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DNA binding specificity and cytotoxicity of novel antitumor agent Ge132 derivatives

Guoqiang Shangguan,[a] Feifei Xing,[a] Xiaogang Qu,[a,*] Jianhua Mao,[b] Dan Zhao,[b] Xuejian Zhao[b] and Jinsong Ren[a]

[a]*Division of Biological Inorganic Chemistry, Key Laboratory of Rare Earth Chemistry and Physics, Graduate School of the Chinese Academy of Sciences, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China*

[b]*Prostate Diseases Prevention and Treatment Research Center, Jilin University, Changchun, Jilin 130021, China*

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Abstract—A series of Ge132 derivatives have shown enhanced antitumor activity. Previous studies suggest that DNA can be their primary target. Here we show direct evidence that two newly synthesized Ge132 derivatives can intercalate into DNA. Unexpected methyl substitution effect of the novel derivatives on DNA sequence selectivity and cytotoxicity was observed.

DNA is an important cellular target for many anticancer agents. The design and synthesis of small molecules that target particular DNA sequences and control gene expressions are of considerable interests in chemistry, biology, and medicine.[1–6] Germanium is a naturally occurring element. Organogermanium compounds, typical carboxyethylgermanium sesquioxide (Ge132) and 2-aza-8-germanspiro decane-2-propamine-8,8-diethyl-*N*,*N*-dimethyl dichloride (spirogermanium), have been in the clinic trails.[7–16] Evidence suggests that Ge132 not only possesses antitumor activity, but also increases interferon production with almost no detectable sign of cytotoxicity.[12,15,16] Ge132, even as a supplement, has been used as an immuno-stimulant and enhanced the natural killer cell activity and interferon levels.[12,16] Encouraged by these promising results, researchers developed the idea that the incorporation of a germanium residue into compounds of fundamental biological importance would enhance their antitumor activity to a significant degree, while their toxicity would be reduced at the same time.[12,15,16] A series of Ge132 derivatives has shown activity against different types of cancer cells.[12–16] However, the organogermanium anticancer mechanism still remains to be elucidated. Here we show that Ge132 can enhance their complex DNA binding affinity by more than 400-fold. The newly synthesized Ge132 derivatives (Scheme 1) can intercalate into DNA and inhibit the PC-3M[17] proliferation. The primary results from flow cytometry and fluorescence microscopy suggest that these compounds could cause apoptosis. Unexpected methyl substitution effect on cytotoxicity and DNA binding affinity of novel antitumor agent Ge132 derivatives was observed. To the best of our knowledge, there is no previous report showing that Ge132 derivatives can intercalate into DNA and inhibit cell proliferation.

Table 1 summarizes the inhibition effects of the two compounds on the PC-3M proliferation. The inhibition is not only concentration and time dependent, but also methyl substitution related. The only difference of these two compounds is with or without the methyl group in the linking chain of the germanium and quinoline (Scheme 1).

Without the methyl group, compound **12a** has stronger inhibition effect than compound **12b**. The IC_{50} of compound **12a** was 10 μM, 3-fold lower than that of compound **12b**, which was measured by MTT method.[18,19] Cell morphology studies indicate that 10 μM **12a** or 30 μM **12b** can cause cell irregularity, shrinking, and even fragmentation (Supplementary Fig. 1b), and flow

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* Corresponding author. Tel./fax: +86 4315262656; e-mail: xqu@ciac.jl.cn

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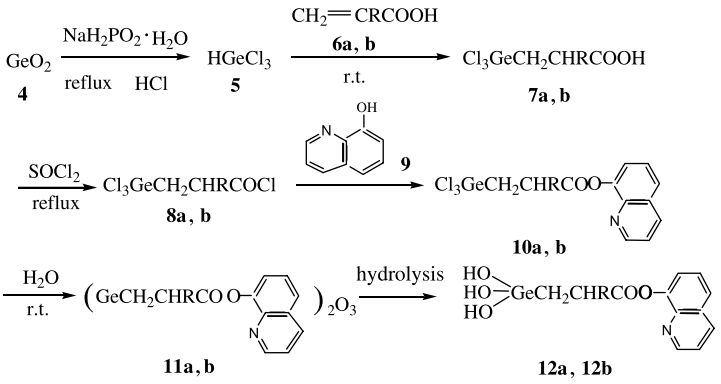

Scheme 1. Reagents: (a) R = H; (b) R = CH_3.

Table 1. Summary of the inhibition effect of the two compounds on PC-3M proliferation

Concentration (μM)	24 h		48 h		72 h	
	12a	**12b**	**12a**	**12b**	**12a**	**12b**
10[a]	0.34[b]	0.09	0.69	0.32	0.62	0.58
30	0.48	0.30	0.74	0.45	0.74	0.69
60	0.50	0.20	0.74	0.43	0.78	0.67

[a] The compound concentration we used.
[b] The inhibition fraction at the given concentration.

cytometeric data (Supplementary Fig. 1c and d) showed that the cell number in G_0/G_1 and G_2/M phase was decreased but increased dramatically in S phase in the presence of the compounds. As shown in Supplementary Figure 1d, the sub-diploid peak (Apoptosis Peak) before G_1 Peak was observed inferring that DNA was cleaved and released from the nuclei. Cell death was observed under microscopy by acridine orange staining (Supplementary Fig. 1e and f), consistent with the treatment with hydroxyurea leading to the appearance of a hypo-diploid DNA content peak (sub-G1) characteristic of the apoptotic cell population.[20] Therefore, the newly synthesized Ge132 derivatives may cause the cell cycle to be halted at the S phase and inhibited DNA synthesis. Further studies on the influence of DNA topoisomerase I and II are ongoing. DNA can be the primary target for our newly synthesized Ge132-quinoline complexes, and we carried out their DNA binding studies at the molecular level.

UV–vis and fluorescence spectroscopy, circular dichroism, continuous variation method, and viscosity measurements were used to study the nature of the interaction between the compounds and the DNA.[5,6,21–25] The interaction of the compounds with DNA causes a marked hypochromism and bathrochromic shift of around 5 nm. Fluorescence intensity of the compounds was enhanced when bound to DNA[5,21,24,25] owing to the perturbation of the quinoline chromophore moiety upon binding to DNA (Supplementary data). CD spectra for DNA molecules showed no conformational change in the presence of the compounds (data not shown), consistent with only 2 °C increase of DNA melting temperature (T_m) when the compound bound[5] (Supplementary data). We are aware that small DNA intercalators usually significantly increase the DNA melting temperature, and the DNA melting temperature in their presence is proportional to their binding affinity and inversely proportional to their DNA binding site size. However, in our case, these compounds do not significantly increase the DNA melting temperature, similar to the indolocarbazole antitumor drug NB506 and its derivatives, which was reported to intercalate into DNA and slightly increase DNA melting temperature.[25] The reason for this discrepancy is not clear. Further studies on their DNA binding are ongoing in our laboratory.

The DNA binding affinities were measured using the intrinsic fluorescence of the quinoline chromophore.[5,25] As shown in Figure 1a, Ge132 greatly enhanced Ge132-quinoline complexes bound to DNA because the DNA binding affinity of 8-hydroxyquinoline ($1.4 \times 10^3\ M^{-1}$) was 100-fold weaker than compound **12b**, 400-fold weaker than compound **12a**. Interestingly, the methyl substitution effect was also observed in the binding studies. Nonlinear least squares analysis of the fluorescence titration curves[5,6,25] shown in Figure 1a yielding the binding constant of compound **12a**, which was without methyl group in the linking chain (Scheme 1), was $6.45 \times 10^5\ M^{-1}$, 5-fold larger than that of compound **12b**, which was with the methyl group, $1.32 \times 10^5\ M^{-1}$, demonstrating that the replacement of the methyl group in the linking chain facilitates the compound binding to DNA. The methyl group may cause stereo hindrance for the Ge132 derivatives binding to DNA. It is consistent with the cytotoxicity data that the IC_{50} of compound **12a** was 3-fold lower than that of compound **12b**. Previous studies have shown that anthracycline and rebecamycin derivatives have amino substitution effect on their DNA intercalation.[5] The binding affinity can be 10-fold stronger than the one without amino group since the amino group can have favorable interactions with DNA minor grooves.[5] Continuous variation method was used to unravel the binding stoichiometry of our newly synthesized compounds bound to DNA. The experiments show breaks at the compound fraction of 0.37 (Fig. 1b), indicating one compound molecule covers 1.7 base pairs.

Ligand-binding specificity is desired for developing of the new anticancer drugs. Compound **12a** shows strong GC sequence preference. Its GC duplex binding affinity, $2.1 \times 10^5\ M^{-1}$, is 8.5-fold stronger than binding to AT

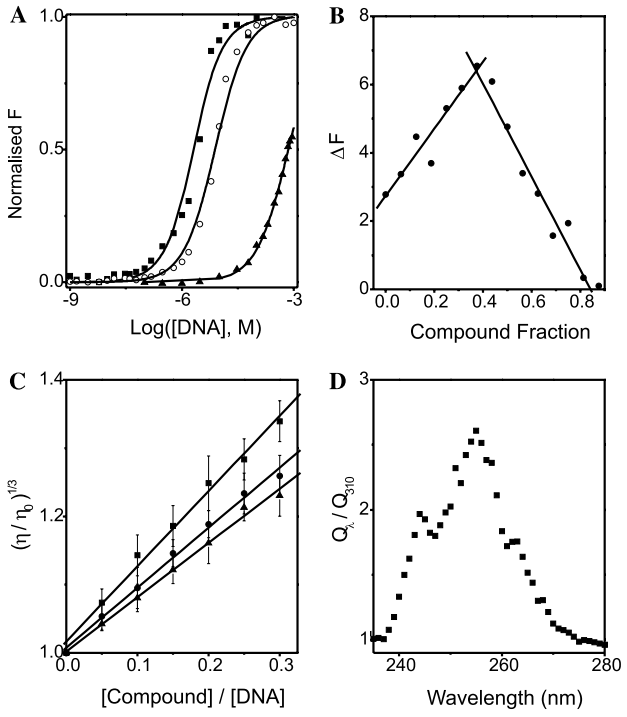

Figure 1. (A) Fluorescence titrations for the interaction of compound **12a** (squares), **12b** (circles), and 8-hydroxyl quinoline (triangles) with calf thymus DNA. The normalized fluorescence intensity, $\theta = (F - F_0)/(F_b - F_0)$, is shown as a function of total DNA concentration. The concentration of ligand was kept constant at 5 μM while the DNA concentration was varied between 1 mM and 0.001 μM bp. The curve fittings were carried out using nonlinear least-squares analysis. (B) Job plot of compound **12a** bound to DNA. Total concentration of compound **12a** and DNA was held constant at 80 μM over the course of the titration. (C) Viscosity measurements of compound **12a** and **12b** bound to DNA in BPE buffer. The cubed root of the relative viscosity was plotted against the ratio of bound compound per DNA base pair. Squares: Ethidium bromide; circles: compound **12a**; upper triangles: compound **12b**. (D) Contact energy transfer from DNA to the bound compound. Relative fluorescence quantum yield of bound versus free ligand is plotted against excitation wavelength.[5]

duplex, $2.5 \times 10^4\ M^{-1}$ (Supplementary data). For compound **12b**, however, equal binding affinity was found for GC ($1.7 \times 10^5\ M^{-1}$) and AT ($1.6 \times 10^5\ M^{-1}$) duplex DNA, indicating that the methyl group in the linking chain not only influences their DNA binding affinity but also modulates their DNA sequence specificity.

Hydrodynamic studies, especially viscosity, have been considered as the most reliable means of inferring the binding mode of agents that interact with DNA when the high-resolution structural data is not available.[22,23] It is well known that intercalators increase DNA solution viscosity while groove binders induce a decrease in viscosity.[22,23] Figure 1c shows the classical plots of the cube root of the relative viscosity versus the binding ratio (bound drug/DNA bp), demonstrating DNA solution viscosity is increased and verifying that compounds **12a** and **12b** bind to DNA by intercalation. Within experimental errors, the slope of the fitting lines is about 0.87 and 0.8, respectively, consistent with the slope of 1.05 for the proven intercalators ethidium run as controls in the experiment.[22,23] These two complexes can also decrease ethidium bromide (EB) fluorescence (data not shown) by displacement of DNA-bound EB in which the method has been widely used to establish DNA binding mode.[26] Another evidence to support intercalation is the unambiguous contact energy transfer between DNA bases and the compound shown in Figure 1d. When ligands intercalate into DNA, DNA molecules can efficiently transfer energy to the excited fluorophore due to their favorable close contacts and orientation of the donor–accepter dipoles.[5,22] Therefore, based on DNA solution viscosity measurements and contact energy transfer results, Ge132 quinoline derivatives are DNA intercalators.

In summary, the incorporation of antitumor agent Ge132 into pharmacophore quinoline compounds can enhance their DNA binding affinity by more than 400-fold. The methyl substitution effect of these two compounds was observed in DNA binding studies and IC_{50} measurements. The methyl group in the linking chain between germanium and the chromophore moiety not only affects compound DNA binding affinity, but also modulates their DNA sequence selectivity. Without the methyl group, their GC duplex binding affinity can be 8.5-fold stronger than binding to AT duplex. No such sequence selectivity exists for the one with the methyl group. The structural details of these DNA complexes are clearly needed. To the best of our knowledge, this is the first report to show Ge132 derivatives can intercalate into DNA and these compounds inhibit cell proliferation. These results provide new insights into the design of the incorporation of antitumor agent Ge132 into pharmacophore moiety that target specific DNA sequences. We are currently focusing on further defining the role of germanium derivatives in their recognition of multistrand DNA, such as triplex and tetraplex DNA.

Acknowledgments

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Supplementary data

Experimental section, Scheme 1, Cell morphology change and flow cytometeric data, Fluorescence spectroscopy, Table of GC duplex and AT duplex binding constants, and Table of DNA melting data at 1:2 ratio of [compound]/[DNA] in BPE buffer. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.04.053.

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