and the basis for this site discrimination. The initial study here involved setting up a crystallographic platform to observe adduct formation in crystals of the nucleosome core, and it was a surprise to find that platinum drugs could form adducts even at the nucleosome center, where histone contacts with the DNA, and thus constraints on DNA conformational changes, are greatest.[1,3,41] This led us to propose that central regions in the nucleosome may comprise favorable sites for drug design, since being least accessible to protein factors, like the DNA repair machinery,[42,43] adducts that form here may be more efficacious.[40,41]

We ended up conducting a number of structural and biochemical studies on platinum agents, as well as other transition and heavy metal species.[21,22,33,44] This showed that adduct formation by platinum drugs is governed by steric access to the guanine N7 coordinating group (Fig. 3c–e).[33] Thus the site selectivity is dependent on solvent accessibility, which we could demonstrate is modulated by specific double helix conformational features that are dictated by the interplay between DNA sequence and protein binding. As such we could establish that this is a feature common to coordinating metals and also likely many other DNA-attacking agents. In the case of the platinum drugs and other cross-linking agents, however, the initial reaction involves formation of a single coordinate bond, the 'monofunctional' adduct, which can undergo a second reaction to generate the 'bifunctional' adduct (cross-link). For the classic platinum drugs, it appears that it is only the bifunctional adducts that are therapeutically active, and we could show that the same DNA conformational features that promote fast monofunctional adduct formation can oppose chelation to generate the cross-link, which helped explain the observation of long-lived but ineffective monofunctional adducts.[33,34,45,46]

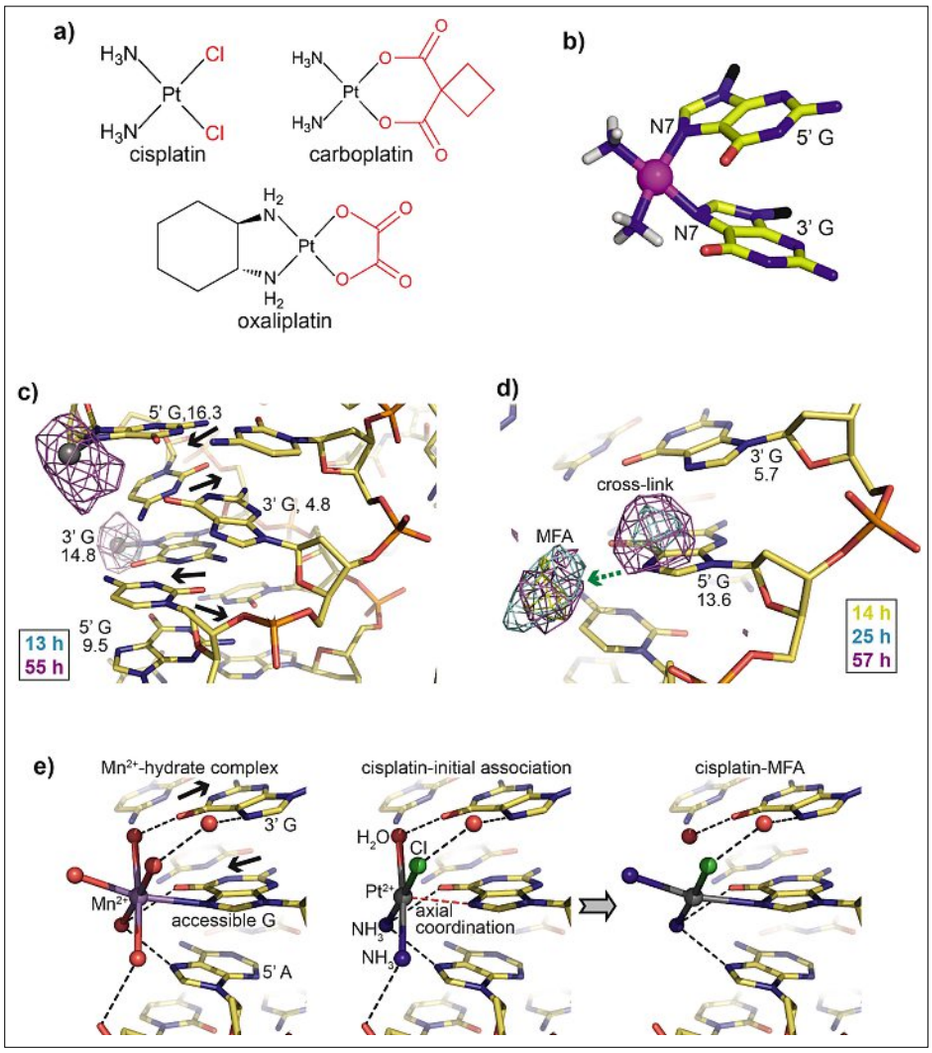

Fig. 3. Platinum agent adduct formation and site selectivity. (a) Chemical structures of the classic platinum drugs. Leaving groups (susceptible to aquation) are shown in red. (b) A cisplatin cross-link at a GG dinucleotide from the oligonucleotide DNA X-ray crystal structure.[31,32] (c,d) Platinum adduct formation in the nucleosome core.[33] Anomalous difference electron density maps show the locations of platinum atoms (silver spheres in c) resulting from treatment of crystals with compound for different durations. Values correspond to the solvent accessible surface area for the N7 atom (ASA_{N7}, Å^2) of the respective guanine base (overall average = 10.7 ± 3.5 Å^2). (c) Alternating dinucleotide shift (displacement of base pairs into the major and minor grooves; arrows) causes pronounced fluctuation in consecutive ASA_{N7} values over this GCGC element. Platinum adducts can be seen to form only at the guanine bases protruding into the solvent. (d) Oxaliplatin treatments for 14, 25 and 57 hours reveal monofunctional adduct (MFA) forming initially at the solvent accessible 5' guanine of this GG dinucleotide element, which can be seen to 'slowly' chelate to the 3' guanine to generate cross-link. (e) A model for monofunctional adduct formation by cisplatin, illustrating the commonality with transition metal-hydrate coordination in solvent access to the guanine N7 group. The central guanine base of this AGG element has an elevated ASA_{N7} as a result of shift at both the 5' and 3' sides (arrows), which favours metal bond formation.

Epigenetics for Finding Better Metal-based Drugs

Knowing that the small size and planar geometry of traditional platinum compounds allows them to form adducts at essentially any solvent accessible guanine bases, such indiscriminateness must contribute to the shortcomings of this class of anticancer agents. In considering how to design more site selective DNA attacking agents, aiming at the nucleosome core could have two potential advantages, in that for one there is a targeting potential that goes beyond the DNA sequence alone, because there are additionally unique double helix conformational features and an associated histone protein context. And secondly, hitting the nucleosome core regions of the genome can yield a distinct cellular impact. For instance, nucleotide excision repair of cisplatin adducts is inhibited when the lesions reside within the nucleosome core,[42] making adduct accumulation at such locations potentially more efficacious.

We started looking into designing nucleosome site-selective DNA adduct-forming agents after discovering that certain DNA sequences yield a phenomenal kink distortion at a site close to the nucleosome center.[15] This is a consequence of a unique histone binding motif at this location that acts as a clamp to impose an exceptionally narrow minor groove.[16] In fact, we found that the extreme kinking into the minor groove can also predispose intercalation at the unstacked base pairs of the major groove face, which creates a hotspot for DNA alkylation in the nucleosome by an intercalating epoxide antitumor species.[47] We took this as a lead and have made some progress in designing more stable, selective and potent therapeutic candidates.

The interest in finding compounds that can recognize DNA conformational features that are unique to the nucleosome core stems from the fact that the binding of such agents would be influenced by nucleosome positioning. That is, the same DNA element positioned at different histone binding sites, or instead in a linker region, would have distinct adduct formation potential. In this way, the activity of such agents could be sensitive to distinctions in nucleosome positioning that are cell-type and cell-status dependent. In fact, because of the pronounced changes in the gene expression profiles of cancer cells relative to healthy cells, one finds distinctions between the two cell types for all kinds of epigenetic features.[48–50] Depending on the particular gene and its activation status, there can be alterations in nucleosome positioning/occupancy as well as compaction state, in addition to DNA methylation, histone variant composition, and histone post-translational modifications. This translates to at least five classes of distinguishing chromatin features, and it suggests there may be many potential cancer-specific nucleosome targets in the genome.

The extensive epigenetic changes in chromatin structure and chemistry associated with the transformed state suggest a number of possibilities for selective disruption of cancer cell livelihood. Interestingly, this goes well beyond the DNA, since histone alterations can influence not only interactions with a multitude of regulatory factors but can also have a direct impact on nucleosome structure and dynamics. Traditionally, it has been assumed that reactive metal-based agents exert their therapeutic effect by forming DNA adducts, but recent work has challenged this view and in fact suggested that the histone proteins may emerge as a medicinal target.[51–55]

To circumvent the side effects associated with platinum drugs, much recent effort has focused on discovering improved compounds based on alternative metals.[56] Certain ruthenium agents have been showing promise from their specific activities against different cancers in combination

with having favorable toxicity and clearance profiles.[57] We found that one such compound, the antimetastasis ruthenium agent, RAPTA-C,[58,59] is associated with the protein component of chromatin in treated cancer cells and forms adducts in the nucleosome core at specific histone protein sites *in vitro* (Fig. 4).[52] Strikingly, a similar ruthenium-cymene compound, RAED-C,[61,62] differing from RAPTA-C in only a seemingly modest ligand substitution, associates predominantly with the DNA.[60] We established that the difference in site selectivity comes down to a steric obstacle posed by the slightly bulkier carrier ligand of RAPTA-C, which disfavors DNA adduct formation. Interestingly, RAED-C is highly cytotoxic and has antiprimary tumor activity, whereas RAPTA-C has very low cytotoxicity. We proposed that the DNA lesions are largely responsible for the cytotoxic effects, whereas protein adducts may be linked to the antimetastatic activities of RAPTA-C. Moreover, the selectivity for specific structural features elicited by these two agents suggests that there is indeed potential for targeting distinguishing epigenetic characteristics of cancer cells.

Swiss-Singaporean Synergy

Although Switzerland is about a 12-hour flight from Singapore, there are direct connections for us – in both the research and airline sense. Having become an expert at exploiting synchrotron facilities while at the ETH, and resigned to the superb operations at the Swiss Light Source, we still go to collect our X-ray diffraction data in Switzerland. Moreover, most of our collaborators outside of Singapore are at Swiss institutes, and even those within Singapore often share similar connections with past experiences or present collaborations in Switzerland.

I think the strong academic links between Singapore and Switzerland are no coincidence, because of the, structural and functional as we might say, commonalities between the two countries. In fact, just before we made the move over from Zürich, to get a perspective on life in Singapore, we met with a Swiss family who had lived there with their five children for many years. The couple summarily described Singapore as 'the Switzerland of Asia'. And I gather this comes down to the fact that both countries, because of their small size and population, have evolved analogous niche economies that allow them to be very affluent. In the same sense, for academics, Singapore and Switzerland continue to invest heavily in research and yet because of their small size relative to academic giants like the US, UK, Germany and Japan, they can be most competitive by specializing in just a few specific domains. I believe in this regard the two countries can capitalize on similarities and synergistic differences in continued collaboration to achieve excellence in defined research arenas.

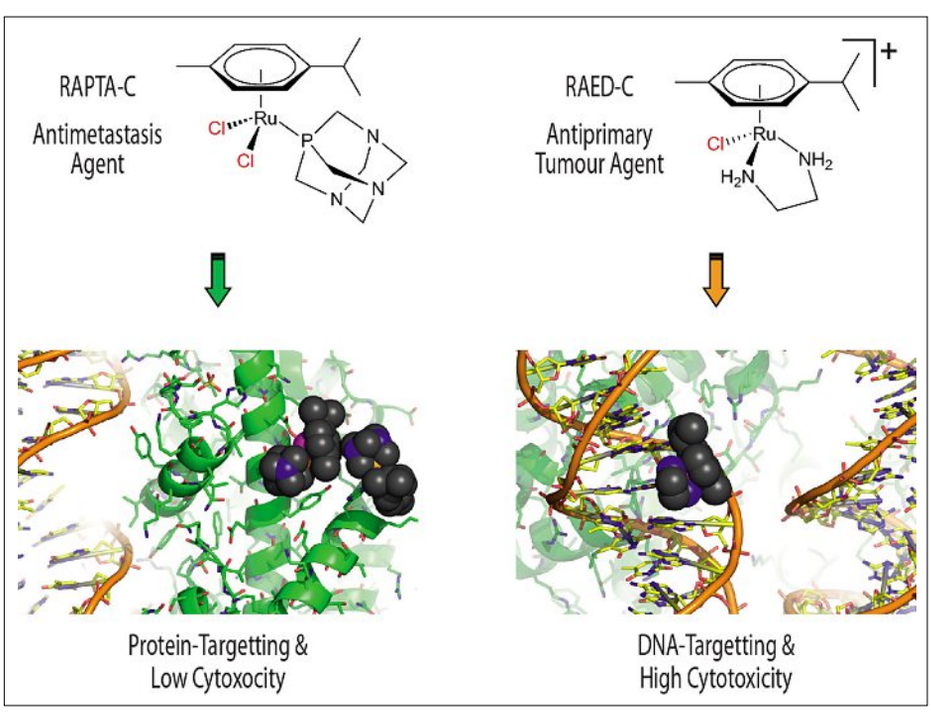

Fig. 4. Ligand substitutions between ruthenium-arene compounds can control protein versus DNA targeting and anticancer activity.[52,60] Molecular models correspond to X-ray crystal structures of the nucleosome core treated with RAPTA-C, [(η^6-*p*-cymene)Ru(1,3,5-triaza-7-phosphaadamantane)Cl$_2$], or RAED-C, [(η^6-*p*-cymene)Ru(ethylene-diamine)Cl]PF$_6$. Ruthenium compound adducts appear in space filling representation with black carbon atoms. Histone proteins and DNA are shown with green and orange backbones, respectively.

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Competing Financial Interests

The author declares no competing financial interests.

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