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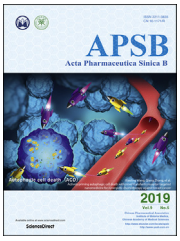

ORIGINAL ARTICLE

Orthogonal assays for the identification of inhibitors of the single-stranded nucleic acid binding protein YB-1

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Abstract We have previously shown that high expression of the nucleic acid binding factor YB-1 is strongly associated with poor prognosis in a variety of cancer types. The 3-dimensional protein structure of YB-1 has yet to be determined and its role in transcriptional regulation remains elusive. Drug targeting of transcription factors is often thought to be difficult and there are very few published high-throughput screening approaches. YB-1 predominantly binds to single-stranded nucleic acids, adding further difficulty to drug discovery. Therefore, we have developed two novel screening assays to detect compounds that interfere with the transcriptional activation properties of YB-1, both of which may be generalizable to screen for inhibitors of other nucleic acid binding molecules. The first approach is a cell-

Abbreviations: cDNA, complementary DNA; CSD, cold shock domain; CTD, C-terminal domain; DMSO, dimethylsulfoxide; dsDNA, double-stranded DNA; E2F1, E2F transcription factor 1; *EGR1*, early growth response 1; HTS, high-throughput screening; NTD, N-terminal domain; shRNA, short-hairpin RNA; siRNA, small-interfering RNA; ssDNA, single-stranded DNA; YB-1, Y-box binding protein-1; *YBX1*, Y-box binding protein gene 1

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based luciferase reporter gene assay that measures the level of activation of a fragment of the *E2F1* promoter by YB-1. The second approach is a novel application of the AlphaScreen system, to detect interference of YB-1 interaction with a single-stranded DNA binding site. These complementary assays examine YB-1 binding to two discrete nucleic acid sequences using two different luminescent signal outputs and were employed sequentially to screen 7360 small molecule compounds leading to the identification of three putative YB-1 inhibitors.

1. Introduction

Y-box binding protein-1 (YB-1) is a multifunctional nucleic acid binding protein that preferentially binds single-stranded DNA (ssDNA) and RNA. It also binds double stranded DNA (dsDNA). YB-1 regulates gene expression at both transcriptional and translational levels[1–3] and is involved in the splicing, packaging and stabilization of mRNA, as well as DNA replication and repair[4]. These diverse functions appear to share a common theme of direct or indirect nucleic acid binding, through which YB-1 can influence a multitude of cellular processes that are disturbed during cancer[5].

YB-1 appears to be a driver of many cancer types including tumors of the breast, ovary, intestine, lung, liver, prostate, skin and blood[5,6]. In breast cancer, the highest expression levels of Y-box binding protein gene 1 (*YBX1*), the RNA that encodes YB-1, are found in the most aggressive and rapidly proliferating tumor subtypes[7]. *YBX1* RNA levels provide a significant indicator of breast cancer patient prognosis[8–10] and in a rapidly proliferating breast cancer cell line, YB-1 promotes resistance to paclitaxel *via* its downstream target early growth response 1 (*EGR1*)[11]. In addition to breast cancer, small-interfering (si)RNA-mediated knockdown of YB-1 inhibits the growth of tumor cell lines of several other histological types[7,12]. For example, *in vitro* short-hairpin (sh)RNA-mediated knockdown of YB-1 reduces melanoma cell proliferation, migration and invasion, decreases drug resistance, and increases apoptosis[13]. Conversely, increased expression of YB-1 correlates with melanoma progression[13,14] and epithelial-to-mesenchymal transition[15].

YB-1 has been shown to preferentially transactivate genes encoding proteins involved in cellular proliferation[16], including cyclins[17], E2F transcription factor 1 (E2F1) targets and E2F family members[7], and is highly expressed in tumors with a high mitotic index[7] or resistant to chemotherapy[18].

YB-1 consists of a short, 51-residue N-terminal domain (NTD), a 78-residue cold shock domain (CSD), and a large, 195-residue C-terminal domain (CTD)[19]. The CSD is evolutionarily conserved with homologues found across mammalian species like primates, rodents, rabbits, bats and cats[4]. While a prediction of the YB-1 structure was recently made[4], only the CSD structure has been determined using NMR[20]. The 3-dimentional (3-D) structure of the NTD and CTD are still unknown, possibly because they are usually disordered, only becoming rigid upon ligand binding, and may vary when bound to different ligands. This lack of a rigid structure may enhance YB-1's capacity to interact specifically with a variety of ligands[6]. However, without 3-D structures of the NTD and CTD, it is not possible to conduct rational and structure-based drug design[21]. Therefore, we developed functional assays to identify compounds that inhibit YB-1 activity.

2. Materials and methods

2.1. Cell culture

HCT116 (colon cancer; American Type Culture Collection (ATCC), Manassas, VI, USA) and MDA-MB-231 (breast cancer; ATCC) cells were cultured in RPMI 1640 (ThermoFisher, Waltham, MA, USA) supplemented with 5% (*v*/*v*) fetal bovine serum (FBS) and 1% (*v*/*v*) penicillin-streptomycin-glutamine (PSG; ThermoFisher). A375 (melanoma; ATCC) cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM; ThermoFisher) supplemented with 5% (*v*/*v*) FBS (ThermoFisher) and 1% (*v*/*v*) PSG. Cells were grown in a humidified incubator at 37 °C with 5% (*v*/*v*) CO_2.

2.2. Reporter gene assay

A 728 base-pair *E2F1* promoter fragment[22] was cloned into the pGL4.17 vector (Promega, Fitchburg, WI, USA) upstream of a firefly luciferase reporter gene to create the pGL4.17-E2F1-728 plasmid. Cloning was confirmed by restriction digest and sequencing.

To establish a cell-based luminescence assay capable of measuring the activity of YB-1, HCT116 cells were transfected with the pGL4.17-E2F1-728 plasmid. Through this promoter fragment, endogenous YB-1 activates transcription of the luciferase reporter gene[7]. In addition to YB-1, the transcription factor E2F1 autonomously binds and increases the activity of the promoter of its encoding gene *E2F1*[22]. Increased transcription of the luciferase gene leads to a greater amount of luciferase protein, which is proportional to the amount of luminescence produced as a result of bioluminescent reactions catalyzed by the activity of luciferase upon addition of its substrate.

An inhibitor of YB-1 activity was required as a control to validate this *E2F1* promoter: luciferase reporter gene assay. YB-1 has previously been shown to strongly bind ($K_d \sim 4$ nmol/L) to a promoter fragment of the human γ-globin genes either in cells or cell-free systems[23,24]. This sequence has been used to isolate YB-1 from cellular extracts by affinity purification[23]. A decoy oligonucleotide containing the same sequence (5′-CCTCCCACCCTCCC-CACCCTCCCCACCCTCCCC-3′) was constructed and used in excess molar amounts to be bound by YB-1, thereby mimicking or modeling inhibition of YB-1 binding to other nucleic acids.

HCT116 cells were seeded into 100 mm cell culture dishes 12–18 h prior to transfection with 8 μg of pGL4.17-E2F1–728 plasmid DNA by Lipofectamine 3000 (ThermoFisher). A parallel transfection was performed with this plasmid and 5 nmol of decoy oligonucleotide. After incubation for 6 h at 37 °C, cells were re-suspended and dispensed into 384-well plates at 8000 cells/well.

Eight hours thereafter, screening compounds in dimethylsulfoxide (DMSO) were dispensed by robot [final concentration of DMSO was 0.5% (*v*/*v*)]. An equivalent amount of DMSO without compound was added to control wells containing transfected cells with or without decoy oligonucleotide. Thirty-six hours after transfection, each well received 30 μL of SteadyGlo luciferase Substrate (Promega), incubated at room temperature for 20 min and measured for luminescence using an EnSpire® Multimode Plate Reader (PerkinElmer, Boston, MA, USA). IC_{50} concentrations were calculated by fitting data to dose-response equations, and then calculating concentrations at which the relative luminescent signal is 50% of that of the control wells.

2.3. *AlphaScreen assay*

An AlphaScreen assay system was adapted to screen compounds that inhibit YB-1 binding to ssDNA, which is a biotinylated oligonucleotide containing a 3 × repeat of the promoter fragment of human γ-globin genes.

AlphaScreen acceptor beads (PerkinElmer) were conjugated, according to the manufacturer's instructions, to a polyclonal sheep anti-YB-1 antibody generated as previously described[23]. Fifty μL AlphaScreen reactions were performed in 96-well OptiPlates (PerkinElmer) using PBS with 0.2% (*w*/*v*) bovine serum albumin (MilliporeSigma, Burlington, MA, USA) buffer. The reactions were set up as follows, with final reaction concentrations given in parentheses. Dispensed into each well was 20 μL of buffer containing purified YB-1 protein[23] (40 fmol/L), with control wells also receiving decoy oligonucleotide (1 pmol/L). After 30 min incubation at room temperature, each well received 10 μL of buffer containing antibody-conjugated AlphaScreen acceptor beads (20 μg/mL) and the biotinylated 3 × repeat oligonucleotide (2.5 fmol/L). Plates were then incubated in darkness for 60 min at room temperature before addition of 20 μL of buffer containing streptavidin-coated AlphaScreen donor beads (20 μg/mL; PerkinElmer). Following another 60 min incubation in the dark, plates were read on the Enspire® Multimode Plate Reader, with excitation and emission detection wavelengths of 680 and 570 nm, respectively. IC_{50} concentrations were calculated by fitting data to dose-response equations, and then calculating concentrations at 50% of the maximal response. As a complementary screen to eliminate false positives, an AlphaScreen TruHits Kit (PerkinElmer) was used according to the manufacturer's instructions. This kit consists of acceptor beads and donor beads that form a complex *via* a streptavidin to biotin interaction which results in emission of a luminescent signal. This signal can be reduced by compounds capable of interfering with these fundamental AlphaScreen assay system components.

2.4. *Screening compounds*

For the primary screening, 7360 small molecule compounds from the Chinese National Compound Library in Shanghai (http://en.cncl.org.cn/) were used. For compounds of interest that were re-ordered for evaluation experiments, compound identity and purity were confirmed by nuclear magnetic resonance (NMR) and mass spectra.

2.5. *Computational filtering*

Computational filtering was performed on compound structures to remove samples possessing specified traits or containing certain substructures. Filters were applied using the SYBYL-X 2.11 software (Certara, Princeton, NJ, USA) with compound structure inputted in Structure Data File (SDF) format. The first filter eliminated compounds containing substructures identified as pan-assay interference compounds (PAINS)[25]. Five increasingly stringent filters were applied to eliminate groups unfavorable for drug development, such as groups with toxicity, poor pharmacokinetic behavior or that are highly electrophilic. The filters applied, from least stringent to most stringent, were: WEHI_93K, Baell 2013 Filters 1, 2 and 3, and the CTX filter[26].

2.6. *Cell enumeration assay*

Cell lines A375, MDA-MB-231 or HCT116 were seeded at approximately 2000 cells/well into 96-well plates. After allowing 2 h for adhesion, cells were treated with a range of concentrations of three putative YB-1 inhibitors identified during the high-throughput screening (HTS) campaign. Each of these was diluted in DMSO, with DMSO without compound added to control cells [final concentration of DMSO was 0.5% (*v*/*v*)]. DNA content was measured, as a surrogate for adherent live cell numbers, using a SYBR Green I-based fluorimetric assay as described previously[11]. In brief, cells were incubated with diluted compound or DMSO only for 24, 48 or 72 h at 37 °C, medium was then removed and plates frozen at –80 °C. Plates were thawed before each well received SYBR Green I Nucleic Acid Gel Stain (ThermoFisher) diluted 1:4000 (*v*/*v*) in lysis buffer [10 mmol/L Tris–HCl pH 8.0, 140 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% (*v*/*v*) Triton X-100, 0.1% (*w*/*v*) sodium deoxycholate, 0.1% (*w*/*v*) sodium dodecyl sulfate (SDS)]. After 8 h of incubation at room temperature, fluorescence was measured with excitation/emission wavelengths of 485 nm/535 nm to derive a signal proportional to cell number for each well. IC_{50} concentrations were calculated by fitting data to dose-response equations, and then calculating compound concentrations at 50% of the control cell signal.

2.7. *Reverse transcription-quantitative PCR*

Six-well tissue culture plates were seeded with 160,000 MDA-MB231 cells. After allowing 2 h for adhesion, cells were treated with three putative YB-1 inhibitors (20–160 μmol/L) before incubation at 37 °C. Equivalent volumes of DMSO were added to control wells to a final concentration of 0.5% (*v*/*v*). Ten h after addition of compounds, medium was removed and plates were frozen at −80 °C. Cellular RNA was extracted using TRIzol reagent (ThermoFisher) according to the manufacturer's instructions.

cDNA was synthesized in reverse transcription reactions using SuperScript IV (ThermoFisher) according to the manufacturer's instructions. Upon completion of reverse transcription, reactions were diluted 1:3 prior to 1 μL being used in each 10 μL reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Primers were used to amplify *EGR1* and *LAMIN* transcripts as previously described[11]. RT-qPCR was performed using SYBR Select Master Mix (ThermoFisher) according to the manufacturer's instructions on a QuantStudio 12 K Flex Real-Time PCR system (ThermoFisher). Data were analyzed by normalizing to the reference transcript (*LAMIN*) and the level of expression relative to the DMSO-treated control cells was calculated using the $2^{\Delta ddCt}$ method[27].

3. Results

In order to discover potential YB-1 inhibitors, two complementary assays were developed to detect modulation of its transcription

factor activity or inhibition of its binding to a specific nucleic acid sequence. The first assay was based on the transcriptional activation of the *E2F1* promoter by YB-1[7] and employed a cell-based luciferase reporter gene system to screen for inhibitors. The compounds identified by this method were then tested in a novel AlphaScreen assay, using a single-stranded oligonucleotide which YB-1 is known to bind with high affinity[23,24], in order to identify compounds that also interfere with YB-1 binding to this sequence. The sequential HTS process involving 7360 compounds is shown in Fig. 1.

3.1. *Primary screening*

In HCT116 cells transfected with the pGL4.17-E2F1-728 plasmid, we observed that co-transfection of increasing concentrations of the decoy oligonucleotide reduced the level of *E2F1* promoter-driven luciferase activity (Fig. 2A). As the decoy oligonucleotide concentration increased, the luciferase activity decreased, but at a diminishing rate. This, along with the saturation of this effect at the highest oligonucleotide concentrations, suggests that the decoy oligonucleotide competed with the *E2F1* promoter for binding by YB-1, and thus decreased transcriptional activation of the *E2F1* promoter by YB-1. Maximal inhibition was observed with 800 nmol/L of decoy oligonucleotide, so subsequent screening experiments used 1 μmol/L of decoy oligonucleotide, exceeding the concentration of maximal inhibition, as a control to generate a luminescent signal that was designated "100% inhibited". To exhibit luminescent signal representing reporter gene activity that has not been inhibited, *i.e.*, 0% inhibition, we used cells transfected with only the pGL4.17-E2F1-728 plasmid without compound treatment. The decrease in luminescent signal effected by each compound was mapped as a percentage inhibition between these two controls representing 0% and 100% inhibition.

In total, 7360 compounds were screened on twenty-three 384-well plates (Fig. 1 and Supporting Information Table S1) at concentrations of 10 μmol/L. The Z' factor defined by Zhang et al.[28] was used to evaluate assay quality[29]. The Z' factors for the HTS

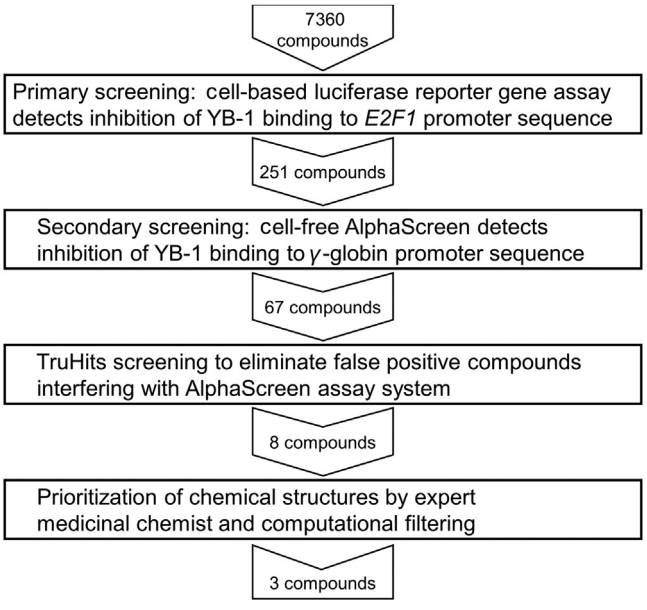


Figure 1 Process of screening for compounds that inhibit YB-1 nucleic acid binding. A sequential screening approach was used to reduce 7360 starting compounds to three putative YB-1 inhibitors.

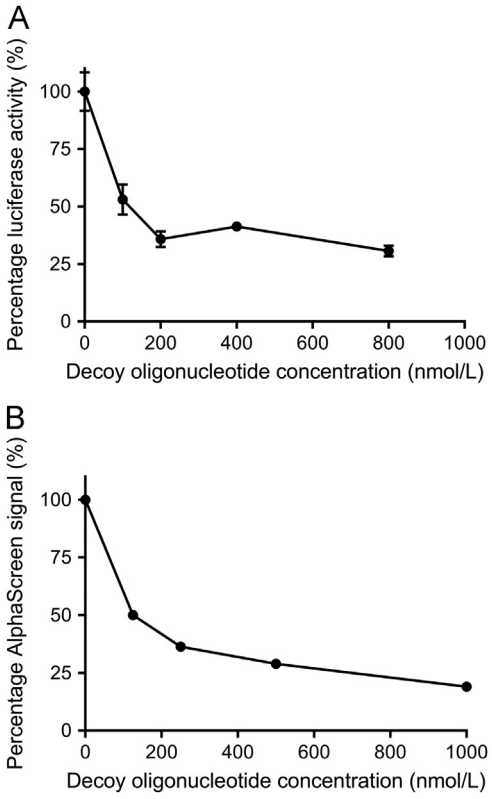


Figure 2 Competitive inhibition of *E2F1* promoter activation (as measured by luciferase activity) and YB-1 binding to oligonucleotide (as measured by AlphaScreen signal) by the decoy oligonucleotide. In the luciferase and AlphaScreen assays, the decoy oligonucleotide competes with other nucleic acid sequences for binding by YB-1. (A) YB-1 activation of the *E2F1* promoter decreases with increasing concentrations of decoy oligonucleotide. Data shown are the mean of three replicates at each decoy oligonucleotide concentration within one experiment, with error bars indicating standard error of the mean (SEM). Results shown here are representative of three independent experiments. (B) YB-1 interaction with the binding oligonucleotide decreases with increasing concentrations of the decoy oligonucleotide, resulting in decreasing AlphaScreen signal. Data shown are the mean of two replicates at each decoy oligonucleotide concentration within one experiment. Results shown here are representative of five independent experiments.

campaign involving 23 plates were within the range of 0 to 0.5 and transfection was performed in six batches. There was strong evidence that the Z' factor was significantly affected by transfection batch ($^{***}P=0.000191$), and was negatively correlated to the standard deviation of the 0% inhibited control well signal (correlation coefficient $=-0.94$).

Compounds were prioritized for advancement to the secondary screening if their reduction of luciferase signal was at least 90% of that achieved by 1 μmol/L of the decoy oligonucleotide. For plates where five or fewer compounds were identified, the luminescent signal threshold was lowered to 80%. Each 384-well plate containing 320 compounds identified up to 26 hits, to give a total of 272 hit compounds that were prioritized for advancement. They were then re-tested to confirm inhibition of the *E2F1* promoter activity in this assay, from which, 251 compounds proceeded into the secondary screening (Table S1).

3.2. Secondary screening

An *in vitro* AlphaScreen assay was developed and used as an orthogonal, secondary screening for 251 initial hits. In contrast to the cell-based assay which used endogenous YB-1, this method utilized purified YB-1 incubated with an oligonucleotide concatemer containing three repeat sequences of the "decoy" oligonucleotide. In South-Western dot blot-based analysis, this concatemer is capable of increasing signal by stabilizing the interaction between protein and DNA[30]. It was confirmed in our study that the concatemer bound by YB-1 protein produces a higher signal than that of the single-repeat decoy oligonucleotide (data not shown).

We observed that the AlphaScreen signal decreased with addition of an increasing concentration of a decoy oligonucleotide (Fig. 2B), suggesting that this sequence competes with the oligonucleotide concatemer for binding by YB-1. A maximal level of signal inhibition occurred with 1 μmol/L decoy oligonucleotide and so this concentration was used in subsequent screening experiments as a "100% inhibition" control. The 251 initial hits were then tested in three sets (Fig. 1), each set contained eight 0% inhibition and four 100% inhibition control reactions. The decrease in signal caused by each compound was calculated as a percentage inhibition between these two controls. The Z' factor was calculated from the controls in each set and found to be 0.20, 0.37 and 0.56, respectively. The calculated percentage of signal reduction for the three sets was 33.2%. Sixty-seven compounds that reduced the luminescent signal by $>50\%$ were selected for further assessment. They were screened in the TruHits assay to eliminate false positives (Fig. 1) resulting in 8 confirmed hits (Table S1).

3.3. Computational filtering

To prioritize the 8 compounds identified above for suitability for drug development, their structures were subjected to computational filters (Fig. 1). The first filter eliminated compounds containing substructures identified as PAINS[25], which would indicate they are likely false positives. All eight compounds passed this PAINS filter. Then five increasingly stringent filters were applied to eliminate groups unfavorable for drug development, such as groups with toxicity, poor pharmacokinetic behavior or are highly electrophilic. These filters, from least stringent to most stringent, were: WEHI_93K, Baell 2013 Filters 1, 2 and 3, and the CTX filter[26]. The three least stringent filters passed 7 of the 8 structures, eliminating one compound as overly chemically reactive. None of the compounds passed the two most stringent filters, Baell 2013 Filter 3 and CTX, however this did not exclude them from further investigation (see Section discussions).

Next, medicinal chemistry expertise was utilized and as a result, a further three compounds were recognized as belonging to classes known to undergo colloidal aggregation, suggesting that they may still be false positives despite passing the TruHits assay and the PAINS filter. A further compound was identified as containing a bond vulnerable to hydrolysis, requiring modification if the compound was to progress as a drug lead. The remaining three compounds (RUS0207-A006, RUS0202-G005, and JK0395-B007, structures shown in Table 1) were assessed as being unlikely to be residual false positives, with structures sufficiently drug-like to warrant continued investigation as putative drug leads.

3.4. Functionality evaluation

In order to confirm the bioactivity of these three putative YB-1 inhibitors, they were independently re-synthesized and then tested in the same *E2F1* promoter:luciferase reporter gene and AlphaScreen assays at a range of concentrations to generate dose-response curves. It was shown that both luciferase activity (Fig. 3) and AlphaScreen signal (Fig. 4) were decreased with increasing concentrations of the compounds. In both assays, JK0395-B007 elicited the greatest signal reduction and AlphaScreen was more sensitive to low concentrations (6.25 and 12.5 μmol/L) of this compound than either of the other two.

Next, we studied the effect of these compounds on cancer cell proliferation to verify if they exert action consistent with reduction of YB-1 activity *in vitro*[7,11]. A375, HCT116 and MDA-MB-231 cells were grown for 72 h in the presence of the three compounds and the results (Fig. 5) showed that RUS0207-A006 and JK0395-B007 inhibited the proliferation of all three cell lines, with MDA-MB-231 cells appearing most sensitive. RUS0202-G005 did not display any significant inhibitory effect on these cell lines.

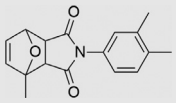
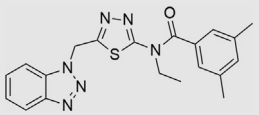
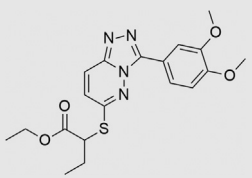
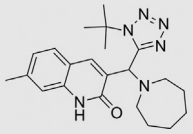
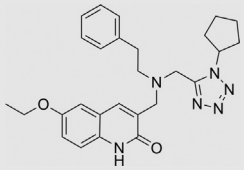
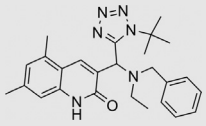
In order to determine whether these compounds were acting on-target, we further analyzed their effect on the transcription of a downstream mRNA target of YB-1. *EGR1* was selected for this purpose since previous studies have demonstrated that *EGR1* mRNA levels increase with reduction of YB-1 and/or *YBX1* mRNA in MDA-MB-231 cells, with *EGR1* postulated as a downstream target of YB-1[11]. The three compounds, at 20–160 μmol/L concentrations, were incubated with MDA-MB-231 cells and the levels of *EGR1* mRNA were found to increase in proportion to increasing concentrations of RUS0207-A006 and JK0395-B007 (Fig. 6). These effects were consistent with the hypothesis that these two compounds were acting as YB-1 inhibitors in cells.

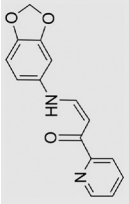
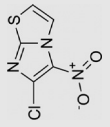
4. Discussions

The present study aimed at discovering sequential orthogonal assays to identify potential inhibitors of YB-1 *via* a pilot screening of a compound library. A novel AlphaScreen assay for protein: ssDNA interaction was developed and could be further adapted to detect binding of ssDNA or RNA by other nucleic acid binding proteins. As an ssDNA- and RNA-binding transcription factor with a dynamic and flexible protein structure, YB-1 is an unusual and potentially difficult target. Therefore, we screened a collection of diverse small molecules that cover a broader chemical space than some more focused libraries[31]. Of the 7360 compounds screened in the primary assay, 251 proceeded to the secondary AlphaScreen followed by the supplementary TruHits assay to eliminate false positive hits (Fig. 1). Prioritization of the remaining compounds identified three putative YB-1 inhibitors that were further evaluated for their effect on tumor cell growth *in vitro* and on the mRNA level of an YB-1 target. Two of them caused a dose-dependent increase in the expression of *EGR1,* a downstream target of YB-1.

The *E2F1* promoter:luciferase reporter gene assay was used as the primary screening due to its relatively low cost per reaction and its ability to identify compounds that not only inhibit YB-1 binding to *E2F1* promoter, but may also perturb the interaction between YB-1 and E2F1 or other protein co-factors. Theoretically, this cell-based assay has the advantage of discovering compounds that are biologically relevant for future *in vivo* studies. For example, only compounds that passed the cell membrane would be active. We also observed that Z' factors were significantly affected by each batch of transfected cells, suggesting variabilities in transfection efficiency. While the percentage inhibition of signal

Table 1 Chemical structures and bioactivities of eight hit compounds identified by screening.

Compd.	CAS registry number	Structure	Chemical formula	Molecular weight	IC_{50} Reporter gene assay (μmol/L)	IC_{50} Alpha Screen assay (μmol/L)	Percentage inhibition (%) in primary screening (reporter gene assay)	Percentage inhibition (%) in secondary screening (Alpha Screen)	Percentage non-specific inhibition (%) in TruHits screening to eliminate false positives	Medicinal chemist's annotation
RUS0207-A006	497917-11-0		$C_{17}H_{17}NO_3$	283.322	73	41	76	71	13	Related to compound BMS-641988, a novel androgen receptor antagonist for the treatment of prostate cancer Possible co-polymer
RUS0202-G005	602283-51-2		$C_{20}H_{20}N_6OS$	392.477	59	27	99	65	−24	Class known to have antifungal/antimicrobial activity
JK0395-B007	852437-97-9		$C_{19}H_{22}N_4O_4S$	402.467	30	25	80	59	13	Triazolopyridazines patented as protein kinase inhibitors, both broadly, and specifically for inhibition of LRRK2. Inhibition of GABA-A also published
RUS0016-B003	460073-26-1		$C_{22}H_{30}N_6O$	394.513	–	–	103	90	−9	Class known to undergo colloidal aggregation (dominant mechanism for artifactual inhibition of proteins*), patented for altering eukaryote lifespan
RUS0020-D011	460070-80-8		$C_{27}H_{32}N_6O_2$	472.582	–	–	103	58	16	Class known to undergo colloidal aggregation (dominant mechanism for artifactual inhibition of proteins*), patented for altering eukaryote lifespan
RUS0028-G002	516450-12-7		$C_{26}H_{32}N_6O$	444.572	–	–	104	81	13	Predicted to aggregate by Aggregator Advisor Database*, patented as inhibitors of lymphoid tyrosine phosphatise and urea channel protein

RUS0116-H006	1164455-04-2		$C_{15}H_{12}N_2O_3$	268.267	–	–	96	50	6	Patented for altering eukaryote lifespan, related to compounds as antitumor agents against human cancer cell lines. May not be stable *in vivo*, as it contains an easily hydrolyzed bond
RUS0116-G009	23576-89-8		$C_5H_2ClN_3O_2S$	203.606	–	–	101	148	10	Nitro group too chemically reactive, positive in Ames mutagenicity assay

The three putative YB-1 inhibitors (RUS0207-A006, RUS0202-G005 and JK0395-B007) are shown in the top three rows with estimated IC_{50} values. *www.advisor.bkslab.org. –Not applicable.

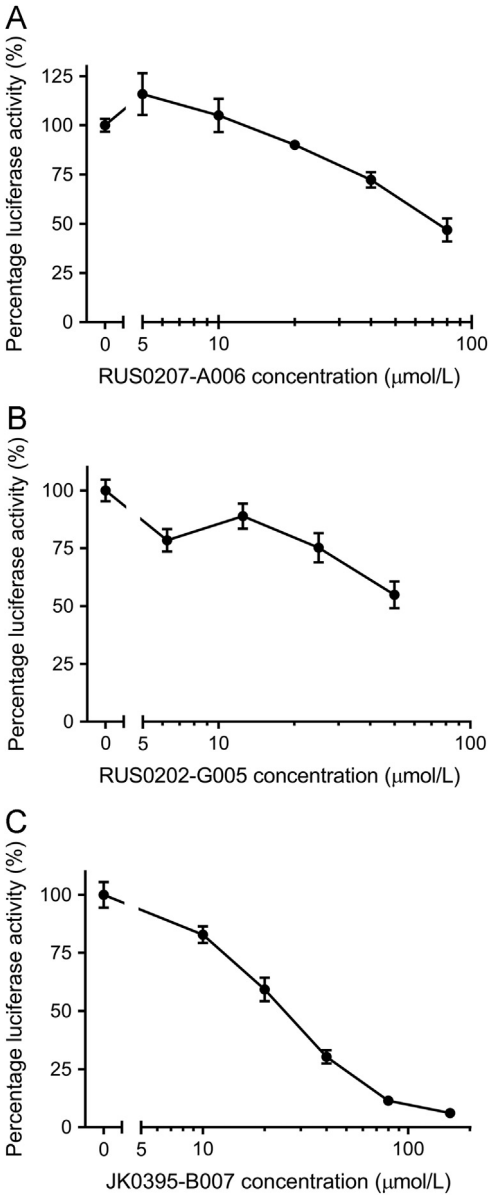


Figure 3 Effect of the three putative YB-1 inhibitors on *E2F1* promoter:luciferase reporter gene activity in cancer cell lines. Results shown here are representative of two independent experiments. (A) Effect of RUS0207-A006 on luciferase activity in A375 cells (IC_{50} = 73 μmol/L). Data shown are the mean of four replicates at each compound concentration within one experiment, with error bars indicating SEM. (B) Effect of RUS0202-G005 on luciferase activity in HCT116 cells (IC_{50} = 59 μmol/L). Data shown are the mean of three replicates at each compound concentration within one experiment, with error bars indicating SEM. (C) Effect of JK0395-B007 on luciferase activity in HCT116 cells (IC_{50} = 30 μmol/L). Data shown are the mean of three replicates at each compound concentration within one experiment, with error bars indicating SEM.

exhibited by each compound was calculated relative to the control wells on each screening plate, there may be some difficulty comparing these values across batches. However, within a single plate the values can be expected to be relative and comparable. Therefore, the threshold for identifying hit compounds was lowered when five or fewer compounds were identified in a single

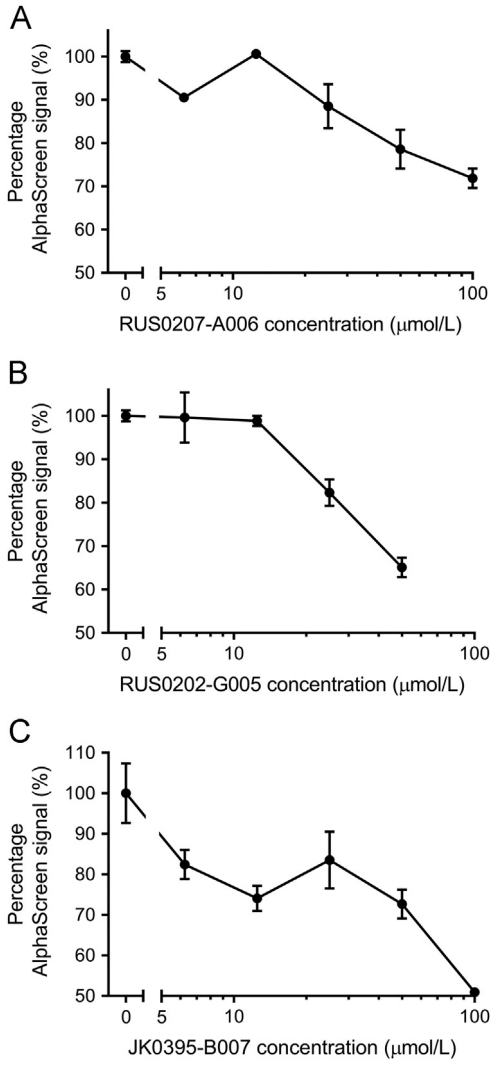


Figure 4 Effects of the three putative YB-1 inhibitors on YB-1 binding to decoy oligonucleotide, tested at a range of concentrations by AlphaScreen assay. Data shown are the mean of two replicates at each compound concentration within single experiment, with error bars indicating range. Results shown here are representative of two independent experiments. (A) RUS0207-A006 (IC_{50} = 41 μmol/L). (B) RUS0202-G005 (IC_{50} = 27 μmol/L). (C) JK0395-B007 (IC_{50} = 25 μmol/L).

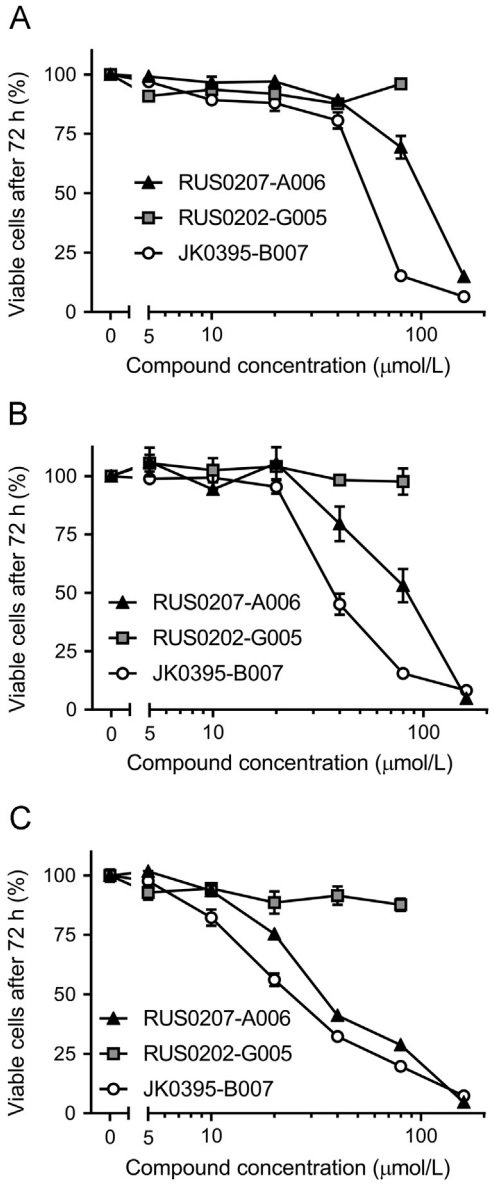


Figure 5 Effects of the three putative YB-1 inhibitors on the growth of three cancer cell lines. Measurement of percentage viable cells, based on DNA content, was performed after treatment with a concentration range of each compound for 72 h. Data were normalized and plotted relative to DMSO-treated control (no compound) cells and expressed as means ± standard error of at least three replicates within a single experiment. Results shown here are representative of two independent experiments. (A) A375 cells. RUS0207-A006 IC_{50} = 102 μmol/L, JK0395-B007 IC_{50} = 50 μmol/L. (B) HCT116 cells. RUS0207-A006 IC_{50} = 85 μmol/L, JK0395-B007 IC_{50} = 38 μmol/L. (C) MDA-MB-231 cells. RUS0207-A006 IC_{50} = 38 μmol/L, JK0395-B007 IC_{50} = 30 μmol/L.

plate in order to capture compounds that show the greatest signal inhibition relative to the other compounds on the same plate. This use of a lower selection threshold in the primary luciferase screen when ≤ 5 compounds were identified in a single plate was justified by the fact that all selected compounds would be subjected to secondary screens using the AlphaScreen system.

While *E2F1* promoter activity and cell proliferation rate are dependent upon E2F1 and YB-1 protein activity, many other factors also exert influences. Compounds that impact cell proliferation or other cellular processes, such as translation, will affect luminescent signal. Before the primary screening was run, it was expected that some compounds would inhibit luminescent signal independent of E2F1 or YB-1 protein inhibition. For this reason, the YB-1-specific AlphaScreen assay was designed as an orthogonal secondary screening to filter out compounds not associated with YB-1 (including E2F1 inhibitors). Key components differed between the two assays, creating complementation that improved the ability to identify YB-1 inhibitors. Each assay used a different YB-1 binding site sequence: a single-stranded oligonucleotide sequence derived from a promoter of human γ-globin genes was used in the AlphaScreen assay while a fragment of the human *E2F1* promoter was used in the luciferase reporter gene assay. Additionally, the primary and secondary screenings employed different sources of YB-1: the AlphaScreen assay utilized purified

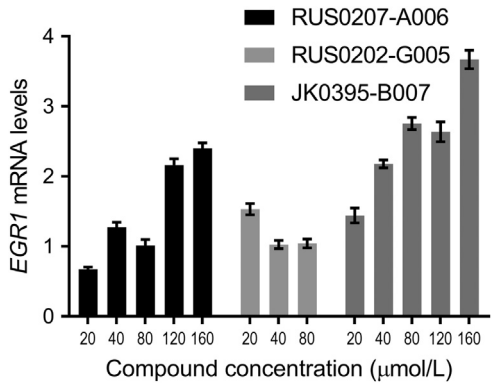


Figure 6 *EGR1* mRNA levels, a downstream target of YB-1, following incubation of MDA-MB-231 cells for 10 h with the three putative YB-1 inhibitors at a range of concentrations from 20 μmol/L to 160 μmol/L. Data were quantitated relative to *EGR1* mRNA levels in untreated cells and expressed as means ± standard error of three replicates within one experiment. For 20 μmol/L concentrations of RUS0207-A006 and JK0395-B007, the *EGR1* mRNA levels are significantly lower than *EGR1* mRNA levels at all higher concentrations ($P < 0.05$, unpaired Student's *t*-test). Results shown here are representative of two independent experiments. Note: RUS0202-G005 was used at 20 to 80 μmol/L only as it precipitates into solution at concentrations above.

YB-1, while the luciferase reporter gene assay used endogenous YB-1 within the HCT116 cells.

An advantage of our AlphaScreen assay is its adaptability. AlphaScreen is based on a signal emitted when a series of binding interactions brings two types of beads into close proximity and was originally developed for detecting protein to protein interactions[32]. We adapted this system to screen potential YB-1 inhibitors. While AlphaScreen was used to detect protein binding to dsDNA or RNA[33–36], only very recently it has been adapted to detect protein binding to ssDNA[37]. We initially used a similar approach but made some modifications during the process of iterative experimental development. We observed a high background noise when using protein A-coated acceptor beads and addressed this by chemically conjugating YB-1 antibody directly to Acceptor beads. Background noise was further reduced by addition of bovine serum albumin to our assay buffer. Additionally, to increase signal, a concatemer oligonucleotide containing three repeats of an YB-1 binding site was used rather than an oligonucleotide containing a single binding site. These improvements may have been required because of the flexible protein structure of YB-1, a trait that may be shared by other ssDNA-binding transcription factors. The human γ-globin promoter fragment ssDNA oligonucleotide was chosen due to the high affinity of its binding to YB-1[23,24]. However, other ssDNA oligonucleotide sequences were tried in this assay during development, as well as an RNA oligonucleotide. The oligonucleotide sequence can be changed to specifically focus on YB-1 binding. Therefore, this adaptable assay may have utility for the screening with other ssDNA binding proteins, or even with RNA binding proteins, thereby expanding the use of this powerful technology[37].

The present AlphaScreen assay had two uses, *i.e.*, counter-screening as well as selectivity screening. As a counter-screening, it removed false positives. Clearly, our application of two separate, orthogonal screening systems significantly reduced the number of false positives to only those that disrupt both assay systems, such as light-absorbing compound aggregates. As a selectivity screening, the AlphaScreen assay discriminated compounds that decreased signal in the primary screening *via* inhibition of YB-1 binding to DNA from compounds that reduced activation of the *E2F1* promoter *via* interaction with other proteins, such as E2F1.

The TruHits kit should be considered as an essential supplement for identifying false positives that interfere with the assay system, such as light scatterers, color quenchers, singlet oxygen quenchers and biotin mimetics. Despite the primary and secondary screenings, some of such compounds did pass through both screenings before being picked up by the TruHits kit. It should be noted that other classes of false positives exist that are not identified by the TruHits kit, such as compounds that compete with the AlphaScreen acceptor bead to bind YB-1, or those that undergo colloidal aggregation to impede assay signal. The former would not be expected to pass the primary screening. To eliminate the latter, we relied upon medicinal chemistry expertise.

None of the eight confirmed hits identified in the secondary screening passed the two most stringent computational filters, Baell 2013 Filter 3 and CTX. Baell 2013 Filter 3 represents a progressive tightening of the criteria used in Filters 1 and 2. The high stringency of Filter 3 and CTX means that they should not absolutely exclude compounds from development into drug leads. While a compound that does pass these filters would be an especially good candidate for drug development, compounds that do not pass could still be suitable for further investigation. For example, it may be possible to find more drug-like, but still active, analogues of these compounds.

Computational filtering was supplemented with the medicinal chemistry expertise that selected three putative YB-1 inhibitors. When tested in cancer cell lines, two of them inhibited proliferation and increased *EGR1* mRNA levels—both activities consistent with suppression of YB-1 activity. It is possible that the third compound inhibited YB-1 *via* a mechanism distinct and perhaps more subtle than that of the other two compounds, such as inhibition of YB-1 polymerization[38] rather than nucleic acid binding. It may also be possible that signal outputs of the assays used during this round of *in vitro* experiments were not as sensitive as the two screening assays originally used to identify this compound. It may be possible to investigate the inhibition of YB-1 polymerization by performing AlphaScreen experiments using variable concentrations of YB-1, which may suggest mechanisms of inhibition particular to compound RUS0202-G005, or the seemingly biphasic inhibitor profile of compound JK0395-B007 in Fig. 4C. The signal reduction caused in AlphaScreen assays by compounds that inhibit YB-1 polymerization would be expected to be more sensitive to decreasing concentrations of YB-1.

At present, the cold shock domain is the only domain of YB-1 with a determined structure[20]. Computational docking simulations may be able to dock the compound structures to this domain. A flexible structure may underlie the capacity of YB-1 to interact specifically with a variety of ligands[6], and the structure or folding of YB-1 may be altered by these three putative YB-1 inhibitors.

5. Conclusions

We report here the development of novel AlphaScreen and luciferase reporter gene assays for the discovery of novel small-molecule inhibitors of the transcription factor YB-1. Applying these assays to screen a collection of 7360 compounds yielded

three putative YB-1 inhibitors. Consistent with YB-1 inhibition, two of them reduced growth of three cancer cell lines *in vitro* and also increased expression of *EGR1,* a downstream target of YB-1. Follow-up studies are required to verify the present findings using additional experimental techniques, including animal models.

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Appendix A. Supporting information

Supporting data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2018.12.011.

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