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Mithramycin forms a stable dimeric complex by chelating with Fe(II): DNA-interacting characteristics, cellular permeation and cytotoxicity

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ABSTRACT

Mith (mithramycin) forms a 2:1 stoichiometry drug–metal complex through the chelation with Fe(II) ion as studied using circular dichroism spectroscopy. The binding affinity between Mith and Fe(II) is much greater than other divalent metal ions, including Mg(II), Zn(II), Co(II), Ni(II) and Mn(II). The [$(Mith)_2$–Fe(II)] complex binds to DNA and induces a conformational change of DNA. Kinetic analysis of surface plasmon resonance studies revealed that the [$(Mith)_2$–Fe(II)] complex binds to DNA duplex with higher affinity compared with the [$(Mith)_2$–Mg(II)] complex. A molecular model of the Mith-DNA–Metal(II) complex is presented. DNA-break assay showed that the [$(Mith)_2$–Fe(II)] complex was capable of promoting the one-strand cleavage of plasmid DNA in the presence of hydrogen peroxide. Intracellular Fe(II) assays and fluorescence microscopy studies using K562 indicated that this dimer complex maintains its structural integrity and permeates into the inside of K562 cells, respectively. The [$(Mith)_2$–Fe(II)] complex exhibited higher cytotoxicity than the drug alone in some cancer cell lines, probably related to its higher DNA-binding and cleavage activity. Evidences obtained in this study suggest that the biological effects caused by the [$(Mith)_2$–Fe(II)] complex may be further explored in the future.

INTRODUCTION

The anticancer antibiotic mithramycin (plicamycin®) (Mith) belongs to the aureolic family isolated from *Streptomyces griseus* (1). Mith contains five sugar rings by using sugar residues A and C connected to an aglycon chromophore via O-glycosidic bonds, with the A–B disaccharide on one side and C–D–E trisaccharide on the other (Figure 1). This drug is a DNA-binding antitumor agent, which has been used clinically in several cancer therapies and Paget's disease (2–4). The antitumor property of Mith is probably associated with its inhibitory effects on replication and transcription of tumor cells (5,6). Recently, Fibach *et al*. (7) revealed that Mith could induce fetal hemoglobin production in normal and thalassemic human erythroid precursor cells, and suggested that Mith may be used as a therapeutic agent in certain neoplastic diseases.

Previous optical spectroscopy and footprinting studies revealed that Mith forms a dimer mediated by a single divalent metal ion, such as Mg(II) and Zn(II), which binds to DNA duplex around the GC-rich sequences that is at least 3 bp long (8–11). The structures of the metal-coordinated Mith dimer bound with DNA duplex have been analyzed using NMR by Patel and Shafer groups (12–14) who proposed that the binding of Mith to DNA leads the local structure into A-DNA conformation. Consequently, Mith may interfere with the transcription of genes that bear GC-rich motifs in their promoters, such as *c-myc* proto-oncogene (15).

Metal ions play an indispensable role in the actions of some synthetic and natural metalloantibiotics, and are involved in specific interactions of these antibiotics with biomolecules. A number of anticancer drugs complexed with metal ion are capable of inducing cancer cell death via DNA backbone cleavage (7). Some of these drugs are believed to be activated through a redox system, with the generation of hydroxyl free radicals. For example, pepleomycin (PEP) and bleomycin (BLM) belong to a class of important clinical anticancer drugs that cleave DNA at GpT or GpC sites. DNA degradation caused by PEP and BLM requires prior activation of the drug with molecular oxygen and certain metals ions, such as Fe(II) or Co(III). The close connection between DNA binding (or DNA damage) and inhibition of cell proliferation

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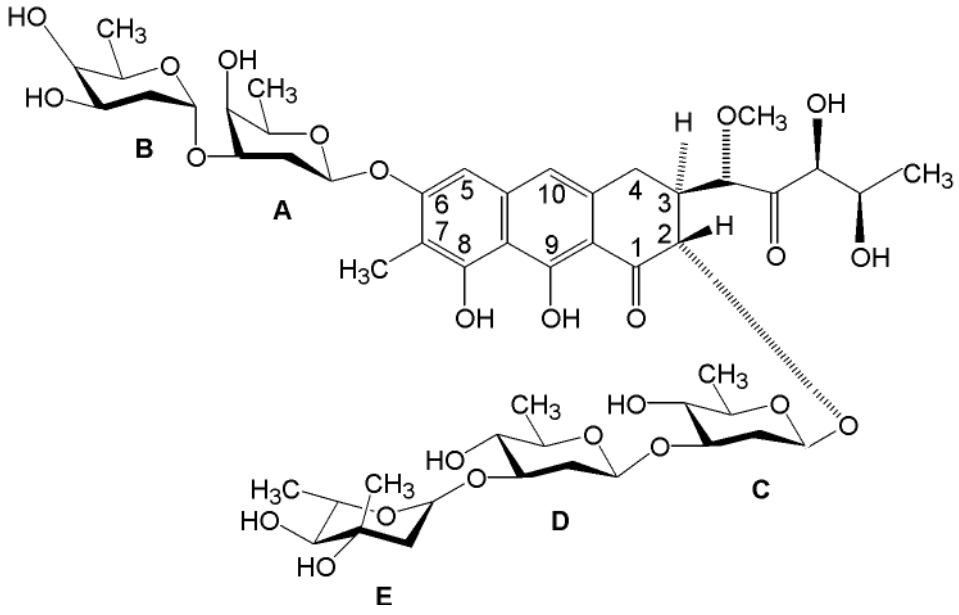

Figure 1. Chemical structure of mithramycin (Mith).

has suggested that Fe(II)–drug complexes with strong DNA-binding affinity are useful in the inhibition of tumor cell growth (7).

Mg(II)-coordinated Mith dimer was formed in the absence of DNA at concentrations of Mg(II) in the high-mM range (9). In addition to Mg(II), other divalent cations with radius <0.85 Å are candidates for promoting drug dimer formation as well (8,16). In this study, we investigated the ability of Mith to form a dimer complex in the presence of Fe(II) (and other metal ions) and its DNA-interacting property. The cleavage effects of the [$(Mith)_2$–Fe(II)] complex on DNA and the integrity and cellular permeation of the [$(Mith)_2$–Fe(II)] complex in cells were evaluated. Finally, the cytotoxicity of the stable [$(Mith)_2$–Fe(II)] complex toward cancer cell lines appeared to be higher than the drug alone, making it possible for the development of Fe(II) derivative of Mith as a potential target in the future.

MATERIALS AND METHODS

The synthetic DNA oligonucleotides were purified using gel electrophoresis. Mith was purchased from Sigma Chemical Co. (St Louis, MO). Absorbance measurements were carried out in a quartz cuvette using a Hitachi U-2000 spectrophotometer. The concentrations of Mith were estimated using extinction coefficients of 10 000 M^{-1} cm^{-1} at 400 nm. The concentrations of oligonucleotides were determined according to Beer's law ($A = \varepsilon bc$; where A is the optical density at 260 nm, ε is the extinction coefficient, b is the cell path length (1 cm) and c is the DNA concentration).

Molecular modeling

Molecular modeling was carried out on a Silicon Graphics workstation. Previous studies have shown that the Chro–DNA and Mith–DNA complexes have global similarities, as well as local differences (14,17). In addition, the coordination geometry surrounding the metal ion has been defined in the crystal structure of the [$(Chro)_2$–Mg(II)] complex bound to a DNA duplex. In this study, we use the Chro–DNA duplex crystal structure as a template to construct a plausible Mith–DNA complex by using the molecular modeling programs including Insight II and CNS. There are several differences between Chro and Mith, including the functional groups on sugar rings, sugar chirality and sugar–sugar linkages. Based on the [$(Mith)_2$–Mg(II)]-d$(TGGCCA)_2$ NMR structure, we modify the side chain of sugar, the A–B and D–E sugar linkages, as well as in the chirality of the E sugar of the initial model (14). Some distance restraints between Mith and DNA, obtained from the [$(Mith)_2$–Mg(II)]-d$(TGGCCA)_2$ NMR structure, were added to the refinement using the procedure implemented in CNS. The DNA force-field parameters of Parkinson *et al*. (18) were used. The quality of the model geometry was evaluated by r.m.s. derivation of bond length and bond angle.

CD experiments

The circular dichroism (CD) spectra were collected between 520 and 200 nm with bandwidth at 1 nm interval using a JASCO-720 CD spectropolarimeter. All spectra were the average of five runs. Method of CD spectral analysis was described previously (19). The data corresponding to the titration of Mith with metal (II) (normalized intensity change at λ_{275} versus metal concentrations) were fitted using a non-linear least squares plot. The evaluation of the dissociation equilibrium constant (K_d) between Mith and Metal(II) is measured as follows:

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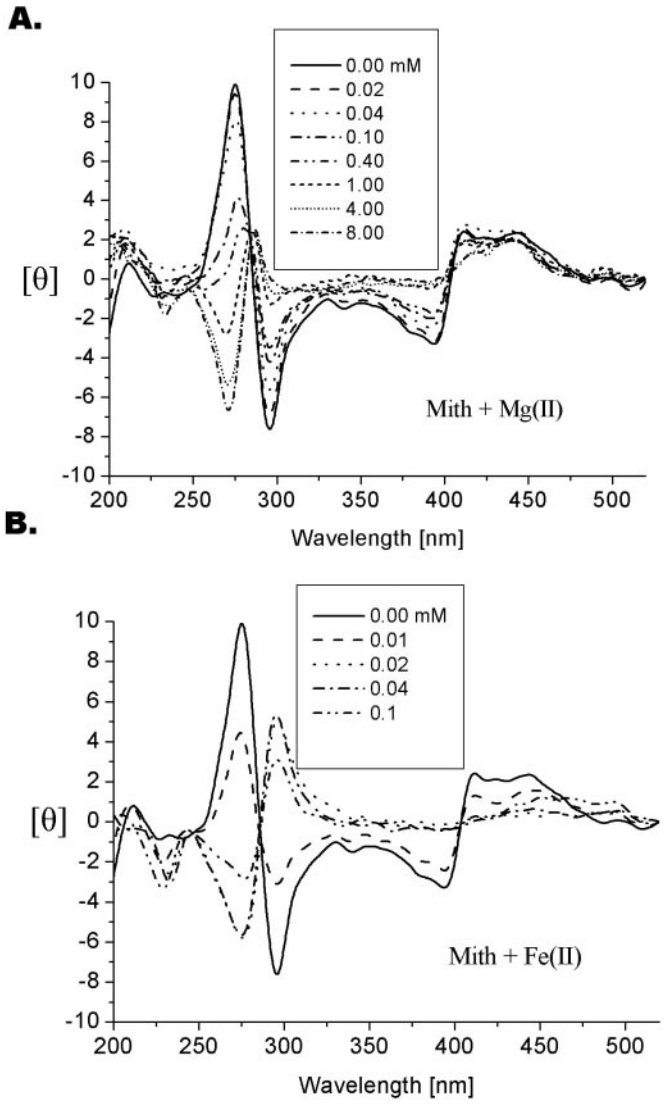

Figure 2. CD spectra of (**A**) Mith in the presence of Mg(II) with concentrations varying from 0 to 8 mM at 25°C (bottom). CD spectra of (**B**) Mith in the presence of Fe(II) with concentration varying from 0 to 0.1 mM (bottom) at 25°C. The drug concentration is 40 μM, buffered by 20 mM sodium-cacodylate at pH 7.3. The two isobestic points at 285 and 402 nm remain basically unchanged between the two complexes' association with Mg(II) and Fe(II) ions.

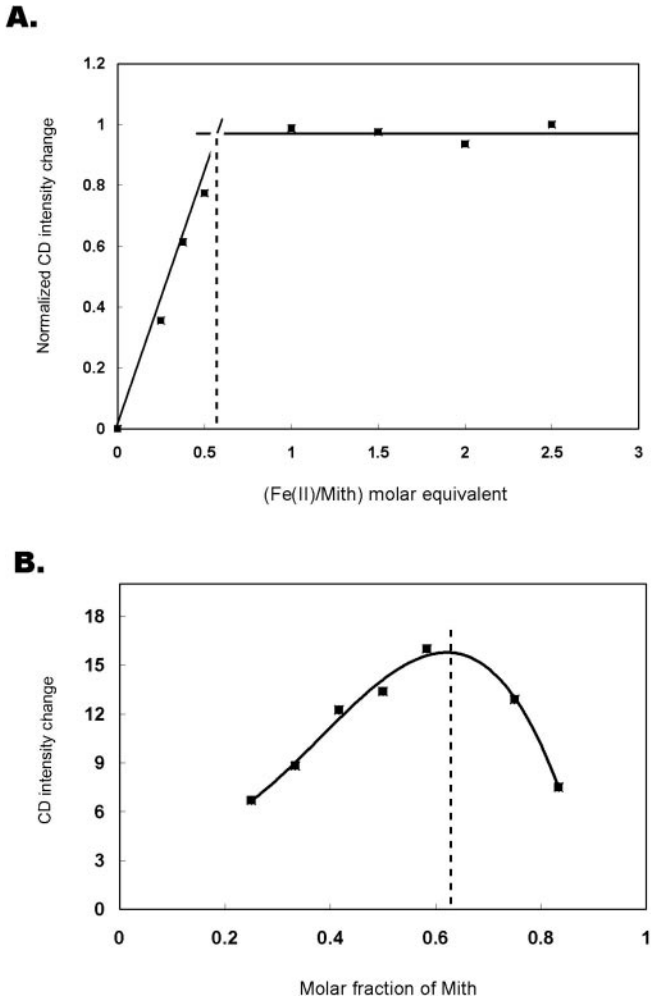

Figure 3. (**A**) CD titration of Mith against Fe(II) with normalized CD intensities change at 275 nm versus molar equivalents of [Fe(II)–Mith] at 25°C. The drug concentration is 40 μM, buffered by 20 mM sodium-cacodylate at pH 7.3. The two dashed lines in the figure represent the initial binding curve of Fe(II) to mithramycin and the one reaching the plateau. (**B**) Job-type titration plots for Fe(II)–Mith in 20 mM sodium-cacodylate at pH 7.3 at 25°C. The total concentration of Mith and metal was set at 60 μM.

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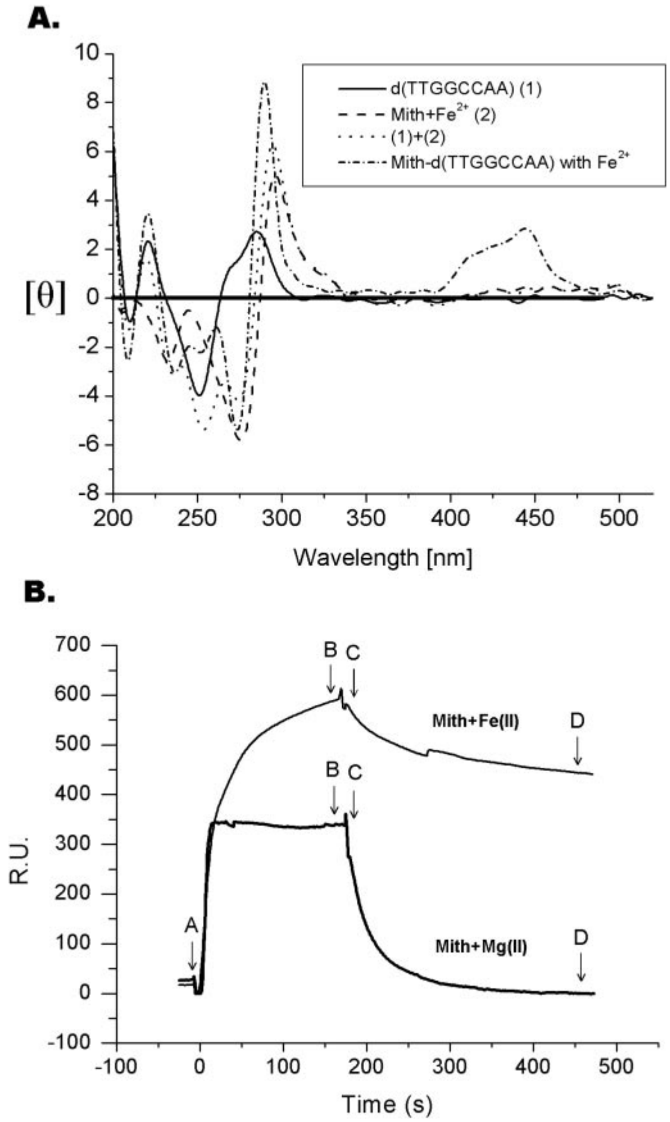

Figure 4. (**A**) Comparison of the CD spectra of the [(Mith)$_2$–Fe(II)] complex, d(TTGGCCAA)$_2$, [(Mith)$_2$–Fe(II)]-d(TTGGCCAA)$_2$ complex and the sum of the CD spectra of [(Mith)$_2$–Fe(II)] plus d(TTGGCCAA)$_2$ in 20 mM sodium-cacodylate buffer at pH 7.3 with 100 mM NaCl at 25°C. (**B**) Sensorgram of Mith–DNA interaction between immobilized hairpin DNA duplex and the target [(Mith)$_2$–Fe(II)] and [(Mith)$_2$–Mg(II)] complexes by subtracting the reference of control. The concentration of each target is 10 µM, in 50 mM NaCl, buffered by 30 mM Tris–HCl (pH 7.3) at 20°C. The start (A) and end (B) point of the association and the start (C) and end (D) point of the dissociation are indicated by arrows.

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Table 1. Binding parameters between Mith dimer complex and hairpin DNA in the presence of Mg(II) and Fe(II) determined by SPR

Dimer form	k_a (M^{-1} s^{-1})[a]	k_d (s^{-1})[a]	K^a (M^{-1})[b]
$(Mith)_2$–Fe(II)	0.37×10^3	7.55×10^{-4}	4.9×10^5
$(Mith)_2$–Mg(II)	1.6×10^3	2.86×10^{-2}	0.56×10^5

[a]Typical error for the k_a and k_d values are 20 and 10%, respectively.
[b]K_a value obtained from k_a divided by k_d.

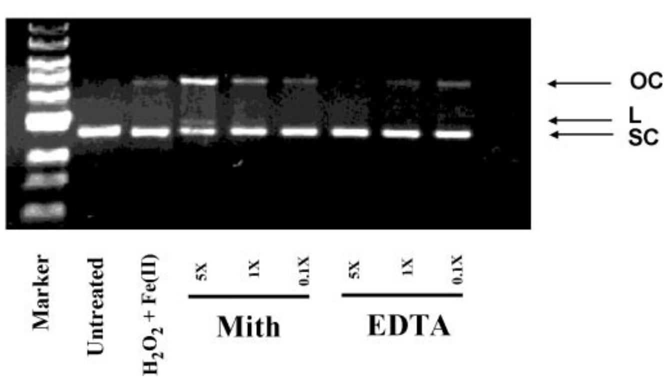

Figure 5. The effects of EDTA and Mith on integrity of the supercoiled plasmid DNA (pET-21b) when incubated in the presence of Fe(II) and hydrogen peroxide. The ratios of drugs versus Fe(II) are represented as 5×, 1× and 0.1× for 5:1, 1:1 and 1:10, respectively. The assays were carried out as described in Materials and Methods.

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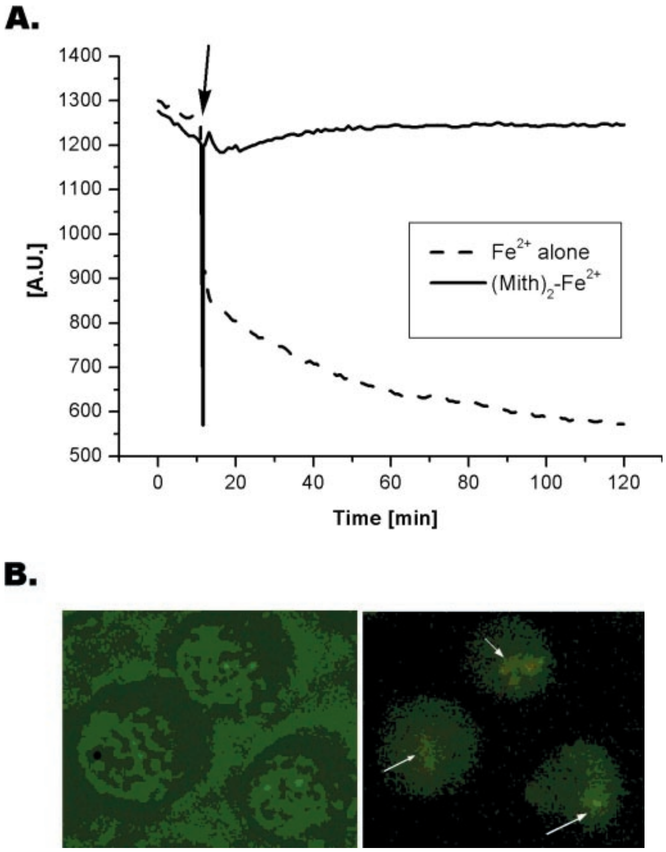

Figure 6. (**A**) The effect of the [$(Mith)_2$–Fe(II)] complex on calcein-detectable cellular Fe(II) content. The fluorescence of calcein-loaded K562 cells were treated with two different compounds as denoted by arrows, including 30 μM $FeCl_2$ (control), and 10 μM [$(Mith)_2$–Fe(II)] complex, buffered by PBS buffer at pH 7.3. (**B**) Subcellular locations of the [$(Mith)_2$–Fe(II)] complex are detected by phase contrast (left) and fluorescence (right) microscope. The location of cell nucleus was indicated by arrows.

Table 2. IC_{50} (μM) of Mith and the $(Mith)_2$–Fe(II) complex in various cancer cell lines at 24 and 48 h

Cell line type	Mith 24 h	48 h	$(Mith)_2$–Fe(II) 24 h	48 h
HepG2	>10	10 (±2)	>10	1 (±0.2)
Hep3B	1 (±0.2)	0.1 (±0.02)	0.4 (±0.1)	0.1 (±0.03)
K562	>10	0.3 (±0.1)	5 (±0.3)	0.1 (±0.05)
MCF-7	>10	0.4 (±0.1)	>10	0.35 (±0.1)

IC_{50} indicates 50% inhibitory concentration.

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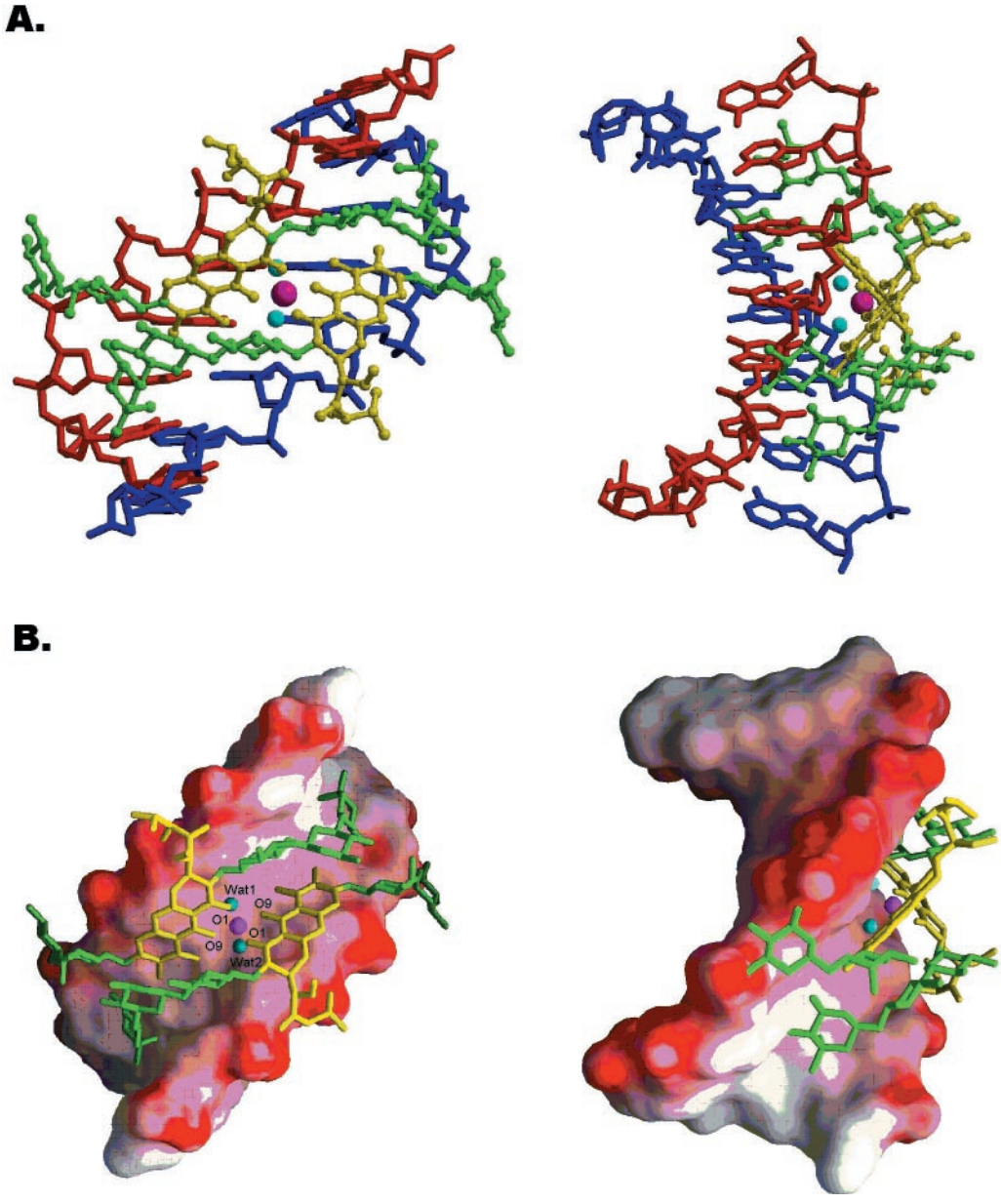

Figure 7. (**A**) The drawings of the metal(II)-coordinated [Mith-(TTGGCCAA)]$_2$ model viewed from the minor groove (left) and backbone direction, Mith containing chromophore (yellow) and saccharide (green) in ball and stick, and DNA in skeletal line drawing. (**B**) Surface representation of the metal(II)-coordinated [Mith-(TTGGCCAA)]$_2$ model shown with DNA electrostatic potentials viewed from the minor groove (left) and backbone (right). The metal(II) and waters are depicted in pink and blue, respectively.

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