Biochemistry and Biophysics Reports 5 (2016) 346–352

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Blocking the binding of WT1 to *bcl-2* promoter by G-quadruplex ligand SYUIQ-FM05

Yun-Xia Xiong [a,1], Ai-Chun Chen [a,1], Pei-Fen Yao [a], De-Ying Zeng [a], Yu-Jing Lu [b], Jia-Heng Tan [a], Zhi-Shu Huang [a], Tian-Miao Ou [a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China
[b] Faculty of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, PR China

ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form
21 December 2015
Accepted 29 December 2015
Available online 14 January 2016

Keywords:
WT1
Quindoline derivative
Bcl-2 gene
G-quadruplex forming sequence
Blocking binding

ABSTRACT

At present, *wt1*, a Wilms' tumor suppressor gene, is recognized as a critical regulator of tumorigenesis and a potential therapeutic target. WT1 shows the ability to regulate the transcription of *bcl-2* by binding to a GC-rich region in the promoter, which can then fold into a special DNA secondary structure called the G-quadruplex. This function merits the exploration of the effect of a G-quadruplex ligand on the binding and subsequent regulation of WT1 on the *bcl-2* promoter. In the present study, WT1 was found to bind to the double strand containing the G-quadruplex-forming sequence of the *bcl-2* promoter. However, the G-quadruplex ligand SYUIQ-FM05 effectively blocked this binding by interacting with the GC-rich sequence. Our new findings are significant in the exploration of new strategies to block WT1's transcriptional regulation for cancer-cell treatment.

1. Introduction

The human Wilms' tumor 1 (*wt1*) gene, located at chromosome 11p13, was originally identified as a tumor suppressor in Wilms' tumor in 1990 [1]. Because of its overexpression in hematologic malignancies and a variety of solid cancers, but not in the healthy adjacent tissues, *wt1* is currently considered as a potential oncogene [2,3] and a promising target for cancer therapy. *wt1* is a prognostic biomarker in uterine sarcoma [4] and in acute myeloid leukemia (AML) [5] because of its high correlation with cancer growth and relapse. The knockdown of WT1 effectively induces the apoptosis of leukemic cells [6] and inhibits malignant cell growth [7]. WT1 peptide vaccination of clinical patients with myeloid malignancies and several solid cancers has shown positive outcomes [8]. All of the substantial investigations implicate WT1 as a critical regulator of tumorigenesis and a potential therapeutic target.

The *wt1* gene encodes a zinc-finger transcription factor that can be translated into at least 36 isoforms by alternative mRNA splicing. All of these isoforms have four Cys2-His2 zinc fingers on their C-terminus [9]. The alternative splice sites at the end of exon 9 leads to the insertion of three amino acids (KTS) between zinc fingers 3 and 4, thereby forming two kinds of zinc-finger domains termed as ZF−KTS and ZF+KTS [10]. The ZF+KTS isoform usually binds to RNA and tends to function in the post-transcriptional regulatory processes [11], whereas the ZF−KTS isoform binds to DNA more strongly and is more active in transcriptional regulation [12]. By recognizing the consensus site 5′–GNGNGGGNG–3′, WT1 was reported as a transcriptional regulator that targets at least 137 genes. As such, WT1 plays important roles in cell processes, including cell differentiation, cell adhesion, apoptosis, angiogenesis, and immune function [13,14]. Interestingly, the GC-rich sites recognized by WT1 were reported to be remarkably associated with the potential G-quadruplex-forming motifs, which carry a signature motif of $G_{\geq 3}N_XG_{\geq 3}N_XG_{\geq 3}N_XG_{\geq 3}N_X$, in humans, chimpanzees, mice, and rats [15]. This observation implies the possible role of WT1 in transcriptional regulation through G-quadruplex elements. A well-known example is a 39 bp GC-rich element (Pu39) located upstream of the P1 promoter of the *bcl-2* oncogene (Fig. 1). In our study, we select a native sequence Pu59 from the *bcl-2* promoter; Pu39 sequence locates in the center of Pu59 and a 10-nucleotide flanking sequence was added on each side of Pu39. This region can be bound directly by the WT1 protein and plays an important role in the regulation of *bcl-2* gene transcription [16]. This guanine-rich sequence also has the potential to form a G-quadruplex structure and might cause the down-regulation of

Abbreviations: CD, circular dichroism; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry; RT-PCR, reverse transcription polymerase chain reaction; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine

* Corresponding author.
E-mail address: outianm@mail.sysu.edu.cn (T.-M. Ou).
[1] These authors contribute equally to this manuscript.

http://dx.doi.org/10.1016/j.bbrep.2015.12.014

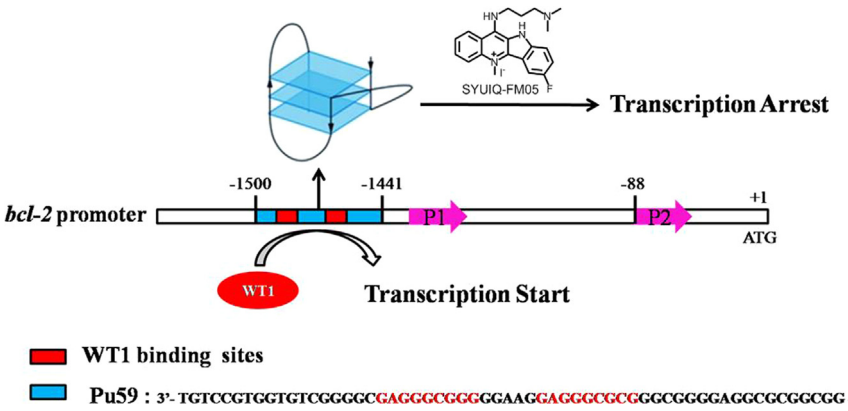

Fig. 1. WT1 binding sites in the *bcl-2* promoter and the G-quadruplex-forming sequence in the *bcl-2* promoter. Two conservative binding sites of WT1 exist in the Pu59 region (red region). The binding of WT1 to the *bcl-2* promoter will activate *bcl-2* transcription. The Pu59 region (blue region) in the *bcl-2* promoter *bcl-2* can form a G-quadruplex structure and be stabilized by compound SYUIQ-FM05. The resulting structure would lead to transcriptional arrest. The relationship among WT1, the Pu59 region, and the G-quadruplex ligand SYUIQ-FM05 will be further illustrated in this paper. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bcl-2 transcription. Various G-quadruplex ligands have been reported possessing different functions, such as inhibition oncogenes' transcription, interfering with the telomere's function, and repressing tumor cell growth [17]. The high correlation between the WT1 binding sites and the G-quadruplex-forming site was noted. Hence, we explore whether or not WT1 can bind to the G-quadruplex structure. The second reason for our research is related to the positive roles that WT1 plays in leukemogenesis, in which it promotes tumor cell survival and affect clinical outcomes [18]. Moreover, *bcl-2* is reported to be transcriptionally upregulated by WT1 [19]. The coexpression of WT1 and BCL-2 is associated with response and long-term outcome of AMLs [20]. Therefore, we attempt to study the mechanism of the compound interfering with the transcriptional regulation of WT1.

In the present research, a variety of methods, including the electrophoretic mobility shift assay (EMSA), fluorescent studies, isothermal titration calorimetry (ITC), chromatin immunoprecipitation (Ch-IP), and circular dichroism (CD) were applied. For the first time, we demonstrated that WT1 cannot bind to the G-quadruplex structure. We identified a G-quadruplex ligand that could block WT1 binding by interaction with the GC-rich region containing the G-quadruplex-forming sequence in the promoter region of *bcl-2* gene. These new findings are significant in the development of new strategies to block WT1's transcriptional regulation for the treatment of leukemia cells.

2. Materials and methods

2.1. Materials

All chemicals were obtained from commercial sources. All oligomers were purchased from Invitrogen (China). The enzymes used for reverse transcription and PCR were purchased from TaKaRa (China), and the enzymes used for plasmid construction were purchased from Fermentas (USA). The compound was synthesized by our group as described previously [21] and was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mM as the stock solution. HL-60 (human promyelocytic leukemia cell lines) were obtained from the American Type Culture Collection and preserved at our laboratory. HL-60 cells were cultured in 1640 medium supplemented with 10% fetal bovine serum and 5% CO_2 at 37 °C. The antibodies employed in the study include WT1 polyclonal antibody (sc-192, Santa Cruz, CA, USA) and anti-rabbit IgG–horseradish peroxidase (#7074, Cell Signaling, MA, USA).

2.2. Plasmid construction and protein purification

Total mRNAs were isolated from the HL-60 cells using the RNAiso Plus reagent (TaKaRa, China) following the manufacturer's protocol. Then, 500 ng of total RNA was reverse transcribed in a total volume of 20 μL PCR mixture using the M-MLV reverse transcriptase (TaKaRa, China) and oligo $d(T)_{18}$ primer (TaKaRa, China). After reverse transcription, the cDNA was subjected to PCR for the amplification of the C-terminus of WT1 (amino acids 312–449) containing the four zinc fingers using primers ZF-A (5′–GGAATTCTCAAAGCGCCAGCTGGAG–3′) and ZF-S (5′–GAATTCCATATGTCGGCATCTGAGACCAG–3′). PCR was performed on a Bio-Rad T100 Thermal Cycler using Dreamtaq PCR kit (Thermo Scientific). The program proceeded as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation, annealing, and extension (95 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min). The PCR products were verified using a 2% agarose gel. The PCR products digested by NdeI and EcoRI were inserted into a pET-28a (+) vector (Novagen, TX, USA) for pET28a-(ZF−KTS) plasmid construction.

All reconstructed plasmids were sequenced and confirmed by BLAST on NCBI.

The *E. coli* strain BL21 (DE3) cells were transformed with pET-28a-(ZF-KTS) plasmid for overexpression and purification of the recombinant protein. Cells were first grown at 37 °C in 0.2 l of LB medium containing kanamycin (50 ng/mL) to an OD600 of 0.6 before addition of 0.1 mM IPTG to induce protein expression. Cells were then grown at 15 °C for 18 h before harvested by centrifugation. Cell pellet was resuspended in 40 mL of start buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl) and lysed using a SCIENTZ-II D sonicator (SCIENTZ). The insoluble debris was removed by centrifugation, and the clear supernatant was used for protein purification after filtered through a 0.45 μm filter. Protein purification was carried out using the Hi-Trap column (GE Healthcare) according to the protocol supplied by the manufacturer. The purified protein fractions were filtered and stored in 20 mM Tris, 100 mM KCl, 1 mM DTT, and 10% glycerol, pH 7.4. The purified protein was identified by MALDI-TOF-TOF (Ultraflex TOF/TOF III) and Mascot Search and the purity were verified by SDS-polyacrylamide gel electrophoresis.

2.3. Electrophoretic mobility shift assay (EMSA)

The oligomers were diluted from stock solution to the indicated concentration in Tris–HCl buffer (10 mM, pH 7.4) with or without 100 mM KCl, and then annealed by heating to 95 °C for 5 min, and