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Preferential DNA Cleavage under Anaerobic Conditions by a DNA-Binding Ruthenium Dimer

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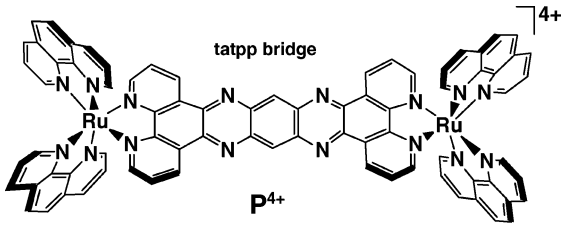
In the absence of dioxygen, the cationic complex $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ ($\mathbf{P}^{4+}$) undergoes in situ reduction by glutathione (GSH) to form a species that induces DNA cleavage. Exposure to air strongly attenuates the cleavage activity, even in the presence of a large excess of reducing agent (e.g., 40 equiv of GSH per $\mathbf{P}^{4+}$), suggesting that the complex may be useful in targeting cells with a low-oxygen microenvironment (hypoxia) for destruction via DNA cleavage. The active species is identified as the doubly reduced, doubly protonated complex $\mathbf{H_2P}^{4+}$, and a carbon-based radical species is implicated in the cleavage action. We postulate that the dioxygen concentration regulates the degree to which the carbon radical forms and thus regulates the DNA cleavage activity.

The use of transition-metal complexes in medicine has enjoyed extensive attention given the tremendous success of cisplatin as a chemotherapeutic agent[1] and the ability of many metal complexes to interact with and damage cellular structures, particularly DNA.[2−7]

A large number of DNA-cleaving metal complexes function via the activation of dioxygen (O_2) to generate reactive oxygen species (ROS), such as hydroxyl and superoxide radicals.[8,9] These ROS are ultimately responsible for the DNA cleavage. Others, including cisplatin and certain photoactivated,[10−14] oxidizing,[15,16] or hydrolyzing complexes,[8] do not require O_2 to function, but they are also insensitive to the cellular [O_2]. Compounds that show enhanced cleavage activity under a low-oxygen microenvironment (hypoxia) are rare[17−21] but offer a unique mechanism to target tumor cells under such conditions. These hypoxic tumor cells are often the most resistant to radiotherapy[22,23] and chemotherapy[24,25] and the most susceptible toward metastasis,[26,27] making this subpopulation a particularly attractive chemotherapeutic target.



We have discovered that the cationic ruthenium dimer $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ ($\mathbf{P}^{4+}$; tatpp = 9,11,20,22-tetraazatetrapyrido[3,2-*a*:2′,3′-*c*:3″,2″-*l*:2‴,3‴-*n*]pentacene and phen = 1,10-phenanthroline) shown above (water

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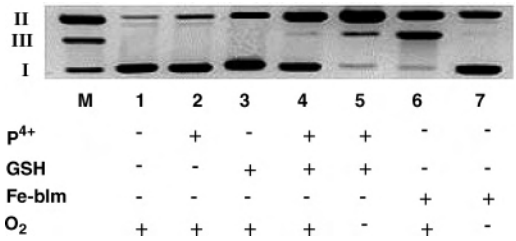

Figure 1. Cleavage of supercoiled pUC18 DNA (0.154 mM) by $\mathbf{P}^{4+}$ in a 7 mM Na_3PO_4 buffer (pH 7.0) at 25 °C: lane M, marker lane containing forms I−III DNA; lane 1, DNA control; lane 2, DNA + $\mathbf{P}^{4+}$ (0.0128 mM); lane 3, DNA + GSH (0.512 mM); lane 4, DNA + GSH (0.256 mM) + $\mathbf{P}^{4+}$ (0.0128 mM) under aerobic conditions; lane 5, same as lane 4 under anaerobic conditions; lane 6, DNA + GSH (0.512 mM) + Fe-Blm (0.0128 mM) under aerobic conditions; lane 7, same as lane 6 under anaerobic conditions. The incubation time for all of these cases was limited to 1 h.

soluble as the chloride salt) not only induces DNA cleavage in the presence of mild reducing agents but shows enhanced activity under anaerobic conditions. The fact that exposure to air attenuates the cleavage activity suggests that ROS are not responsible for the observed cleavage and that such a complex might be useful in targeting cells under hypoxic conditions. Complex $\mathbf{P}^{4+}$ is known to intercalate and bind DNA tightly ($K_b = 1.1 \times 10^7\ M^{-1}$ at 25 mM NaCl).[28,29] The strong interaction with DNA is not unusual for this class of cationic complexes, and it has a number of structural similarities to many known metallointercalators,[13,14,30−33] including those that are known to thread their way through the DNA double helix.[34]

The ability of $\mathbf{P}^{4+}$ to cut DNA was examined by following the conversion of supercoiled plasmid DNA (form I) to the circular form (form II) or linear form (form III) using agarose gel electrophoresis to separate the products (experimental details given in the Supporting Information). As shown in Figure 1, $\mathbf{P}^{4+}$ alone does not cause appreciable DNA cleavage (lane 2); however, the addition of a mild reducing agent such as glutathione (GSH) leads to cleavage activity (lanes 4 and 5). However, the yield of cleavage products is clearly higher under anaerobic conditions (compare lane 4 vs lane 5). Yields of cleavage products (forms II + III) under aerobic and anaerobic conditions are 55% and 97%, respectively.[35] The appearance of linear DNA in lane 5 appears to result from sequential single-strand (ss) cuts, not double-strand (ds) cleavage; thus, the overall cleavage activity is ss scission.

Given the importance of exclusion of trace O_2 as playing a role in the observed cleavage activity, a positive control

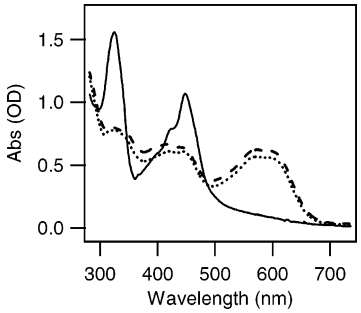

Figure 2. Absorption spectra of $\mathbf{P}^{4+}$ (12.8 μM) before (solid line) and after (dotted line) the addition of 10 equiv of GSH in an anaerobic 7 mM Na_3PO_4 buffer (pH 7.0). The dashed line is the absorption spectrum of $\mathbf{H_2P}^{4+}$ in MeCN when prepared by stoichiometric cobaltocene reduction and trifluoroacetic acid protonation.[42]

was included. Under anaerobic conditions, iron(II) bleomycin (Fe-Blm) is known to induce DNA nicks but not ds cuts. When exposed to O_2, however, Fe-Blm is an effective ds nuclease.[36−38] As seen in Figure 1, lane 6, Fe-Blm in the presence of O_2 causes extensive DNA ds breaks, whereas when O_2 was excluded (lane 7), only ss nicking was observed. These studies were carried out side by side with the $\mathbf{P}^{4+}$ cleavage experiments, demonstrating unequivocally that $\mathbf{P}^{4+}$ is a more effective DNA cleaving agent under reducing and hypoxic conditions. Because of the known photoreactivity of $\mathbf{P}^{4+}$,[39−41] all experiments were conducted in the dark so that photochemically induced cleavage reactions could be ruled out. All cleavage experiments were conducted under low-light conditions and control experiments conducted in the dark or under ambient laboratory lighting gave identical results, showing that this cleavage reaction is not a photochemical reaction.

We have previously examined the redox chemistry of $\mathbf{P}^{4+}$ in water at various pHs by electrochemical, spectroelectrochemical, and chemical reduction methods.[39−41] Reaction 1 shows the two reduction products of $\mathbf{P}^{4+}$ in water at pH 7.0. The redox reactions are reversible and easily followed by visible spectroscopy because distinct changes in the absorption spectrum are observed for each reduction and protonation event. It is readily apparent that GSH reacts with $\mathbf{P}^{4+}$ as the solution color changes from yellow to green upon the

$$\mathbf{P}^{4+} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \mathbf{P}^{3+} \underset{-e^- - 2H^+}{\overset{+e^- + 2H^+}{\rightleftharpoons}} \mathbf{H_2P}^{4+} \qquad (1)$$

addition of GSH. As seen in Figure 2, the absorption spectra of $\mathbf{P}^{4+}$ in an aqueous buffer (pH 7.0) after the addition of GSH is identical with that of $\mathbf{H_2P}^{4+}$, as prepared by stoichiometric reduction and protonation.[40,42] Thus, it appears

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(35) Yields determined from densiometry in which the DNA in each lane was summed to 100%. Yields corrected for the amount of cleavage product in lane 1 (10%) and the relative staining ability of ethidium bromide for forms I−III DNA (see Barton, J. K.; Raphael, A. L. *J. Am. Chem. Soc.* **1984**, *106*, 2172−2176).
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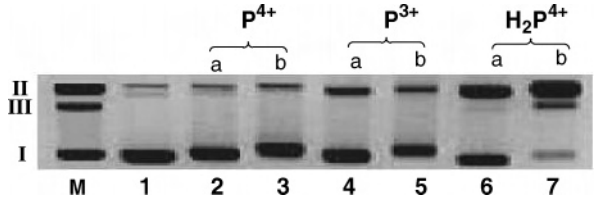

Figure 3. Agarose gel of supercoiled pUC18 DNA (0.154 mM) in the presence of $\mathbf{P}^{4+}$, $\mathbf{P}^{3+}$, and $\mathbf{H_2P}^{4+}$. All incubations were performed under anaerobic conditions with an incubation time of 2 h at 25 °C. The ratio of complex to DNA-bp was (a) 0.083 or (b) 0.20 as indicated above each lane: lane M, marker; lane 1, DNA control; lane 2, DNA + $\mathbf{P}^{4+}$ (0.0128 mM); lane 3, DNA + $\mathbf{P}^{4+}$ (0.0307 mM), lane 4, DNA + $\mathbf{P}^{3+}$ (0.0128 mM); lane 5, DNA + $\mathbf{P}^{3+}$ (0.0307 mM), lane 6, DNA + $\mathbf{H_2P}^{4+}$ (0.0128 mM); lane 7, DNA + $\mathbf{H_2P}^{4+}$ (0.0307 mM).

that $\mathbf{P}^{4+}$ is a prodrug, which is converted to $\mathbf{H_2P}^{4+}$ by in situ reduction.

In order to identify the chemical species responsible for the observed anaerobic cleavage and to rule out participation by glutathyl radical species, we examined the cleavage activity of $\mathbf{P}^{4+}$, $\mathbf{P}^{3+}$, and $\mathbf{H_2P}^{4+}$ (see the Supporting Information for synthetic procedures) under anaerobic conditions without GSH present.[40,42]

As seen in Figure 3, $\mathbf{P}^{4+}$ does not induce appreciable DNA cleavage under anaerobic conditions (lanes 2 and 3). $\mathbf{P}^{3+}$ (lanes 4 and 5) does show some enhanced nicking ability; however, $\mathbf{H_2P}^{4+}$ is clearly the most potent nicking agent (lanes 6 and 7). As seen in lanes 6 and 7 (Figure 3), the amount of DNA cleavage increases with increased [$\mathbf{H_2P}^{4+}$], as would be expected if this complex were the actual cleaving agent. Thus, one simple explanation for the attenuated cleaving activity under aerobic conditions would be reoxidation of $\mathbf{H_2P}^{4+}$ to $\mathbf{P}^{4+}$. Exposure of an aqueous solution of $\mathbf{H_2P}^{4+}$ to air is known to result in a rapid reoxidation of this complex to $\mathbf{P}^{4+}$, as measured by UV−visible absorption spectroscopy.[39]

The mechanism of DNA cleavage is still unclear; however, Yamaguchi and co-workers have shown that dihydropyrazines cleave DNA by both oxygen-dependent and -independent pathways.[43−46] $\mathbf{H_2P}^{4+}$ contains the dihydropyrazine substructure and thus could function in a similar manner with high-DNA-binding affinity, further enhancing its activity. For dihydropyrazines, the oxygen-independent cleavage activity is attributed to the formation of a carbon-based radical on the drihydropyrazine moiety. Thus, we speculate that DNA-bound $\mathbf{H_2P}^{4+}$ is cleaving DNA in a similar manner via the generation of reactive carbon-based radicals that are in close proximity to the DNA because of the tight DNA binding.

To test this hypothesis, the cleavage activity of $\mathbf{H_2P}^{4+}$ was examined in the presence of various radical-trapping and metal-complexing reagents. Dimethyl sulfoxide (DMSO) is an effective scavenger of diffusible oxygen-based radicals such as $^{\bullet}$OH and superoxide.[47,48] On the other hand, 2,2,6,6-tetramethyl-1-piperdinyloxy (TEMPO) is an effective scavenger of carbon radicals or metal-based radicals but is ineffective with oxygen-based radicals.[49,50] As seen in Figure 4, the addition of up to 5% DMSO by volume has no effect on the cleavage activity (lane 3), whereas the addition of 2 mM TEMPO stops most of the DNA cleavage (lane 4). These data clearly support the role of carbon-based radicals in the cleavage mechanism. Yamaguchi and co-workers postulated that trace metals ions, such as copper(II), activated the dihydropyrazines to the reactive form.[43−46] This does not seem to be the case here because added ethylenediaminetetraacetic acid (EDTA), at concentrations up to 1 mM, has little effect on the cleavage activity (Figure 4, lane 5) of $\mathbf{H_2P}^{4+}$. We are further investigating this unusual behavior and hope to elucidate the cleavage mechanism with the help of electron paramagnetic resonance spectroscopy. We note that the carbon-based radical species would likely be very reactive toward O_2 in solution, and this "quenching" reaction could also explain the observed sensitivity of this cleavage activity to oxygen.

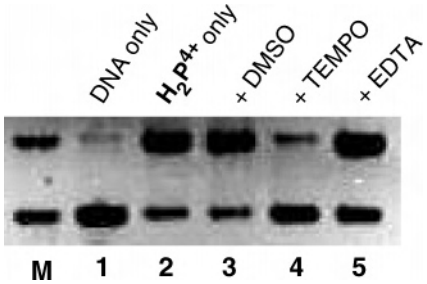

Figure 4. Agarose gel of supercoiled pUC18 DNA (0.154 mM) in the presence of $\mathbf{H_2P}^{4+}$ (0.0256 mM). All incubations were performed in a 7 mM Na_3PO_4 buffer (pH 7.0) at 25 °C under anaerobic conditions with an incubation time of 2 h. The ratio of complex to DNA-bp was 0.16: lane M, marker lane; lane 1, DNA control; lane 2, DNA + $\mathbf{H_2P}^{4+}$; lane 3, DNA + $\mathbf{H_2P}^{4+}$ + DMSO (0.64 M); lane 4, DNA + $\mathbf{H_2P}^{4+}$ + TEMPO (2.04 mM); lane 5, DNA + $\mathbf{H_2P}^{4+}$ + EDTA (1.02 mM).

To our knowledge, this is the first example of a metal complex with potentiated DNA cleavage activity under hypoxic conditions, suggesting potential therapeutic applications. Future studies will establish the mode of action and its effects on tumor cells in vitro.

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Supporting Information Available: Procedures for the DNA cleavage assay, synthetic methods for the preparation of [**P**][Cl]$_3$ and [$\mathbf{H_2P}$][Cl]$_4$, and the absorption spectrum of GSH-reduced $\mathbf{P}^{4+}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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