**Development of a FOXM1-DBD Binding Assay for High-Throughput Screening Using TR-FRET Assay**

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Electrophoretic mobility shift assay (EMSA) technology has been widely employed for the analysis of transcription factors such as Forkhead box protein M1 (FOXM1). However, the application of high-throughput screening (HTS) in performing, such analyses are limited as it uses time consuming electrophoresis procedure and radioisotopes. In this study, we developed a FOXM1-DNA binding domain (DBD) binding assay based on time-resolved fluorescence energy transfer (TR-FRET) that enables HTS for the inhibitors of FOXM1–DNA interaction. This assay was robust, highly reproducible and could be easily miniaturized into 384-well plate format. The signal-to-background (S/B) ratio and Z’ factor were calculated as 7.46 and 0.74, respectively, via a series of optimization of the assay conditions. A pilot library screening of 1019 natural compounds was performed using the FOXM1-DBD binding assay. Five hit compounds, namely, ACILXm, BRN5, gangaleoidin, leoidin, and roemerine were identified as the inhibitors of FOXM1. In a cell viability assay, it was demonstrated that cell proliferation of FOXM1 overexpressed cell lines was suppressed in cell lines such as MDA-MB-231 and MCF-7 by five hit compounds. These results indicate that developed FOXM1-DBD binding assay can be applied to highly efficiency HTS of compound libraries.

Key words Forkhead box protein M1; protein–DNA interaction; high-throughput screening

INTRODUCTION

FOXM1, a member of the Forkhead Box (Fox) transcription factor family, contributes to the activation of gene associated with cell proliferation and differentiation. To date, over 40 Fox proteins have been discovered in humans, which are subdivided into consecutive alphabetic designations. These Fox-subfamilies share almost no sequence similarity and are only linked via a conserved DNA-binding domain. FOXM1 protein contains three main functional components which are the N-terminal repressor domain, a central DNA-binding domain called Forkhead or winged-helix domain, and C-terminal transcriptional transactivation domain. The FOXM1 DNA binding domain (FOXM1-DBD) preferentially binds the ‘C/TAAACA’ promoter regions, the tandem repeats of a core consensus recognition sequence. Among Fox-subfamilies, FOXM1 is considered to be an attractive anti-cancer drug target as it participates in mitosis, cell cycle progression and other signal pathways leading to tumorigenesis. Elevated expression of FOXM1 is observed in conditions leading to uncontrolled cell growth, for instance, during the development of tumors. The expression of FOXM1 is scarcely detected when the cell is at rest or in its final differentiated state. Its expression is closely associated with the proliferative potential of the cell. It also regulates the transcription factors required for cell-cycle progression such as Cdc25c, Plk1, cyclin B2. There is a positive correlation between increased FOXM1 and poor prognosis in cancer patients. Experiments with FOXM1 transgenic mice have demonstrated that abnormal expression of FOXM1 accelerates proliferation and increases the number of tumor cells in lung tumor animal models. Similarly, the deletion of the FOXM1 gene inhibits the formation of 3-methylcholanthrene/butylated hydroxytoluene or urethane-induced lung tumors in Mx-Cre Foxm1−/− mice. Despite the therapeutic potential, inhibitors of FOXM1 have rarely been reported. Previous studies have reported compound that selectively inhibit FOXM1, such as FDL-6. However, more potent and selective inhibitors of FOXM1 are required.

Transcription factors, including FOXM1, are not considered suitable as drug targets as not many ways are known for the application of a protein-DNA binding assay for high-throughput screening (HTS). Electrophoretic mobility shift assay (EMSA) has been consistently used to determine if a protein can bind to a given DNA sequence or if a small molecule can disrupt protein-DNA binding. EMSA-based FOXM1 assay has been widely accepted, as it quantifies the binding activity of FOXM1. However, there is a lot of limitations in performing HTS using this analysis. EMSAs involve electrophoresis using multiple heterogeneous processes, such as several incubation and washing steps. In addition, it also includes the use of radioisotope along with safety concerns, and waste disposal process. Recently, instead of radioisotope, IR fluorescent dyes (IRDye) are used for protein or nucleic acid labeling, which are designed for use in a variety of biological detection application including EMSA. In addition to EMSA, other techniques have been developed to detect DNA–protein interactions such as molecular beacon assay using oligonucleotide hybridization probes, exonuclease footprinting...
assay using fluorescence resonance energy transfer (FRET) probe or SYBR Green probe\(^{15,16}\) and FRET-based DNA probe assay.\(^{17}\) These probe-based DNA–protein interaction assay has recently attracted great interests as a tool for transcription factor studies and HTS although there are limitations of custom labeling process. However, only few probe-based DNA–protein interaction systems are commercially available, and still need a major expansion of probe or biosensor in various transcription factor for drug discovery. In addition, although fluorescence polarization (FP) assay has been applied to detect DNA–protein interaction,\(^{18}\) it is well known that all homogenous assay formats using fluorescein are subject to interference from intrinsically fluorescent compounds or from scattered light.

The time-resolved fluorescence resonance energy transfer (TR-FRET)-based methods used in this study has come to be noted as a method of identifying the inhibitors of protein–DNA interaction with HTS.\(^{18}\) To the best of our knowledge, because we know that the TR-FRET-based FOXM1-DBD binding assay has never been investigated before, we applied this binding assay method to high efficiency screening for inhibitors of FOXM1–DNA interaction. In this study, we developed a TR-FRET-based FOXM1-DBD binding assay and screened 1019 natural compounds to identify inhibitors of FOXM1–DNA interactions.

**MATERIALS AND METHODS**

**Materials** Biotinylated oligomer (sense: 5'-bionin-triethylene glycol spacer (TEG)-AAAACAACAACAAATC-3' and antisense: 5'-GATTGTGGTTTGTTGTT-3') were purchased from Bioneer (Daejeon, South Korea). The double stranded DNA (dsDNA) binding domain (DBD) of FOXM1 tagged with his-tidine (His)\(^6\) was prepared by Dr. Sang Joo Shin (Yonsei University, Seoul, Korea). The TR-FRET reaction reagents such as anti-His\(\times 6\)-cryptate and Streptavidin-XL665 were obtained from Cisbio (Codolet, France). FDI-6 as reference were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Assay Set-Up** The FOXM1-DBD binding assay was performed on 384-well plates (Corning Life Science, Lowell, MA, U.S.A.). His\(\times 6\)-tagged FOXM1-DBD, biotinylated dsDNA, and compounds were diluted in 5\times binding buffer containing 2.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM MgCl\(_2\), 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 20% glycerol, and 2.5 mM dithiothreitol (DTT). His-tagged FOXM1-DBD and biotinylated dsDNA were mixed at final concentrations of 200 and 50 nM, respectively. Before dsDNA was added, the compounds were preincubated with FOXM1-DBD in 10 \(\mu\)L for 10 min. The binding reactions were performed at 25°C for 30 min. And then 4 nM antibody and 250 nM of Streptavidin-XL665 diluted in detection buffer (5 mM EDTA, 200 mM NaCl, 50 mM N-(2-hydroxyethyl)piperazine-N'2-ethanesulfonic acid (HEPES) pH 7.5, and 0.02% Brij-35) were added and induced reaction at 25°C for 1 h. We detected the TR-FRET signals using a multi-label reader (Envision, Perki nElmer, Inc., Waltham, MA, U.S.A.), with an excitation wavelength (320 nm) and an emission wavelengths (615 and 665 nm) options. The positive control signal was obtained from the binding reaction in the presence of 200 nM FOXM1-DBD, 50 nM biotinylated dsDNA for 30 min. The negative control was defined in the absence of FOXM1-DBD.

**Pilot Library Screening of Natural Compounds** The Korea Chemical Bank (KCB) collected natural products or natural product-like compounds similar to terpenes, flavonooids, stilbene, alkaloids, lignans, and saponins from about hundred kinds of plants, and selected a library of 1019 natural compounds based on molecular diversity criteria. The FOXM1 inhibitory effect of the natural product library was measured for % inhibition at a final concentration of 10 \(\mu\)M in a reaction volume of 20 \(\mu\)L, and compounds with 50% or more inhibition are considered hits. The Z\(^\prime\) factor and the signal-to-background (S/B) ratio were determined using columns land 2 (negative controls) and columns 3 and 4 (positive controls) in 32 wells of each plate, respectively.

**Cell Culture and Proliferation Assays** MDA-MB-231 and MCF-7 cells, the FOXM1 overexpressing breast cancer cell lines, were maintained at 1 x 10\(^6\) cells/mL in Dulbecco's modified Eagle's medium (High Glucose) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 \(\mu\)g/mL) and l-glutamine (2 mM) in a 37°C humidified atmosphere containing 5% CO\(_2\) and 95% air. For cell viability assay, the cells (5 x 10\(^5\) cells/well) in 96-well plates were treated with 30 \(\mu\)M test compounds for the indicated periods. WST [water soluble terazolium salt, 20 \(\mu\)g/mL; 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium] solution was added to 100 \(\mu\)L of cell and incubated for 3 h. The absorbance was measured with multi-label reader (Envision, PerkinElmer, Inc., Waltham, MA, U.S.A.) at 450 nm. The absorbance of formazan formed in control cells with dimethyl sulfoxide (DMSO) treated only was considered to be 100% of the viability.

**Real-Time Quantitative PCR Analysis** The mRNA expression level of FOXM1 and FOXM1 downstream target was determined by real-time quantitative PCR service in COSMO genetech (Sungsoo, Seoul, Korea). MDA-MB-231 cells were cultured and treated with 30 \(\mu\)g/mL of five hit compounds, similar to the proliferation assay. Total RNA was extracted from cultured cells using an RNaseasy mini kit (Qiagen, Valencia, CA, U.S.A.). Gene expression was measured by real-time quantitative PCR (RT-qPCR) using a Bio-Rad CFX96 system (Hercules, CA, U.S.A.). CDNA

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Statistical Analysis All values were expressed as mean ± standard deviation (S.D.). The data were analyzed by one-way ANOVA, followed by Dunnett’s tests for multiple comparisons (Sigma Stat, Jandel Co., San Rafael, CA, U.S.A.). Concentration–response curves were analyzed by nonlinear regression functions using PRISM version 5.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). For the Z’ factor, representing the quality indication of the assay itself, it was analyzed as $1-\frac{[3 \times \text{S.D.}_c]+[3 \times \text{S.D.}_n]}{\text{Mean}_c+\text{Mean}_n}$. S.D. c+ and S.D. c− are the standard deviations of the positive and negative control signals, respectively, and Mean c+ and Mean c− represent the means of the TR-FRET counts of the positive and negative control signals, respectively. In all comparisons, statistically significances were defined as $p$-values $<0.05$.

RESULTS

Development of the HTS Assays The TR-FRET-based binding assay measures a FRET pair between a donor and a receptor labeled with fluorophore. We generated a quaternary complex of His×6-tagged FOXM1-DBD, oligomeric DNA, a europium (Eu) cryptate-labeled anti-His×6 monoclonal anti-

Fig. 1. Schematic of FOXM1-DBD Binding Assay

The TR-FRET-based FOXM1 binding assay was performed using the FRET pair between the donor Eu fluorophore and a receptor XL665 fluorophore following the generation of a quaternary complex of oligomeric DNA, His×6-tagged FOXM1-DBD, streptavidin-XL665 and a europium (Eu) cryptate-labeled anti-His×6 monoclonal antibody. The FRET signaling is related to the generation of quaternary complex, thus binding of oligomer and FOXM1-DBD can be monitored.

Fig. 2. Optimization of the Working Concentration of FOXM1-DBD (A, B) and Oligomer DNA (C, D) in TR-FRET Based FOXM1-DBD Binding Assay

(A) An increasing amount of FOXM1-DBD (16–1000 nM) was titrated with 100 nM Oligomer DNA (Bio-TEG-dsDNA) for 30 min in the absence (△) or the presence (●). (B) Specific binding to measure the $K_d$ value and $B_{max}$ was obtained by subtracting nonspecific binding (▲) from total binding signals (★). (C) A various concentration of oligomer DNA (8–500 nM) was incubated with 200 nM FOXM1-DBD (200 nM) for 30 min in the absence (△) or the presence (●). (D) Specific binding to measure the $K_d$ value and $B_{max}$ was obtained by subtracting nonspecific binding (▲) from total binding signals (★).
body, and streptavidin-XL665 and then performed the FOXM1 binding assay based on TR-FRET using a FRET pair between a donor Eu fluorophore and a receptor XL665 fluorophore (Fig. 1). Initially, FOXM1-DBD titrations were implemented at different type of concentrations (from 16 to 1000 nM) using 100 nM biotinylated dsDNA to determine the optimal FOXM1-DBD concentration. We confirmed that the concentration of FOXM1-DBD, which has sufficient separation between total and non-specific binding (Fig. 2A), affects the TR-FRET count, and the maximum specific binding was reached at 1000 nM of FOXM1-DBD concentration. We confirmed that the concentration of FOXM1-DBD, which has sufficient separation between total and non-specific binding (Fig. 2A), affects the TR-FRET count, and the maximum specific binding was reached at 1000 nM of FOXM1-DBD concentration. A typical dose–response binding curves was shown with increasing dsDNA concentration, and the $K_d$ and $B_{max}$ of substrates was analyzed to be 45.67 ± 5.05 nM and 40120 ± 1680 TR-FRET counts, respectively. Therefore, in further experiments, the FOXM1-DBD and dsDNA concentrations were set to 200 and 50 nM, respectively.

**Assay Quality Control and Validity** In the high-throughput format of TR-FRET-based FOXM1-DBD binding assays, quality control metrics was assessed as $Z'$ factor and S/B ratio through the comparison of positive and negative controls in wells (Fig. 3). The S/B ratio represents the degree of the separation of positive and negative control data, and the $Z'$ factor is a standard commonly used to assess the quality of a binding assay with a screening tool that involves many comparisons. The S/B ratio and the $Z'$ factor of the TR-FRET-based FOXM1-DBD binding assays were analyzed to be 0.73 and 7.46, respectively. The results of the developed TR-FRET-based FOXM1-DBD binding assay are expected to be applicable to HTS with high reproducibility and sensitivity.

The inhibitory response was measured by reaction with 200 nM FOXM1-DBD (○) or with buffer (●). The positive control signal (○) was obtained from the binding reaction in the presence of 200 nM FOXM1-DBD, 50 nM biotinylated dsDNA for 30 min. The negative control (●) was defined in the absence of FOXM1-DBD.

**Pilot Library Screening to Identify FOXM1-DBD Inhibitors** We performed pilot library screening of 1,019 natural compounds to identify inhibitors of FOXM1-DBD binding. In runs with the high-throughput format, the S/B ratio was 2.83 ± 0.55 and $Z'$ factor was 0.52 ± 0.07, which is an indication of assay robustness. It was identified that a total of 15 hit compounds showed more than 60% inhibition at 10 µM (Fig. 5). Among these, five compounds with an inhibition rate of more than 80% such as ACILXN, BRN5, ganaleoidin, leoidin, and roemerine were selected to measure the IC$_{50}$ value. These compounds had an IC$_{50}$ value in the range of 1.3 to 8.7 µM (Fig. 6, Table 1).
Anti-proliferation Effects of Hits Classified MDA-MB-231 and MCF-7 cells, the FOXM1 overexpressing breast cancer cell lines, were treated with five hit compounds at 30 µM for 24, 36, and 72 h to determine their anti-proliferation effects. As shown in Fig. 7, AC1LXM, BRN5 and roemerine demonstrated more potent inhibition of cellular proliferation than that by FDI-6. Gangaleoidin and leoidin showed similar effects with FDI-6.

Effects of Hits on FOXM1 and Its Downstream Target Expression at the Transcriptional Level

The effect of five hit compounds on mRNA expression of FOXM1c (auto-regulation) and the well-known FOXM1 downstream target such as CCNB1 and CDC25b were investigated in MDA-MB-231 cells. A decrease in FOXM1 transcription was observed in AC1LXM, BRN5, gangaleoidin and leoidin-treated groups. In the downstream transcriptional analysis such as CCNB1 and CDC25B, the transcription of CCNB1 was decreased in AC1LXM, BRN5, gangaleoidin and leoidin treated groups and the transcription of CDC25 was decreased in AC1LXM and BRN5 treated groups (Fig. 8).

DISCUSSION

In this study, we developed a FOXM1-DBD binding assay applying TR-FRET and searched for FOXM1-DBD inhibitors through a pilot library screening of 1019 natural compounds.

TR-FRET-based techniques have been introduced into development of assays to analyze enzyme activity such as kinases, peptidases, ubiquitin ligases, and deubiquitinases. These techniques are characterized by excellent decay times and have high stability because it can efficiently distinguish short-term background fluorescence observed in small molecules or compounds found in biological fluids based on the long-term fluorescence emission half-life of lanthanides. Thus, time-resolved fluorescence signals can be measured very reliably and reproducibly. Its application results in an excellent

Table 1. Significant Hits from Pilot Library Screening

| Compound  | Description | TR-FRET-based FOXM1-DBD binding assay | % Inhibition at 10µM | IC50 value (µM) |
|-----------|-------------|---------------------------------------|----------------------|----------------|
| AC1LXM    |             |                                       | 100.6                | 1.3 ± 0.4      |
| BRN5      |             |                                       | 80.2                 | 8.7 ± 1.3      |
| Gangaleoidin |        |                                       | 90.7                 | 2.8 ± 0.7      |
| Leoidin   |             |                                       | 89.3                 | 4.3 ± 0.8      |
| Roemerine |             |                                       | 82.8                 | 8.7 ± 1.4      |
| FDI-6     | FOXM1-DBD binding inhibitor (reference) | 90.1                 | 4.2 ± 1.1        |

Fig. 5. Pilot Screening of a 1019 Natural Compounds Library
Scatter plots of 1019 natural compounds for % inhibition of FOXM1-DBD binding.

Fig. 6. Structures of AC1LXM, BRN5, Gangaleoidin, Leoidin, and Roemerine
Five compounds including AC1LXM, BRN5, gangaleoidin, leoidin, and roemerine that exhibited >80% inhibition in TR-FRET-based FOXM1-DBD binding assay.
S/B ratio and superior sensitivity of the analysis. In this study, to develop the TR-FRET-based FOXM1-DBD binding assay, an approach was taken using a FRET pair between the donor Eu fluorophore and a receptor XL665 fluorophore. This pair generated from a quaternary complex, which consisted of a dsDNA comprising tandem repeats of a consensus sequence, a His$_6$-tagged FOXM1-DBD, streptavidin-XL665 and an Eu-labeled anti-His$_6$ monoclonal antibody. The dsDNA probes, 5'-biotin-TEG-AAA CAA ACA AAC AAT C-3' and 5'-GAT TGT TTG GTT GTT T-3', contain tandem repeats known as FOXM1 recognition motif on DNA that activate proliferation- and differentiation-associated genes. A biotin and TEG are connected to the 5' end of the forward strand for binding streptavidin-XL665 and minimizing steric hindrance between the biotin moiety and the oligomer, respectively. In an optimized assay condition, concentrations of 200 nM FOXM1-DBD, 50 nM biotin-TEG-oligomer, 62.5 nM XL665, and 2.5 ng/well of Eu-tagged His-antibody demonstrated a reasonable S/B of 7.46 and Z' factor of 0.74. These S/B and Z' factor mean that it showed high sensitivity and reproducibility suitable for HTS assays. In a typical assay, the unlabeled dsDNA showed a concentration-dependent inhibitory response during increasing unlabeled dsDNA to a pre-bound complex between FOXM1-DBD and labeled dsDNA probe. This demonstrated the specificity and reversibility of dsDNA and FOXM1-DBD binding. The IC$_{50}$ values of FDI-6 as a known FOXM1-DBD binding inhibitor was 4.24 ± 1.07 µM, which is better than previously reported value based on EMSA (22.5 ± 12.3 µM). These results might be attributed to the different sensitivity of both TR-FRET and EMSA-based assay. The TR-FRET-based FOXM1-DBD binding assay reduced the time required to perform assay and increased reproducibility because of the mix-and-read approach, resulting in significantly higher reliability than the EMSA-based assay. Thus, the TR-FRET based FOXM1-DBD binding assay showed an alternative approach for HTS to detect the inhibitors of FOXM1–DNA interaction. This TR-FRET based FOXM1-DBD binding assay was applied to pilot library screening and identified hit compounds capable of inhibiting the FOXM1–DNA interaction. Screening was done with an S/B ratio value of 2.83 ± 0.55 and a Z' factors of 0.51 ± 0.07, suggesting that HTS was performed with sufficient sensitivity and reproducibility. Five hit compounds (AC1LXM, BRN5, gangaleoidin, leoidin, and roemerine) from the 15 initial hit compounds exhibited a typical concentration-dependent inhibitory response with an IC$_{50}$ ranging from 1.3 to 8.7 µM. BRN5 and roemerine possess anticancer activities, such as the suppression of pancreatic cancer proliferation and cytotoxic activity in human breast or colon cancer cells (MCF7 or HCT116), respectively. The anti-cancer effects of AC1LXM, leoidin and gangaleoidin has not yet been reported. However, tetrahydroisoquinoline-based compounds, such as AC1LXN derivatives, exhibited anti-tumor activity targeting microtubule polymerization and broad spectrum of...
cytotoxic activity against various cancer cells. In addition, leoinid derivatives, namely, physciosproin and panarin demonstrate anti-cancer activity in colorectal cancer cells and prostate carcinoma DU-145 cells, respectively. In cell proliferation assay performed with MDA-MB 231 and MCF-7 breast cancer cells that demonstrate a highly expression of FOXM1, all five compounds inhibited breast cancer cells proliferation. AC1LXM, BRN5 and roemerine more potently inhibited cell proliferation than FDI-6, and gangaleoidin and leoidin showed similar effects to FDI-6 in cell viability assays. Next, we observed changes in mRNA expression levels of FOXM1c as well as target genes by inhibiting the binding between FOXM1 and target DNA. However, in the case of roemerine, it does not affect gene expression. Therefore, it is possible that some unknown off-target effects of roemerine may contribute to suppressing breast cancer cell proliferation. The TR-FRET-based binding assay exhibits different efficacy than the cell-based proliferation assay and mRNA expression qPCR assays because of the physicochemical properties of hit compounds such as cell membrane permeability, solubility and stability in media. Since FOXM1 is an important transcription factor for mitosis implementation, cell cycle progression and tumorigenesis, it can reduce breast cancer proliferation by inducing cell cycle arrest and mitotic inhibition by inhibiting of FOXM1–DNA interaction. Therefore, out findings suggest that AC1LXM, BRN5, gangaleoidin and leoidin can inhibit breast cancer cell proliferation by inhibiting FOXM1–DNA interaction.

In summary, we screened FOXM1–DNA interaction inhibitors using TR-FRET-based FOXM1–DBD binding assay and identified five potent FOXM1–DNA interaction inhibitors. Such TR-FRET-based FOXM1–DBD binding assay is considered a powerful tool for screening direct protein–DNA interactions.

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Conflict of Interest The authors declare no conflict of interest.

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