Biochimica et Biophysica Acta 1840 (2014) 3123–3130

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

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

Lijuan Su, Huaqin Zheng, Zeng Li, Jun Qiu, Siqi Chen, Jinggong Liu, Tian-Miao Ou, Jia-Heng Tan, Lian-Quan Gu, Zhi-Shu Huang, Ding Li *

*School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou University City, 132 Waihuan East Road, Guangzhou 510006, PR China*

ARTICLE INFO

*Article history:*
Received 16 April 2014
Received in revised form 23 June 2014
Accepted 2 July 2014
Available online 10 July 2014

*Keywords:*
RNA polymerase I
Nucleolin
*c-Myc*
G-quadruplex
Transcriptional regulation
Cancer

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

* Corresponding author. Tel.: +86 20 3994 3058.
*E-mail address:* liding@mail.sysu.edu.cn (D. Li).

http://dx.doi.org/10.1016/j.bbagen.2014.07.004
0304-4165/

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