Supramolecular Metal Drug

DOI: 10.1002/anie.201004471

Noncovalent DNA-Binding Metallo-Supramolecular Cylinders Prevent DNA Transactions in vitro**

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Processing of DNA information, through replication or transcription, is mediated by proteins which bind mainly to the DNA major groove through specific subunits such as helix-turn-helix, zinc fingers and leucine zippers.[1] There have been many efforts to synthesize artificial molecules which could interact with DNA to interfere with or control biological processes.[2] DNA replication, when unregulated or uncontrolled, results in many pathological states such as cancer, and is also inherent in viral and bacterial infections. Thus many compounds used in anticancer and antiviral therapy are able to inhibit or block DNA replication, such as platinum drugs[3] and nucleoside analogues.[4]

In this context we have recently developed supramolecular tetracationic cylinders consisting of three bis(pyridylimine) ligand (L) strands wrapped in a helical fashion about two iron(II) or ruthenium(II) centers (Figure 1). Their size and shape are very similar to those of protein zinc fingers and the cylinders have proven to be remarkable agents with unique DNA recognition properties: They can not only bind strongly and noncovalently in the major groove of DNA, inducing substantial and unprecedented intramolecular DNA coiling in natural polymeric DNAs,[5] but still more excitingly we have also shown that they can bind at the heart of Y-shaped DNA 3-way junctions (3WJ).[6] Such a mode of DNA recognition is without precedent and quite distinct from the classical modes of DNA interaction.[2,7] Yet what is particularly tantalizing is that the DNA replication fork is a form of Y-shaped junction. Indeed whenever the DNA duplex is opened to process the genetic information (replication or transcription) a Y-shaped junction is created. Gel studies have confirmed that different Y-shaped junctions can be recognized[8] (including biologically relevant ones like splayed duplexes, as the replication fork) as well as the perfectly paired 3-way junctions. Agents that recognize this particular type of "unusual" DNA structure (by shape-fit structural specificity as opposed to traditional sequence specificity) and interfere with replication or transcription processes could be a very powerful tool not only in the fundamental study of the processes such as replication but also in the modulation of cell cycle control.

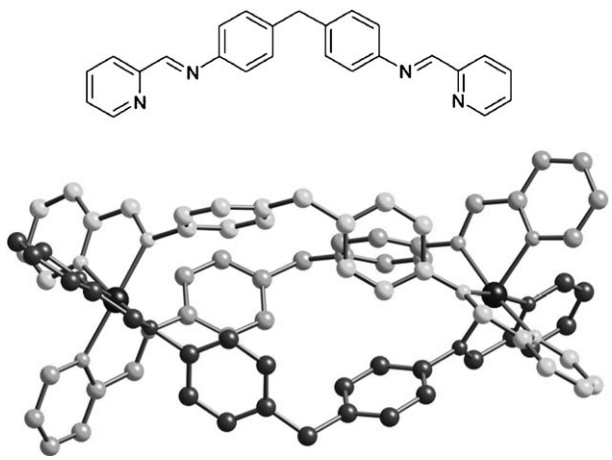

Figure 1. Bis(pyridylimine) ligand L and the structure of the tetracationic M_2L_3 cylinders formed from that ligand.[5e,9a,12]

These metallo-supramolecular cylinders induce cytostasis (at low micromolar concentrations) and, at slightly higher concentrations, apoptosis in various cell lines.[9] They are not only taken up but seem to concentrate in cells, with their killing potential unusually related to amount of compound added not to concentration. We hypothesize that these biological effects are a consequence of cylinders binding to Y-shaped junctions such as DNA replication forks and preventing DNA processing. To provide further support for this we wished to probe whether the cylinders would affect DNA transactions in an in vitro experiment. While there is no simple way to study DNA-replication in vitro, polymerase chain reaction (PCR)[11] provides a similar set of DNA transactions,[10] is a DNA replication process involving a DNA polymerase, and is readily accessible. Herein we report the effects of the cylinders on the PCR reaction and demonstrate that they do indeed interfere with the binding of the polymerase to the DNA highlighting the potential for inhibition of DNA replication.

In the first experiment we adopted a PCR-based assay to evaluate whether the ruthenium cylinder $[Ru_2L_3]^{4+}$ would interfere with the amplification of DNA. The DNA transaction is Taq DNA polymerase (a thermostable DNA polymerase) recognizing and elongating a primer-DNA double strand. The ruthenium(II) complex was selected as

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[**] This work was supported by the EU DNAREC Marie Curie Training Site (MEST-CT-2005-020842, C.D. and A.L.). We thank Rachel Kershaw for supplying the mouse cDNA and Prof. J. Kevin Chipman for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004471.

the focus for these PCR experiments because of its robust inert nature which is compatible with the relatively harsh (at least by mammalian biology standards) conditions involved in PCR.

To initiate our investigations we performed PCR reactions using a mouse cDNA as substrate, with two primers specific for a 223 base pair (bp) region, together with the Taq DNA polymerase and the four standard deoxynucleotides (C, G, A, T). Agarose gel electrophoresis of the reaction products after 35 cycles, followed by ethidium bromide staining and UV visualization, showed a single DNA product of the predicted length (Figure 2A, lane 1). The reaction was also conducted

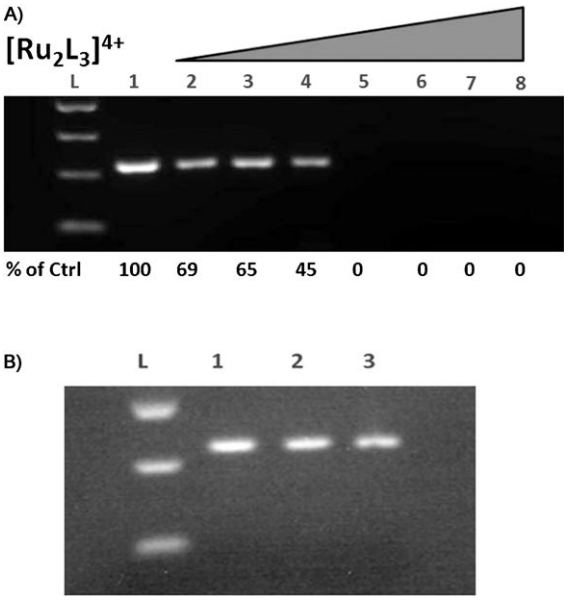


Figure 2. A) Inhibition of the PCR by ruthenium cylinder. L: 100 bp oligonucleotide ladders; 1: control; 2: ruthenium cylinder 0.1 μM; 3: ruthenium cylinder 0.5 μM; 4: ruthenium cylinder 1 μM; 5: ruthenium cylinder 1.5 μM; 6: ruthenium cylinder 2 μM; 7: ruthenium cylinder 2.5 μM; 8: ruthenium cylinder 3 μM. Band intensities were reported (below the picture) as percentage of the control. B) Incubation of ruthenium cylinder with PCR products. L: 100 bp oligonucleotide ladders; 1: control (no ruthenium cylinder); 2: ruthenium cylinder 20 μM incubated 5 min with DNA; 3: ruthenium cylinder 20 μM incubated 50 min with DNA.

in the presence of increasing concentrations of the ruthenium cylinder (Figure 2A, lanes 2–8). The ruthenium cylinder inhibits drastically the DNA amplification even at the lowest concentration, 0.1 μM, and it blocks completely the PCR at 1.5 μM (Figure 2A). A separate control experiment (Figure 2B) confirms that the ruthenium cylinder does not interfere with the visualization of the PCR products, which confirms that the absence of bands corresponding to amplified DNA is a consequence of PCR inhibition not visualization.

If the cylinder is inhibiting the formation of the DNA-Taq complex (and subsequent elongation) then higher concentrations of Taq DNA polymerase and DNA substrate should affect the inhibition. To test this we probed the effect of DNA substrate and Taq polymerase concentrations at a fixed 1.5 μM concentration of ruthenium cylinder, selected as the lowest concentration which blocked completely the DNA amplification in the previous assay. Increasing concentrations of Taq DNA polymerase (Figure 3; lanes 2–5) reduce the inhibition

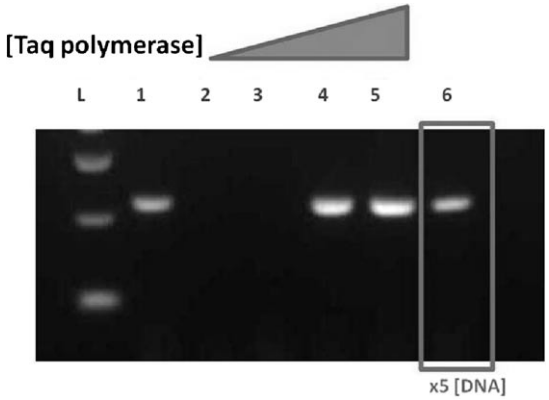
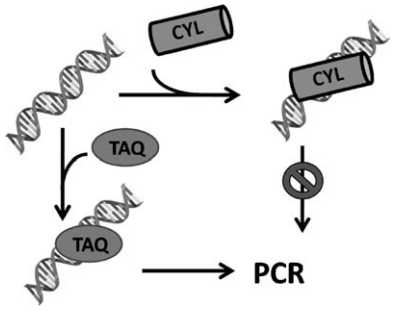


Figure 3. Effect of Taq DNA polymerase and DNA template on the inhibition by ruthenium cylinder. L: ladders; 1: control; 2: Taq polymerase 0.15 μM; 3: Taq polymerase 0.3 μM; 4: Taq polymerase 0.6 μM; 5: Taq polymerase 1.5 μM; 6: Taq polymerase 0.15 μM and 5× DNA (1 μg); samples 2–6: ruthenium cylinder 1.5 μM.

effect of ruthenium cylinder on the PCR. Increasing by five-fold the cDNA used in the PCR (Figure 3; lane 6) also led to a reduction in inhibition.

These results are consistent with the ruthenium cylinder and Taq DNA polymerase competing in binding DNA. Thus a higher concentration of enzyme or DNA promotes the amplification of DNA (Scheme 1).



Scheme 1. Increasing the concentration of Taq DNA polymerase and DNA itself, the formation of the complex DNA–Taq DNA polymerase is promoted favoring the PCR. When ruthenium cylinder is bound to DNA it prevents the amplification.

To obtain further support for the mechanism proposed in Scheme 1, we have studied the interactions between the components using gel electrophoresis techniques. Binding of a protein to DNA fragments leads to a reduction in the electrophoretic mobility of the fragments in a non-denaturating polyacrylamide or agarose gel. Thus a double strand DNA (dsDNA) bound to a Taq DNA polymerase will run slower than the same but naked DNA. Two complementary oligonucleotides of 20 and 43 bases were chosen as a model of a

primer attached to a DNA. The shorter one was ^{32}P labeled. These two complementary strands were incubated for 10 min at room temperature with the Taq DNA polymerase with increasing concentrations of ruthenium cylinder, and then polyacrylamide gel electrophoresis was performed and the bands were visualized by phosphorus imaging plate scanner (Figure 4). Increasing concentrations of ruthenium cylinder

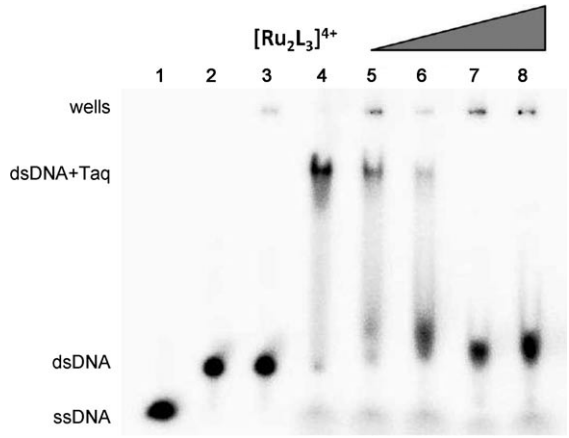


Figure 4. Gel mobility assay in presence of ruthenium cylinder. 1: 20-base single strand (ss) DNA; 2: 20-base and 43-base ssDNA; 3: 20-base and 43-base ssDNA, 2.5 μM ruthenium cylinder; 4: 20-base and 43-base ssDNA, 3 μM Taq DNA polymerase; 5: 20-base and 43-base ssDNA, 3 μM Taq DNA polymerase, 2.5 μM ruthenium cylinder; 6: 20-base and 43-base ssDNA, 3 μM Taq DNA polymerase, 5 μM ruthenium cylinder; 7: 20-base and 43-base ssDNA, 3 μM Taq DNA polymerase, 10 μM ruthenium cylinder; 8: 20-base and 43-base ssDNA, 3 μM Taq DNA polymerase, 15 μM ruthenium cylinder.

reduce the intensities of the bands corresponding to the Taq–dsDNA complex and raise the intensities of the bands corresponding to the naked dsDNA. This result confirms that ruthenium cylinder displaces the Taq DNA polymerase from the double strand DNA even at the lowest concentration used (2.5 μM).

The same experiment was repeated replacing the ruthenium cylinder with the corresponding iron cylinder (Supporting Information, Figure S1). This also proved to be a strong displacer of the Taq DNA polymerase from DNA, even more than ruthenium cylinder. The iron cylinder does not, however, inhibit the PCR reaction, because the PCR reaction contains a DNA denaturation step at 94 °C and the iron(II) cylinder does not survive those conditions. Consequently, even at high concentrations of iron cylinder, the PCR is unaffected (Figure S2). Nevertheless the displacement of the Taq polymerase in the gel electrophoresis experiment, indicates that under ambient body-temperature conditions the iron cylinder should be able to interfere with DNA transactions just as the ruthenium cylinder. This is consistent with the observations of the activity of both cylinders in cell lines.[9]

The PCR assays demonstrate that the ruthenium cylinder can inhibit DNA polymerase transactions in vitro at low concentration (0.1 μM). Indeed the cylinder blocks completely the PCR at 1.5 μM. The gel assays demonstrate that the cylinder displaces the polymerase from the DNA: on adding the metallo-cylinders to a double strand DNA complexed to the Taq DNA polymerase, the enzyme is displaced from the double strand. This would prevent the polymerase from elongating the DNA and explain the inhibition.

Although the PCR experiments focus on the ruthenium(II) cylinder, the gel experiments indicate that the corresponding iron(II) cylinder can similarly interfere with Taq binding to dsDNA. These experiments provide a key bridge to link the remarkable DNA-binding properties of the cylinders observed in vitro with the cytostasis and apoptosis inducing activity of the compounds observed in cells. They provide crucial evidence that the non-covalent DNA binding of the cylinders can indeed affect the ability of proteins to process the DNA information.

Experimental Section

$[Ru_2L_3]Cl_4$ and $[Fe_2L_3]Cl_4$ were prepared according to our published procedures.[9a,12]

PCR-based assays: All PCR reagents were purchased from Bioline. PCR reactions were performed in 50 μl using the following buffer conditions: 1 × NH_4 reaction buffer, $MgCl_2$ (1.5 mM), dNTP (3 mM), Taq DNA polymerase (5U), two GAPDH primers (Alta Bioscience) (0.4 μM each) forward (5′-AACTTTGGCATTGTGGAAGG-3′) and reverse (5′-ACACATTGGGGGTAGGAACA-3′), cDNA substrate (200 ng) obtained by reverse transcription of mRNA extracted from mouse embryonic fibroblast cells. Increasing concentrations of ruthenium cylinders (from 0.1 μM up to 3 μM) were incubated with PCR solutions for 5 min before starting the PCR cycles. After Taq polymerase activation at 95 °C for 5 min, 35 cycles of PCR were performed as follows: denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and synthesis at 72 °C for 3 min. A final polymerization step was performed at 72 °C for 5 min. The products were loaded on 2.5 % agarose gel and the images were acquired by UV lamp (UVITEC) and analyzed by software UVi pro Platinum.

The second PCR assay was performed in the same conditions apart from the ruthenium cylinder concentration (in all solutions it was 1.5 μM) Taq DNA polymerase concentration (increased up to 10 times) and cDNA (increased in the last assay solution 5 times).

Gel mobility assay: Solutions containing 0.2 μM of the ^{32}P labeled oligonucleotide (5′-CAACCATCCCCTCTCGCACC-3′), 50 mM KCl, 50 mM Tris-HCl, 0.1 mM dithiothreitol (DTT) were prepared. A 43-base oligonucleotide (5′-TAAAAAATGGGGTTGAGGTGCGAGAGGGGATGGTTGATGGCAA-3′) was added to form a double strand DNA (both oligonucleotides were purchased from Eurofins MGW Operon). Increasing concentrations of ruthenium and iron cylinder (from 2.5 μM up to 15 μM) were added to the solutions after incubating the two strands with the Taq DNA polymerase. All solutions were loaded on a 6 % polyacrylamide gel ($MgCl_2$ 1 mM and glycerol 2.5 %) running at room temperature with 0.5 × TBE buffer (0.045 M Tris-borate pH 8.3, 1 mM Na_2EDTA in distillated and deionized water, purchased from National Diagnostic) for 2.5 h and the voltage set at 120 V. The results were visualized by phosphorus imaging plate scanner and analyzed by the software Quantity One.

Received: July 21, 2010
Published online: October 8, 2010

Keywords: cancer · helical structures · metallodrugs · PCR inhibition · supramolecular chemistry

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