SUPPLEMENT

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Suppression of bromodomain-containing protein 4 by shRNA: A new approach for cancer treatment

Abstract

Purpose: MYC is a transcription factor coding gene that is believed to control 15% of the genes in the entire human genome. The central role of *c-MYC* in cancer pathogenesis makes it a major therapeutic target in field of anticancer agent development.

Methods: We targeted the acetyl-lysine binding modules or bromodomains, which are associated with *c-MYC* transcriptional activation.

Results: Sequence specific inhibition of BET bromodomains with small hairpin RNAs (shRNAs) resulted in cessation of cellular proliferation in different cancer cell lines. Unlike previous studies on inhibition of bromodomains with selective small-molecule inhibitors, our study revealed the significant role of BET bromodomains in solid tumours and also highlighted the ease of RNA interference (RNAi) methodology for inhibition of bromodomain translation.

Conclusion: The degree of influence of BET bromodomain inhibition on proliferation in five cancer cell lines established it as the major target in malignancies characterized by activation of *c-MYC*.

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Cancer, one of the most lethal diseases worldwide, is caused by alterations in a wide range of genes. Chemotherapy, radiotherapy, immunotherapy and surgery are the methods currently used for cancer treatment, although gene therapy is a new promising option. For the development of a cancer, a single genetic change is rarely sufficient. Most evidence points to a multistep process of sequential alterations in several, often many, proto-oncogenes, tumour-suppressor genes or miRNA genes in cancer cells. Proto-oncogenes encode proteins that function to stimulate cell division, inhibit cell differentiation and halt cell death. Mutations on these genes cause normal cells to become cancerous and these mutations are typically dominant in nature [1, 2]. Examples of proto-oncogenes include *RAS*, *WNT*, *MYC*, *ERK* and *TRK*.

MYC is a regulator gene that codes for a transcription factor. In the human genome, *MYC* is believed to regulate expression of 15% of all genes [3] through binding on enhancer box sequences and recruiting histone acetyltransferases, in addition to transcription factor role [4]. A mutated but constitutively expressed version of *MYC* is found in many cancers. This leads to the unregulated expression of many genes, some of which are involved in cell proliferation, and results in the development of cancer [5]. *MYC* is critical to the development of most cases of Burkitt lymphoma [6] and has also been found in carcinoma of the colon, breast, cervix, lung and stomach [5]. *MYC* is thus viewed as a promising target for anti-cancer drugs [7].

MYC transcriptional function is based on the disruption of chromatin-dependent signal transduction [8]. The transcription of *MYC* is related, both locally and globally, to an increase in histone-lysine side-chain acetylation. Histone acetylation means a covalent modification of chromatin regionally associated with transcriptional activation [9] [10]. The acetylation of histone proteins templates the assembly for high-ordered transcriptional complexes through the recruitment of proteins with one or more acetyl-lysine-binding modules or bromodomains [11] [12]. Members of the bromodomain and extra-terminal domain (BET) subfamily of human bromodomain proteins (BRD2, BRD3, and BRD4) associate themselves with the acetylated chromatin and facilitate the activation of transcriptional factors by increasing the molarity of the recruited transcriptional activators [13].

Bromodomain-containing protein 4 (BRD4) is a BET family member that is transcribed by the *BRD4* gene [14][15]. BRD4 includes two bromodomains, which are responsible for the detection of acetylated lysine residues [16]. The C-terminal domain of BRD4 has been shown to be a factor in promoting transcription through interaction with the transcription

elongation factor P-TEFb and RNA polymerase II [17][18][19].

The expression of *MYC* often requires BRD4 and other "tumour driving" oncogenes in hematologic cancers, including multiple myeloma and acute myelogenous leukemia. BRD4 is a major target of BET inhibitors [20], a class of pharmaceuticals currently being evaluated in clinical trials.

The major goal of our study is elucidation of *c-MYC* transcriptional modulation by inhibition of BET bromodomain through RNA interference (RNAi). RNA interference (RNAi) is an important process for specific gene knockdown, in which RNA molecules inhibit gene expression by causing the destruction of targeted mRNA molecules. Short hairpin RNA (shRNA) is an artificial RNA molecule, with a tight hairpin turn, that can be used to silence target gene expression via RNAi [21,22] and also has a relatively low rate of degradation and turnover. Due to the ability of shRNA to provide specific, long-lasting, gene silencing there has been great interest in using shRNA for gene therapy applications.

Methods

shRNA Design and Plasmid Construction:

To knockdown of *BRD4* gene expression in different cell lines, RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (Clontech) was used. Two target sites in *BRD4* gene sequence were selected by using RNAi Target Sequence Selector (Clontech) and two shRNA constructs were designed by using shRNA Sequence Designer (Clontech). The designed constructs contained *MluI* restriction enzyme recognition and restriction site. shRNA for luciferase (*shRNA-Luc*) was used as negative control in this study. shRNA oligonucleotide sequences were as follows:

shRNA-BRD4#1 forward 5'

GATCCAGCTGAACCTCCCTG
ATTATTCAAGAGATAATCAGGGAGGTTTCAGCTTT
TTTTACGCGTG 3';

shRNA-BRD4#1 reverse 5'

AATTCACGCGTAAAAAAGCTGAACCTCCCTGAT
TATCTCTTGAATAATCAGGGAGGTTCA GCTG 3';

shRNA-BRD4#2 forward 5'

GATCCGCTGGAATGCTCAGGAATGTTTCAAGAGA
ACAT TCCTGAGCATTCAGTTT TTTTACG CGTG 3';
and,

shRNA-BRD4#2 reverse 5'

AATTCACGCGTAAAA
AACTGGAATGCYCAGGAATGTTC
TCTTGAAACATTCCTGAGCATTCACGCG 3'.

Cloning shRNA Constructs into pSIREN-RetroQ-ZsGreen Plasmid

shRNA constructs were obtained in the ssDNA oligonucleotide form. Each purified oligonucleotide was re-suspended in TE buffer to a concentration of 100 μ M. Oligonucleotides were mixed with the top strand and the bottom strand at a 1:1 ratio. Mixtures were heated to 95°C for 30 secs to remove all secondary structure, then heated at 72°C for 2 min, 37°C for 2 min and 25°C for 2 min. Annealed oligonucleotides were ligated with pSIREN-RetroQ ZsGreen plasmid then diluted with TE buffer to obtain a concentration of 0.5 μ M. For each ligation, the following reagents were combined in a microfuge tube: 2 μ l linearized pSIREN vector (25 ng/ μ l), 1 μ l diluted, annealed oligonucleotide (0.5 μ M), 2 μ l 10X T4 DNA ligase buffer, 9.5 μ l nuclease-free H₂O and 0.5 μ l T4 DNA ligase (400 U/ μ l). Reaction mixtures were incubated for 2 h at 22°C.

Transformation of pSIREN-RetroQ-ZsGreen/shRNA-BRD4#1,2 Plasmid

Escherichia coli strain BL21 was used in the study. Competent cells were thawed on ice for 10 min and 2 μ l of the ligation mixture was added to 100 μ l of cell suspension. Tubes were mixed gently to ensure even distribution of the DNA solution. The transformation mixtures (ligation sample + cells) were incubated on ice for 30 min. The tubes were heated for precisely 60 secs in a water bath at 37°C without shaking. The tubes were removed from the water bath, placed them directly on ice for 2 min, 500 μ l of SOC medium was added to each tube and then tubes were incubated at 37°C for 60 min while mixing at 250 rpm. Tubes were centrifuged at 4500 rpm and 500 μ l supernatant were removed from each tube. The pellet was re-suspended with the remaining supernatant and 100 μ l from each transformation mixture was plated on selective medium containing of 50 μ g/ml ampicillin. Cells were incubated at 37°C for 16 h.

Plasmid DNA Isolation

After incubation at 37°C for 16 h, plasmid DNAs were isolated from bacteria using the Nucleospin Plasmid Isolation Kit (Macherey-Nagel). Incubated cells were first centrifuged 4500 rpm for 5 min and supernatant was removed. The pellet was re-suspended with 250 μ l resuspension buffer and 250 μ l lysis buffer added after 2 min incubation at room temperature. Neutralization buffer (350 μ l) was added and samples centrifuged at 4500 rpm for 5 min. Supernatant was transferred to the spin column by pipetting and the samples

were centrifuged for 1 min and the flow-through was discarded. The wash solution (500 μ L) was then added to the spin column, tubes were centrifuged for 30-60 secs and the flow-through was discarded. The wash procedure was repeated using 500 μ L of the wash solution. The flow-through discard and centrifuged for an additional 1 min. Spin column was transferred into a fresh 1.5 mL microcentrifuge tube and 50 μ L of the elution buffer was added to the center of spin column membrane. The spin column was incubated for 2 min at room temperature, centrifuged for 2 min discarded from microfuge tube.

Cloning control

To control the cloning, isolated plasmid DNAs were digested with *MluI* and *XhoI* restriction enzymes. For each digestion, were combined the following reagents in a microfuge tube: 500 ng isolated plasmid DNA, 2 μ l Neb Cut Smart buffer, 16 μ l nuclease-free H₂O and 0.5 μ l *MluI* and 0.5 μ l *XhoI* enzymes. Reaction mixtures were incubated for 2 h at 37°C. Plasmid DNA fragments after digestion were separated on a 1% agarose gel by electrophoresis and visualized using ethidium bromide staining.

Cell Culture and Transfection

Cell lines used in this study were Human Embryonic Kidney Cell 293T (ATCC® CRL-3216™), Pancreas Cancer Cell AsPC-1 (ATCC® CRL-1682™), Liver Cancer Cell Hep G2 [HEPG2] (ATCC® HB-8065™), Colorectal Cancer Cell HT-29 (ATCC® HTB-38™), Cervix Cancer Cell HeLa (ATCC® CCL-2™) and Breast Cancer Cell MCF7 (ATCC® HTB-22™). HEK 293T cells were cultured with DMEM. AsPC-1 cells were cultured with RPMI. HT-29 cells were cultured with McCoy's 5a Medium. Hep G2, MCF7 and HeLa cells were cultured in EMEM. In addition, insulin was added into EMEM for MCF-7 cells. 10% heat-inactivated fetal bovine serum (Hyclone), 1% L-glutamine 200 mM (Hyclone), 1% penicillin/streptomycin 100U/mL (Hyclone), and 2.5 μ g/mL plasmocin prophylactic (Invivogen, Toulouse, France) were supplemented in each medium. Cultures were maintained in a 5% CO₂ humidified incubator at 37°C. Isolated pSIREN-RetroQ-ZsGreen plasmids were transfected using polyethylenimine (PEI). Transfection optimization was made for each cell line and 2 μ g PEI was used per 1 μ g plasmid DNA.

RNA Isolation from HEK 293T Cells

After 48 h transfection, total RNA was isolated from HEK293T cells. The medium was removed and the cells were washed with PBS. Cells were then treated with TRIzol and transferred to a microfuge tube from the well plate. Chloroform was added to microfuge tube and the tube was vortexed. Samples were centrifuged at 15K rpm for 10 min, the aqueous phase was transferred into a new tube and 1000 μ l 100 % ethanol was added. Samples were centrifuged at 15K rpm for 15 min and supernatant was removed. Ethanol (70%) was added to the pellet and samples were centrifuged at 15K rpm for 5 min. Supernatant was removed and pellet was dried for 5 min. The pellet was resuspended with DEPC-treated water.

FIGURE 1. Agarose gel view of digested PSIREN-shRNA BRD4#1,2 to control cloning.

FIGURE 2. . Effects of *pSIREN-shRNA-BRD4#1,2* constructs on expression level of BRD4 in HEK293T cells compared with control group.

cDNA Production and RT-PCR

cDNA was produced from isolated RNA by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fischer #K1622). For each cDNA production, the following reagents were combined in a microfuge tube: 100 ng total RNA, 1 μ l oligo dT primer, 12 μ l nuclease-free water, 4 μ l 5X reaction buffer, 1 μ l RiboLock RNase Inhibitor (20 U/ μ L), 2 μ l 10 mM dNTP Mix and 1 μ l Revert Aid M-MuLV RT (200 U/ μ L). Samples were incubated for 60 min at 42°C and the reaction was terminated by heating at 70°C for 5 min.

Specific gene expression was performed Real Time PCR using Applied System StepOnePlus™ Real-Time PCR Systems. A real-time PCR reaction mixture was 6 μ l. The following mixture was prepared in each optical tube: 5 μ l SYBR Green Mix (2x), 1 μ l HEK293T cell cDNA, 1 μ l reverse primer (5 pmol/ μ l), 1 μ l reverse primer (5 pmol/ μ l), 2 μ l H₂O.

XTT Cell Viability Assay

Cell viability was measured using the Cell Proliferation Kit II (XTT) (Roche, Penzberg, Germany). ASPC-1 cells were seeded at a cell density of 5–63/cm² and cultivated at 37°C in 5% CO₂. Cell proliferation was estimated with the XTT assay after 48 h transfection. AsPC-1 cells that were transfected with the pSIREN plasmid were seeded onto 96-well plates as 3000 cells/well in 0.1 mL RPMI 1640 medium (n=8) and incubated for 48 hours. XTT was used to measure cell viability 48 hours after transfection. Absorbance at 480 nm was measured using the Varioscan Flash Multimode Microplate Reader (Thermo Scientific).

xCELLigence Cell Proliferation Assay

Cell proliferation was monitored using RTCA xCELLigence system for four different cancer cell lines: Hep G2, HeLa, HT-29 and MCF7. Medium (100 μ l) was added into each E-plate well and incubated at 25°C for 15 min. Transfected and wild type cells were seeded in a determined number into E-plate wells: HeLa 7500 cells, MCF7 30,000 cells, HT29 20,000 cells and HepG2 7500 cells. Cells were incubated and monitored for 48 h using RTCA xCELLigence system and software.

FIGURE 3. The inhibitory effect of *pSIREN-shRNA-BRD4#1* construct on proliferation of different cancer cells. Green curves are transfected cancer cells with *pSIREN-shRNA-BRD4#1* and red curves are wild type cancer cells.

Results

To synthesize shRNAs intracellularly, specific vector RNAi-Ready pSIREN-RetroQ-ZsGreen Vector was used. This vector constitutively expresses a *Zoanthus* sp. green fluorescent protein (ZsGreen). The fluorescent marker enabled us to directly monitor the delivery efficiency of plasmid using fluorescence microscopy. Insert was cloned under stream of U6 promoter – RNA polymerase III controlled promoter – in order to transcribe it *in vitro* under the control of RNA polymerase III (Pol III). To confirm the cloning step, a cut-check experiment was performed with *MluI* and *XhoI* enzymes. The *MluI* restriction site has been engineered into the shRNA oligonucleotides and is not found on the vector itself, so that a diagnostic digest can be used to verify that the pSIREN vector contains the shRNA insert. After digestion, the expected lengths of obtained fragments were 1321 bp and 5307 bp. Gel electrophoresis confirmed the expected results (Figure 1).

To demonstrate that *BRD4* mRNA could be silenced with shRNA transcribed *in vitro* in a sequence-specific manner, three different construct-synthesizing shRNAs were incorporated into vectors and transfected to HEK293T

healthy cell lines. Two of these constructs were homologous, targeting different regions of *BRD4* mRNA (*shRNA-BRD4#1* and *shRNA-BRD4#2*) and the other construct was non-homologous, targeting *Luc* mRNA (*shRNA-Luc*). Silencing of *BRD4* using directed shRNAs, validated by RT-PCR analysis, revealed a marked decrease in *BRD4* transcription, especially in cells transfected with *shRNA-BRD4#1*, when compared with *shRNA-BRD4#2*. We obtained approximately 65% decrease in expression level of *BRD4* by using *shBRD4#1* (Figure 2).

In order to prove that inhibition of *BRD4* expression influenced proliferation of cancer cells, we quantified cellular proliferation using a xCELLigence® RTCA DP instrument over the course of 48 hours (Figure 3). Four different cancer cell lines, all expressing *BRD4* mRNA, were used for this part of the study. We firstly evaluated *BRD4* expression among cell lines available in our laboratory by integrating a publicly available compendia of gene expression data sets. To verify *BRD4* expression, cDNA synthesized from isolated RNAs found in cellular lysates of these cell lines was amplified using *BRD4* specific primers. Using gel electrophoresis data (not shown), cell lines with appropriate fragment lengths were

FIGURE 4. The inhibitory effect of *pSIREN-shRNA-BRD4#1,2* constructs on viability of AsPC-1 cells.

chosen to proceed to further experiments: HepG2 (hepatocellular carcinoma), HeLa (cervical adenocarcinoma), MCF7 (breast cancer) and HT-29 (colorectal adenocarcinoma).

Our experiments showed that shRNA targeting of *BRD4* revealed marked arrest of cellular proliferation in four cancer lines over the 48 hours. AsPC-1 cell line viability assay performed using XTT Cell Viability Assay showed a noticeable increase in the ratio of living cells transfected with *shRNA-Luc* compared with *BRD4* shRNA transfected cells (Figure 4).

Discussion

For more than 15 years, since the discovery of endogenous cellular process RNAi in *Caenorhabditis elegans*, researchers have used this methodology for hundreds of genetic screens. The discovery of RNAi made it possible to study new genes and gene networks that are involved in cell proliferation, viability, epigenetics and drug resistance. The most exciting feature of this methodology is that it induces sequence-specific gene silencing, making it a powerful tool for manipulation of gene expression. But several endogenous mechanisms, including dsRNA-activated protein kinase, PKR, in mammalian cells, fetters the use of RNAi. In our study, sequence-specific inhibition of BRD4 with shRNA was achieved, as validated by RT-PCR, and caused cell-cycle arrest in cervical, colorectal, hepatocellular, breast and pancreatic cancer cell lines. Based on these findings, we conclude that the RNAi methodology can be used for efficient inhibition of BRD4 and can also decrease cellular proliferation rates in all cell lines tested, indicating the potential therapeutic aspect of BRD4 suppression in solid tumour cell lines. Consistent with

our results from solid tumours, BRD4 is known to play a significant role in disease maintenance in acute myeloid leukemia (AML) and in therapeutic strategy in MM. Previous studies on specific inhibition of the acetyl-lysine binding domain, BRD4, with its small molecule competitive inhibitor JQ-1, showed arrest of cell cycle and initiation of apoptosis. Suppression of BRD4 with JQ1 or shRNA led to strong antileukaemic effects.

The other important aspect of this study was that we took advantage of RNA interference for inhibition of BRD4. By using RNAi, any gene sequence can be targeted for suppression at the translational level. In addition, our study shows that RNAi can be used for the targeting of new genes in the treatment of cancer. Previous studies showed that MYC oncoprotein transcription is under the control of BRD4; thus, inhibition of BRD4 will downregulate MYC levels, which will lead to suppression of the MYC pathway in cancer cells. Successful downregulation of MYC mRNA and protein levels following the inhibition of BRD4 has been confirmed by several studies. Based on these findings, we conclude that shRNA targeting BRD4 shows its anti-tumour effect by suppression of MYC transcription and reduction in expression of MYC target genes. In conclusion, our study establishes a promising therapeutic strategy, BRD4 inhibition in solid tumours, and reveals the potential power of RNAi for targeting the genes responsible for cancer.

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