Solution structures and effects of a platinum compound successively bound MYC G-quadruplex

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Abstract
G-quadruplex (G4) structures play integral roles in modulating biological functions and can be regulated by small molecules. The MYC gene is critical during tumor initiation and malignant progression, in which G4 acts as an important modulation motif. Herein, we reported the MYC promoter G4 recognized by a platinum(II) compound Pt-phen. Two Pt-phen–MYC G4 complex structures in 5 mM K+ were determined by NMR. The Pt-phen first strongly binds the 3′-end of MYC G4 to form a 1:1 3′-end binding complex and then binds G4 to form a 2:1 complex with more Pt-phen. In the complexes, the Pt-phen molecules are well-defined and stack over four bases at the G-tetrad for a highly extensive π–π interaction, with the Pt atom aligning with the center of the G-tetrad. The flanking residues were observed to rearrange and cover on top of Pt-phen to stabilize the whole complex. We further demonstrated that Pt-phen targets G4 DNA in living cells and represses MYC gene expression in cancer cells. Our work elucidated the structural basis of ligand binding to MYC promoter G4. The platinum compound bound G4 includes multiple complexes formation, providing insights into the design of metal ligands targeting oncogene G4 DNA.

Graphical abstract

Introduction
Structural investigation of nucleic acid secondary structure is a thriving area of research, and many kinds of DNA have been studied to date (1–3). G-quadruplexes (G4s) are noncanonical nucleic acid secondary structures formed by stacked G-tetrads with Hoogsteen hydrogen bonding and cations (4). G-rich sequences able to form G4 structures are prevalent in biologically significant regions, such as human telomeres and oncogene promoter regions (5–7). Their formation and stabilization are involved in many important physiological and pathological processes and the development of cancer or other diseases (8–10), receiving great attention as potential biomedical
targets for drug development (11). The MYC proto-oncogene, as one of the research hotspots in molecular biology in recent years, has proven to be important in the malignant progression of many human tumors (12). The MYC promoter nuclelease hypersensitive element (NHE) III region controls 80–95% of MYC expression and can form DNA G4 structures (13,14). As a result, inhibition of MYC transcription by G4 structures is an attractive strategy for targeting the MYC gene for anticancer therapy (15,16).

Of great interest is that the G4 has structural diversity, which points to a variety of potential applications in nanodevices (17) and controlling G4 functions in biological settings (18,19). Sequencing of G4 indicates approximately 700 000 DNA sequences in the human genome that can form various G4 structures (20), which challenges our understanding of G4s. Small molecules that can selectively target G4 structures have the ability to regulate gene expressions. Studies have shown that the accessible binding of G4 are complicated and can be affected by cations, molecular crowding conditions or small molecule modifications (21–23). Thus, it is crucial to elucidate the structural information and binding process on the interaction mechanisms of ligand/G4 to provide a structural basis for designing G4-targeting agents, which is important for understanding the biological functions of G4 (24–27). Moreover, the binding modes of metal ligands bound to G4s elucidated by ligand/G4 complex structures are rather sparse (28–31). Previous studies have demonstrated that NMR technology is a powerful tool for exploring and characterizing the binding of G4s with small molecules at the structural level (32–34).

Herein, we report that the platinum(II) compound Pt(1,10-phenanthroline)2(PF6)2 (Pt-phen) (35) strongly binds the MYC promoter G4 (36) (Figure 1A and B). Using the NMR method, the complex structures of Myc1234 G4 bound to Pt-phen are unambiguously characterized under 5 mM K+ mM solution. The molecular structures of Pt-phen–Myc1234 G4 complexes show that the Pt-phen well-stacks at the terminal G-tetrads and allows rearranging of flanking residues. Cellular studies demonstrated that Pt-phen can target G4 DNA in living cells and further effectively repress MYC gene expression in cancer cells. This paper presents a work showing both structural basis and functional regulation of MYC G4 recognition by a platinum ligand.

Materials and methods

Sample preparation

DNA sequences were obtained from Sangon Biotech Co., Ltd (Shanghai) and purified by HPLC. The 8%–enrichment site-specific 15N-labeled oligonucleotides were synthesized using 15N-labeled dG-phosphoramidite from Cambridge Isotope Laboratories (USA). DNA oligonucleotides in buffer were annealed to form the G4 structures by heating at 95°C for 5 min and then slowly cooling to room temperature overnight. The concentration of DNA was tested by Nanodrop 200/200c (Thermo Science). The Pt-phen ligand was synthesized as described in our previous report (35).

NMR experiments

The NMR experiments were performed on Bruker AVIII 600/700 MHz spectrometers equipped with a cryoprobe. DNA samples were prepared in D2O/H2O (10%/90%) or D2O (99.8%). The concentration of DNA samples was 0.1–1.0 mM. In the 1D NMR titration experiments, after each addition of Pt-phen, the solution was stirred and allowed to equilibrate for 10 min. The imino H1 protons of G-tetrad guanines were assigned by the 1H-15N HMBC experiments. The 2D COSY, TOCSY and NOESY experiments were collected at different temperatures, 5, 15 and 25°C, for the Pt-phen–Myc1234 complexes. The NOE mixing times were 50–300 ms, and the TOCSY mixing times were 40 ms. 1H-13C HSQC experiments were performed with an optimized 1J(C,H) of 190 Hz. Water suppression techniques (WATERGATE or presaturation) were used for H2O NMR samples. Peak assignments and integrations were performed by Sparky (UCSF).

NOE-distance restrained molecular dynamics simulation

Proton distances were obtained based on the NOE integrated at NOEY spectra with ±20% variance. The distance of Me-H6 (2.99 Å) in thymine was set as the reference. The X-PLOR program and Accelrys Discovery Studio 2.5.5 were used to perform the structure calculations. The Pt-phen molecule was optimized using Gaussian 03, and the Pt-phen topology and parameters were generated by hand. The starting models of the 1:1 and 2:1 Pt-phen–Myc1234 complexes were constructed in the Discovery Studio program based on the NOE
data. NOE-restrained simulation annealing refinement calculations were conducted in the XPLOR program. The whole system was put into energy minimization of 1000 steps and then equilibrated at 1000 K for 10 ps. Then, the molecular dynamics and cooling simulation were started with a 25 K reduction in temperature, each cycle with 1000 time-steps of 2 fs, until the temperature reached 300 K. Finally, the structure systems were subjected to energy minimization of 1000 steps. In Accelrys Discovery Studio, these models were first minimized and equilibrated and then soaked into a 15 Å water layer, and a dynamic simulation was run with the CHARMM force field. The best structures were selected based on the minimum energy and number of NOE violations.

CD spectroscopy
CD spectra were measured in a J-810 spectropolarimeter (JASCO, Japan) at room temperature. The wavelength range of 220–360 nm, 1 cm optical path length, and 200 nm/min scan speed were set. The buffer used to prepare the DNA G4 samples was 10 mM Tris-HCl in the presence of different concentrations of KCl and NaCl (pH 7.4). The DNA concentration was 3 μM. After each addition of Pt-phen, the samples were stirred and equilibrated for 30 min. Data analysis was performed using Origin 8.5 (OriginLab Corp.).

Native polyacrylamide gel electrophoresis (PAGE)
The native gel contained 18% acrylamide with 1 × TBE buffer (pH 8.0). DNA G4 samples were prepared in a buffer comprising 10 mM Tris-HCl and different concentrations of KCl (100, 50 and 10 mM) with a pH of 7.4. Different ratios of Pt-phen were added to DNA G4 samples. Each sample included 0.3 mM DNA. GelRed was used for DNA band staining. The gels were photographed in a FluorChem M imager (Protein-Simple).

Fluorescence lifetime imaging microscopy (FLIM)
FLIM imaging of NBTE (4,4′,4″-nitrotris(benzene-4,1-diyil)tris(1-ethylpyridin-1-ium)iodide) (30) and Pt-phen was performed using Zeiss LSM 880 NLO multiphoton microscopes equipped with a bh TCSPC FLIM system (Becker & Hickl GmbH, Berlin, Germany). The two-photo excitation of samples was performed by a femtosecond Ti:sapphire laser (Coherent Chameleon), and the emission was collected using a Zeiss BiG-2 GaAsP detector. The TCSPC module type is an SPC-150 module, and the resolution of the images is 512 × 512 pixels. For FLIM imaging, HeLa cells were incubated with 20 μM NBTE and Pt-phen (or PDS) at 37°C for 24 h, and the control group was incubated with only 20 μM NBTE. Then, the dish was washed three times with ice-cold PBS and detected by the FLIM system. The wavelength for two-photon excitation of NBTE was 810 nm, and the emission was recorded from 545 to 590 nm. The acquisition time was sufficient to obtain a better SNR. Lifetime data were fitted to a tri-exponential function for each pixel using SPC-Image software.

Cell viability assay
The cytotoxicity of Pt-phen toward different cell lines was determined by MTT assay. The cells were seeded in 96-well plates (Corning) and grown overnight. Then, the cells were treated with a series of concentrations of Pt-phen and cisplatin. After incubation for 44 h, 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) solution (5 mg/mL in PBS) was added to each well. The plates were incubated for an additional 4 h before the media was carefully removed, and DMSO was added (150 μL per well). After shaking for 5 min, the absorbance at 595 nm was measured using a microplate reader (Infinite M 200 Pro, Tecan, Männedorf, Switzerland). Each experiment was repeated at least three times to obtain the mean values.

Western blotting
The HeLa cells were seeded into 60 mm tissue culture dishes and incubated under a 5% CO2 atmosphere at 25°C for 24 h. Cells were treated with 5.0 μM and 10.0 μM Pt-phen for different times. After pelleting, cells were solubilized in 1× RIPA buffer supplemented with 1× protease inhibitor cocktail (Roche, USA). The nuclear and intracellular organelles were disrupted by sonication five times for 10 seconds each. After incubation for 20 min at 4°C, the suspensions were separated by centrifugation at 13 000 rpm for 10 min at 4°C, and the supernatant was collected. The protein bands were detected using anti-MYC (D84C12, Cell Signaling Technology, USA) and anti-GAPDH (FL-335, Santa Cruz Biotechnology, USA) antibodies. An equal amount of protein (20 μg) was electrophoresed on a 12% SDS–PAGE gel and transferred to a 0.45 μm PVDF membrane (Millipore, USA) at 200 V for 2 h. The membrane was blocked with QuickBlock Western buffer (Biyuntian, China) for 1 h at room temperature. The membrane was incubated overnight at 4°C with the primary antibodies anti-MYC (9E10, Santa Cruz Biotechnology, USA) and anti-GAPDH (FL-335, Santa Cruz Biotechnology, USA). After three washes in TBST, the membrane was incubated with the appropriate HRP-conjugated secondary antibody at room temperature for 2 h. After another three washes in TBST, developing reagent (Biyuntian, China) was added to the membrane. The Western photo was taken by GeneGnomeXRQ (Syngene, UK).

Real-time quantitative PCT (RT-qPCR)
RT-qPCR experiments were performed in the cells. RNA was extracted with RNAiso Plus (TAKARA) and converted to complementary DNA with an Eco M-MLV RT Kit with gDNA Clean for Qpcr II (Accurate Biotechnology (Hunan) Co. Ltd). Real-time quantitative PCR (qPCR) was conducted in triplicate using the LightCycler 480 system (Roche Diagnostics, IN, USA). The following primers were used: c-myc (forward primer: 5′-GCTGCTTAGACGCTGGATT-3′; reverse primer: 5′-TCTCCCTCGTCGAGTAGA-3′) and GAPDH (forward primer: 5′-GGTCTGCTCCCTGACTCTCAACA-3′; reverse primer: 5′-GGTCTGCTTACGAAAAATCGTGTG-3′). The specificity of each PCR product was measured and controlled using the melting curve. The 2−ΔΔCt method was used to calculate the relative gene expression levels in which the amount of MYC mRNA was normalized to an endogenous reference (GAPDH), where Ct represents the threshold cycle.

Results
Pt-phen strongly binds the MYC G4
We first studied the Pt-phen binding to Myc1234 G4 by NMR (Supplementary Table S1). 1D 1H NMR titration experiments of Myc1234 G4 and Pt-phen were first performed (Figure 1C and Supplementary Figure S1A). Under 5 mM K+ and
10 mM Na\(^+\) conditions, upon titration of Pt-phen, a new set of sharp imino proton signals appeared (blue asterisks) at a low 0.5:1 Pt-phen/Myc1234 ratio, suggesting the presence of a first well-defined Pt-phen–Myc1234 complex. After adding more Pt-phen, another new set of sharp imino protons for the second Pt-phen–Myc1234 complex emerged (green asterisks). And the free DNA, first and second Pt-phen–Myc1234 complexes coexist at the 1:1 Pt-phen/Myc1234 ratio. Upon reaching the 2:1 ratio, only well-resolved peaks from the second complex were observed, illustrating the formation of a single dominating complex structure. Under 100 mM K\(^+\) and 10 mM Na\(^+\) condition, the 1D NMR titration spectra displayed similar binding process (Supplementary Figure S1B). At the 0.5:1 ratio, one complex emerged with new peaks, and at higher than 4:1 ratio, the NMR spectra showing the same pattern as that at the high ratio of 5 mM K\(^+\) condition, implying forming a same second complex. As presented by the NMR titrations, Pt-phen shows strongly interaction with Myc1234 G4 and forms multiple stable complex structures during binding. We have also performed the 1D NMR titration experiments of Pt-phen with the published Myc14/23, MycA2A14 and Myc1245 G4 structures in 100 mM K\(^+\) (Supplementary Figure S2 and Supplementary Table S1). The NMR spectra of these three G4 DNA broadened after adding 0.5:1 and 1:1 ratio of Pt-phen. With more Pt-phen, the clear peaks of complex structures were observed at 2:1 ratio, illustrating the formation of a single dominating structure. The NMR spectra of these three G4 DNA at 2:1 ratio suggested a 2:1 Pt-phen/G4 complex existed, which is similar to that of Myc1234 G4 (Supplementary Figure S1). The results revealed that Pt-phen could recognize the MYC promoter G4s and form stable complex structures.

Pt-phen-bound MYC G4 was also visualized by PAGE under native conditions (Supplementary Figure S3). In the gel, the free Myc1234 G4 DNA formed a major monomeric structure and the Pt-phen binding keeps the MYC G4 structure in the monomeric state. Next we used CD to test their interactions. The CD spectrum of free Pt-phen shows no signal (Supplementary Figure S4). Myc1234 G4 forms a parallel-stranded structure under 5 or 100 mM K\(^+\) conditions (Supplementary Figure S1), as indicated by the characteristic maximum at 260 nm and minimum at 240 nm in the CD spectra. After titration with high ratio of Pt-phen, a shoulder at around 290 nm became notable. We then measured the UV spectra of free Pt-phen in both DMSO and aqueous solution (Supplementary Figure S4). Pt-phen shows a clear UV absorption at 280 nm (Supplementary Figure S3). Therefore, the changes observed in the CD spectrum might be induced CD effects of Pt-phen upon binding to the chiral DNA because it absorbs strongly at 280 nm in the UV/Vis spectrum. In order to clarify the accurate structure and binding mechanism of Pt-phen with Myc1234 G4, we further performed 2D NMR analysis and confirmed the unchangeable parallel-folding of MYC G4 structure.

Solution structure of the 1:1 3′-end binding Pt-phen–MYC G4 complex in 5 mM K\(^+\)

As the 1D NMR spectra show distinct peaks which seem to be suitable for NMR structural analysis, we decided to solve the Pt-phen–Myc1234 complex structures to elucidate how Pt-phen binds the Myc1234 conformation. We collected 2D NOESY, TOCSY and COSY NMR spectra in both 5 and 100 mM K\(^+\) solution. After analysis, we found that in the 100 mM K\(^+\) solution, there are more than two species co-existing in the system at the 0.5:1 ratio. Because the dimerization of MYC G4 is preferred under high salt concentrations, there might be dimer structure existed (37). Due to the overlap of NMR signals, NMR spectra are difficult to be accurately assigned. And we found the 2D NMR spectra in 5 mM K\(^+\) solution has much better spectra quality, thus we chose the spectra at 0.5:1 and 2:1 Pt-phen/Myc1234 ratios in 5 mM K\(^+\) solutions for NMR structure determination. Complete sets of 2D NOESY, TOCSY and COSY NMR spectra of these complexes in both water and D\(_2\)O at temperatures of 5, 15 and 25°C were collected.

NMR spectral assignments of the Pt-phen–Myc1234 complex at a 0.5:1 ratio were made in the co-presence of two molecular species, as the free and bound Myc1234 G4 co-existed at a 0.5:1 ratio (Supplementary Figures S6–S13). The imino protons of Myc1234 G4 at a 0.5:1 ratio were unambiguously assigned by \(^1\)H–\(^15\)N HMQC experiments (Supplementary Figure S7). Based on the imino proton assignments, we assigned other protons of bound Myc1234 G4 through NOE connections using NOESY, TOCSY and COSY (Figure 2A, B, Supplementary Figure S8–S11, and Supplementary Table S2). The protons of free and bound Pt-phen molecules were also unambiguously assigned by COSY and NOEY experiments (Supplementary Figure S6, S8–S9). As shown in the COSY results, a weak H3/H1 cross-peak was shown in the free Pt-phen but was lost in the complex with G4 DNA, which was probably because of the decrease in the Pt-phen concentration from 10 to 0.4 mM. After obtaining the complete assignment of this complex at a 0.5:1 ratio, we found intermolecular NOE between the protons of Pt-phen and the Myc1234 3′-end (Supplementary Table S3), which implies specific binding of Pt-phen at the 3′-end of Myc1234.

To validate the folding of MYC G4 in the complex, we measured the \(^1\)H–\(^13\)C HSQC for this 1:1 Pt-phen–Myc1234 complex (Supplementary Figure S12). Tetrade guanine residues with syn-glycosidic conformation are easily distinguishable from anti-guanines due to the characteristic down-field shift of C8 resonance by about 4 ppm (38). Therefore, the \(^1\)H–\(^13\)C HSQC can unambiguously determine the syn/anti- conformations of tetrade guanines and thus the folding of a G4. All tetrade guanine C8–H8 cross-peaks in the HSQC spectrum are located in the anti-conformation region, as shown by their characteristic \(^1\)H and \(^13\)C chemical shifts. This supports that the bound Myc1234 G4 in this complex retains the parallel-stranded G4 conformation.

We calculated the solution structure of this 1:1 Pt-phen–Myc1234 complex at a 0.5:1 ratio in 5 mM K\(^+\) conditions based on the NOE data (Figure 2C and Table 1) and selected 10 lowest-energy refined complex structures (Supplementary Figure S14).

As shown in the 1:1 3′-end binding complex structure, the Pt-phen stacks over four bases, G5, G9, G14 and G18, of the 3′-G-tetrad for a highly extensive π–π interaction (Figure 2D). The Pt-phen molecule is well defined and covers the 3′ terminal with the Pt atom aligning with the center of the G-tetrad. One phenanthroline ring of Pt-phen covers the bases of G5 and G18, and another covers the bases of G9 and G14 by observing clear NOE cross peaks. Compared to the free Myc1234 G4 structure, the flanking residues were rearranged due to Pt-phen binding. At the 3′-end binding pocket, the T19 at the 3′-flanking stacks on top of one phenanthroline ring of Pt-phen to stabilize the complex (Figure 2E).
**Figure 2.** The 2D NMR spectra and the solution structure of the 1:1 3′-end binding Pt-phen–MYC G4 complex (PDB Code: 9B6Z). (A) The expanded H8/H6-H1′ region of the NOESY spectrum at 0.5:1 Pt-phen/Myc1234 ratio, 5 mM K+, 10 mM Na+, pH 7.0, 25°C. (B) The expanded H8/H6/H2-H1 region of the NOESY spectrum of the same ion condition at 25°C. The DNA concentration is 600 μM. The G-tetrad G1-H1-G8 cross-peaks of free Myc1234 are coloured in black, and that of the 1:1 5′-end binding complex are coloured in blue. Intermolecular cross-peaks of the Pt-phen and 3′-G-tetrad are coloured in purple. (C) The stereo view of the representative structure of the 1:1 3′-end binding complex. (D, E) Two different views of the Pt-phen-induced binding pockets at the 3′-end. In the structures, guanines, adenines and thymines are coloured in pink, green, and blue, respectively. Pt-phen molecule is coloured in purple.

<table>
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<th>Table 1. Structural statistics for the solution structure of the 1:1 3′-end binding Pt-phen–Myc1234 complex</th>
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<td>Myc1234</td>
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**Solution structure of the 2:1 Pt-phen–MYC G4 complex**

Next, we unambiguously assigned all protons of the NMR spectra at a 2:1 Pt-phen/Myc1234 ratio in 5 mM K+ solution (Figure 3A-B, S15–S21 and Supplementary Table S4–S5). The complex was determined to be a 2:1 Pt-phen–Myc1234 G4 complex. The Myc1234 G4 in the 2:1 complex adopted the same parallel folding as the free Myc1234 G4. Intermolecular NOEs between the protons of Pt-phen with the 5′-end and 3′-end of Myc1234 were observed, revealing the second Pt-phen molecule interacting with the 5′-end of Myc1234.

We calculated the 2:1 complex structure based on the NOE data (Table 2) and presented the ensemble of 10 lowest-energy structures (Supplementary Figure S22). As shown by the representative structure (Figure 3C), two Pt-phen molecules bind the MYC G4, with one Pt-phen stacked on the 5′-G-tetrad and the other stacked on the 3′-G-tetrad. The 3′-end binding pocket is the same as that in the 1:1 3′-end binding complex above (Figures 2D, E and 3C). At the 5′-end of MYC G4, the Pt-phen molecule is well defined and matches the 5′-G-tetrad.
with the Pt atom aligning with the center of the G-tetrad (Figure 3D). One phenanthroline ring of Pt-phen covers the base of G3 and G7, and another covers the bases of G12 and G16. The A2 at the 5′-flanking stacks on top of one phenanthroline ring of Pt-phen, and the T1 folds back and covers another phenanthroline ring to stabilize the complex structure (Figure 3E).

Therefore, in 5 mM K+ solution, the Pt-phen first strongly binds at the 3′-end of MYC G4 with higher affinity to form a 1:1 complex. Further adding more Pt-phen, the Pt-phen molecule continues to bind the 5′-end to form the 2:1 complex. Solving the NMR structures allows us to elucidate the binding process and interaction mechanism of the Pt-phen with Myc1234 G4 (Figure 4).

Pt-phen targets G4 in living cells and represses MYC gene expression in cancer cells

MYC transcription inhibition can be achieved by targeting the MYC promoter DNA G4 structures (13). To explore the cellular effects of Pt-phen on MYC gene expression, we performed several experiments. The fluorescence lifetime imaging microscopy (FLIM) experiment was performed in the HeLa cell line to understand the cellular targeting of Pt-phen (Figure 5A). We used our recently reported fluorescence lifetime probe, NBTE (24), which can detect the G4 binding ligand in living cells through FLIM competitive

![Figure 3](https://example.com/fig3.png)

**Figure 3.** The 2D NMR spectra and the solution structure of the 2:1 Pt-phen–MYC G4 complex (PDB Code: 8YU3). (A) The expanded H8/H6-H1′ region of the NOESY spectra of 2:1 complex at 4.1 Pt-phen/Myc1234 ratio, 5 mM K+, 10 mM Na+, pH 7.0, 25°C. (B) The expanded H8/H6-H2-H1 region of the NOESY spectrum of 2:1 complex at 25°C. The DNA concentration is 800 μM. The G-tetrad G12-G13-G8-G16 cross-peaks of the 2:1 complex are shown. Intermolecular cross-peaks of the Pt-phen and G-tetrad are coloured in purple. The ‘a’ and ‘b’ of Pt-phen represent two ligands bound at 5′- and 3′-end. (C) The stereo view of the representative structure of the 2:1 complex. (D, E) Two different views of the Pt-phen-induced binding pockets at the 5′-end. In the structures, guanines, adenines and thymines are coloured in pink, green and blue, respectively. Pt-phen molecule is coloured in purple.

### Table 2. Structural statistics for the solution structure of the 2:1 Pt-phen–Myc1234 complex

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experiments, as proven by the results of canonical G4 binder PDS (Supplementary Figure S23). In the FLIM, the ratio value of DNA peak area to total area counting from the lifetime distribution reflects the relative percentage of the photon counts contributed by different DNA. As we can see from FLIM competitive results, the ratio of G4 peak area decreased from 41 ± 2% to 30 ± 2% after we added Pt-phen, implying that Pt-phen can partially replace NBTE and bind G4 DNAs. Therefore, we demonstrated that Pt-phen can target G4 structures in living cells.

Next, we conducted MTT, RT–qPCR and Western blotting assays to investigate G4-targeted MYC gene repression by Pt-phen. The MTT experiments showed that Pt-phen has moderate cytotoxicity against various cancer and normal cell lines (Supplementary Table S6). Then, we performed RT–qPCR and Western blotting assays in the HeLa cancer cell line. We chose two Pt-phen concentrations, 5 and 10 μM, which are lower than the IC_{50} value of Pt-phen in HeLa cells (17.33 ± 1.04 μM). We found that Pt-phen could effectively inhibit MYC expression. The MYC mRNA levels were significantly reduced.
below 40% after 48-hour Pt-phen incubation at both 5 and 10 μM in a time-dependent manner, as shown by the RT-qPCR results (Figure 5B). Consistently, MYC protein expression levels were decreased significantly after 48 hours of Pt-phen treatment at 5 μM and 36 h at 10 μM (Figure 5C). Considerably lower MYC gene expression was observed with increased Pt-phen treatment time. In the MCF-10A normal cell line (Supplementary Figure S24), the MYC mRNA levels were slightly reduced at 5μM and decreased below 40% after 60-hour Pt-phen incubation at 10 μM. And the MYC protein expression levels were not changed at 5μM and decreased after 60-hour Pt-phen treatment at 10 μM. Thus, the inhibition of MYC expression in the MCF-10A normal cell was observed to be weaker than that in the HeLa cancer cell, which might be because the amount of G4 structures in normal cells is less than that in cancer cells (24). The results indicated that Pt-phen presents a little bit cancer selectivity. These results demonstrated that Pt-phen functions as an effective MYC repressor in cancer cells, likely by binding and stabilizing the MYC promoter G4 structures to inhibit transcription.

**Discussion**

In-depth knowledge of the NMR complex structures of Pt-phen and Myc1234 G4 provides accurate binding details for explaining the G4 interaction mechanism. Myc1234 G4 in parallel-stranded folding has short flanking segments and stretched-out side loop residues, whose ligand binding pockets can form by the short 5’ or 3’ flanking segments. Structural studies revealed two binding modes of small-molecule ligands recognizing MYC promoter G4s. (i) The ligands possess a large aromatic moiety and bind to 5’- or 3’-end of G4 through extensive π-π stacking interactions, such as TMPyP4 (25), Phen-DC3 (26) and DAOTA-M2 (27). Pt-phen is also a planar platinum ligand that can strongly interact MYC G4 and stack at the external G-tetrads. (ii) The crescent-shaped ligands with low molecular weight recruit a 5’- or 3’-flexible flanking residue to form a ligand-base layer stacking on the 5’- or 3’-external G-tetrad, such as BMVC (33), quinoline (39), PEQ (40) and DC-34 (34). By determination of the NMR complex structures, we were able to reveal the binding process of Pt-phen bound Myc1234 G4. In the 5 mM K+ Pt-phen molecule first binds at the 3’-G-tetrad and then binds at the 5’-G-tetrad of MYC promoter G4. Thus, the 3’-G-tetrad of MYC G4 has higher affinity for Pt-phen than 5’-G-tetrad. As shown by our complex structures, Pt-phen possesses a large planar surface for extensive π-π stacking, which is distinct from the crescent small molecules that can recruit the flexible flanking residues (33).

In this study, we observed that the binding of ligand to Myc1234 G4 is sensitive to salt concentrations. In the 5 mM K+, the platinum compound Pt-phen clearly has two binding sites of 3’-G-tetrad and 5’-G-tetrad. While in the 100 mM K+ solution, we found the binding process of Pt-phen and MYC G4 is far more complicated. There are more than two complex species co-existing in the system. The NMR spectra are overlapping and hard to separate. As previous reported, the MYC G4 prefers to form dimer structure under high salt conditions (37), we suggested the formation of dimer or higher order complex structures in the 100 mM K+, which might be elucidated after experimental condition optimization in the further research. This study also revealed that common CD spectroscopy is insufficient for describing the structural features and transitions of nucleic acid secondary structures. As shown in the CD spectra, after adding Pt-phen, the CD signal displayed characteristics of the hybrid-type G4 structure. However, as elucidated by the NMR method, Pt-phen bound Myc1234 G4 remains parallel G4 folding.

In the NMR structures, the elucidated high-resolution complex structures of metal ligands bound to G4s are rare. There are six published platinum compound/G4 structures (30,31,41–43), two ruthenium compound/G4 structures (44), and one gold compound/G4 structure (29). The binuclear gold(III) compound Aurox6 is reported to use its planar coordination structure to recognize hybrid-2-G4 via π-π stacking (29). Structural studies revealed that the ruthenium(II) compounds bind diastereoselectively to antiparallel folded telomeric G4 (44). Our lab has reported the platinum compounds specifically bound to G4s and determined their interaction complex structures in recent years. The platinum compounds recognize G4 mainly through three modes. (i) The platinum units conjugate G4 targeting ligands to specifically bind G4s, such as NDI-Pt(dien) (30) and PyPDS-cisplatin (45), which conjugates G4 binder NDI and PyPDS with platinum moieties to realize G4 selectivity. (ii) The planar platinum compounds strongly bind G4s, such as triangle Pt-tripod (31) and platinum supramolecular square (22,46), which stack on top of external G-tetrads via π-π interaction. (iii) The structure-self-adaptive platinum compounds could change the molecular structure through adjusting the coordination ligands upon G4 binding, such as PtI (42), which selectively recognizes G4 through adaptive structural change from non-planar into planar structure by Cl–dissociation and coordination ligands rotation. Herein, the Pt-phen in this paper belongs to the second type. Due to its planar and positive-charged properties, Pt-phen acts on MYC G4 with high affinity and exhibits multipoint binding to the 5’- or 3’-end of G4. Because of its rigid structure and steric hindrance, Pt-phen can specifically bind G4 DNA instead of duplex DNA. Pt-phen is also susceptible to environmental influences upon MYC G4 binding, such as ionic strength and viscosity. The determination of two Pt-phen–MYC G4 complex structures provided the structural basis for the future design of small molecules recognizing parallel-stranded MYC promoter G4, inspiring the strategy of MYC promoter G4 targeting and function regulation for anticancer treatment.

Previous studies showed that the MYC NHE III1 sequence, which controls 80–95% of MYC transcription, coexists with different parallel G4 conformations (13). And the G4 content in cancer cells is 4-fold that in normal cells (25). In this study, we demonstrated through FLIM experiments that Pt-phen can target G4 structures in living cells. Furthermore, we found that the MYC mRNA levels and protein expression levels were significantly reduced after Pt-phen incubation, as shown by the RT-qPCR and Western blotting results. These results demonstrated that Pt-phen is an effective MYC repressor, likely by binding and stabilizing the MYC promoter G4 structures to inhibit the transcription. The inhibition of MYC expression in the cancer cell line suggested a potential anticancer therapy through targeting MYC G4.

**Conclusion**

In summary, we found that the platinum(II) compound Pt-phen can strongly interact with the Myc1234 G4 and form two complex structures in 5 mM K+ concentrations. The
structures revealed that Pt-phen has higher affinity for the 3'-terminal G-tetrad of MYC G4 than the 5'-terminal. Pt-phen first strongly binds the 3'-G-tetrad to form a 1:1 3'-end binding complex, upon adding more ligands, a 2:1 complex was stabilized. In the complex, the Pt-phen molecules stack over four bases at the center of G-tetrad through π-π interaction, with the rearranged flanking residues of MYC G4 covering the Pt-phen molecule and further stabilizing the complex. We found the binding process of platinum compound and G4 is far more complicated, forming multiple complexes. After unambiguously characterizing the structural details of Pt-phen with MYC G4, we further revealed that Pt-phen binds G4 DNA in living cells and effectively inhibits the expression of MYC gene. By elucidation of these two complex structures, we provided the structural basis for MYC G4-targeting small molecules, inspiring the future design of drugs targeting MYC G4 for anticancer treatment.

Data availability
The coordinates for structures of the 1:1 3'-end binding Pt-phen–MyC1234 G4 complex (PDB code: 9B6Z) and 2:1 Pt-phen–MyC1234 G4 complex (PDB code: 8YU3) have been deposited in the Protein Data Bank.

Supplementary data
Supplementary Data are available at NAR Online.

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Conflict of interest statement
The authors declare no conflict of interest.

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