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Mechanistic studies on the anticancer activity of 2,4-disubstituted quinazoline derivative

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ABSTRACT

Background: Accelerated proliferation of solid tumor and hematologic cancer cells is related to accelerated transcription of ribosomal DNA by the RNA polymerase I to produce elevated level of ribosomal RNA. Therefore, down-regulation of RNA polymerase I transcription in cancer cells is an important anticancer therapeutic strategy.
Methods: A variety of methods were used, including cloning, expression and purification of protein, electrophoretic mobility shift assay (EMSA), circular dichroic (CD) spectroscopy, CD-melting, isothermal titration calorimetry (ITC), chromatin immunoprecipitation (Ch-IP), RNA interference, RT-PCR, Western blot, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell assay.
Results: Our results showed that 2,4-disubstituted quinazoline derivative **Sysu12d** could down-regulate *c-myc* through stabilization of *c-myc* promoter G-quadruplex, resulting in down-regulation of nucleolin expression. **Sysu12d** could also disrupt nucleolin/G-quadruplex complex. Both of the above contributed to the down-regulation of ribosomal RNA synthesis, followed by activation of p53 and then cancer cell apoptosis.
Conclusions: These mechanistic studies set up the basis for further development of **Sysu12d** as a new type of lead compound for cancer treatment.
General significance: 2,4-Disubstituted quinazoline derivatives may have multi-functional effect for cancer treatment.

1. Introduction

Ribosome biogenesis is a fundamental cellular process, which plays an important role in cellular growth and proliferation [1]. RNA polymerase I is a multi-protein complex directly responsible for the transcription of ribosomal RNA gene (rDNA) to give a precursor transcript 45S pre-rRNA. It can be then processed to generate 18S, 5.8S, and 28S rRNAs, which associate with ribosomal proteins to form the ribosomal subunit [2]. It has been established that hyperactivated transcription of rDNA by RNA polymerase I is correlated with cancer [3]. It has been known that RNA polymerase I transcription is regulated by various oncogenes and tumor suppressors, including tumor suppressor p53. There is an extensive bidirectional crosstalk between RNA polymerase I and p53 [4]. On one hand, activation of p53 is known to repress RNA polymerase I transcription through disruption of pre-initiation complex formation [5,6]; on the other hand, RNA polymerase I transcription negatively regulates p53 activation through sequestration of ribosomal proteins in the nucleolus [7]. RNA polymerase I is aberrantly activated in cancer cells, therefore, selective inactivation or down-regulation of RNA polymerase I transcription may offer a general therapeutic strategy to block cancer cell proliferation.

It has been shown that antitumor agent actinomycin D could effectively inhibit elongation of RNA polymerase I transcription [8]. CX-3543 [9] is an antitumor bioactive compound developed by Cylene Pharmaceuticals, which has been shown to down-regulate hyperactivated RNA polymerase I transcription by disrupting the formation of nucleolin/rDNA G-quadruplex complex. Nucleolin is a multifunctional protein localized primarily in the nucleolus [10], which is highly expressed in rapidly dividing cells and cancer cells. Nucleolin is connected with rDNA *in vivo* and is required for rRNA synthesis [11], since its knockdown has been shown to specifically inactivate RNA polymerase I-driven transcription [12]. Thus both nucleolin depletion and disruption of nucleolin/rDNA G-quadruplex complex may inhibit aberrant polymerase I transcription in cancer cells.

CX-3543 is a selective RNA polymerase I inhibitor as mentioned above [9], but failed in phase II clinical trials because of its bioviability problem [13]. Quinazoline derivatives have shown significant antiviral

Abbreviations: CD, circular dichroism; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; FAM, 6-carboxyfluorescein; ITC, isothermal titration calorimetry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NHE III1, nuclear hypersensitivity element III1; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcription polymerase chain reaction; VP-16, etoposide

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and anticancer activities, and so far thirty one quinazoline drugs have been in the market [14]. For example, CB3717 has been used as an anti-neoplastic agent [15,16] in cancer chemotherapy. Quinazoline derivatives, such as gefitinib, used mainly for non-small-cell carcinoma, have been used widely in the market with demonstrated good bioviability [17], therefore, we attempted to screen and discover a quinazoline derivative to down-regulate RNA polymerase I transcription or as a RNA polymerase I inhibitor. Previously, we have studied the interaction of 2,4-disubstituted quinazoline derivatives with the G-quadruplex DNA in the telomere [18]. We have found that some 2,4-disubstituted quinazoline derivatives could induce and stabilize the G-quadruplex structure formation, and inhibit telomerase in the cancer cell lines. In the present research, we found that one of these 2,4-disubstituted quinazoline derivatives (**Sysu12d**) could reduce the nucleolin protein expression level and disrupt the interaction of nucleolin with rDNA, which both contributed the down-regulation of RNA polymerase I transcription, resulting in activation of p53 and apoptosis of tumor cells.

2. Materials and methods

2.1. Cells, antibodies, oligomers, siRNAs, and reagents

HeLa (cervical cancer cell line), A549 (adenocarcinomic human alveolar basal epithelial cell line), CA46 (lymphoma cell line), HL-60 (human promyelocytic leukemia cell line), and HEK293 (human embryonic kidney 293 cell) were obtained from the American Type Culture Collection (ATCC), and preserved in our lab. The cell culture was maintained at 37 °C under humidified atmosphere with 5% CO_2 in complete DMEM, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

All antibodies were commercially available as shown in the following: β-actin rabbit mAb (Cat. No. 4970, Cell Signaling), *c-myc* rabbit mAb (Cat. No. 1472-1, Epitomics), nucleolin mouse polyclonal antibody (Abcam ab13541), p53 mouse polyclonal antibody mAb (5969-1, Epitomics), Bcl-2 rabbit mAb (Cat. No. 1017-S Epitomics), bax rabbit mAb (Cat. No. 1063-S, Epitomics), IgG-HRP (Cat. No. 7074, Cell Signaling), and anti-mouse IgG-HRP (Cat. No. 7076, Cell Signaling).

All oligomers were purchased from Invitrogen. The sequences of the oligomers used for EMSA, CD, CD-melting, and competitive FRET-melting are listed in Table 1. The sequences of the PCR primers used in PCR-stop assay, ChIP, and RT-PCR are shown in Table S1. For experiments where G-quadruplexes were needed, oligomers were heated at 95 °C for 10 min and slowly cooled to room temperature in the presence of 100 mM KCl. For EMSA, oligomer was 5′-FAM labeled. For competitive FRET-melting, oligomer was 5′-FAM and 3′-TAMRA dual labeled.

c-Myc stealth siRNAs (5′-AACGUUAGCUUCACCAACATT-3′, 5′-UGUU GGUGAAGCUAACGUUTT-3′) were purchased from RiboBio (Guangzhou). Stealth RNAi™ siRNA Negative Control Med GC (Cat. No. 12935-300) was purchased from Invitrogen. All enzymes used for reverse transcription and PCR were purchased from TaKaRa.

2.2. CD titrations and melting

The oligomer pu27 at a final concentration of 5 μM was resuspended in CD binding buffer (20 mM Tris–HCl, pH 7.0) with varying amounts of **Sysu12d**. The samples were incubated at room temperature for 4 h. The CD spectra were recorded on Jasco J-810 spectropolarimeter. The CD spectra were obtained by taking the average of two scans made from 220 to 340 nm with blank buffer spectra contribution subtracted.

For melting studies [19], 5 μM pu27 in buffer (10 mM sodium cacodylate, 100 mM LiCl, pH 7.2) was annealed by heating at 95 °C for 5 min followed by gradual cooling to room temperature. 5 μM **Sysu12d** was added to prepared G-quadruplex and incubated for 2 h. Thermal melting was monitored at 263 nm at the heating rate of 1 °C/min. The melting temperatures were determined through curve fitting of melting profiles using Origin 7.0.

Table 1
Sequences of oligomers used in this study.

Name	Sequence (5′–3′)
Pu 27	TGGGGAGGGTGGGGAGGGTGGGGAAGG
Fpu18T	5′-FAM-AGGGTGGAAAGGGTGGGG-TAMRA-3′
ds26	CAATCGGATCGAATTCGATCCGATTG
6534NT	GGGGGCGGGAACCCCCGGGCGCCTGTGGG

2.3. PCR-stop assay

PCR-stop assay was performed following the protocol of previous study [20]. Sequences of the test oligomers are included in the Supporting information. Test oligomer (Pu27, 5′-TGGGGAGGGTGGGG AGGGTGGGGAAGG-3′) was amplified with a complementary oligomer (Pu27rev) overlapping the last G-repeat, and *Taq* polymerase extension resulted in the formation of a 43 bp PCR product. Pu27mut (5′-TGGGGA GGGTGGAAAGGGTGGGGAAGG-3′) is a mutant oligomer of Pu27, which may not form G-quadruplex. To rule out the possibility that a ligand itself affects the activity of *Taq* polymerase, a parallel control experiment with oligomer Pu27mut was carried out. The reactions were performed in 1× PCR buffer, containing 10 μmol of each pair of oligomers, 0.16 mM dNTP, 2.5 U *Taq* polymerase, and a certain concentration of **Sysu12d**. The reaction mixtures were incubated in a thermo-cycler, with the following cycling conditions: 94 °C for 3 min, followed by 10 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The amplified products were resolved on 15% nondenaturing polyacrylamide gels in 1× TBE followed by Gel Red staining.

2.4. Western blot

The cellular lysates were prepared as described previously [21], and protein concentrations were determined by using BCA protein assay kit (Pierce). Proteins were resolved by using SDS-PAGE and then transferred to PVDF membranes. The blots were blocked with 5% defatted milk for 2 h at room temperature, and then probed with primary antibodies against β-actin, *c-myc*, nucleolin, p53, Bax, Bcl-2, caspase-3, and PARP (1:1000) at 4 °C overnight. After 3 washes, the blots were subsequently incubated with corresponding secondary antibodies (1:2000) for 1 h at room temperature. The blots were visualized by using chemiluminescence, and blot images were acquired by using Tanon-4200SF gel imaging system (Shanghai).

2.5. Protein expression, purification, identification, and quantification

The pET-28a-nucleolin fusion plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen) for protein expression. Protein expression was induced in the presence of 0.1 mM IPTG for 14 h at 16 °C, 160 rpm. The cells were lysed using a SCIENTZ-II D sonicator (SCIENTZ) with the addition of complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche). Recombinant protein was purified by using HisTrap HP columns (GE Healthcare) to apparent homogeneity, following the manufacturer's protocol. The purified protein was dialyzed in a dialysis buffer (20 mM Tris, 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM DTT, 5% glycerol, pH 7.4) for electrophoretic mobility shift assay. The protein purity was analyzed by using SDS-PAGE. The concentration of purified protein was determined by using BCA protein assay kit (Pierce).

2.6. Electrophoretic mobility shift assay (EMSA)

Briefly, to determine the effect of **Sysu12d** on the binding of nucleolin to rDNA, 5 μM 5′-FAM-labeled rDNA oligonucleotide 6534NT

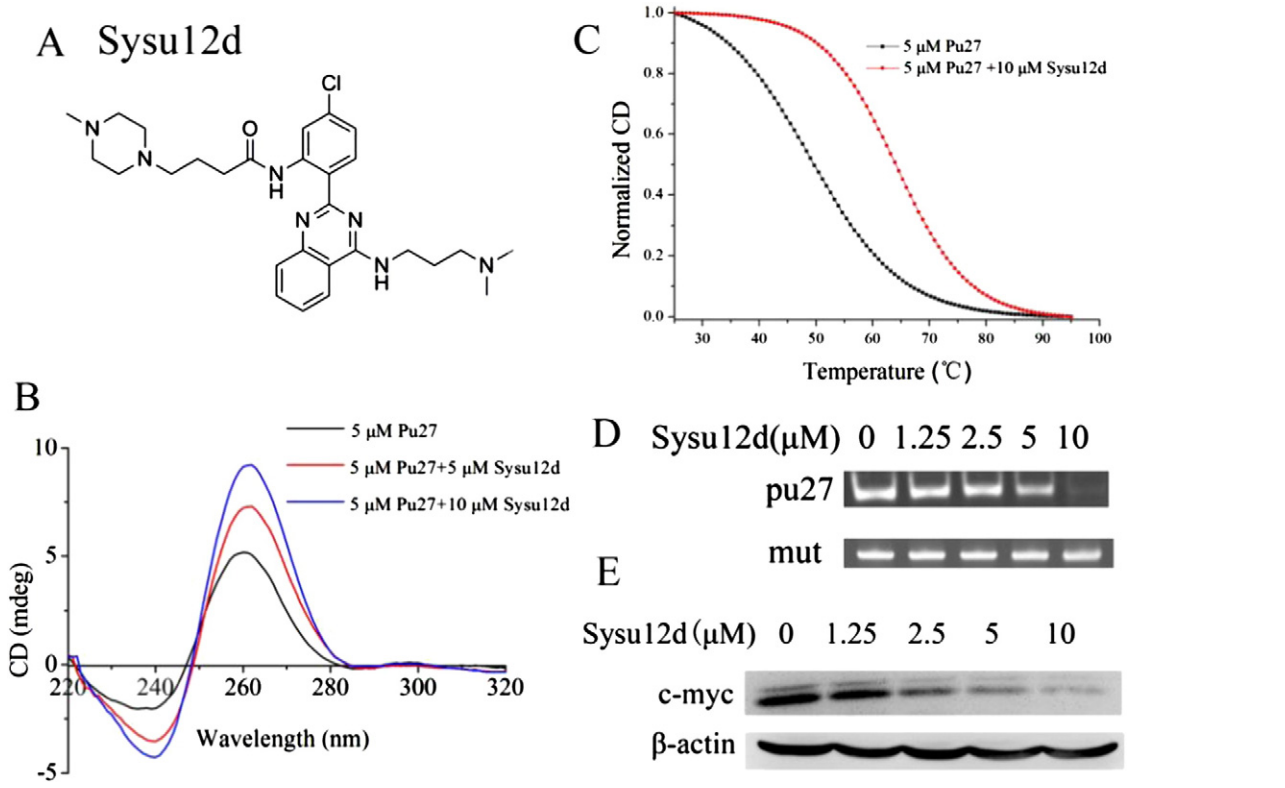

Fig. 1. Down-regulation of c-MYC by **Sysu12d** through the G-quadruplex. (A) Structure of compound **Sysu12d**. (B) CD spectra analysis for incubation of **Sysu12d** with pu27 for the study of *c-myc* G-quadruplex formation. (C) CD melting curves of *c-myc* DNA (5 μM, 10 mM sodium cacodylate, 100 mM LiCl, pH 7.2) with 10 μM **Sysu12d**. (D) PCR-stop assay. (E) Western blotting analysis of c-MYC protein level.

(5′-GGGCGGGGGGGGCGGGGGG-3′) which is a putative G-quadruplex forming sequence in nonrepetitive regions of ribosomal DNA was mixed with 10 μM recombinant nucleolin and various concentrations (0.3, 1, 3 μM) of compound **Sysu12d** in EMSA binding buffer (60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol), and the mixture was incubated for 2 h at 37 °C in a final volume of 10 μL. The resulting mixture was electrophoresed on 8–10% native polyacrylamide gels at 120 V for 1 h in the presence of ice-cooled 0.5× TBE buffer.

2.7. Chromatin immunoprecipitation

Chromatin immunoprecipitation (Ch-IP) was performed using Magna ChIPTM Kit (Millipore) following the manufacturer's protocol. HeLa cells (2×10^5) were cultured for 2 days and then treated for an additional 6 h with 10 μM **Sysu12d**. The treatment of the cells with DMSO was served as a control. Ch-IP-class nucleolin primary antibody (Abcam ab13541) was used to immunoprecipitate chromatin in HeLa cells. Rabbit IgG was used for mock immunoprecipitation. Briefly, cells were fixed with 1% formaldehyde for 10 min and then lysed. Chromatin was sheared to an average size of 0.5 kb using a SCIENTZ-II D sonicator (SCIENTZ), and 10% of the lysate was removed as input. ChIP was performed overnight at 4 °C, and immune complexes were collected using protein A magnetic beads provided by the kit. After extensive washing, the DNA was extracted from immunoprecipitated chromatin. Immunoprecipitated DNA samples were amplified by using PCR. The primer sequences used here were for rDNA-48NT: −48, 5′-CCCG GGGGAGGTATATCTTT-3′ and 5′-CCAACCTCTCCGACGACA-3′; rDNA-2957NT: +2907, 5′-GACGTGTGGCGTGGGTCGAC-3′ and 5′-GACGGG AGGCAGCGACCGG-3′; rDNA-13079NT: +12855, 5′-ACCTGGCGCTAA ACCATTCGT-3′ and 5′- GGACAAACCCTTGTGTCGAGG-3′.

2.8. RNA interference

siRNAs (5′-AACGUUAGCUUCACCAACATT-3′, 5′-UGUUGGUGAAGC UAACGUUTT-3′) targeting *c-myc* were diluted to 50 nM final concentration. Non-specific siRNA (Cat. No. 12935-300) was used as a negative control. For the untreated control, transfection reagent with media only was used. HeLa cells (2×10^5 per well of a 6-well plate) were cultured in antibiotic-free DMEM with 10% FBS. siRNA of *c-myc* was transfected followed by cell incubation for 24 h. The controls included samples transfected with non-specific siRNA or transfection reagent only. Cells were harvested 24 h after transfection for Western blot analysis.

2.9. Reverse transcription-PCR

Total RNA was used as a template for reverse transcription using the following protocol: each 20 μL reaction contained 1× M-MLV buffer, 500 μM dNTPs, 100 pmol oligo dT18 primer, 100 units of M-MLV reverse transcriptase, DEPC in water (DEPC-H_2O), and 1 μg total RNA. The mixtures were incubated at 42 °C for 60 min for reverse transcription, and then at 92 °C for 10 min. After reverse transcription, PCR was performed according to the followed protocol: each 20 μL reaction contained 1× PCR buffer, 500 μM dNTPs, 0.2 μM β-actin primers, 2 μM 45s pre-RNA primers, 1 unit of *Taq* DNA polymerase, 0.1% DEPC-H_2O, and 2 μL of the cDNA template. The reactions were incubated in a thermocycler, with the following cycling conditions: 94 °C for 3 min, followed by 24 cycles of 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s. Amplified products were resolved on 1.5% agarose gel, and photos were taken on a Gel Doc 2000 Imager System. The primer sequences used here were: β-actin, 5′-CTGGAACGGTGAAGGTGACA-3′ and 5′-

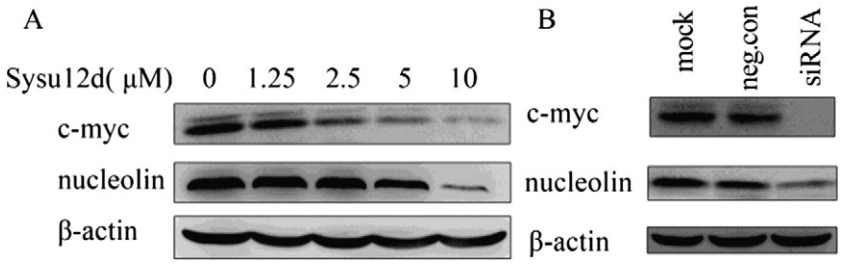

Fig. 2. Regulation of nucleolin by c-MYC. (A) Western blotting analysis of c-MYC and nucleolin after 6 h treatment with **Sysu12d**. (B) Western blot analysis of c-MYC and nucleolin protein levels upon *c-myc* gene depletion induced by *c-myc* siRNAs in HeLa cells. Mock stands for a control experiment with the cells transfected with transfection reagent only. Neg.con stands for a negative control experiment with the cells transfected with non-specific siRNA of *c-myc*. siRNA stands for the experiment with the cells transfected with specific siRNA of *c-myc*.

AAGGGACTTCTGTAACAACGCA-3′; 45s-pre-RNA, 5′-CCGCGCTCTACCTT ACCTACCT-3′ and 5′-GCATGGCTTAATCTTTGAGACAAG-3′.

2.10. Isothermal titration calorimetry (ITC)

ITC experiments were performed in a VP-ITC titration calorimeter (MicroCal, Northampton, MA). Before loading, the solutions were thoroughly degassed. The reference cell was filled with the degassed buffer. The preformed 6543NT (5′-GGGGGCGGGAACCCCCGGGCGCCTGTGGG-3′) G-quadruplex DNA (7 μM) which is a putative G-quadruplex forming sequence (PQS) in nonrepetitive regions of ribosomal DNA was kept in the sample cell, and a syringe with a volume of 300 μL was filled with the ligand **Sysu12d** (300 μM) in the same buffer. The ligand solution was added sequentially in 10 μL aliquots (for a total of 28 injections with a duration of 10 s each) at 4 min intervals at 20 °C. In control experiments, the heats of dilution were determined in parallel experiments by injecting ligand solution of the same concentration in the same buffer. The respective heats of dilution were subtracted from the corresponding binding experiments prior to curve fitting. The thermograms (integrated heat/injection data) obtained in the ITC experiments were fitted with an appropriate model in Origin 7.0. For the ITC experiment of **Sysu12d** with recombinant nucleolin, the protein nucleolin (10 μM) was kept in the sample cell, and a syringe with a volume of 300 μL was filled with the ligand (200 μM) in the same buffer. Other experimental process was the same as that described above.

2.11. Cell growth inhibition assay

The growth inhibitory effect of **Sysu12d** toward five different human cancer cell lines, including HeLa (cervical cancer cell line), A549 (adenocarcinomic human alveolar basal epithelial cell line), CA46 (lymphoma cell line), HL-60 (human promyelocytic leukemia cell line), and HEK293 (human embryonic kidney 293 cell), was evaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] method. The cells were plated at a density of 1×10^4 per well in 96-well microplates, and allowed to incubate overnight. **Sysu12d** was added to the wells at increasing concentrations (0–100 μM). After 48 h, each well was treated with 20 μL 5 mg/mL MTT solution, and the cells were further incubated at 37 °C for 4 h. At the end of the incubation, the untransformed MTT was removed, and 150 μL DMSO was added. The microplates were well shaken to dissolve the formazan dye, and the absorbance at 570 nm was measured using a microplate-reader (Bio-Tek). All drug doses were parallel tested in triplicate, and the IC_{50} values were derived from the mean OD values of the triplicate tests *versus* drug concentration curves.

3. Results and discussion

3.1. 2,4-Disubstituted quinazoline derivative *Sysu12d* suppressed c-myc gene expression in HeLa cells through stabilization of c-myc promoter G-quadruplex

G-quadruplexes are DNA secondary structures formed from planar arrangements of four guanines stabilized by Hoogsteen hydrogen bonding and monovalent cations [22]. The nuclear hypersensitivity element III1 (NHE III1), upstream of the P1 promoter of *c-myc*, controls 80–90% of the transcriptional activation of this gene [17]. The NHE III1, a G (guanine)-rich strand of the DNA containing a 27 base pair sequence (pu27, 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′), can form intramolecular G-quadruplex structure and function as a transcriptional repressor element [23]. The transcription of *c-myc* can be repressed through stabilizing the G-quadruplex by using specific G-quadruplex binding ligands. 2,4-Disubstituted quinazoline derivatives have been reported as telomere G-quadruplex binding ligands [18]. In the present research, we further studied their effect on *c-myc* gene expression in HeLa cells.

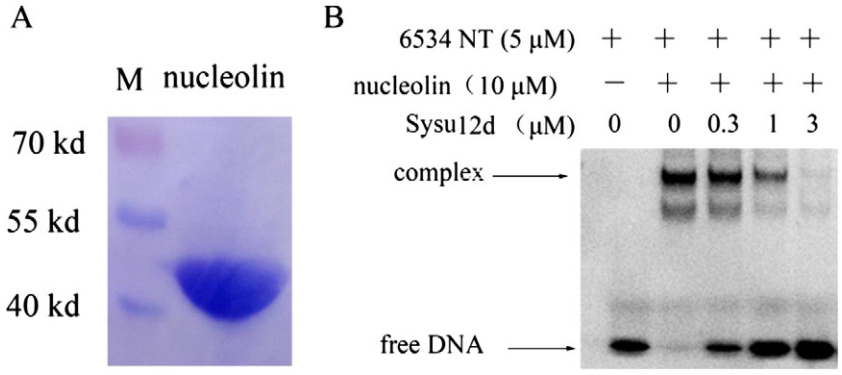

Fig. 3. The effect of **Sysu12d** on the binding between nucleolin and rDNA. (A) SDS-PAGE of purified human recombinant nucleolin. Lane 1, standard protein molecular weight marker; lane 2, human recombinant nucleolin. The gel was stained with Coomassie blue. (B) The *in vitro* effect of compound **Sysu12d** on the binding between nucleolin and rDNA was studied with EMSA.

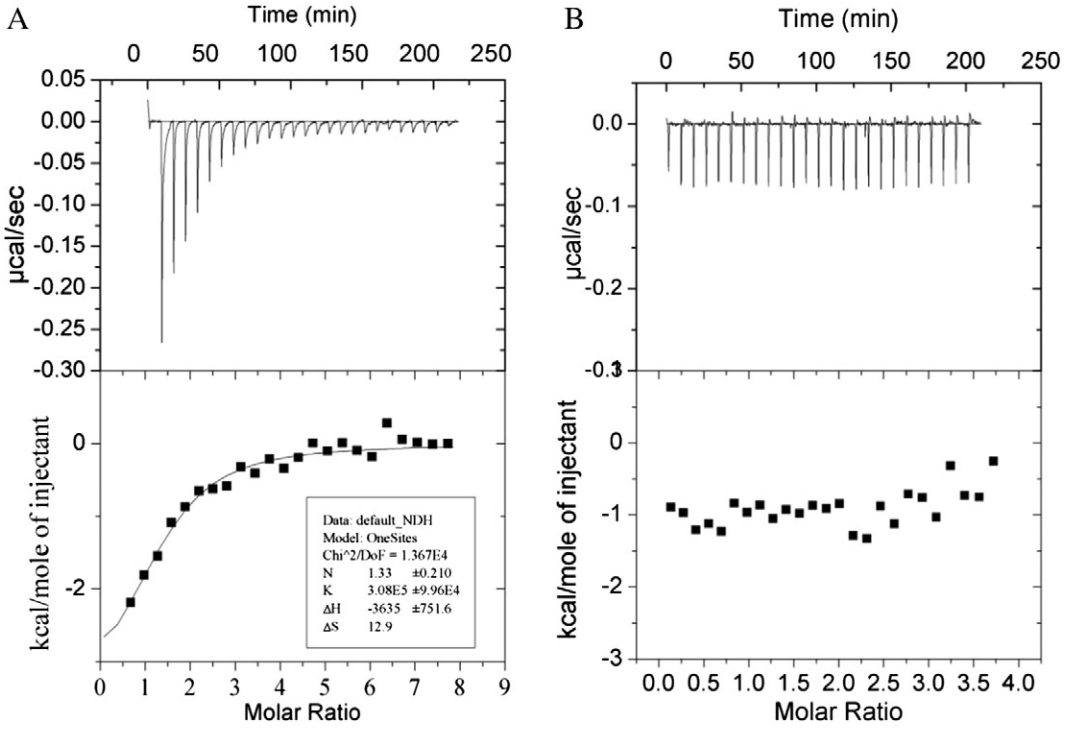

Fig. 4. ITC experiment for titration of 6534NT G-quadruplex (A) or nucleolin (B) with **Sysu12d**.

We found that one of these 2,4-disubstituted quinazoline derivatives (**Sysu12d**, Fig. 1A) effectively down-regulated *c-myc* gene expression in HeLa cells.

Our CD results (Fig. 1B) showed that incubation of **Sysu12d** with single-strand pu27 resulted in a dose-dependent increase of the positive peak at 262 nm. This result indicated that 2,4-disubstituted quinazoline derivative **Sysu12d** could interact with *c-myc* G-quadruplex. Our further study showed that **Sysu12d** can stabilize the *c-myc* G-quadruplex (Fig. 1C) with enhanced melting temperature (ΔTm) of 15 °C. To further demonstrate that **Sysu12d** is selective for *c-myc* G-quadruplex over duplex-DNA (ds26, 5′-CAATCGGATCGAATTCGATCCGATTG-3′), competitive FRET-melting was carried out in the presence of varying amounts of competitor ds26, and the thermal stabilization of Fpu18T by **Sysu12d** had only slight change, as shown in Fig. S2 and Table S2.

Then, we studied the effect of **Sysu12d** on amplification of *c-myc* promoter Pu27 (5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′) by using PCR-stop assay. In the presence of a ligand that can stabilize G-quadruplex structure of a test oligomer, PCR can be inhibited leading to reduced PCR product. The compound of various concentrations (1.25, 2.5, 5.0, and 10.0 μM) was used, and the PCR products were separated with PAGE and stained with Gel Red. As shown in Fig. 1D, **Sysu12d** reduced the level of PCR product in a dose-dependent manner, possibly due to its ability of stabilizing *c-myc* promoter Pu27 G-quadruplex (Fig. 1D). For comparison, a parallel control experiment was carried out with an oligomer pu27mut (5′-TGGGGAGGGTGG**AA**AGGG TGGGGAAGG-3′) that could not form the G-quadruplex, and the result was also shown in Fig. 1D.

Next, we investigated the effect of **Sysu12d** on *c-myc* gene expression *in cellulo*, and found that **Sysu12d** could effectively down-regulate *c-myc* gene expression in HeLa cells, as shown in Fig. 1E. β-Actin is a widely used internal control protein whose expression was not affected by **Sysu12d** in the present study. Our results indicated that **Sysu12d** down-regulated *c-myc* gene expression in HeLa cells by stabilization of *c-myc* promoter Pu27 G-quadruplex.

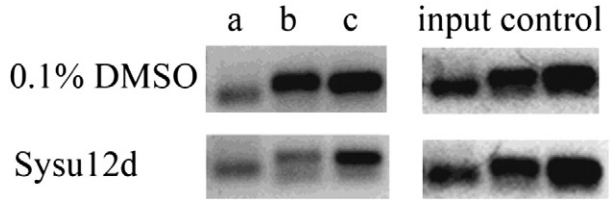

Fig. 5. The *in vivo* effect of compound **Sysu12d** studied with ChIP assays in HeLa cells treated with 0.1% DMSO as a control or 10 μM **Sysu12d** for 6 h. Immunoprecipitated DNA samples were amplified with PCR to show nucleolin occupancy of rDNA G-quadruplex. (a) rDNA-48NT, a rDNA region containing no putative G-quadruplex forming sequence; (b) rDNA-2957NT, a rDNA region containing putative G-quadruplex forming sequence; (c) rDNA-13079NT, a rDNA region containing putative G-quadruplex forming sequence. 10% of the cell lysate without antibody addition was used as input control. The input was used to ensure equal amount of ChIP DNA in the control group (0.1% DMSO) and the experimental group (**Sysu12d**), which can also rule out the possibility of operating error.

*3.2. The effect of **Sysu12d** on c-myc expression further down-regulated expression of nucleolin*

The *c-myc* proto-oncogene has been shown to direct a diverse array of biological activities, including cell cycle progression, apoptosis, and differentiation. It is believed that MYC can affect this wide variety of activities by functioning as a regulator of gene transcription. It has also been reported that MYC activates transcription of nucleolin [23]. In the present study, after 6 h treatment of HeLa cells with varying concentrations of **Sysu12d**, the MYC protein level reduced in a dose-dependent manner, as shown in Fig. 2A. In the mean time, the protein level of nucleolin also reduced in a dose-dependent manner, as shown in Fig. 2A. It is possible that down-regulation of *c-myc* caused the down-regulation of nucleolin. For the purpose of verifying this hypothesis, siRNA technique was employed. *c-Myc* siRNA treatment introduced apparent silencing of *c-myc* gene expression, as shown in Fig. 2B. Interestingly, the silencing of *c-myc* induced significant decrease of nucleolin protein expression level in the mean time, as shown in Fig. 2B. This result indicated that the down-regulation of *c-myc* upon treatment with **Sysu12d** resulted in down-regulation of nucleolin.

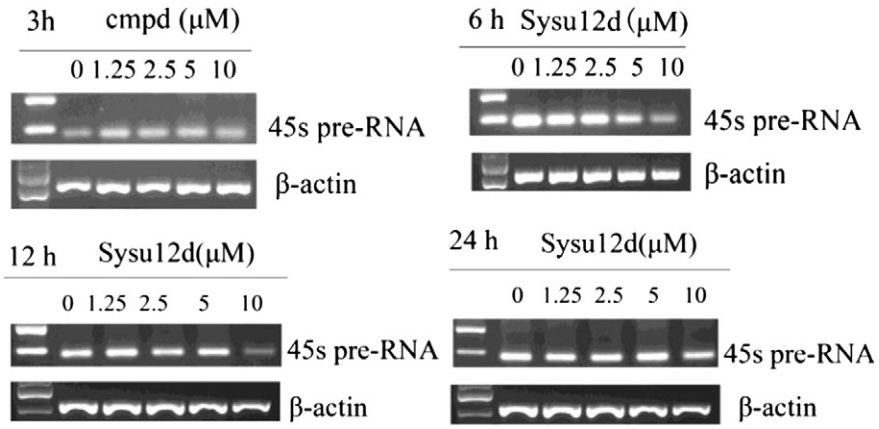

Fig. 6. Sysu12d down-regulated polymerase I transcription in HeLa cells (70% confluent). The cells were treated with different concentrations of **Sysu12d** for various time periods followed by cell lysis, RNA purification, and first strand cDNA synthesis. The cDNA generated was analyzed by using PCR.

3.3. *Sysu12d* disrupted the complex of nucleolin/G-quadruplex formed on rDNA

The prevalence of putative G-quadruplex forming sequence (PQS) has been reported in non-repetitive regions of the human genome [25–27]. The formation and dissociation of the G-quadruplex was regulated by G-quadruplex binding proteins *in vivo*. Nucleolin is an important G-quadruplex binding protein, and its binding complex with rDNA was investigated in the present study. The protein was purified to apparent homogeneity as shown in Fig. 3A, which was incubated with fluorescence-labeled human rDNA 6534NT (GGGGGCGGGAACCC CCGGGCGCCTGTGGG) in the absence or presence of different concentrations of compound **Sysu12d**. 6534NT is a putative G-quadruplex forming sequence (PQS) in nonrepetitive region of rDNA, and our present CD study indicated that 6534NT could form G-quadruplex structure (Fig. S3). As shown in Fig. 3B, **Sysu12d** could disrupt nucleolin/G-quadruplex complexes with micromolar inhibition constants, as determined by electrophoretic mobility shift assays.

To further investigate why **Sysu12d** can disrupt nucleolin/G-quadruplex complexes *in vitro*, isothermal titration calorimetry (ITC) experiment was carried out. By determining heat changes associated with binding between any two in-solution biomolecules, ITC can measure all binding parameters, including binding affinity (K_D), reaction stoichiometry (n), enthalpy (ΔH), and entropy (ΔS). Here, ITC was used to study and compare the binding of **Sysu12d** with 6534NT G-quadruplex and with nucleolin. As shown in Fig. 4, **Sysu12d** bound well with 6534NT G-quadruplex, and the K_D value for their interaction was determined to be 3.3 μM. In comparison, **Sysu12d** showed no specific binding interaction with protein nucleolin (Fig. 4B). These results indicated that **Sysu12d** could compete with nucleolin for 6534NT G-quadruplex binding site.

To probe the physical occupancy of the nucleolin to rDNA in cells, we performed a chromatin immunoprecipitation (ChIP) assay according to the procedure employed for CX-3543 [9] that has been in phase II clinical trial. Two regions (rDNA-2957 and rDNA-13079) containing putative G-quadruplex forming sequences (PQSs) in the non-template strand of rDNA and one corresponding region (48NT) that is not PQS were selected for the ChIP assay. As shown in Fig. 5, treatment of HeLa cells with 10 μM **Sysu12d** for 6 h caused significant dissociation of nucleolin from both rDNA-2957 and rDNA-13079 containing PQSs in the rDNA, while it had little effect on the occupancy of nucleolin at the rDNA-48 that is not PQS. It should be noted that this investigation was carried out after 6 h treatment of the cells with 10 μM **Sysu12d**, and therefore the expression of nucleolin should be down-regulated as described above. However, our results still showed that compound **Sysu12d** indeed dissociated nucleolin from rDNA. This result indicated that compound **Sysu12d** could interfere with the binding between the nucleolin and the human rDNA *in cellulo*, which might further down-regulate the transcription of RNA polymerase I [9].

3.4. The effect of *Sysu12d* on the complex of nucleolin/G-quadruplex further down-regulated rRNA synthesis

It has been reported that both nucleolin depletion and disruption of the nucleolin/rDNA G-quadruplex complex may inhibit aberrant polymerase I transcription in cancer cells [9,12]. Therefore, we further investigated the effect of **Sysu12d** on rDNA transcription on human-derived cell lines including HeLa cervical epithelial carcinoma and normal cell line HEK293. RT-PCR experiment was carried out to study the effect of **Sysu12d** on HeLa cells after different time period of incubations, as shown in Fig. 6. We found that **Sysu12d** repressed RNA polymerase I transcription mostly efficiently after 6 h treatment. It indicated that **Sysu12d** reacted with target G-quadruplex rapidly, resulting in significant repression of rRNA synthesis within 6 h. In comparison, the normal cell line HEK293 displayed relatively lower sensitivity to the treatment of **Sysu12d** compared to cervical epithelial carcinoma HeLa after 6 h treatment, as shown in Fig. 7. Based on previous reports, our present

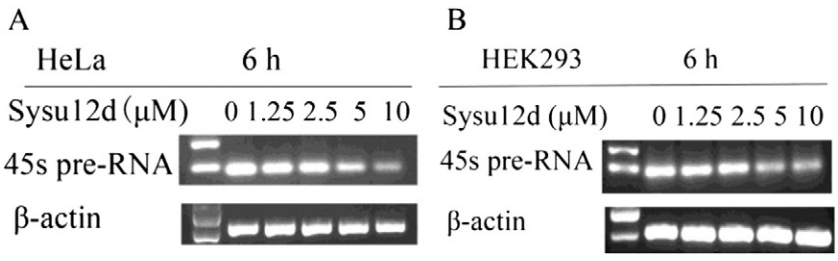

Fig. 7. Sysu12d down-regulated polymerase I transcription in HeLa cells more efficiently compared with that in HEK293 cells. Actively growing HeLa cells and HEK293 cells (70% confluent) were treated with different concentrations of **Sysu12d** for 6 h followed by cell lysis, RNA purification, and first strand cDNA synthesis. The cDNA generated was analyzed by using PCR.

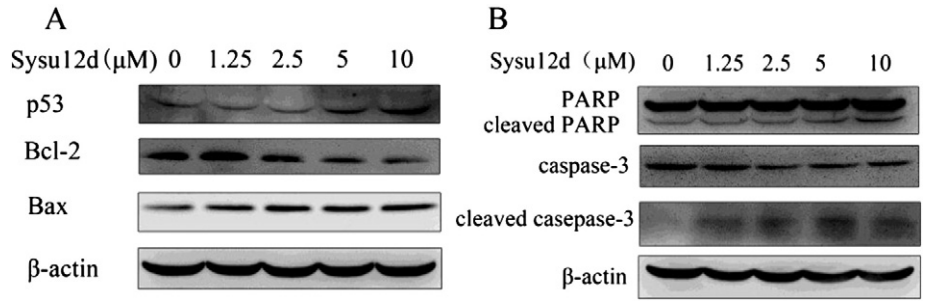

Fig. 8. Sysu12d activated p53 and induced apoptosis. (A) Effect of **Sysu12d** on p53 stabilization in HeLa cells after 6 h treatment. (B) HeLa cells were treated with various doses of **Sysu12d** for 6 h, and the induction of apoptosis was measured by monitoring activation of caspase-3 and PARP cleavage.

data indicated that the effect of **Sysu12d** on the complex of nucleolin/G-quadruplex further down-regulated rRNA synthesis.

3.5. *Sysu12d activated p53 and induced cell apoptosis*

To further understand the mechanism for the effect of **Sysu12d**, we studied some factors related to cancer cell apoptosis. It has been reported that down-regulation of polymerase I transcription in cancer cell lines can cause nucleolar stress resulting in stabilization of p53 and induction of p53-dependent apoptosis [28]. Therefore, we investigated whether **Sysu12d** could promote the stabilization of p53. For this purpose, we performed a dose-dependent analysis for the effect of **Sysu12d** on the level of p53 after its incubation with HeLa cells for 6 h, as shown in Fig. 8A. It is possible that down-regulation of rRNA synthesis by **Sysu12d** led to the stabilization of p53, which was followed by induction of apoptosis, as judged by caspase-3 activation and cleavage of poly(ADP-ribose) polymerase (PARP), as shown in Fig. 8B.

It has been known that DNA damage can result in γ-H2AX phosphorylation also leading to p53 activation [29]. For example, actinomycin D has previously shown to induce DNA double-stranded breaks consequently causing γ-H2AX phosphorylation and p53 activation [30]. In order to rule out this possible mechanism for **Sysu12d**, we tested phosphorylation of histone H2AX at Ser139 after its incubation with HeLa cells. Fig. 9 showed that **Sysu12d** had no noticeable effect on phosphorylation of H2AX at Ser139, in comparison with a positive control vp-16, a known DNA-damaging agent.

4. Discussion

As the role of excessive rRNA synthesis in tumorigenesis comes to be better understood [31–33], targeting aberrant transcription of rDNA catalyzed by RNA polymerase I should be a very promising approach for anti-cancer chemotherapeutics [34,35]. Consistent with this concept, it has been reported that low doses of antitumor agent actinomycin D inhibited rRNA synthesis [8]. Other clinically approved antitumor drugs such as cisplatin [36], mitomycin C [37], 5-fluorouracil [38], and temsirolimus [39] could disrupt ribosome biogenesis to some extent. Recently, two new selective RNA polymerase I transcription inhibitors

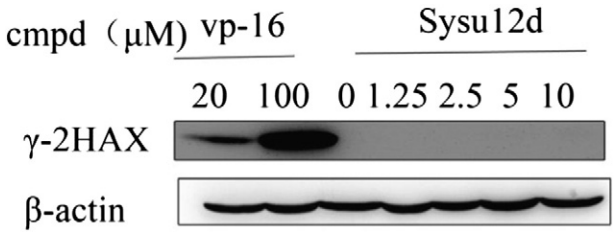

Fig. 9. Sysu12d didn't trigger phosphorylation of H2AX. HeLa cells were treated with various concentrations of **Sysu12d** and vp-16 for 6 h. Cellular proteomes were isolated and the phosphorylation status of H2AX (γ-H2AX) and β-actin levels were analyzed by using Western blot.

(CX3543 [9] and CX5461 [40,41]) in the clinical trials designed by Cylene Pharmaceuticals have been shown to specifically inhibit rDNA transcription. CX-3543 (quarfloxin) has been discontinued from phase II clinical trials for the treatment of neuroendocrine and carcinoid tumors because of its bioviability problem [13]. It should be noted that quinazoline derivatives have been previously used as medicine with good bioviability. For example, gefitinib [42] is one member of quinazoline derivatives as the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which is a marketed drug with excellent oral bioviability in patients [17]. Since **Sysu12d** is also a quinazoline derivative, it is possible that compound **Sysu12d** as a lead compound could be further developed for future clinical trial without bioviability problem.

It has been shown previously that 2,4-disubstituted quinazoline derivatives could well recognize G-quadruplex and have high selectivity toward G-quadruplex over duplex DNA [18]. 2,4-Disubstituted quinazoline derivatives have been shown to significantly stabilize the G-quadruplex structure formation, and inhibit telomerase in the cancer cell lines. In this study, we demonstrated for the first time that 2,4-disubstituted quinazoline derivative **Sysu12d** could efficiently down-regulate RNA polymerase I transcription.

Sysu12d could stabilize *c-myc* G-quadruplex, resulting in down-regulation of *c-myc* gene expression. Nucleolin is an abundant nucleolar and multifunctional protein involved in rRNA processing and ribosomal assembly [43–47]. It has been reported that MYC activates transcription of nucleolin and BN51 *via* E-box located in their first introns [24]. Our data showed that down-regulation of *c-myc* reduced the expression level of nucleolin (Fig. 2B). It has been shown that the knockdown of nucleolin can down-regulate polymerase I-driven transcription [48], therefore, in the present study, the down-regulation of nucleolin could contribute to the reduced level of polymerase I-driven transcription. Nucleolin can bind to and stabilize rDNA G-quadruplex structure [49], and thus increase the rate of polymerase I transcription. Therefore, disruption of nucleolin/rDNA G-quadruplex complex may down-regulate aberrant polymerase I transcription in cancer cells. Our data showed that **Sysu12d** indeed displaced nucleolin from rDNA G-quadruplex (Figs. 3B, 5). In this study, our results suggested that both nucleolin depletion and disruption of nucleolin/rDNA G-quadruplex complex contributed to the down-regulation of polymerase I transcription in cancer cells.

Sysu12d exhibited a broad-spectrum of anti-proliferative activity for cancer cells (Table 2). Besides, we analyzed if down-regulation of RNA polymerase I transcription by **Sysu12d** correlated with p53 accumulation, since the down-regulation of RNA polymerase I transcription has been previously reported to cause nucleolar stress that leads to

Table 2
MTT assay for the effect of **Sysu12d** on different cancer cells.

	A549	HeLa	HL60	HEK293	CA46
IC_{50} (μM)	3.1	4.1	5.0	6.3	11.2

stabilization of p53 and induction of p53-dependent apoptosis [28]. Our present data possibly indicated that the down-regulation of RNA polymerase I transcription by **Sysu12d** caused activation of P53 (Fig. 8A), which induced the down-regulation of Bcl_2 and up-regulation of Bax (Fig. 8A), resulting in cancer cell apoptosis (Fig. 8B).

In summary, our results showed that **Sysu12d** could down-regulate *c-myc* through stabilization of *c-myc* promoter G-quadruplex, resulting in down-regulation of nucleolin expression. **Sysu12d** could also disrupt nucleolin/G-quadruplex complex. Both of the above contributed to the down-regulation of rRNA synthesis, followed by activation of p53 and then cancer cell apoptosis. These results set up the basis for further development of **Sysu12d** as a new type of lead compound for cancer treatment. This study also indicated that 2,4-disubstituted quinazoline derivatives may have multi-functional effect for cancer treatment.

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Appendix A. Supplementary data

Supporting Information Available: Experimental methods for competitive FRET-melting and UV experiment, sequences of primers used for PCR in this study (Supplementary Table 1), stabilization temperatures (ΔTm) determined with FRET-melting (Supplementary Table 2), UV spectrum of 50 μM **Sysu12d** (Supplementary Figure 1), competitive FRET results for **Sysu12d** without and with excess of duplex DNA competitor (ds26) (Supplementary Figure 2), and CD spectra of 5 μM 6534NT alone and with 100 mM K^+ (Supplementary Figure 3). Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.07.004.

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