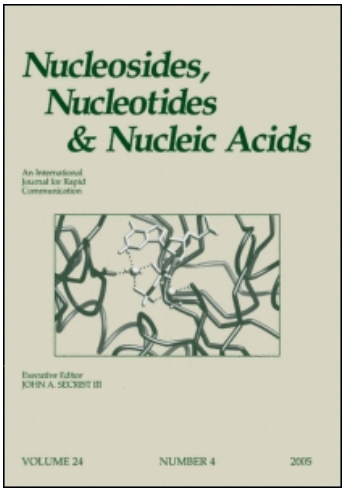

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QUADRUPLEX-DUPLEX MOTIFS AS NEW TOPOISOMERASE I INHIBITORS

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□ *In this article, 13 short chains that can form G-quadruplex and quadruplex-duplex motif have been designed. Fourteen oligonucleotides, including 13 short chains as well as a reference short chain all have certain level of inhibition to topoisomerase I, whether or not they form G-quadruplex and quadruplex-duplex motif, and the G-quadruplex and quadruplex-duplex motif show better activity than single short chain. The result confirmed that after forming G-quadruplex and quadruplex-duplex motif these 14 oligonucleotides are competitive inhibition, that is, through the priority binding with the topoisomerase I and precluding from its binding with the normal substrate pBR322 and, therefore, inhibiting the next reaction.*

Keywords Topoisomerase I; G-quadruplex; quadruplex-duplex motif

INTRODUCTION

Malignant tumor is a common, frequently occurring disease that seriously affects human health, and its related research has always been the focus of many countries around the world in the field of life science.[1]. Chemotherapy is one of the major solutions used in malignant clinical treatment. However, the treatment of anticancer drugs is not satisfactory, and often leads to side effects and drug resistance. Therefore, the research of

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new types of high-efficiency and low-toxicity anticancer drugs is significant to the treatment of malignant tumors, and is becoming one of the most active research area in contemporary chemical biology.[2]

The two fundamental enzyme complexes involved in DNA winding and unwinding are topoisomerases I and II.[3,4] Topoisomerase is most important intracellular target enzyme of many antineoplastic agents, thus, it is important to explore its potential role in such therapy. Topoisomerase-targeting anticancer drugs can be divided into two broad classes that vary widely in their mechanisms of action. There are topoisomerase inhibitors and topoisomerase poisons.[5,6] Topoisomerase inhibitors bind to the enzyme prior to DNA binding and inhibit the formation of the cleavable complex. A topoisomerase poison does not interfere with the cleavable complex formation but binds to and stabilizes the DNA-topoisomerase intermediate after it has been formed, thus, prohibiting the release and resealing of the DNA strand.

Most of oligonucleotide drugs showed biological activity through complementary target gene and inhibition of gene expression.[7,8] However some studies show that the G-rich oligodeoxynucleotides can inhibit cell growth through the integration with proteins inside cells. Bates[9] showed that this type of G-rich oligonucleotides can form G-quadruplex structure that shows good inhibition of cell proliferation activity, and this activity is relevant to its integration with the protein Nucleolin inside of nucleus.

G-quadruplexes are multistranded structures held together by square planes of four guanines interacting by forming Hoogsteen hydrogen bonds.[10] They can block the action of telomeres, which are found at the ends of eukaryotic chromosomes and protect chromosome ends from base-pair loss and end-to-end fusion.[11,12] Many intramolecular DNA G-quadruplex structures have been reported, for example, the basket-type and the chair-type. As well, a series of G-quadruplex is shown to inhibit thrombin, and quadruplex-duplex motifs can enhance the inhibition activity.[13] Recently, research about G-quadruplex and topoisomerase have been reported.[14,15] This article investigates whether quadruplex-duplex motif can also bind to topoisomerase I and prevent cleavage of duplex DNA. 14 oligonucleotides (13 short chains as well as a reference short chain), which can successfully form G-quadruplex structure or quadruplex-duplex motif under reaction conditions have biological activities towards topoisomerases I in vitro whether or not they are formed G-quadruplex and quadruplex-duplex motif. The sequences of all oligonucleotides used in this study are reported in Table 1. The basic structure of the quadruplex-duplex motif is shown in Figure 1.

RESULTS AND DISCUSSION

Measurement of Relaxation Activity of Topoisomerase I

Since the 3′ and 5′ of the chair-type G-quadruplex are at the two sides of the structure, this chair-type G-quadruplex may be able to bind with

TABLE 1 Sequences of oligonucleotides and the IC50 of oligonucleotides before and after formed G-quadruplex and quadruplex-duplex motif. Results are the average of three independent experiments

Oligonucleotide	Sequence of Oligonucleotide	IC50 of Oligonucleotide (μM) after formed G-quadruplex
S4	CTACTGGTTGGTGTGGTTGGGTAG	2.13 ± 0.34
S5	ACTACTGGTTGGTGTGGTTGGGTAGT	0.79 ± 0.037
S6	GACTACTGGTTGGTGTGGTTGGGTAGTC	0.7 ± 0.049
S7	TGACTACTGGTTGGTGTGGTTGGGTAGTCA	1.32 ± 0.098
S8	GTGACTACTGGTTGGTGTGGTTGGGTAGTCAC	1.23 ± 0.15
LO	GACTACGGTTGGTGTGGTTGGGTAGTC	2.49 ± 0.17
LA	GACTACAGGTTGGTGTGGTTGGGTAGTC	0.55 ± 0.09
LC	GACTACCGGTTGGTGTGGTTGGGTAGTC	2.67 ± 0.1
LG	GACTACGGGTTGGTGTGGTTGGGTAGTC	0.28 ± 0.028
H3	GACTACTGGTTGGTGTGGTTGGGTAGTCTT	0.36 ± 0.014
H5	TTGACTACTGGTTGGTGTGGTTGGGTAGTC	0.41 ± 0.017
TBA	GGTTGGTGTGGTTGG	2.58 ± 0.33
ODN	GGTTGGGGGTGGTGGG	1.44 ± 0.05
LG-H3	GACTACGGGTTGGTGTGGTTGGGTAGTCTT	0.14 ± 0.03

double stranded DNA better. The inhibition activities to topoisomerase I of 14 oligonucleotides, which are modified from **TBA** with the chair-type structure, is investigated in this article. The **ODN**, which is reported to inhibit topoisomerase, is chosen as the control. It has been confirmed that, by improving the base types as well as the order of the oligonucleotide, the IC50 values of the inhibition of these quadruplex-duplex motifs to topoisomerase I can dramatically reduce (Table 1). As well, the results indicate that

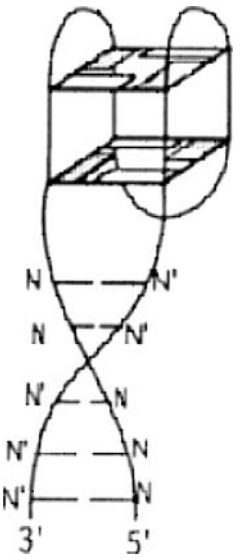

FIGURE 1 The basic structure of the quadruplex-duplex motif.

all the oligonucleotides strongly inhibit the DNA relaxation activity of topoisomerase I (see Figures 2–6), whether or not they are formed quadruplex-duplex motif. The IC50 value of the best inhibitory effect of quadruplex-duplex motif **LG** is 0.28 μM, which has improved by nearly 5 times than the reference oligonucleotide. **LA**, **H3**, and **H5** are also very effective to topoisomerase I. The inhibitory activities of these oligonucleotides, which are from G-quadruplex and quadruplex-duplex motif, decrease in the order: **LG** > **H3** > **H5** > **LA** > **S6** > **S5** > **S8** > **S7** > **ODN** > **S4** > **LO** > **TBA** > **LC**.

The structure–activity relationship is comparatively discussed as following:

i) The length of the duplex motif is important to inhibition. The activity is the best when the duplex length is 6, such as **S6** shows better activity than **S4**. It is proprosed that when the length of the duplex motif is 6, quadruplex-duplex motif can bind with topoisomerase I better than the others.
ii) The inhibition activity is the best when the base which connected with a G-quadruplex structure and duplexed structure is G. For example, the inhibitory activities of **LG**, **LA**, **LC** and **LO** decrease in the order: **LG** > **LA** > **LO**> **LC**. It is proprosed that the G-quadruplex, duplex DNA and single-base loop which connect with G-quadruplex and double stranded DNA act with topoisomerase I, in which the single base G has the strongest activity to topisomerase I than the others.
iii) Adding two base T to 3′ or 5′ leads a positive effect on the inhibitory activity against topoismerase I.
iv) Thirteen oligonucleotides all have a certain level of inhibition to topoisomerase I, whether or not they formed G-quadruplex and quadruplex-duplex motif, but the activity is better when these oligonucleotides formed G-quadruplex and quadruplex-duplex motif. According to the result, we have designed another quadruplex-duplex motif **LG-H3** that has the characteristics both of **LG** and **H3**. The IC50 value of **LG-H3 is** 0.14 μM, which is the best result in these quadruplex-duplex motifs (Figure 5D).

It has been confirmed that the inhibitory effect to topoisomerase I of the short chains has been greatly improved when formed into quadruplex-duplex motif. A short-chain **HS6** (GACTACTGTGTAGTC), which cannot form quadruplex-duplex motif but can form loop ring and duplex, has been designed according to **S6**. Our current results indicate that the short-chain **HS6** has biological activities toward topoisomerases I. However, **HS6** could not inhibit the action of topoisomerase I when **HS6** formed into loop ring and duplex (Figure 5C). The results show that a G-quadruplex structure is necessary in this system.

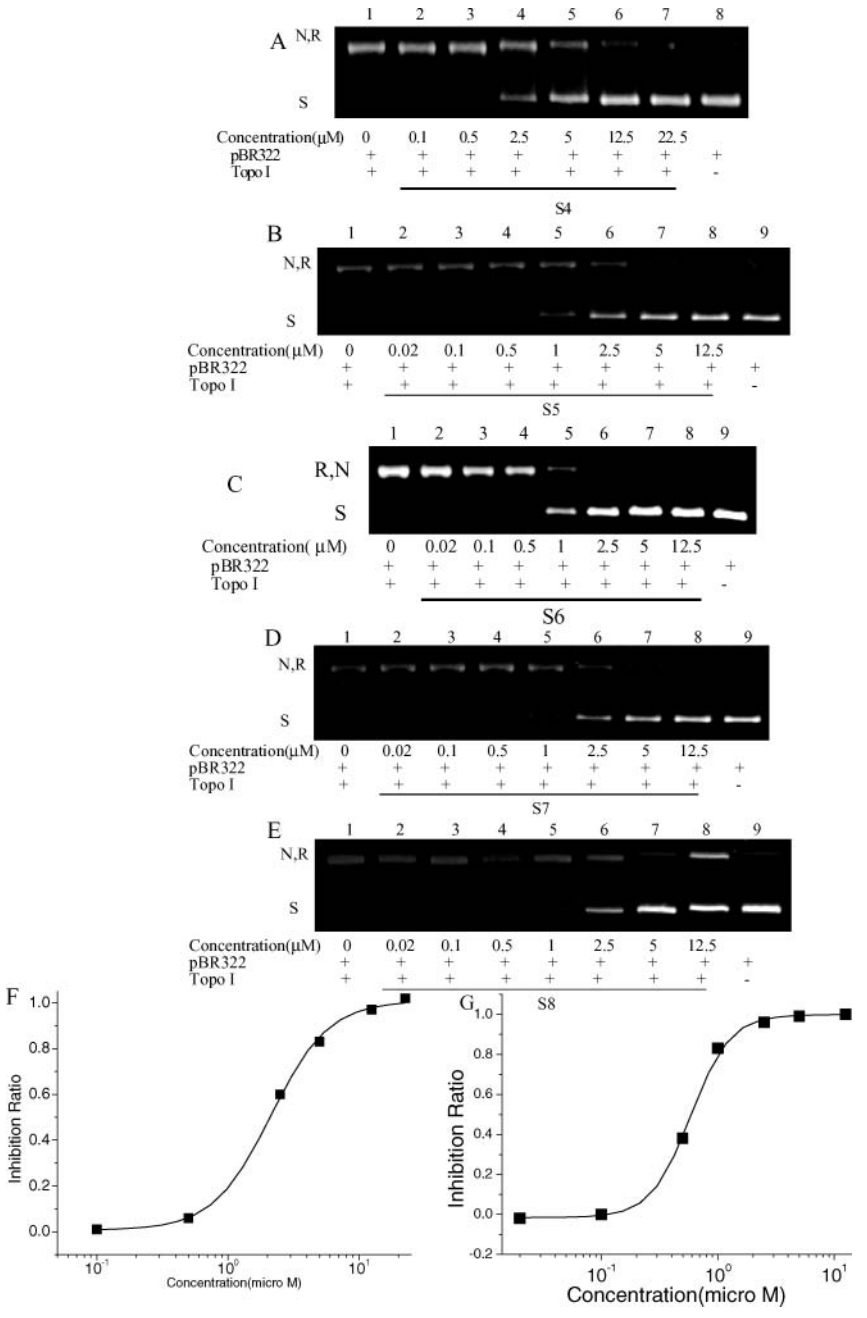

FIGURE 2 Activity inhibition of topoisomerase I by oligonucleotides after formed G-quadruplex and quadruplex-duplex motif. Relaxed DNA (R) and nick DNA (N) slowly migrate in the gel as compared to supercoiled DNA (S). Inhibitory effects of a) **S4**, b) **S5**, c) **S6**, d) **S7**, and e) **S8**. f) The concentration-response curve of **S4** inhibiting the relaxation activity of topoisomerase I. g) The concentration-response curve of **S6** inhibiting the relaxation activity of topoisomerase I.

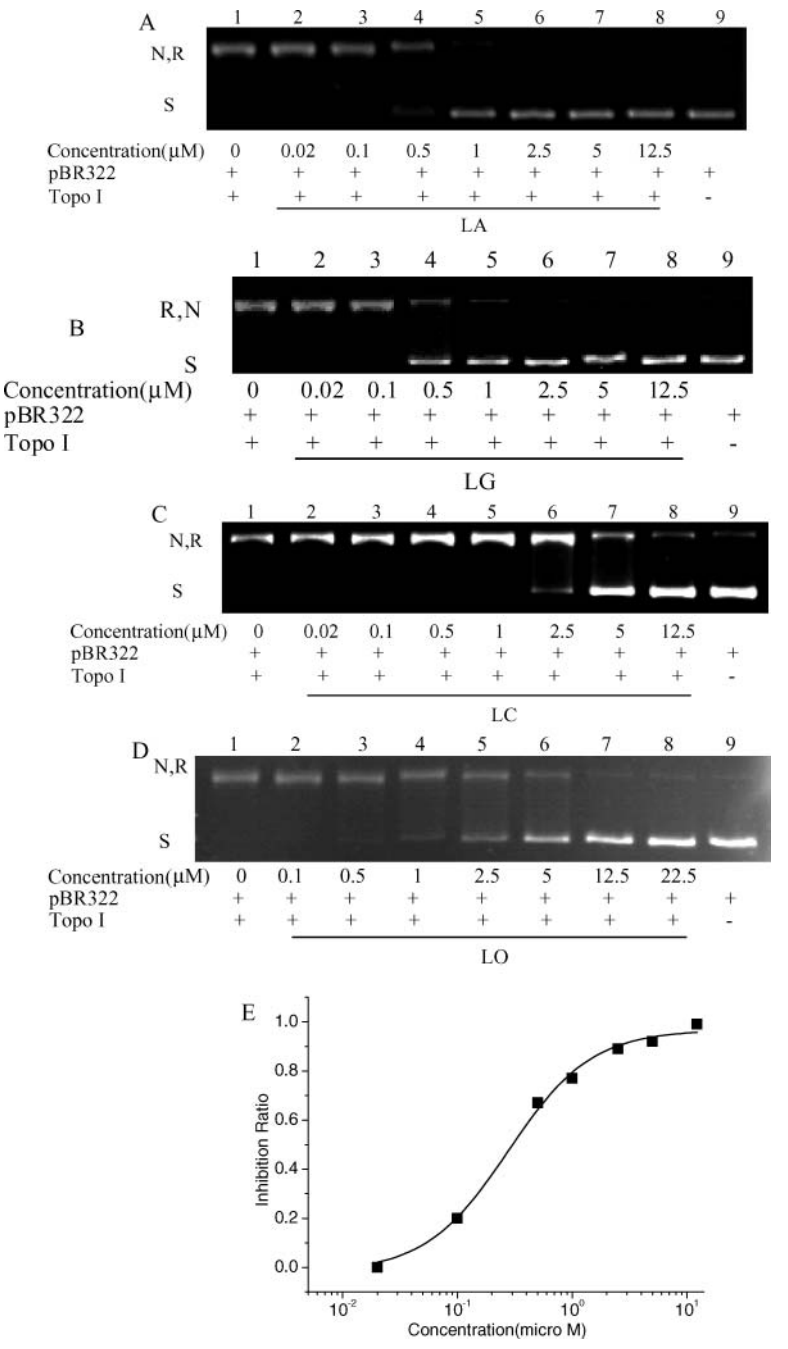

FIGURE 3 Activity inhibition of topoisomerase I by oligonucleotides after formed G-quadruplex and quadruplex-duplex motif. Relaxed DNA (R) and nick DNA (N) slowly migrate in the gel as compared to supercoiled DNA (S). Inhibitory effects of a) **LA**, b) **LG**, c) **LC**, and d) **LO**, e) The concentration-response curve of **LG** inhibiting the relaxation activity of topoisomerase I.

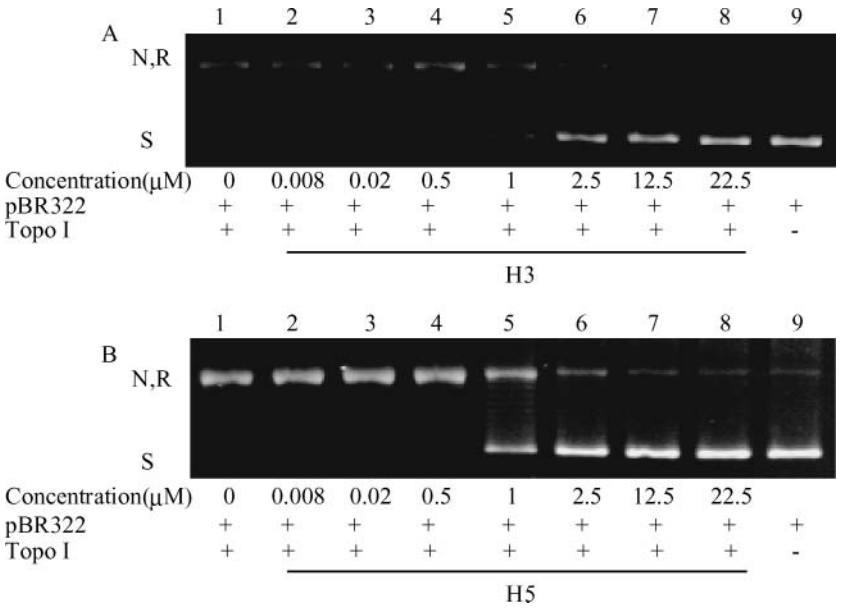

FIGURE 4 Activity inhibition of topoisomerase I by oligonucleotides after formed G-quadruplex and quadruplex-duplex motif. Inhibitory effects of a) **H3** and b) **H5**.

Circular Dichroism Spectroscopy

To investigate whether these short-chain form a G-quadruplex structure, circular dichroism (CD) spectroscopy is used to determine the structure transition of the G-quadruplex.[16] Generally, a positive peak near 265 nm and a negative band around 240 nm are typical features of a parallel G-quadruplex structure. Furthermore, a strong positive peak close to 295 nm and a negative peak near 265 nm are characteristic of antiparallel G-quadruplexes. In the experiments, both **TBA** and **LG** form antiparallel G-quadruplex structure after being heated at 95°C for 5 minutes and then naturally cooled at 20°C in 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl, pH = 7.4 (Figures 6C and 6D). Both **TBA** and **LG** do not form an antiparallel G-quadruplex structure when they were not heated at 95°C for 5 minutes and natural cooled at 20°C. According to the gradient agarose gel assay of **LG**, the IC50 value of single short-chain **LG** is 3.65 μM (Figure 6B). The inhibitory effect to topoisomerase I of the short chains **LG** has been greatly improved, when formed into quadruplex-duplex motif, up to nearly 13 times.

Measurement of Topoisomerase I-Mediated DNA Cleavage

Next, it is important to find out whether these quadruplex-duplex motifs are topoisomerase I inhibitors, or whether they show a similar mechanism with catalytic inhibition of camptothecin (CPT). To investigate the

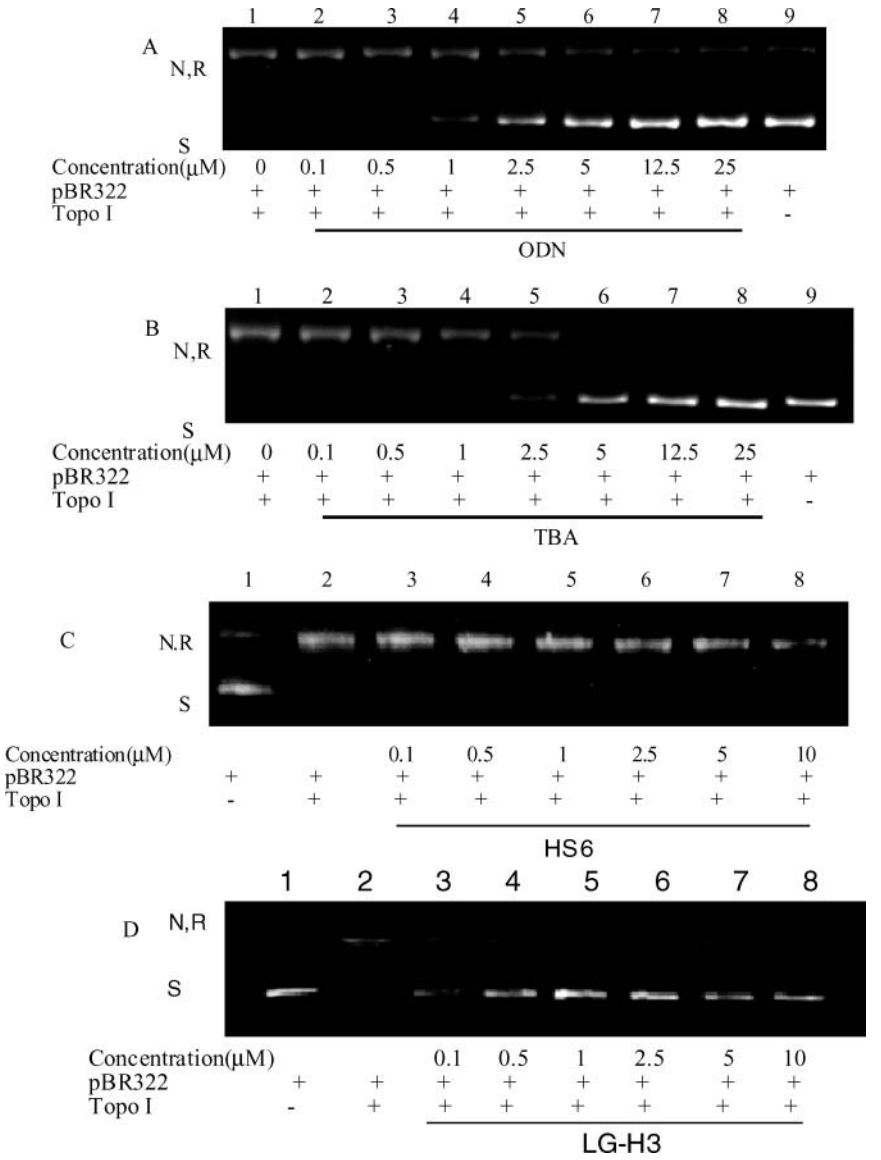

FIGURE 5 Activity inhibition of topoisomerase I by oligonucleotides after formed G-quadruplex and quadruplex-duplex motif. Inhibitory effects of a) **ODN**, b) **TBA**, c) **HS6** after formed loop ring and duplex, and d) of **LG-H3**.

mechanism in detail, we chose to test the generation of a single-strand break (open circular plasmid, marked with N) by using the circular DNA under the reported modified electrophoresis conditions.[17,18] The relaxed DNA and open circular DNA can be disgregated under this experiment conditon. The relaxed DNA which is intercalated by ethidium bromide (EB) can move faster than a negatively supercoiled plasmid.[17,18] The result shows that the positive control CPT can stabilize the cleavable complex

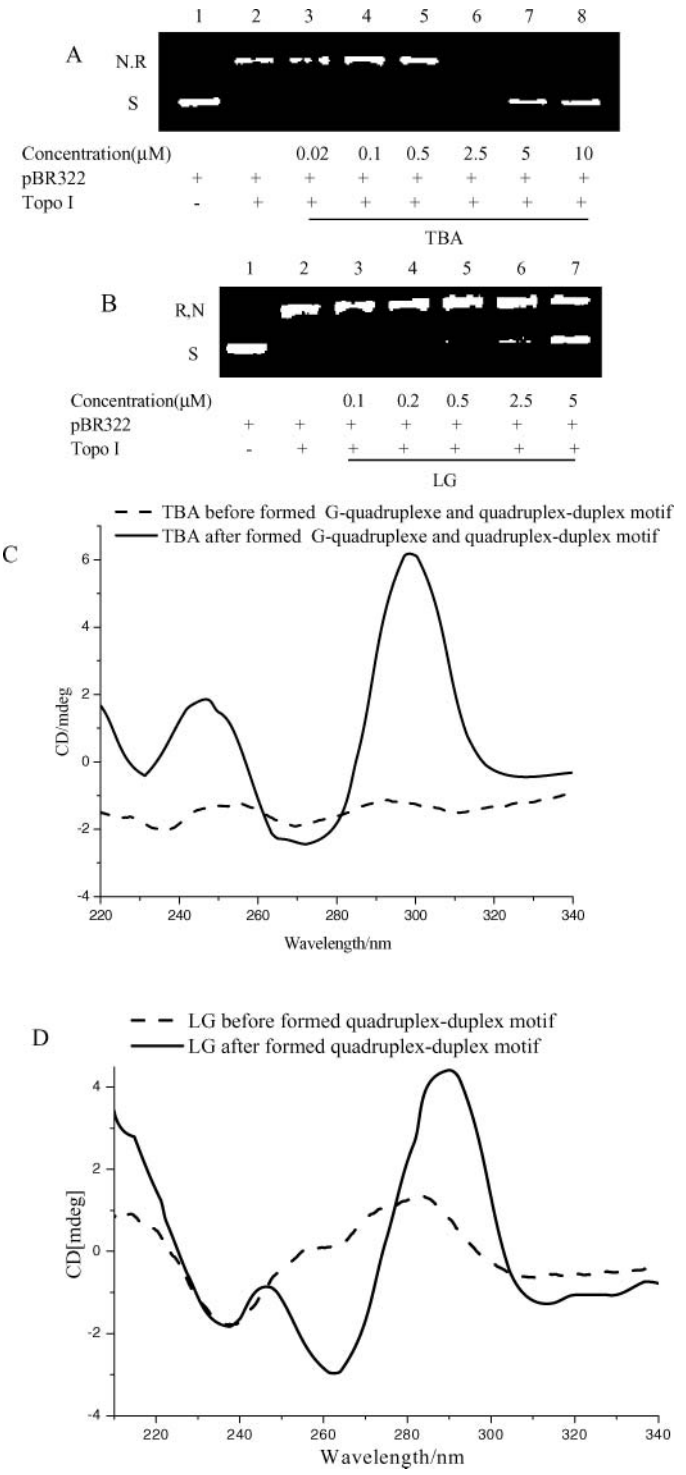

FIGURE 6 Activity inhibition of topoisomerase I by oligonucleotides before formed quadruplex-duplex motif and CD spectroscopy of **TBA** and **LG**. Inhibitory effects of a) **TBA**, b) **LG**, CD spectroscopy, c) **TBA**, and d) **LG**.

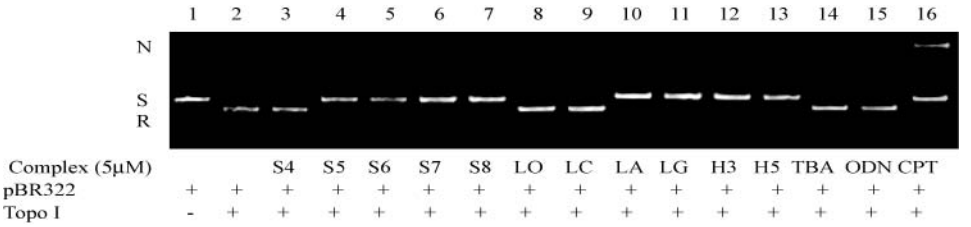

FIGURE 7 Representative image of agarose gel electrophoresis of the cleavage assay. Agarose gel electrophoresis of all the 13 oligonucleotides after formed G-quadruplex and quadruplex-duplex motif.

(Figure7, lanes 16).[14] However, we did not observe the open circular DNA induced by the oligonucleotides tested. The result of S4, LO, LC, TBA, ODN are the same with that of pBR322 added topoisomerase I because of the poor inhibition. However, the result of S5, S6, S7, S8, LA, LG, H3, H5, which have strong inhibition activity are the same with pure pBR322. From this experiment, it can be concluded that the oligonucleotides do inhibit the topoisomerase I-catalyzed DNA relaxation, but not by stabilizing the topoisomerase I-DNA complex. Accordingly, oligonucleotides acting as catalytic inhibitors can interfere in the binding of topoisomerase I to DNA.

Analysis of Topoisomerase-DNA Binding by EMSA (Electrophoretic Mobility Shift Assay)

To investigate whether these oligonucleotides directly interfere with the binding of topoisomerase I to DNA, we used a reported technique (EMSA) to test whether these quadruplex-duplex motifs can inhibit the enzyme-DNA complex formation.[17,18] The catalytic inhibitor CPT, as the positive control, did not interfere with the binding and scission steps of topoisomerase I (Figure 8, lane 16). On the contrary, the results in Figure 8 demonstrated that the oligonucleotides can inhibit the formation of enzyme-DNA complexes. These observations which are inconsistent with the mechanism of action of CPT further support that all oligonucleotides inhibit the DNA-binding step of topoisomerase I.

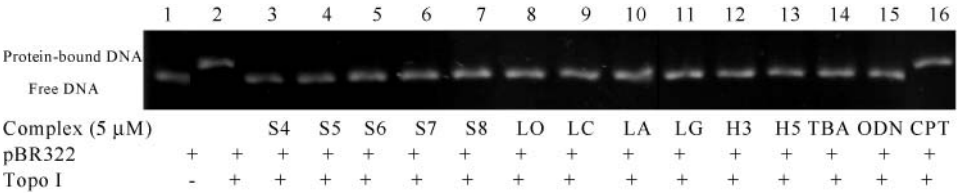

FIGURE 8 EMSAs of topoisomerase I incubated with appropriate DNA. Inhibitory effects of all the 13 oligonucleotides after formed G-quadruplex and quadruplex-duplex motif.

CONCLUSION

Our current results indicated that 14 oligonucleotides, before or after formed G-quadruplex and quadruplex-duplex motif, show activities toward topoisomerases I in vitro. The 14 G-quadruplex and quadruplex-duplex motifs show a stronger activity than that of 14 single short chains, that is, these G-quadruplex and quadruplex-duplex motifs inhibit the next reaction through the priority binding with the topoisomerase I and precluding topoisomerase I from binding with the normal substrate pBR322.

EXPERIMENTAL

CT DNA topoisomerase I (20 U/μl) and supercoiled pBR322 plasmid DNA were purchased from Toyobo (Japan). Camptothecin (CPT) was purchased from Acros (USA). Oligonucleotides were purchased from Invitrogen Technology (China). Concentrations were determined spectrophotometrically using molar extinction coefficients at 260 nm.

Measurement of Relaxation Activity of Topoisomerase I

All the oligonucleotides used in this study were dissolved in 100 μM by super pure water. G-quadruplex or quadruplex-duplex motif were diluted in 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl, pH = 7.4. Oligonucleotides strand equivalent were heated at 95°C for 5 minuutes and then natural cooled at 20°C. G-quadruplex formation was checked by CD experiments. Topoisomerase I functional activity was assayed by relaxation of supercoiled plasmid DNA in a cell-free system. Briefly, each reaction mixture had a total volume of 10 μl containing 0.1 U of Topoisomerase I, 50 ng of supercoiled DNA pBR322, 20 mM *Tris*·HCl buffer (pH 7.5), 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 mg/mL acetylated BSA, and 100 mM KCl, in the presence or absence of short chains dissolved in sp H_2O. Control experiment contained either DNA alone or DNA treated with topoisomerase I only. Incubation at 37°C for a time course was terminated by addition of 0.5% (w/v) SDS and proteinase K. Gel electrophoresis was performed at 5 V/cm in 0.8% (w/v) agarose gels with *TBE* buffer. Subsequently, the gel was stained in 10 μg/ml EB for 15 min and visualized by UV and photographed using a *Vilber Lourmat* video system. The data of experiment is also analysised by *Vilber Lourmat*. IC50 values, defined as the concentration resulting in 50% inhibition toward the relaxation activity of topoisomerase I, were estimated from nonlinear regression analysis of concentration-response curves. Percent inhibition is $(S\text{-}S_0)/(S_{control}\text{-}S_0) \times 100\%$, S is ratio of supercoiled DNA, Scontrol is the ratio of supercoiled DNA in control which only containe pBR322, S_0 is the ratio of supercoiled DNA in control that contain pBR322 treated with topoisomerase I.

Circular Dichroism Spectroscopy

The oligonucleotides were dissolved in super pure water. G-quadruplex or quadruplex-duplex motif were diluted in 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl, pH = 7.4. Oligonucleotides strand were heated at 95°C for 5 minutes and then natural cooled at 20°C. CD spectra were recorded on a Jasco-810 spectropolarimeter (Jasco, USA) by using a quartz cell (1 mm optical path length) and an instrument scanning speed of 100 nm/min with a response time of 1 s, over a wavelength range of 200 to 400 nm. The strand DNA concentration used was 12.5 mm. All CD spectra were baseline corrected for signal contributions due to the buffer.

Measurement of Topoisomerase I-Mediated DNA Cleavage

Reaction mixtures contained excessive enzymes (i.e., 0.2 U of topoisomerase I). Topoisomerase I reactions were performed in topoisomerase I relaxation buffer at 37°C for 30 minutes. Reactions were terminated with 0.5% (w/v) SDS. After digestion with proteinase K, open circular and linear DNAs were separated from the intact supercoiled and the relaxed form by agarose-gel electrophoresis in the presence of 0.5 μg/ml of EB under the same conditions as for the relaxation assay. Gels were visualized by UV and photographed with a *Vilber Lourmat* video system. CPT was used as the control drug.

Analysis of Topoisomerase-DNA-Binding by EMSA

During analysis of topoisomerase I-DNA binding by EMSA, 50 ng of supercoiled DNA pBR322 was incubated with or without excess of topoisomerase I (0.3 U) in the presence of oligonucleotides for 1 minute at 37°C. The reaction was started by addition of DNA. SDS denaturation and proteinase K digestion were omitted. Samples were immediately loaded onto the 0.8% agarose gel with 0.5 μg/ml of EB in TBE buffer and separated by electrophoresis for 6 hours at 2.5 V/cm.

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