Double strand DNA cleavage with a binuclear iron complex†

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Covalently linking two single strand DNA cleaving agents resulted in a new biomimetic binuclear iron complex capable of effecting oxidative double strand DNA cleavage.

Iron bleomycin (Fe–BLM), a potent member of the bleomycin family of anti-tumor glycopeptide antibiotics, is capable of cleaving DNA oxidatively.[1] Its effectiveness as a cytotoxin is proposed to be due to its ability to engage in direct double strand cleavage by delivery of two oxidizing equivalents to the DNA helix,‡ which circumvents the cell's protective repair mechanisms.[2] In the present communication, we present a new strategy involving the covalent tethering of two synthetic single strand cleaving agents (Fig. 1), resulting in the first biomimetic iron-based catalytic DNA cleaving agent capable of effecting oxidative double strand DNA cleavage.[3]

Double strand cleavage results from two successive strand breaks in opposite strands in close proximity to each other.[4] How Fe–BLM achieves this remains a matter of debate;[1e] however, it is clear that a double strand break requires Fe–BLM to deliver two oxidizing equivalents to the DNA double strand,[5] with each strand being cleaved within sixteen base pairs of each other.[4] The importance of this latter aspect is borne out in the many Fe–BLM-mimics, which, although capable of oxidative cleavage of DNA, deliver typically only one oxidizing equivalent to the DNA double strand and, hence, achieve only single strand cleavage.[6]

$[Fe(II)(N4Py)CH_3CN]^{2+}[ClO_4]_2$ (Fe–N4Py, where N4Py = N,N-bis(2-pyridylmethyl)-N-bis(2-pyridyl)methylamine (**1**), Fig. 1)
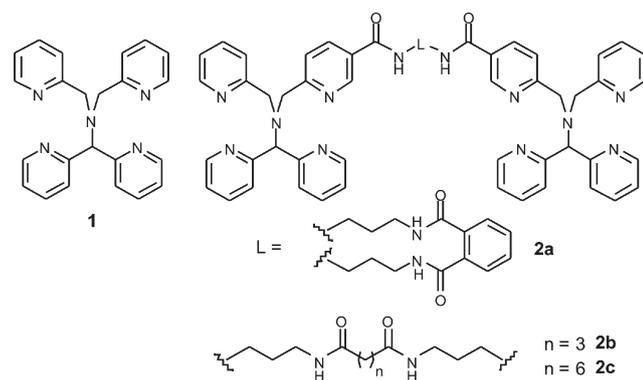
Fig. 1 Ligands used in this study; N4Py (**1**) and ditopic ligands **2a–c**.

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† Electronic supplementary information (ESI) available: Fig. S1–S3, synthesis and characterizations of **2a–c**, experimental procedures for spectroscopic titrations, DNA oxidation and analysis, and preparation of nicked DNA. See DOI: 10.1039/b613469f

has proven itself to be a potent structural and functional model of Fe–BLM.[7] As with Fe–BLM, Fe–N4Py can effect oxidative cleavage of DNA using O_2 as the terminal oxidant without the need for a sacrificial reducing agent.[8] Covalent attachment of a DNA intercalating moiety, *i.e.* a 9-amino acridine, to Fe–N4Py led to a dramatic increase in cleavage efficiency. Analysis of the DNA cleavage products demonstrated that cleavage is initiated by abstraction of the C4′ hydrogen of the deoxyribose.[8] This is typical for DNA cleavage by metal complexes generating reduced oxygen species and is also observed with Fe–BLM.[1c] Yet, despite its activity, the DNA cleavage pattern observed was typical of a single strand cleaving agent, suggesting that Fe–N4Py and its derivatives are capable of delivering only one oxidizing equivalent to DNA. It was envisioned that the covalent tethering of two Fe–N4Py units would produce a new DNA cleaving agent capable of delivering two oxidizing equivalents and, hence, effect double strand DNA cleavage.[9]

The ditopic ligands used in the present study (**2a–c**, Fig. 1) were synthesized by the reaction of a common precursor, propane amine substituted N4Py, with an N-hydroxysuccinimide activated diacid.† The corresponding iron(II) complexes (**3a–c**) were generated *in situ* by complexation to $(NH_4)_2Fe^{II}(SO_4)_2 \cdot 6H_2O$ immediately prior to use. Uptake of two iron(II) ions per ditopic ligand was confirmed from both UV-Vis and ^1H NMR spectroscopic titrations (Fig. S1 and S2†).

The di-iron complexes **3a–c** and the parent mononuclear iron complex Fe–N4Py (**4**) (prepared *in situ* from **1**) were examined in the cleavage of supercoiled pUC18 plasmid DNA (Fig. 2), using 1 μM Fe^{II} and a 1 : 300 stoichiometry of iron with respect to DNA base pairs.† Although DNA cleavage activity was observed in the absence of reducing agents, the reductant dithiothreitol (DTT) was employed to increase the efficiency of the reactions, allowing for lower iron complex concentrations to be used.

Under these conditions, extensive cleavage of DNA was observed for all N4Py-derived complexes, with most of the supercoiled DNA consumed within 30 min (Fig. 2a). However, important differences in the cleavage patterns between the mono- and ditopic complexes were observed. Whereas the parent N4Py iron complex **4** afforded predominantly nicked DNA (lane 4), significant amounts of linear DNA (up to 10% in the case of **3c**), in addition to nicked DNA, were formed with the ditopic complexes **3a–c** (Fig. 2, lanes 5–7). Furthermore, with the ditopic complexes **3a–c** all three forms of DNA are present on the gel at the same time, which is a strong qualitative indication of a direct double strand DNA cleavage pathway.[10]

The time dependence of the activity towards DNA cleavage for both Fe–N4Py (**4**) and **3c** is shown in Fig. 2b (**3a–b**, Fig. S3†). The decrease in the percentage of supercoiled DNA was similar for both **4** and **3c** ($t_{1/2}$ = 9.8 ± 0.8 min and 9.7 ± 0.6 min,
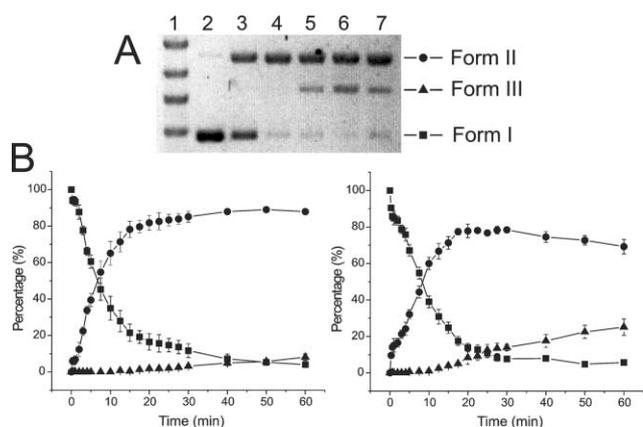
Fig. 2 (a) Cleavage of supercoiled (form I, ■) DNA to give nicked (form II, ●) and linear (form III, ▲) DNA in Tris-HCl (pH 8.0) at 37 °C after 30 min. Concentrations used: 1.0 μM complex (with respect to Fe(II)), 0.1 μg μl^{-1} pUC18 plasmid DNA (300 μM in base pairs) and 1.0 mM DTT; lane 1, marker; lane 2, DNA control; lane 3, $(NH_4)_2Fe^{II}(SO_4)_2 \cdot 6H_2O$; lane 4, **4**; lane 5, **3a**; lane 6, **3b**; lane 7, **3c**. (b) Time profile for cleavage with **4** (left) and **3c** (right). Error bars represent the root mean square (rms) based on three runs. A correction factor of 1.31 was used to compensate for the reduced ethidium bromide uptake capacity of supercoiled DNA.†

respectively). In the early stage of the reaction, this is accompanied by a comparable increase in the percentage of nicked DNA. After approximately 10 min, however, a sudden increase in the formation of linear DNA is observed with the ditopic complex **3c**. Importantly, this is not observed for the parent catalyst **4**.

Statistical analysis was employed to determine the average number of single (n) and double strand breaks (m) per DNA molecule for each sample.† The plots of the number of double strand breaks (m) *versus* the number of single strand breaks (n) are shown for both **3c** and **4** (Fig. 3). The plot for **4** is typical of a single strand cleaving agent, with double strand breaks occurring only after extensive single strand cleavage has taken place.[11] In contrast, the plot of **3c** shows a dramatic increase in the number of double strand breaks with respect to the number of single strand breaks, considerably exceeding that observed for **4**. Taken
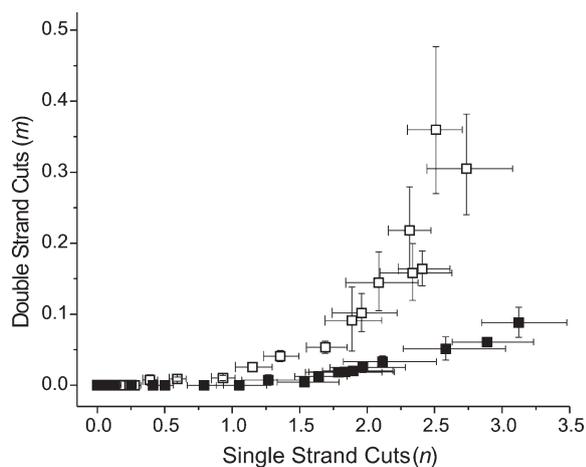
together, these experiments demonstrate unequivocally that **3c** engages in double strand cleavage activity.

It is important to note that the double strand cleavage becomes more pronounced as the reaction progresses. In understanding the reason for this, a key observation is that the rate of conversion of supercoiled DNA is similar for **4** and **3c** (*vide supra*); however, after a significant fraction of supercoiled DNA has been consumed, *i.e.* after approximately 10 min, the double strand cleavage activity increases non-linearly in the case of **3c**. It is apparent that **3c** is much more efficient in effecting double strand cleavage of nicked DNA than of supercoiled DNA. The cleavage of nicked DNA was investigated further to provide additional insight into this phenomenon.

Pure nicked DNA was prepared by treatment of supercoiled pUC18 plasmid DNA with N.BstNBI, a DNA nicking enzyme, which produces four nicks per pUC18 DNA molecule,† and was used in DNA cleavage experiments under standard conditions (*vide supra*, Fig. 4). After 25 min, 30% of linear DNA was observed already with **3c**, which is considerably more than with **4** (10% linear).§ It is apparent, therefore, that complex **3c** prefers cleavage of nicked DNA over supercoiled DNA.

It seems unlikely that the nature of the oxidizing equivalents is different in the cleavage of nicked DNA compared to supercoiled DNA. Rather, we hypothesize that **3c** delivers two oxidizing equivalents, resulting in two strand breaks, to supercoiled DNA, as is the case with nicked DNA. However, in the compact folded

Fig. 3 Number of double strand cuts (m) as a function of single strand cuts (n) per DNA molecule for both **3c** (□) and **4** (■). Error bars represent the maximum and the minimum values of both n and m.†
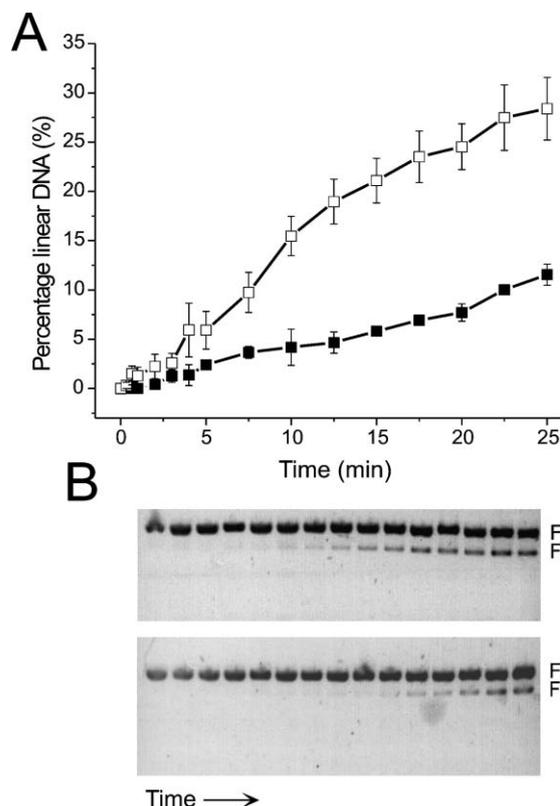
Fig. 4 (a) Time profile of the cleavage of nicked DNA into linear DNA with **3c** (□) and **4** (■). Error bars represent the root mean square (rms) based on three runs. (b) Agarose gel slabs of the reaction of nicked DNA (form II) to linear DNA (form III) with **3c** (upper gel) and **4** (lower gel). Each of the slots corresponds to a point on the graph.

structure of supercoiled DNA, two oxidation events that are close in space are not necessarily close in terms of DNA sequence. When the two strand breaks are too distant in the DNA sequence, the hydrogen bonding network will prevent linearization of the DNA, and, hence, only the formation of nicked DNA is observed. In contrast, nicked DNA has a relaxed open circular structure, in which there is a much greater probability that the two cleavage events will take place in close proximity on opposite strands compared to supercoiled DNA. As a result, direct double strand cleavage by **3c** is observed with nicked DNA.¶ In this respect, the behaviour of **3c** differs from that of Fe–BLM, which effects double strand cleavage of supercoiled DNA. In Fe–BLM, the two oxidizing equivalents are delivered by a single iron complex, which is proposed to hinge around the intercalating bithiazole moiety to cleave both DNA strands sequentially.[1d,e]

In conclusion, a new binuclear iron-based DNA cleaving catalyst was developed by covalently linking two single strand cleaving Fe–N4Py complexes. This biomimetic complex is capable of effecting direct double strand cleavage activity. Following the BLM design, our future efforts will focus on derivatives of **3c** containing a DNA intercalating moiety.

Notes and references

‡ Although single strand cleavage will give rise to double strand breaks on a statistical basis, it is important to note that single strand and direct double strand cleavage represent different cleavage pathways.

§ The first term of a Poisson distribution predicts that the amount of linear DNA has a maximum around 37%.[11] In practice this means that significant amounts of a smear are produced in the gel, which makes quantitative analysis impossible.

¶ This does not exclude that other factors are involved as well. For example, analogous to binuclear copper complexes, which recognize and cleave single strands of DNA extending from a junction,[9] it is also conceivable that **3c** has greater affinity for nicks than **4**. This would give rise to an increased probability of a second single strand cut being generated nearby, resulting in a double strand break.

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