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Citrate inhibition of cisplatin reaction with DNA studied using fluorescently labeled oligonucleotides: implication for selectivity towards guanine†

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The reaction between cisplatin and DNA is conveniently studied using fluorescently labeled oligonucleotides and gel electrophoresis; as an example of application, the inhibition of this reaction by citrate is demonstrated, which might increase selectivity of cisplatin towards guanine over adenine.

Cisplatin is one of the most successful and important anti-cancer drugs.[1–4] It is generally accepted that DNA is the molecular target of cisplatin, forming intrastrand crosslinked guanines,[1] although the exact mechanism is still under debate.[5] A lot has already been learned about the reaction between DNA and cisplatin.[6] The difference in the Cl^- concentration outside (~100 mM) and inside (~4–12 mM) a cell might facilitate dissociation of Cl^- and adding water inside the cell.[1] The aquated product is trapped in the cell to react with various nucleophilic species including DNA.[7,8] Mechanisms related to electron transfer have also been proposed.[9] Due to the lack of appropriate analytical tools to follow cisplatin inside live cells,[5] most studies were carried out in simple buffers. The cellular environment, however, is much more complex containing numerous small molecules, nucleic acids and proteins that compete for cisplatin binding.[10,11] The cisplatin concentration inside cells is estimated to be just nanomolar to low micromolar.[5] Many cellular compounds can tightly bind to cisplatin, leaving little free cisplatin for DNA binding. Examples of such competitors include sulfur containing proteins,[12,13] glutathione (GSH),[14,15] and even inorganic anions.[16] Before cisplatin can react with DNA, it has to be released from these competing ligands.[17]

Many inorganic ions have a high cellular concentration and may affect cisplatin binding to DNA. For example, phosphate, acetate, and carbonate have been shown to bind to cisplatin.[16,18–21] Citrate is an important cellular metabolite but its effect on cisplatin has not been studied. Cellular citrate concentration is high in *Aspergillus niger* (~2–30 mM),[22] and in *Saccharomyces cerevisiae* (~3 mM).[23,24] In human tissues, citrate was reported to be 0.2–0.45 mM.[25] This is likely to be under-estimated since most citrate resides in mitochondria, where it is formed and utilized to make lipids.[26] It has also been suggested that the real target of cisplatin might be the mitochondrial DNA instead of the nuclear DNA,[27] where the role of citrate is even more relevant. Therefore, we are interested in studying the effect of citrate on the reaction between cisplatin and DNA.

Cisplatin binding to DNA has been monitored using HPLC,[28,29] NMR,[30] electrochemistry,[31] mass spectrometry,[32] and elemental analysis.[33] Compared to these methods, gel electrophoresis is more cost-effective and readily accessible to many researchers. It can tolerate complex sample matrices without worrying about clotting of column or spectroscopic interference. Gel electrophoresis has been used to confirm DNA binding to cisplatin in a few reports,[16,28,34] where most employed radioisotope labels or DNA staining dyes for imaging long biological DNA. Given the development and recent applications of covalent fluorophore labels, such advances have not been widely applied to study DNA–cisplatin reaction.[35] Herein, we use gel electrophoresis to follow this reaction in citrate buffer.

FAM (carboxyfluorescein)-labeled DNAs are popular probes because of their low cost and high quantum yield. As an initial test, we employed FAM-labeled 15-mer DNA homopolymers. The DNAs were mixed with increasing concentrations of cisplatin for 16 h and the samples were then loaded into a non-denaturing polyacrylamide gel. A gradual shift of the FAM-A_{15} band with reduced mobility was observed with increasing cisplatin concentration (Fig. 1A), suggesting reaction between this DNA and cisplatin. The Pt-DNA adduct did not migrate as a single band, suggesting the presence of a broad range of products, possibly due to different levels and positions of platination. Fluorescence quenching was also observed, especially at high Pt concentrations. On the other hand, no shift was observed with FAM-T_{15} and its fluorescence just dropped in intensity with increasing cisplatin concentration (Fig. 1B). Reactions also occurred with FAM-C_{15} and its product distribution pattern was quite different, in which discrete bands were observed at low Pt concentrations and the gel smeared at high Pt concentrations (Fig. 1C). Finally, FAM-G_{15} showed a slightly smeared gel even for the initial free DNA, possibly due

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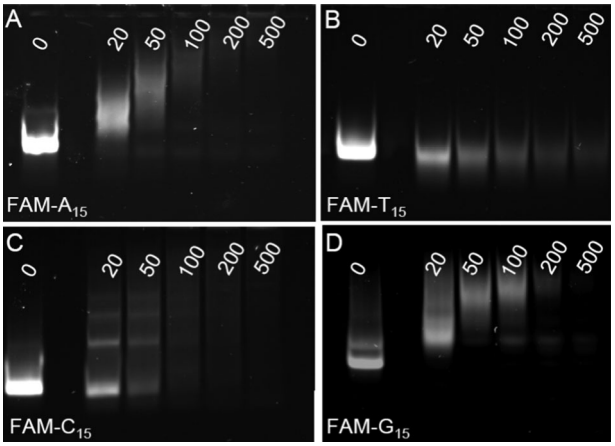

Fig. 1 Gel images of FAM-labeled A_{15} (A), T_{15} (B), C_{15} (C) and G_{15} (D) after reacting with various concentrations of cisplatin in water. The DNA concentrations are 0.5 μM and the numbers marked on each lane are the molar ratio between cisplatin and DNA.

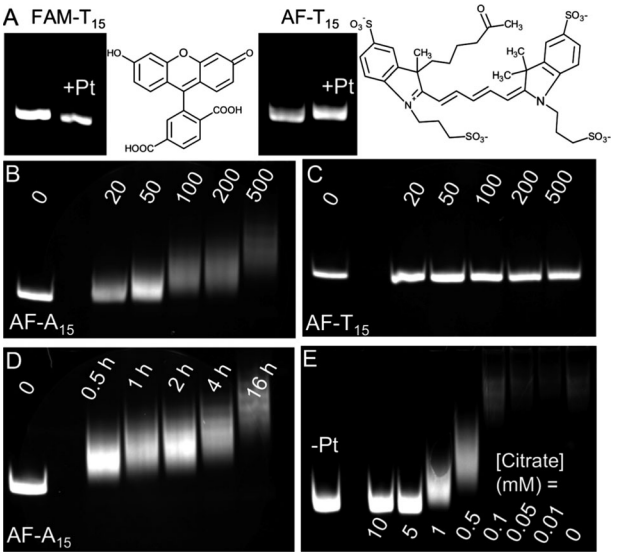

Fig. 2 (A) Gel images of FAM and AF-labeled T_{15} and after cisplatin treatment for 2 h. The structures of these two fluorophores are also shown. Gel images of AF-A_{15} (B), and AF-T_{15} (C) after reacting with various concentrations of cisplatin. The DNA concentrations are 0.5 μM and the numbers marked on each lane are the molar ratio between cisplatin and DNA. (D) Gel images of AF-A_{15} after incubating with cisplatin for various time. Cisplatin concentration = 250 μM. (E) Gel image of AF-A_{15} incubated with various concentrations of citrate (pH 7) for 24 h and then incubated with cisplatin for another 16 h.

to its tendency to form various secondary structures such as inter- and intra-molecular quadruplexes (Fig. 1D). The mass spectrum of FAM-G_{15} showed a few high molecular weight species, consistent with the smeared gel (Fig. S1, ESI†). Addition of cisplatin produced a clear shift, consistent with the fact that cisplatin has high affinity toward guanine.

This initial test suggests that FAM-labeled DNA and gel electrophoresis can be used to study DNA reaction with cisplatin but fluorescence quenching needs to be suppressed. We next tested Alexa Fluor 647 (AF) labeled T_{15}, since AF is known to be a more stable fluorophore. We chose T_{15} because of its low reactivity with cisplatin and therefore its fluorescence intensity can be directly compared. Using a short incubation time of 2 h, ~20% quenching was observed with FAM, while AF was not quenched significantly (Fig. 2A). It needs to be noted that longer incubation can also quench AF, but to a lesser extent than FAM quenching. Therefore, we chose to use AF-labeled DNA for subsequent studies.

First, the cisplatin concentration-dependent study was repeated, in which AF-A_{15} still showed a similar mobility shift but the overall fluorescence intensity was stronger (Fig. 2B). On the other hand, no reaction took place with AF-T_{15} as expected and the band intensity was quite consistent (Fig. 2C). Next a time-dependent study was performed with AF-A_{15}. It is clear that the bands shifted to lower mobility over time (Fig. 2D). We quantified the relative mobility shift by measuring the center of each band and obtained a reaction rate of 0.36 h^{-1} between cisplatin and AF-A_{15} (Fig. S2, ESI†). This rate is comparable with those reported in the literature.[16]

After optimizing the assay conditions, we next studied the reaction in citrate buffers using AF-A_{15}. First, 0.25 mM cisplatin was mixed with various concentrations of citrate for 24 h to allow complex formation. Then AF-A_{15} was added and incubated for another 16 h. In Fig. 2E, the first lane on the left contained the free DNA without cisplatin. All the other lanes contained cisplatin and the citrate concentration was gradually decreased. We observed a gradual mobility shift, which can be pictured as an inhibition curve and the middle point is ~0.5 mM citrate. Since the Pt concentration was 0.25 mM, the inhibition effect by citrate was close to quantitative. Strong free DNA bands were observed with 5 mM citrate, where no cisplatin–DNA adduct was detected. Since cisplatin binding to DNA is thermodynamically stronger than to most other ligands,[17] inhibition was incomplete at low citrate concentrations. After reacting with citrate, negatively charged complexes were formed, which might be a kinetic reason to disfavor the reaction with negatively charged DNA.

Different organelles inside a cell have different pH values. For example, endosomes and lysosomes are acidic and cancer tissues usually also have lower pH.[36] The pH-dependent study is convenient to carry out with citrate since it can be used as a buffer over a wide pH range. Moderate DNA binding to cisplatin was observed only at pH 3 (Fig. 3A), while binding was completely inhibited at higher pH. This might be related to the protonation of citrate at pH 3, thus suppressing its binding to cisplatin (the pK_a values of citrate are 3.14, 4.75 and 6.39). Overall, citrate is a strong inhibitor of cisplatin binding to poly-A DNA over a wide pH range.

In addition to cisplatin, a few other Pt-based drugs have also been approved for clinical use such as oxaliplatin and carboplatin. Next we studied their reaction with DNA in citrate (Fig. 3B). Interestingly, we only observed reaction with cisplatin while no binding was detected with other Pt complexes in 16 h. A moderate reaction with carboplatin was observed only after 48 h (Fig. S3, ESI†). The main difference between cisplatin and carboplatin or oxaliplatin is that the two chloride ligands are replaced by two chelating carboxyl groups. The chloride leaving groups in cisplatin are more labile compared to carboxyl leaving groups, which are moderately stable (Fig. 3C). Therefore, cisplatin can be hydrolyzed and then reacted with citrate, while carboplatin or oxaliplatin does not react with citrate as readily. Mass spectrometry shows the presence of both mono-coordinated and chelated products between citrate and cisplatin (Fig. S4, ESI†).

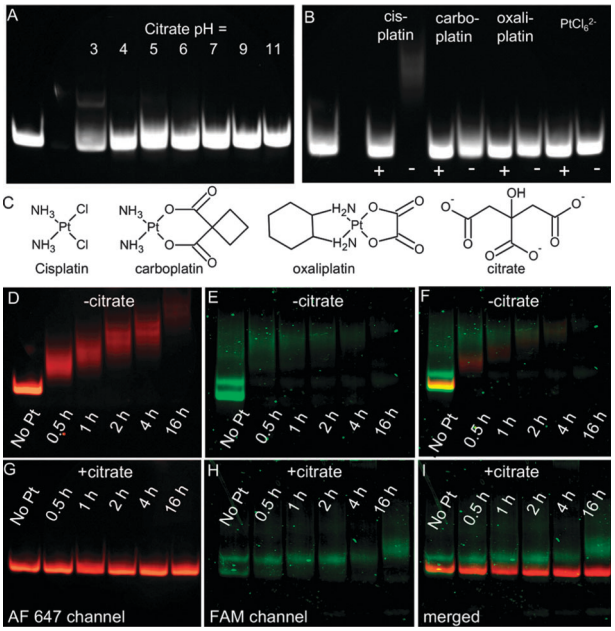

Fig. 3 (A) Inhibition of cisplatin binding to AF-A_{15} as a function of pH in citrate. (B) Reaction of platinum-based compounds with AF-A_{15} in the presence or absence of 10 mM citrate (denoted by the '+' and '−' signs). For all the gels, the first lane on the left is the free DNA without cisplatin. (C) Structures of the platinum drugs and citrate. Mixture of AF-A_{15} and FAM-G_{15} with cisplatin imaged with the AF channel (D, G), the FAM channel (E, H) and the merged (F, I) in the absence of citrate (D–F) or in the presence of 10 mM citrate (G–I) as a function of time.

The abovementioned studies mainly used A_{15} DNA since it forms discrete bands in gels while the G_{15} products smeared more. It needs to be noted that the inhibition effect is less significant for G_{15} (Fig. S5, ESI†). An important advantage of fluorescence is multiplexed detection. With two different fluorophores, we next tested the effect of citrate on reaction selectivity between adenine and guanine. In the absence of citrate, both FAM-G_{15} and AF-A_{15} reacted and the mobility decreased with time (Fig. 3D–F). In the presence of citrate, AF-A_{15} was completely inhibited as expected (Fig. 3G), while FAM-G_{15} still reacted (Fig. 3H), although slower than that in the absence of citrate. The merged band changed from orange to red after 4 h (Fig. 3I), suggesting platination of FAM-G_{15}. Therefore, an interesting role of citrate is to increase the selectivity of cisplatin towards guanine compared to adenine, which might have implications for guanine being the eventual target of cisplatin.[37–39]

In summary, we have employed fluorescently-labeled oligonucleotides for studying the reaction between cisplatin and DNA. Important reaction information such as product distribution, kinetics, and stoichiometry can all be obtained using this simple method. We further showed that citrate is an inhibitor for this reaction but can increase selectivity toward guanine over adenine.

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