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# Double-strand cleavage of DNA by a polyamide-phenazine-di-*N*-oxide conjugate

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ABSTRACT

Phenazine and its derivatives have been widely applied as nucleic acid cleavage agents due to active oxygen activating the C–H bond of the substrate. However, diffusion of oxygen radicals limits their potential applications in the DNA-targeted metal-free drug. Introduction of groove binder moiety such as polyamide enhanced the regional stability of radical molecules and reduced cytotoxicity of the drugs. In this work, we described the design and synthesis of a polyamide-modified phenazine-di-*N*-oxide as a DNA double-strand cleavage agent. The gel assays showed the hybrid conjugates can effectively break DNA double strands in a non-random manner under physiological conditions. The probable binding mode to DNA was investigated by sufficient spectral experiments, revealing weak interaction between hybrid ligand and nucleic acid molecules. The results of our study have implications on the design of groove-binding hybrid molecules as new artificial nucleases and may provide a strategy for developing efficient and safe DNA cleavage reagents.



The tumor microenvironment is now recognized as a major factor that influences not only the response to conventional anti-cancer therapies but also helps define the potential for malignant progression and metastasis.[1–3] In particularly, hypoxia is now considered a fundamentally important characteristic of the tumor microenvironment.[4–6] Representative anticancer drugs activated by bioreduction include quinones and heterocycle-*N*-oxides.[7–9] A leading hypoxic cytotoxin is the benzotriazine-*N*-oxide, tirapazamine (TPZ), which has been shown to have high hypoxic cytotoxicity and has been promoted to clinical trials.[10,11] It is reduced in hypoxic conditions, mainly by cytochrome P450 reductase, although also by xanthine oxidase, aldehyde oxidase, and nitric oxide synthase, to a free radical intermediate. This intermediate induces not only the formation of free radicals in DNA, based on the C4 of the ribose, but also the formation of OH**.** free radical. These radicals produce DNA fragmentation.[12,13]

Recently, a new generation of bioreductive compounds, phenazine-di-*N*-oxide, is the group of hybrid compounds that conjugate an *N*-oxide and π DNA stacking moieties. This kind of derivative could damage hypoxic cells generating **.**OH as TPZ and could interact with DNA before or after the bioreduction process.[14,15] Phenazine-di-*N*-oxide derivative can produce diffusible oxygen radicals responsible for DNA-strand scission under physiological conditions.[16] The proposed mechanism of action involves reductive activation of the phenazine moiety to produce $O_2^{\cdot-}$ or **.**$OH^-$ mediated DNA cleavage depending on aerobic or anaerobic conditions, in the absence of light or metal ions. Hecht and coworkers have also demonstrated that the cleavage activity of phenazine-di-*N*-oxide can be directed to specific sites in DNA via the tethering of oligonucleotides. Antisense oligonucleotides covalently linked to the phenazine-di-*N*-oxide prosthetic group via an aminopropyl chain can provoke DNA-strand scission at the target DNA sequence.[17] As mentioned previously, we reasoned that the linkage of a phenazine-di-*N*-oxide group to a DNA minor groove binding element might plausibly lead to a synthetic endonuclease capable of inducing efficient DNA cleavage.

Polyamides as a wide sequence-specific moiety, has an enhanced capability to function as regulators of transcription in directed applications.[18–23] The efficacious double-strand cleavage modulation has been shown in our earlier work.[24,25] Though it differs somewhat from antisense oligonucleotides with strict DNA-code reading moieties, polyamide limits phenazine reagents in a narrow region to achieve double-strand cleavage. In addition, the introduction of polyamide weakens the cytotoxicity of phenazine-di-*N*-oxide as diffusible oxygen radicals generator. Here, we described the synthesis, DNA-binding and strand cleaving properties of the polyamide-phenazine-di-*N*-oxide hybrid ligand **2** and **3**,

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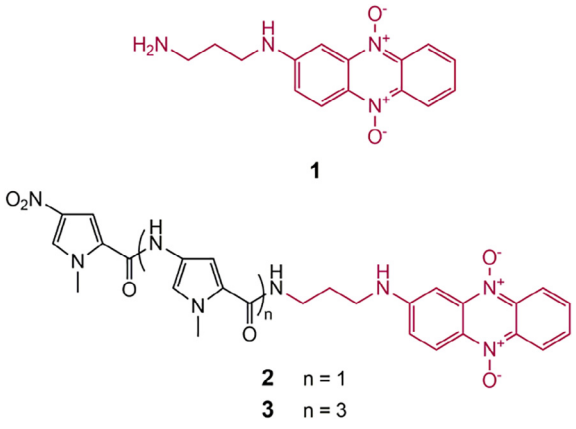
the structure of which was shown in Scheme 1. The preliminary data showed polyamide-phenazine conjugates presented significant difference in double-strand cleavage and DNA binding activity compared with free phenazine derivative. An important realization of this work is the efficacious modulation for phenazine-di-*N*-oxide in cleavage manner. The results may aid in the design and construction of oxygen radicals agents for DNA cleavage with low cytotoxicity.

A simple planar compound phenazine-di—*N*-oxide as a model compound was used to explore the ability of DNA cleavage. Subsequently, a groove-binding molecule containing 2 or 4 pyrrolecarboxamide units was linked to phenazine-5,10-dioxide nucleus at position 2 by a flexible aminopropyl chain. Although phenazine-di-*N*-oxide derivatives cleaved DNA by oxidative manner, small phenazine molecules was effectively fixed on the certain site in double-strand DNA and it resulted in non-random double-strand cleavage. The length of polyamide moieties were used to evaluate phenazine conjugates affinity to DNA molecules. Phenazine-di-*N*-oxide with aminopropyl linker (**1**) as control was applied to compare cleavage and binding activity with polyamide-phenazine di-*N*-oxide (**2** and **3**). The synthetic route was shown in Scheme 2.

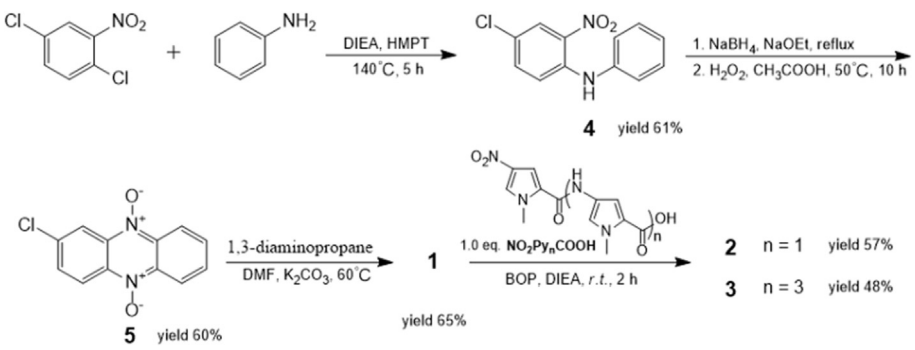
The DNA cleavage ability of conjugate was initially studied by monitoring the conversion of circular supercoiled DNA (Form I), circular relaxed DNA (Form II) and linear DNA (Form III) under physiological conditions (37 °C, pH 7.4). The supercoiled pUC18 plasmid DNA was used as the reaction substrate. Concentration-dependent assay was performed and results were shown in Fig. 1. Nearly 90% supercoiled DNA were gradually converted to nicked ones by increasing the content of **1** while no detectable linear DNA was observed, which indicated only single strand breakage in the presence of **1** over the tested range of concentration (Fig. 1A). Compound **3** has the ability to break plasmid DNA into linear form that implying double-strand breakage, but there could be two different ways in this process, random or non-random cleavage. Thus, Friefelder-Trumbo relationship was used to distinguish the two different manners of linearization.[26,27] The numbers of single and double-strand breaks were conveniently expressed as $n_1$ and $n_2$, respectively. The higher $n_1/n_2$ ratio suggested that linearization process resulted from the random placement of single-strand breaks at closely opposite sites and that a specific concerted mechanism for double-strand breakage may not exist.[28] Thus, the smaller the $n_1/n_2$ value, the higher the linearization activity of an agent in question.[29] A few of linear DNA can be found when the content of **3** was 0.6 mM. Up to 1.2 mM, 8% of Form III was observed with decreasing of $n_1/n_2$ ratio from 101.2 to 18.7 (Fig. 1B). The data showed that non-randomly double-strand cleavage occurred gradually with the increasing of polyamide-phenazine conjugate **3**.

The UV absorption spectra of the compound **3** changed significantly as a result of its binding to calf thymus DNA (Fig. 2A). Monitoring such changes would be helpful in estimating the binding constants. The two absorption bands centered at 295 and 425 nm, corresponding to the polyamide and phenazine oxide, respectively, were shifted by 8 nm when the ligand was fully bound to DNA. The interaction of the ligand with DNA also causes a marked hypochromism at 303 nm. The UV spectra suggested that the two moieties of the hybrid molecule were engaged in the complex with DNA. Subsequently, the titration of compound **3** to ct-DNA was performed in Tris-HCl buffer solution, as shown in Fig. 2B. The apparent binding constant measured for the binding of **3** to calf thymus DNA is $1.2 \times 10^5$ ($M^{-1}$, per nucleotide; n = 3.5). Compared with phenazine-di-*N*-oxide free ligand with $K_a = 2.9 \times 10^5\ M^{-1}$,[30] the affinity of polyamide phenazine conjugate to DNA seemed to be weakened. The different binding modes in the two moieties of the hybrid molecules may lead to such results.

The binding ability of compounds to DNA was reported to affect DNA cleavage in most cases and the three common binding modes including intercalation, grooving binding and electrostatic interaction can be evaluated by fluorescence titration assays based on displacement of ethidium bromide.[31] A competitive displacement assay using polyamide, phenazine (**1**), and polyamide phenazine conjugates (**2** and **3**) as quenchers can characterize the affinity to DNA. To better compare the affinity of these moieties to DNA, fluorescence changes were recorded as shown in Fig. 3. When free 0.2 mM of phenazine (**1**) was added to the EB-DNA system, the fluorescence intensity reduced by about 29%, obtaining $C_{50} = 0.074\ \mu M$. The legible red shift of 34 nm appeared with the increasing of **1**, indicating intense intercalated interaction between DNA and phenazine (Fig. 3A). Similarly, decreasing fluorescence were induced by addition of **2** and **3** under the same conditions, obtaining $C_{50}$




**Scheme 1.** The structures of phenazine-di-*N*-oxide (**1**) and polyamide phenazine-di-*N*-oxide conjugates (**2, 3**).




**Scheme 2.** The synthetic route of compounds **1**–**3**.

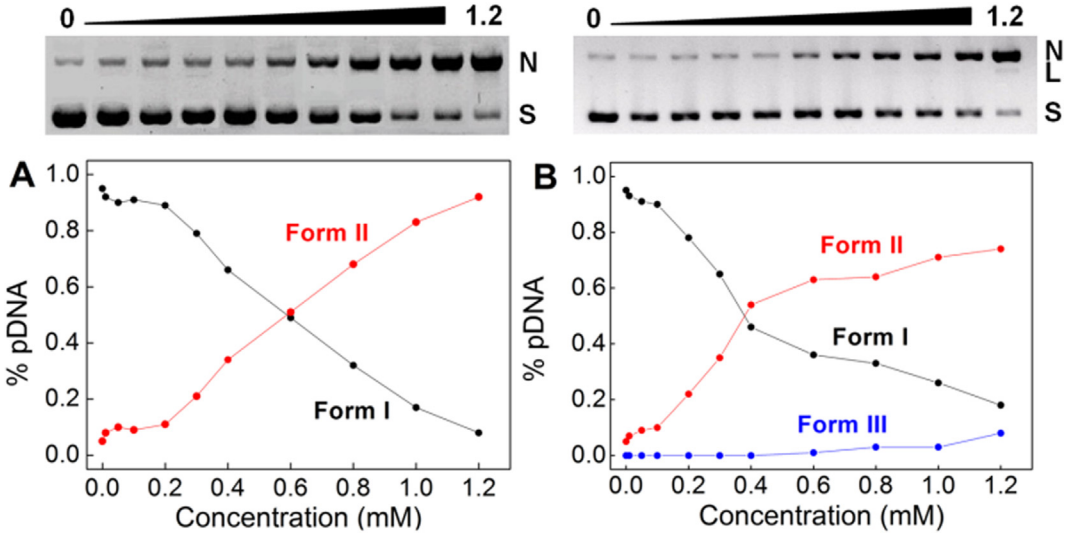



**Fig. 1.** Concentration dependence of pUC18 plasmid DNA cleavage by (a) **1** (b) **3** at 37 °C for 12 h. The range is 10 μM–1.2 mM ligand from left to right. S: supercoiled DNA, N: nicked DNA, L: linear DNA.

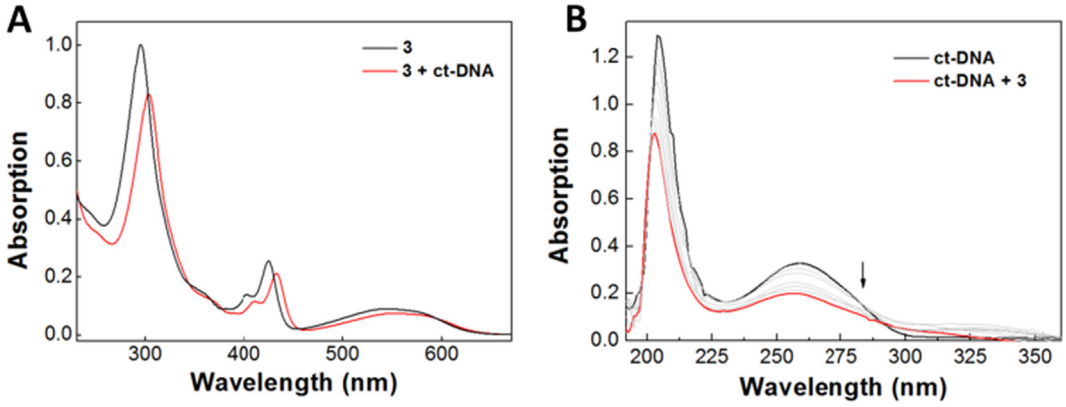



**Fig. 2.** (A) Absorption spectra of phenazine-di-*N*-oxide derivative (**3**) in the absence (black line) and presence (red line) of ct-DNA (500 μM) in 1 mM sodium Tris-HCl buffer, pH 7.4. (B) UV titration assay of ct-DNA (600 μM) by adding **3** (0–120 μM from black line to red line) in 10 mM Tris-HCl buffer, pH 7.4. The spectrum corresponding to the drug bound to DNA was referenced against a DNA solution of exactly the same concentration and was adjusted to a common baseline.

= 0.272 μM with red shift of 23 nm and $C_{50}$ = 0.114 μM with red shift of 25 nm, respectively (Fig. 3B-C). No dramatic decrease of fluorescence was observed when polyamide with COOH terminal was added (Fig. 3D). The fluorescence quenching results were also in accordance with the classical Stern-Volmer equation: $F_0/F = 1 + K_{sv}[Q]$, where $K_{sv}$ represents the binding constant (also namely quenching constant), which reflects the binding ability of the polymer to DNA, and Q is the mole concentration (mol/L) of the polymer.[32] The $K_{sv}$ values of the polymer originated from **1**, **2**, and **3** were obtained as $4.26 \times 10^4\ M^{-1}$, $1.42 \times 10^4\ M^{-1}$ and $2.67 \times 10^4\ M^{-1}$, respectively, showing the order of the EB-displacement ability of **1** > **3** > **2**. Free phenazine (**1**) showed the stronger DNA binding ability than both polyamide phenazine conjugates (**2** and **3**), which were mainly derived from different binding modes of rigid phenazine and flexible polyamide. Also, the data indicated that the affinity to DNA groove depended on the length of polyamide. To increase the polyamide unit could improve the binding activity of the conjugate.

Diverse effects of phenazine derivatives on the DNA secondary structure of double-strand DNA were evaluated by CD measurements. All samples to be tested do not show any CD signal in the studied region. Positive and negative ellipticities centered around 245 and 275 nm arise from the DNA itself, revealing a double-strand structure for the DNA (Fig. 4, black line). Detectable signal changes of CD spectra were induced by **1** at 0.2 mM, including an enhanced negative ellipticity, a flattening of the positive bands and an overall shift of the bands toward higher values of wavelengths (Fig. 4A, red line). The sharp decrease in the positive CD band suggested that DNA was condensed to so-called $\psi$ anomalies and there was a secondary structural change.[33,34] It was, in fact, hypothesized that the $\psi$-DNA was due to a high extent of compaction through intercalated interaction between planar molecules and base pairs. In the case of **3**, no CD signal change was observed at "Cotton effect" region after addition of equimolar **3** to the DNA solution, which indicated that ligand can not lead to changes of base stacking and helicity due to weak interaction. However, a typical DNA-induced CD band appeared at 335 nm, suggesting the polyamide moiety interacted appreciably with DNA minor groove (Fig. 4B). The result was consistent with the original design for groove binding of phenazine derivatives.

Although DNA cleavage by DNA groove-binding molecule conjugates has been extensively studied for the past years, our present efforts attended to the interacted effect on DNA from a new perspective. Limiting radical oxidative molecule range of movement and achieving double-strand cleavage were real intention in current work. The goal was expected to reach by modification of

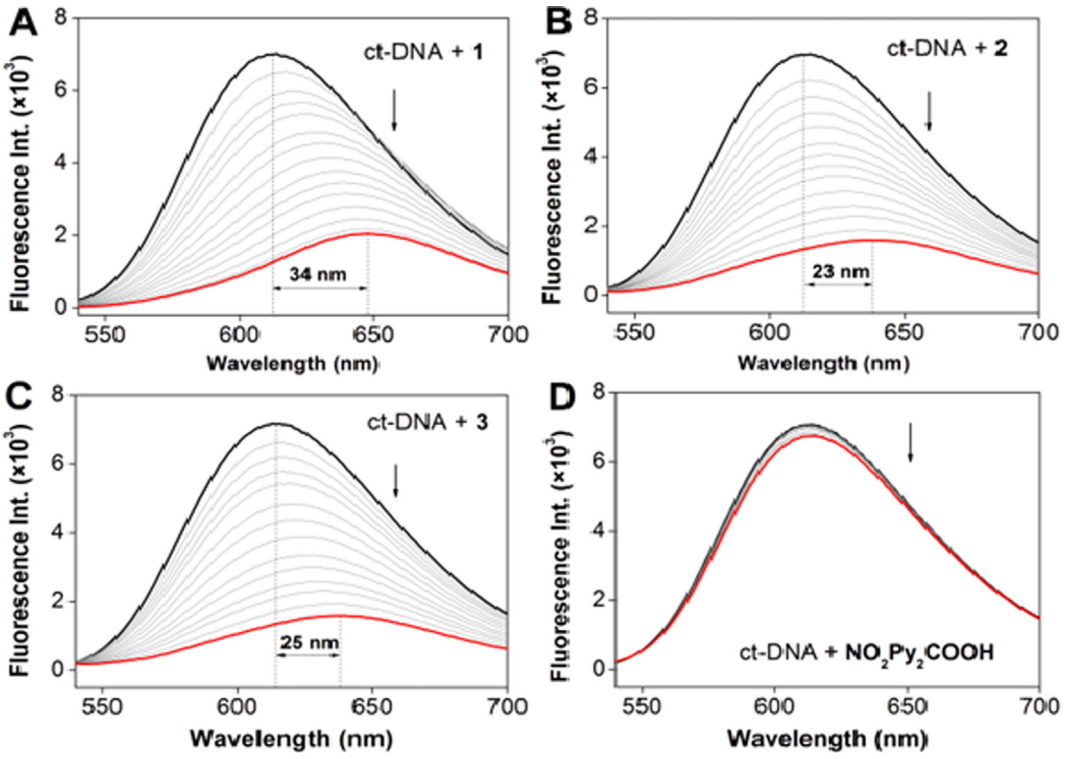



**Fig. 3.** Fluorescence quenching by (A) **1** + ct-DNA; (B) **2** + ct-DNA; (C) **3** + ct-DNA and (D) **$NO_2Py_2COOH$** + ct-DNA at pH 7.4 (10 mM Tris − HCl buffer with 10 mM NaCl) with quencher concentration of 0–0.2 mM (Black line is starting point for titration, and red one for the end).

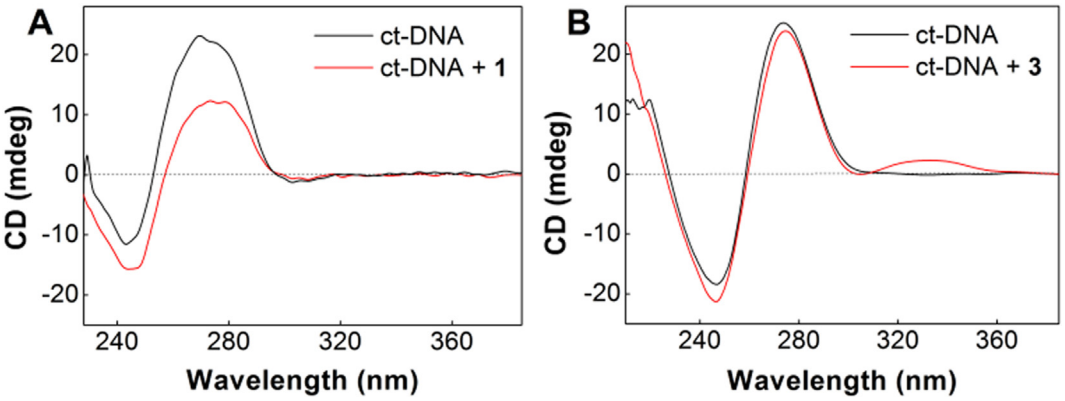



**Fig. 4.** CD spectra of (A) ct-DNA/**1** (0.2 mM) and (B) ct-DNA/**3** (0.2 mM) at the same ratio. The sample was added into 60 μg·mL$^{-1}$ ct-DNA solution (pH 7.4, 10 mM Tris-HCl buffer) at 25 °C.

polyamide as DNA-code reading moiety. The electrophoresis assays clearly demonstrated that plasmid DNA can be linearized in the presence of polyamide-phenazine conjugate at 37 °C pH 7.4, while the plasmid was just converted to nicked DNA by free phenazine-di-*N*-oxide under the same conditions. Polyamide-modified ligand helped better realize DNA linearization with non-random double-strand cleavage ($n_1/n_2$ = 18.7). However, some experimental data from spectroscopy were not consistent with the gel results. The competitive displacement assay presented the higher binding activity of unmodified ligand ($C_{50}$ = 0.074 μM, $K_{sv}$ = 4.26 × 10$^4$ M$^{-1}$) compared to that of polyamide-phenazine conjugate ($C_{50}$ = 0.114 μM, $K_{sv}$ = 2.67 × 10$^4$ M$^{-1}$). Meanwhile, no significant change was observed in the DNA-absorbing wavelength region (230–300 nm) when compound **3** interacted with ct-DNA, and the slight induced signal appearing at 335 nm also did not mean better groove binding to DNA. These findings that the free ligand has better DNA affinity is counterintuitive.

This result might be due to the two moieties of the hybrid molecule with different binding modes and inadequate length in linker. Polyamide can specifically bind to minor groove of double-strand DNA,[35] while the planar phenazine derivative prefer to intercalate into base pair pleated sheet of DNA (Fig. 5A). It is hard to achieve desired and stable binding for both the moieties, which could be attributed to improper linker. Once polyamide rigorously binds to DNA minor groove, it is not easy to adjust suitable spatial conformation for phenazine plane, and vice versa. The CD data do not show persuasive changes that imply strong interaction with DNA groove or base pairs. Thus, it is likely that the hybrid molecule only binds to DNA by weak interaction outside the groove (Fig. 5B), giving the inferior spectral characteristics than unmodified ligand.

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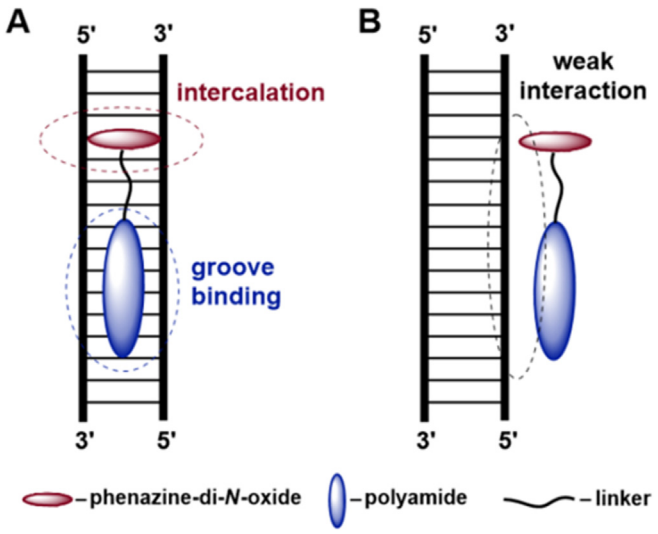


**Fig. 5.** Schematic model to explain the interaction of polyamide-phenazine conjugate with double-strand DNA.

Interestingly, it does not help matters that polyamide-modified phenazine-di-*N*-oxide achieves DNA double-strand cleavage, that is, the introduction of polyamide has positive influence on the manners of DNA cleavage by radical oxidative molecules.

In summary, we showed that polyamide-phenazine conjugates offered a clear enhancement toward DNA cleavage relative to the well-studied phenazine-di-*N*-oxide and possessed the efficient ability for non-random double-strand cleavage. The spectral results indicated that both polyamide and phenazine ligand have the ability for binding to DNA by weak interaction. The structure of linker could have significant effect on binding mode of two moieties in hybrid conjugate. Detailed investigations into optimized linker for high DNA affinity of hybrid molecules are still ongoing. The present work should be of value for rational design of hybrid molecule with high binding activity, as well as efficient strategy for developing artificial nucleases.

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## A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.12.058.

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