DNA Recognition

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Specific Blocking of CREB/DNA Binding by Cyclometalated Platinum(II) Complexes **

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DNA is one of the major targets for anticancer drugs, and, as such, a large proportion of current chemotherapeutic anticancer drugs are DNA-binding agents. Organic molecules such as anthracyclines bind to the DNA duplex by two binding modes, namely DNA intercalation and groove binding.[1] The binding of metal complexes to DNA is well documented.[2] We propose to employ Pt^{II} and Au^{III} ions to assemble organic ligands through metal–ligand coordination into cationic planar structures that have a high level of functionality; Pt^{II} complexes that bear chelating N-donor ligands have been shown by us and other research groups to display anticancer activities.[3–8] Cationic planar structures are known to be bioactive and bind to DNA.[9] However, the synthesis of organic planar cations that have sophisticated structures could be a formidable challenge as this goal could involve multistep synthesis. We have previously developed cyclometalated Pt^{II} complexes that exhibit intercalation[4–7] and minor-groove-binding[5] properties. The DNA-binding reactions of the cyclometalated complexes $[Pt^{II}(C^N^N)L]^{n+}$ (where C^N^N = 6-phenyl-2,2′-bipyridyl), which are structurally analogous to $[Pt^{II}(terpy)Cl]^+$ (terpy = 2,2′;6′,2″-terpyridyl), have been studied.[5–8] Given the structural diversity and the ease with which the $[Pt^{II}(C^N^N)L]^{n+}$ system could be modified, these Pt^{II} complexes could form a class of anticancer agents with tunable biological activities.

Transcription factors are a large class of proteins that bind to specific DNA sequences, thereby controlling the flow of genetic information from DNA to mRNA,[10,11] and are thus critical for normal cellular function. Most therapeutic approaches to targeting transcription factors indirectly alter their activity. Research at the chemistry/biology interface has led to new ways of directly targeting transcription factors, including blocking the transcription factor/DNA interaction by DNA-binding agents.[12,13] The metallointercalator [Λ-1-$Rh^{III}(MGP)_2(phi)]^{5+}$ (MGP = methylguanidium phenanthroline, phi = phenanthrenequinone diimine) and the electrostatic surface binder $[Cr^{III}(salen)(H_2O)_2]^+$ (salen = *N*,*N*′-bis-(salicylidene)ethylenediamine) are rare examples of metal complexes that intercalate DNA in the major groove and inhibit binding of transcription factors AP-1 and Sp1 to their respective consensus DNA sequences.[14] More recently, $[Ru^{II}(phen)_2(dppz)]^{2+}$ and $[Pt^{II}(5,6\text{-}Me_2phen)(S,S\text{-}dach)]^{2+}$ (phen = 1,10-phenanthroline, dppz = dipyrido[3,2-a:2′,3′-c]phenazine, Me_2phen = dimethyl-1,10-phenanthroline, dach = diaminocyclohexane) were found to interfere with the interaction between the transcription factor PU.1 and DNA.[15]

The cAMP response element binding protein (CREB) is a well-characterized transcription factor of the basic leucine zipper family.[16,17] CREB contacts the DNA major groove of the consensus sequences referred to as cAMP response elements (CRE), and thereby activates the transcription of genes related to growth and survival.[18–21] The activation of CREB has been demonstrated in a variety of tumor types, such as acute myeloid/lymphoid leukemia and hepatocellular carcinoma.[22–24] Herein, we report a class of Pt^{II} complexes that effectively inhibit the DNA-binding activity of transcription factors. Notably, a platinum-based DNA major groove binder that specifically blocks CREB/DNA binding has been identified.

The cyclometalated Pt^{II} complexes **1a–b** and **2a–c** (Scheme 1) were synthesized and characterized (see experimental details in the Supporting Information; Figures S1–S6 and Table S1 give photophysical data for complexes **1** and **2** recorded in various solvents). A solution of **1a** in CH_2Cl_2 at 298 K displays a low-energy emission band at $\lambda_{max} = 679$ nm (Figure 1) with vibrational spacing, lifetime, and quantum yield of 1177 cm^{-1}, 26.5 μs, and 0.01, respectively. The energy of this emission is slightly lower than those of the triplet intraligand (^{3}IL) excited states of the Au^I and Pt^{II} pyrenylacetylide complexes (652 and 664 nm, respectively);[25] the emission of **1a** is assigned to the ^{3}IL excited state of 1-((4-isocyano-3,5-diisopropylphenyl)ethynyl)pyrene. As shown in Figure 1, the emission maximum is slightly red-shifted as the solvent is changed from MeOH (672 nm) to MeCN (677 nm) and CH_2Cl_2 (679 nm), that is, in the presence of solvents with lower polarities. Complexes **1b** and **2a–c** exhibit an emission

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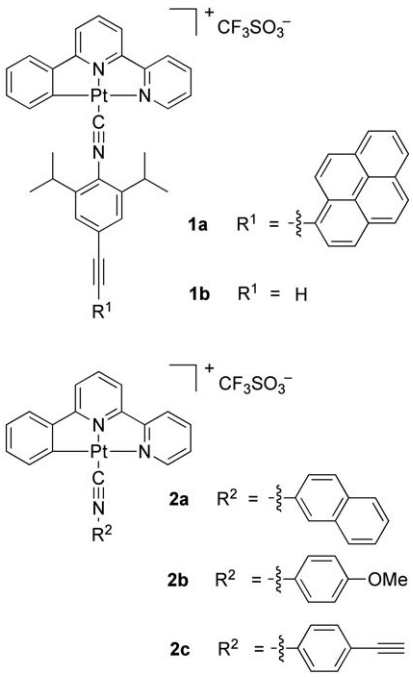

Scheme 1. Structures of $[Pt(C^N^N)(C{\equiv}N\text{-}L)]^+$ (**1a**, **b**, **2a**–**c**).

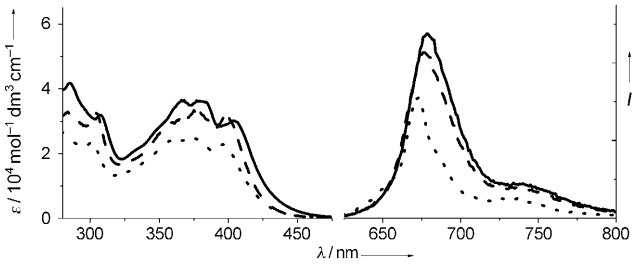

Figure 1. UV/Vis absorption and emission spectra of **1a** in CH_2Cl_2 (——), MeCN (– – –), and MeOH (·····) at 298 K (ca. 5×10^{-5} mol dm^{-3}).

with $\lambda_{max}\approx525$ nm in MeCN solution. With reference to earlier work,[26] the structureless emissions of complexes **2a**–**c** are assigned to triplet metal-to-ligand charge transfer (^{3}MLCT) excited states.

Absorption titration experiments show that all the Pt^{II} complexes bind to calf thymus DNA. The absorption spectrum of **1a** in tris(hydroxymethyl)aminomethane (Tris) buffer shows significant spectral changes upon addition of calf thymus DNA. The binding constant K for **1a** at 298 K was estimated to be 7.4×10^8 mol^{-1} dm^3, as derived from a plot of $D/\Delta\varepsilon_{ap}$ versus D according to the Scatchard equation[27] (the K value for calf thymus DNA determined by isothermal titration calorimetry (ITC) measurements is $(1.7\pm0.12)\times10^7$ mol^{-1} dm^3 (Figure S7)). The K values for **1b** and **2a**–**c** were similarly determined by absorption titration experiments to be 3.0×10^6 and approximately 10^5 mol^{-1} dm^3, respectively (the titration curves are given in Figure S8). All of the Pt^{II} complexes are weakly emissive in Tris buffer. The emission intensity of **1b** and **2b** both increased nearly 20 times upon addition of calf thymus DNA. However, the emission intensity of **1a** did not significantly change upon addition of calf thymus DNA (Figure S9).

The DNA binding modes were examined by gel mobility shift assay, viscosity, and NMR experiments. The results of gel mobility shift assays on a 100 bp DNA ladder showed that the DNA mobility was not affected by **1a** and Hoechst 33342, while a significant reduction in mobility was observed with **1b**, **2a**–**c**, and ethidium bromide, which is the most common intercalator (Figure S10). The intercalative binding modes of **1b** and **2a**–**c** were also confirmed by the increase in viscosity of the DNA solutions upon addition of the complexes (Figure S11).[28] In contrast, **1a** behaves as a DNA major-groove binder. An NMR titration[29] experiment that involved addition of **1a** to the dodecanucleotide d(CAATCCGGATTG)$_2$ revealed significant shifts for the sugar H1′ protons, and the H1′ protons of G_{12} and C_1 exhibited shifts of 0.015 ppm or more (Figure S12). As the sugar H1′ protons are located in the DNA major groove, the observed changes in chemical shifts upon addition of **1a** revealed that this complex specifically binds to the major groove. As a result of its large dimensions (23.1×11.4 Å), complex **1a** behaves as a DNA major-groove binder, whereas **1b** and **2b** were found to behave as DNA intercalators. Furthermore, the emission of **1a** is intraligand in nature, whereas those of **1b** and **2b** are assigned to excited states with ^{3}MLCT character, the intensity of which are sensitive to local environment. Thus, the difference in the emission intensity enhancements upon addition of DNA to solutions of the complexes **1a**, **1b**, and **2b** can be rationalized by the different DNA binding modes and different emissive excited states of these three complexes.

Transcription factors are proteins that typically bind to the grooves of DNA. The transcription factors CREB,[30] NF-κB,[31] and AP-1[14a,32] all bind to the major groove of DNA. We anticipated that the binding of Pt^{II} complexes to the major groove of DNA could block the association of transcription factors with their consensus DNA sequences. The effect of **1a** on the transcription factor/DNA binding activity was examined by an electrophoretic mobility shift assay. Hepatocellular carcinoma (HepG2) nuclear extracts, which contained the CREB, NF-κB, or AP-1 transcription factor, were incubated with their corresponding consensus oligonucleotides in the presence of **1a**. Figure 2 (upper) shows the formation of transcription factor/DNA complexes, which can be recognized by the antibody (Ab). The signal was dramatically reduced upon addition of an excess of unlabeled DNA (UL). In the presence of **1a**, a dose-dependent suppression of CREB/DNA complex formation was observed ($IC_{50}\approx30$ μM), while **1a** did not inhibit the DNA binding of NF-κB and AP-1 to their consensus sequences in HepG2 cell extracts. We then evaluated the impact of **1a** on the transcriptional activity of CREB in HepG2 cells by a luciferase reporter assay. The activities of both NF-κB and AP-1 were also measured. Complex **1a** was found to inhibit the CREB-dependent gene transcription in a dose-dependent manner ($IC_{50}\approx35$ μM), but it did not display a significant effect on the transcriptional activity of NF-κB and AP-1 at concentrations up to 80 μM (Figure 2, lower). Our results revealed that **1a** selectively suppresses CREB-dependent gene transcription, at least in part, by blocking the direct interaction between

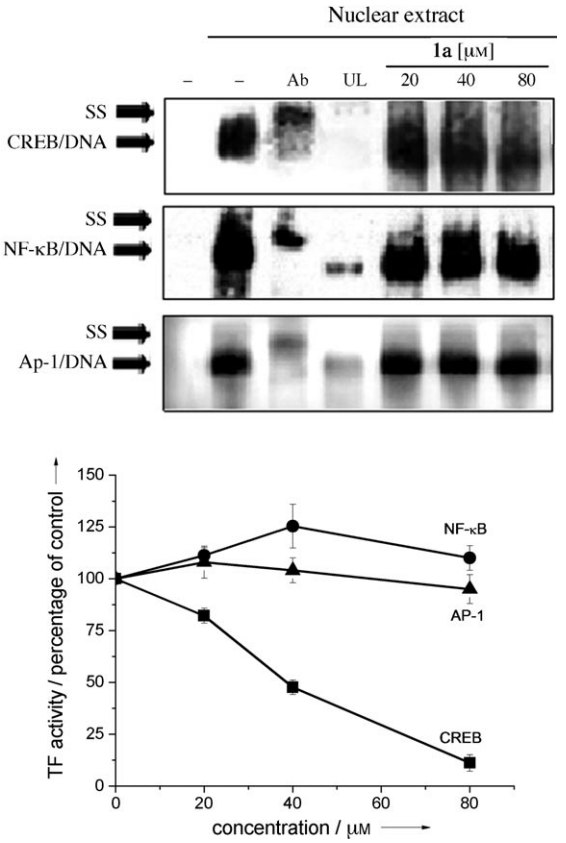

Figure 2. Complex **1a** selectively inhibits CREB activity. Upper: effect of **1a** on CREB, NF-κB, and AP-1–DNA binding activity. SS = supershift. Lower: **1a** inhibits CREB, NF-κB and AP-1-mediated gene transcription in HepG2 cells.

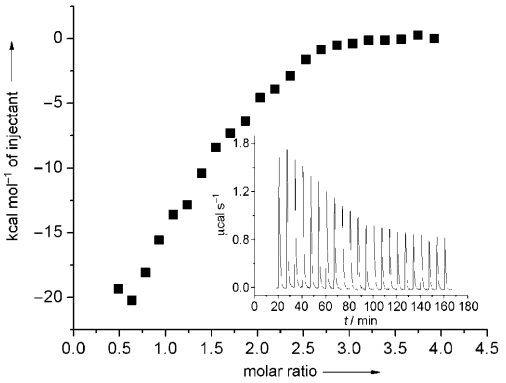

Figure 3. ITC profile for the binding of complex **1a** to CREB consensus DNA sequence at 298 K in TAE buffer at pH 8.0. Raw data is shown in the inset.

CREB and its consensus DNA sequences (CRE). This selectivity is consistent with the result of electrophoretic mobility shift assays. Intriguingly, complexes **1b** and **2b** were found to inhibit CREB, NF-κB, and AP-1-DNA binding, and transcriptional activities with comparable high potencies (Figures S13 and S14), thus suggesting that these two Pt^{II} complexes behave as intercalators that nonspecifically interfere with the DNA binding activities of these proteins, unlike the DNA groove binder **1a**, as described above. The Pt^{II} ion plays a critical role in the inhibitory activity of **1a** as the free ligand (La) shows no significant impact on both CREB/DNA binding and CREB-mediated gene expression at concentrations up to 100 μM (Figures S13-2 and S14-4).

The topologies of the groove regions have been reported to be much more variable and sequence-dependent compared to intercalating binding sites.[33] We therefore examined the sequence selectivity for **1a**. The interaction of this complex with AT-rich, CG-rich, and five consensus DNA sequences of major-groove binding transcription factors (including CREB, NF-κB, AP-1, SP1,[34] and EGR-1[35]) were studied by ITC in order to evaluate the sequence specificity. In each case, the titration curve (Figure 3 and Figure S15) revealed a classical binding behavior with a sigmoidal response. The binding constant of **1a** with CRE, which is a DNA sequence for CREB binding, was calculated to be $(2.3 \pm 0.18) \times 10^7\ mol^{-1}\,m^3$ at 298 K. Notably, **1a** displays a significantly lower binding affinity toward the mutated CRE sequence (see the Supporting Information). The *K* value was determined to be $(8.2 \pm 0.17) \times 10^5\ mol^{-1}\,dm^3$, which is approximately 28 times lower than that of **1a** toward the wild-type CRE sequence (Figure S15-8). Parallel experiments with AT-rich, CG-rich, NF-κB, AP-1, SP1, and EGR-1 consensus 22-mer DNA were performed. The *K* values are approximately 23, 96, 12, 209, 523, and 719 times lower, respectively, than that of **1a** towards the CREB-binding sequence (Table 1). On the contrary, the ITC experiments revealed that complex **1b** exhibits similar affinities towards CREB, NF-κB, and AP-1 consensus DNA sequences (3.2, 5.7, and $2.4 \times 10^6\ mol^{-1}\,dm^3$, respectively; Figure S16). These results reveal the important role of the 1-((4-isocyano-3,5-diisopropylphenyl)ethynyl)pyrene ligand with a large π-surface area in conferring the sequence selectivity to the cyclometalated Pt^{II} complex **1a**, as is consistent with the molecular modeling study (see below). The *K* value of the free ligand (La) towards CRE was determined to be $(2.1 \pm 0.22) \times 10^5\ mol^{-1}\,m^3$, which is 110 times lower than that of **1a**, thus suggesting that the presence of Pt^{II} ion is essential for the biological activity of **1a**.

Table 1: Thermodynamic parameters for the binding of **1a** to DNA sequences.[a]

Oligonucleotide	22-mer DNA sequence	$K\ [mol^{-1}\,dm^3]$
CRE	GACGCGTGACGTCACAACAAGC	$(2.3 \pm 0.18) \times 10^7$
AT	ATAATTAAATTTAAAATTTTAA	$(1.0 \pm 0.11) \times 10^6$
CG	CGCCGGCCCGGGCCCCGGGGCC	$(2.4 \pm 0.26) \times 10^5$
NF-κB	AGTTGAGGGGACTTTCCCAGGC	$(1.9 \pm 0.20) \times 10^6$
AP-1	CGCTTGATGACTCAGCCGGAAG	$(1.1 \pm 0.07) \times 10^5$
SP1	AGCGCCTCTGTGGGCGGGGTCA	$(4.4 \pm 0.15) \times 10^4$
EGR-1	TATGCGGGGGCGGCGCGGCAAT	$(3.2 \pm 0.13) \times 10^4$

[a] Values derived from ITC measurements (pH 8.0, 298 K) in TAE buffer (TAE = Tris-acetate-EDTA; EDTA = ethylenediaminetetraacetic acid).

The different DNA binding modes of **1a**, **1b**, and **2b** can account for the observed DNA sequence selectivity. However, we have not ruled out the possibility that these complexes also suppress the protein activities through direct interactions with proteins, and this area is currently under investigation. We also note that **1a** does not block all major-

groove-binding transcription factors (e.g., NF-κB and AP-1). Apparently, there is no direct relationship between the DNA-binding modes of both the Pt^{II} complexes and transcription factors. Nevertheless, our data revealed that **1a** exhibits sequence selectivity, which could lead to selective inhibition of the binding between transcription factors and their consensus DNA.

Our experimental results are consistent with the results of molecular modeling using the flexible-ligand docking module of ICM-Pro 3.6-1 molecular software (Molsoft).[36] The binding between **1a** and CREB was evaluated from the binding energy (including grid energy), continuum electrostatic, and entropy terms. The X-ray crystal structure of CREB with DNA was downloaded from the Protein Data Bank (PDB code: 1DH3). Analysis of the low-energy metal complex conformations suggests that **1a** binds to the major groove of DNA at the CREB interaction site (Figure 4). We propose that the large size of **1a**, calculated to be 23.1×11.4 Å, together with the flexible rotation of the coordinated

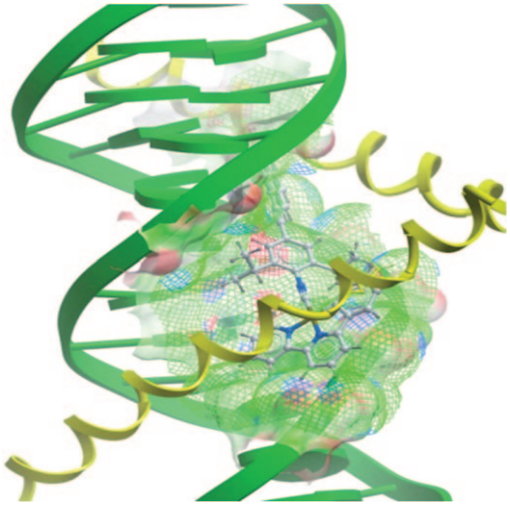

Figure 4. Schematic model of the competition between complex **1a** and CREB on the target DNA. The CREB protein is represented as a ribbon model and is colored yellow, the DNA is colored green, and **1a** is represented as a ball-and-stick model.

isocyanide ligand around the Pt–C≡NR bond, play important roles in allowing the complex to enter and occupy the major groove of the CRE consensus DNA. This binding leads to inhibition of the CREB/DNA interaction and subsequent gene transcription.

The inhibition of human cancer cell growth by **1a** occurs presumably through the regulation of gene expression. It is well documented that CREB regulates cell cycle regulatory genes such as cyclin A1 and cyclin D2.[19,21] Therefore, we used the reverse transcriptase–polymerase chain reaction (RT–PCR) to elucidate the molecular mechanism of CREB inhibition by complex **1a**. The results revealed that complex **1a** significantly suppresses the transcription at the mRNA level of both cyclin A1 and cyclin D2 genes in a dose- and time-dependent manner in forskolin-activated HepG2 cells (Figure 5), thus suggesting that the inhibition of CREB by **1a**

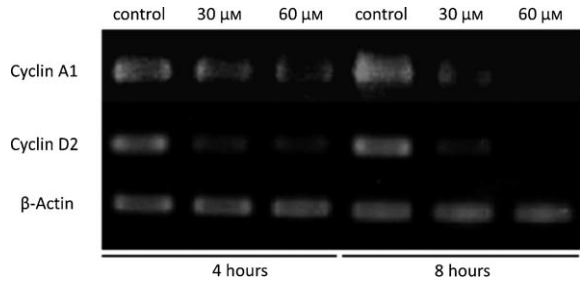

Figure 5. Impact of **1a** on the transcription of cyclin A1 and cyclin D2 in the HepG2 cells. Total RNA were extracted from HepG2 cells treated with DMSO (control) or **1a**. mRNA levels of cyclin A1 and D2 were determined by the reverse transcriptase–polymerase chain reaction.

is accompanied by the down-regulation of cyclin A1 and cyclin D2.

An ICP-MS experiment showed that **1a** readily enters human cells (Figure S17). By using an MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), the in vitro cytotoxicities of **1a** against three human cancer cell lines (HeLa, HepG2, and SUNE1) as well as a normal lung fibroblast cell line (CCD-19 Lu) were evaluated (Table S2). Complex **1a** displays moderate cytotoxicity against the human carcinoma cell lines ($IC_{50} \approx 23$ μM) and was noncytotoxic against CCD-19Lu ($IC_{50} > 100$ μM). The cytotoxicity of **1a** could be, at least in part, due to the down-regulation of the cyclin A1 and cyclin D2 genes that are under the regulation of CREB.

In conclusion, square-planar Pt^{II} complexes can be developed into DNA major-groove binders by a judicious choice of auxiliary ligands. A "proof-of-principle" concept for the blocking of the transcription factor/DNA interaction based on the unique DNA major-groove binding properties of the Pt^{II} complex **1a** has been demonstrated. This complex selectively blocks CREB/DNA binding in both cell-free and cellular assays. The selectivity is, at least in part, due to the higher affinity of **1a** to CREB compared to other consensus sequences. The $[Pt(C^N^N)(C{\equiv}N{-}L)]^+$ scaffold can, in principle, be modified to develop specific gene regulators with superior potency and selectivity while the substituent L is a key structural motif in governing the DNA binding mode and the selectivity of the complexes against transcription factors.

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