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Research Article

Bromodomain Inhibitor JQ1 as a Candidate Therapeutic Agent in Malignant Pleural Mesothelioma

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ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare tumor that develops from the mesothelial linings of the pleural, pericardial, and peritoneal cavities. The actual risk factor for developing the disease is exposure to asbestos in workplace. Bromodomain and extraterminal (BET) domain proteins are epigenetic signaling agents that associate with acetylated histones and expedite the transcription of target genes. This study investigates whether the small molecule BET protein inhibitor JQ1 specifically may be an effective therapy for MPM. Reverse transcriptase polymerase chain reaction methods reveal an inclusive change in gene expression implying that JQ1 is a potential inhibitor which targets the BET proteins. Our results report that JQ1 has tumor-suppressive effects as it significantly ceased cellular activity in MPM cell lines. We predict that JQ1 may be the promising therapy for pleural mesothelioma cancers.

Keywords: Bromodomain and extraterminal domain, JQ1, malignant pleural mesothelioma

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an infrequent type of cancer that affects the pleura (the external lining of the lungs and the internal lining of the chest cavity). MPM includes three main types determined by their appearance under the microscope. MPM includes three main types determined by their appearance under the microscope as: Epithelial MPM which is the most popular form, Sarcomatoid MPM which is meant to be the most aggressive form and Biphasic MPM which mixes both Epithelial and Sarcomatoid MPMs. Pleural mesothelioma is commonly caused by the long-term exposure to asbestos fibers that induce pathogenic changes and eventually lead to cell injury, fibrosis, and possibly cancer. It has been also proposed that aside from asbestos, there are other factors which have a role in the tumor pathogenesis, such as SV40 virus infection and genetic susceptibility.[1]

MPM incidence has been increasing over the world and is anticipated to grow even higher in the following 20 years due to asbestos huge spread exposure throughout the past 10 years or so.[2] It is rated that about 50–80% of pleural malignant mesothelioma in men and 20–30% in women arose in individuals who were exposed to asbestos.[3]

Diagnosis of this disease is pretty much difficult even for some specialists because the symptoms do not appear only after a longer period of exposure to asbestos. So far, the only approved treatment for MPM is the combined therapy of pemetrexed and cisplatin, although currently, there have been many clinical trials determining the efficacy of other drugs.

Bromodomain and extraterminal (BET) domain proteins play an essential role as epigenetic reader molecules that are associated with acetylated histones and aid in the transcription of target genes. Bromodomain is, therefore, an acetyl-lysine-binding motif of about 110 amino acids which are found in various transcription regulators and chromatin-modifying enzymes that are encoded by specific genes.[4] Research has shown that only members of the BET family (BRD2, BRD3, BRD4, and BRDT) may be prospective targets of cancer. These four BET proteins make up a subset of the larger family that consists of 46 proteins which contain 61 BRDs that have been determined in humans.[5]

BET inhibitors are getting to become of huge interest as a potential target due to the prospect that there might be biomarkers which will recognize the patients that may possibly benefit and respond to therapy. Those small molecule inhibitors conquer the acetyl-binding pockets of the bromodomains, leading in their detach from active chromatin and repression of downstream transcriptional targets.[6]

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JQ1 has started its journey in the research world as a novel drug in inhibiting the development of some specific cancers. JQ1 successfully blocks one of the well-known cancer-causing genes of the BET proteins which are BRD4, thereby preventing the early growth of some blood and lung cancers such as leukemia and multiple myeloma. JQ1 is a novel thieno-triazolo-1,4-diazepine having an attached bulky t-butyl ester functional group at carbon 6. This chemical compound is a vigorous, high affinity, selective BET inhibitor. JQ1 detaches BET proteins from chromatin by binding to the acetyl-lysine recognition pocket of BET. JQ1 has been used as a chemical probe to investigate the role of BET in the transcriptional regulation of oncogenesis.[7-9]

BET inhibitors are a new promising treatment for MPM. This study will focus on the effect of the BET inhibitor (JQ1) in MPM. We hypothesized that bromodomains of the BET family are expressed/overexpressed in MPM and hence investigate the effect of the compound JQ1 as an inhibitor of those BET. This appears to be the first study of its kind, as far as I know that reveals the role of inhibiting BET domain proteins with JQ1 in mesothelioma cancers.

MATERIALS AND METHODS

Cell Lines

In this project, two MPM cell lines were treated with the small molecule BET protein inhibitor and were assessed for antiproliferative activity as a response to that drug. The BET inhibitor used in this study was JQ1. The two cell lines used were REN and H226. A brief description of the cell lines as follows:

- REN is an epithelial mesothelioma cell line (fast growth rate)
- NCI-H226 is an epithelial squamous cell carcinoma; mesothelioma cell line (slow growth rate).

Cell Culture

Cell lines were typically grown and preserved at a fixed temperature of 37°C and a humidified environment of 5% CO_2 in a cell incubator. These cell lines were cultured in an appropriate cell culture media RPMI-1640 (Lonza, UK) which was supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 500 U/ml penicillin-streptomycin. Cell lines were regularly passaged in T75 cm^2 flasks (Nunc Flasks obtained from Fisher Scientific, Ireland) as following:

All cell culture procedures were done in laminar flow hoods under sterile conditions. Cell media discarded and cells were washed with 5 ml phosphate buffer solution. Cells were then trypsinized for 5 min at 37°C, and once detached from the flask, media were added to make up a final volume of 10 ml. Cells were pipetted many times and each 1 ml transfer to two new T75 flasks labeled with the cell line, passage number, and date. Further, 9 ml of media was added to each of these two flasks and cells were incubated at 37°C. In this way, cells were split at a ratio of 1:10.

Cell Subculture for Proliferation assays

Before drug treatment, cells were visualized under the microscope (Nikon Corp.) and if found to be of about 70–80% confluent, they were trypsinized with 1.5 ml trypsin-EDTA and 8.5 ml of media to make it up to a final volume of 10 ml. The cells were then counted using a bright-line hemocytometer **(**Sigma, USA**)** and seeded at 3×10^3 cells/well in 96-well plate. This was done in triplicate for both cell lines (REN and H226). The plated cells were kept in the incubator at 37°C.

Treatment of Cells

Once plated, cells were left to recover for 48 h. Subsequently, cells were treated with JQ1 BET domain inhibitor at the following concentrations (0.25 μM, 0.5 μM, 0.75 μM, 1 μM, 15 μM, 20 μM, and 25 μM) for 48 h. 4 μl of dimethyl sulfoxide (DMSO) was added to the control as JQ1 was dissolved in DMSO vehicle. A concentrated stock (18.6 mM) of JQ1 dissolved in DMSO was made and further four stock dilutions (1:25, 1:10, 1:5, and 1:2) were set up from that main concentrated stock. All plates were then incubated for 48 h at 37°C.

5-bromo-2-deoxyuridine (BrdU) Proliferation Assay

A BrdU assay was performed to measure the proliferative activity of the treated cells. Briefly, after cell treatment with JQ1 for 48 h, all media were removed from the triplicate 96-well plate, and subsequently, 10 μl BrdU labeling solution was added to each well. Plates were incubated at 37°C for 4 h followed by the removal of the labeling medium from the wells. 200 μl FixDenat was added to each well and plates were incubated at 25°C for 30 min followed by the removal of FixDenat solution thoroughly. 100 μl anti-BrdU POD solution was added to each well and plates were incubated again at 25°C for 90 min. The antibody was then removed and wells were rinsed 3 times with 200 μl/well washing solution. Finally, washing solution was removed and 100 μl/well substrate solution added to each well. Plates were left for 2–3 min and thence immediately 25 μl/well of 1 M H_2SO_4 was added and measurement of plates was carried out within 5 min. The activity of proliferative cells was measured by enzyme-linked immunosorbent assay at a wavelength of 450 nm using a standard plate reader.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Cell lines and tissue samples

Fresh-frozen mesothelial and benign pleural samples were collected following debulking surgery at Glenfield Hospital, Leicester, and were stored in The Leicestershire Mesothelioma Tissue Bank. Anonymized MPM specimens from 22 patients were transferred to St. James' Hospital, Dublin. Approval for the storage and use of these samples have been obtained from the St. James' Hospital/The Adelaide and Meath Hospital Research Ethics Committee. A panel of mesothelioma cell lines has been assembled and utilized for this project.

RNA isolation

Total RNA was isolated using TRI reagent® (MRC gene) protocol. Samples were mixed and incubated for 5 min at room temperature followed by centrifugation at 13,500 rpm for 5 min. The RNA pellet was left to air dry after the removal of the supernatant and finally resuspended in 30–50 μl of molecular grade water and stored at −80°C until further use.

RNA quantification

NanoDrop 1000 spectrophotometer (Thermo Scientific) was used to measure the purity of the isolated RNA. 1 μl of each RNA sample was loaded separately onto the NanoDrop and analyzed. The quantity of RNA per sample was measured in ng/μl. A 260/280 value of 2.0 indicates the purity of an RNA sample.

Complementary DNA (cDNA) synthesis

cDNA was synthesized using RevertAid RT according to the manufacturer's instructions (Fermentas). Samples were mixed gently, incubated for 90 min at 42°C followed by 10 min at 70°C heating to terminate the reaction. The resultant cDNA was then stored at −20°C until required.

RT-PCR

The cDNA which was synthesized previously was used to detect gene expression. For PCR, the following were mixed in a new tube to set up the PCR reaction: 10 μl (2× GoTaq® Green [Promega]), 2 μl (5 μM of each forward and reverse primers), 6 μl (molecular grade water), and 1 μl (cDNA). 1 μl molecular grade water was added in the place of cDNA for the negative control. PCR samples were loaded on a thermocycler. The PCR cycling conditions involved initial denaturation at 95°C for 5 min, followed by 30–35 cycles of denaturation at 95°C, annealing at the temperature 58°C, extension at 72°C, and a final elongation step at 72°C.

Gel electrophoresis

Agarose gel (2%) was used for loading all PCR products. Agarose (2%) was dissolved in Tris-acetate-EDTA (TAE) in the microwave for 3 min. 4 μl of ethidium bromide was added per 25 ml of agarose solution after being cooled and then poured into a gel tray. The gel was then allowed to set at room temperature for 10–15 min. 4 μl of 100 bp DNA ladder (Fermentas) was loaded onto the gel in parallel with the samples. 8 μl of each PCR product and 2 μl of the corresponding 18S housekeeping gene products were loaded as well. The samples were electrophoresed using an Electrophoresis Unit (Sigma) and 1× TAE was used as a running buffer. The gels were run at 80 volts for 30 min. The resultant bands were visualized and photographed under ultraviolet light using a biospectrum imaging system (Ultra Violet Products, Fusion FX, UK).

Statistical Analysis

Statistical analysis was carried out using two-tailed Student's *t*-test and one-way ANOVA. Data were graphed as mean ± standard error of mean using GraphPad Prism software. Densitometry analysis was performed using TINA software. Results were considered significant when the $P < 0.05$.

RESULTS

Screen of Panel of Mesothelioma Cell Lines for the Expression of BET Domain Proteins

A panel of 34 mesothelioma cell lines was screened for the expression of BRD2 and BRD4 (long and short transcript) variants. All these cell lines are MPMs, except for LP9, Met5A, NP1 and NP2, and P31 and P31CisR. Beta-actin was used as a positive control which is regularly expressed in all cell lines and a negative control was included also. BRD4 long transcript variant shows equal expression in all cell lines, but BRD4 short transcript shows a slightly lower expression in some of the cell lines [Figure 1]. BRD2 also shows an equal expression in all cell lines [Figure 2].

Screen of Primary MPM Patient Cells for the Expression of BET Domain Proteins

A panel of 34 primary mesothelioma cells obtained from patients was examined for the expression of BRD2 and BRD4 (long and short transcript) variants. These patient samples were divided into benign and three histological subtypes of MPM; epithelial, biphasic, and sarcomatoid. Densitometry analysis was performed to identify the differences in expression of the BRDs between benign and tumor tissue following normalization against the 18S rRNA expression for each sample, and a negative control was used in each PCR. BRD2 showed expression across samples [Figure 3a] and was significantly less expressed in the tumor samples compared to benign [Figure 3b and c].

BRD4 long transcript variant appeared to be expressed across the three histological subtype samples and less expressed in benign [Figure 4a] and following statistical analysis, BRD4 long transcript expression was significantly upregulated in

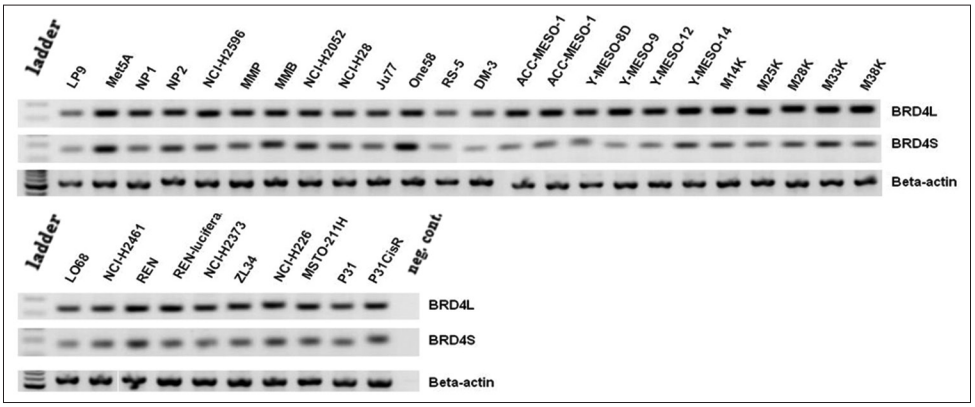

Figure 1: Expression of BRD4 long and short transcript variants in a panel of 34 mesothelioma cell lines. Beta-actin is a positive control. BRD4 long transcript is expressed uniformly in all cell lines and the expression of BRD4 short transcript is relatively less in some of the cell lines

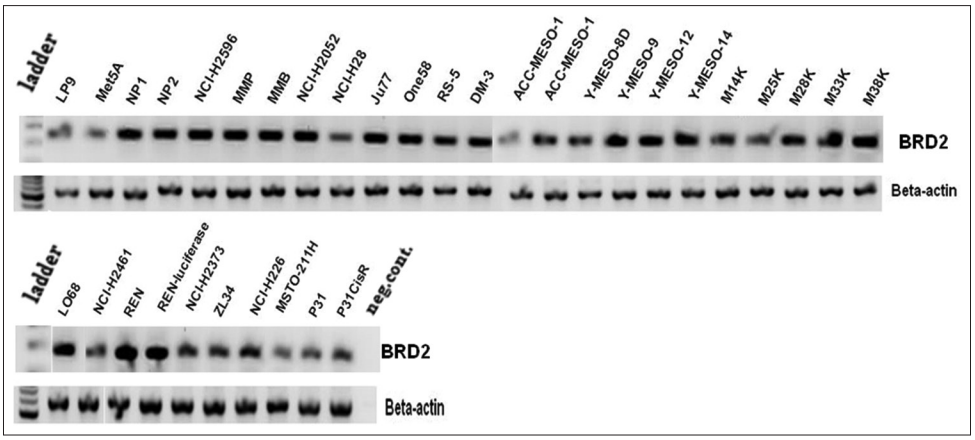

Figure 2: Expression of BRD2 in a panel of 34 mesothelioma cell lines. Beta-actin is a positive control. BRD2 is uniformly expressed in all cell lines

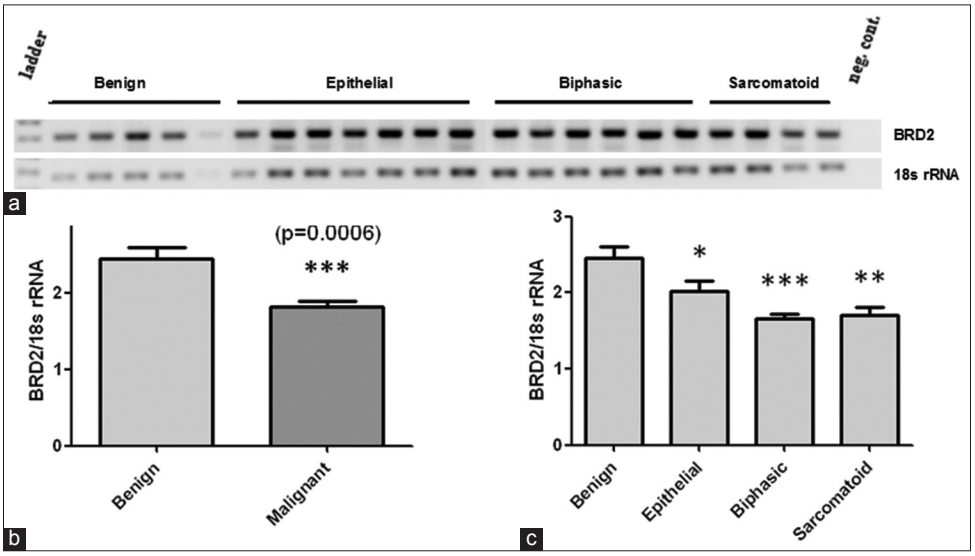

Figure 3: BRD2 expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD2 levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD2 expression level in malignant patients is significantly less compared to benign (***$P < 0.001$, two-tailed t-test). (c) Levels of BRD2 expression in all three histological subtypes is significantly different compared to benign (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

the tumor samples compared to benign [Figure 4b]. BRD4 long transcript expression levels were significantly higher in epithelial and biphasic subtypes compared to benign, while not significant in sarcomatoid [Figure 4c]. On contrary, the expression of BRD4 short transcript variant appears to be almost entirely absent in the benign samples [Figure 5a] and BRD4 short transcript expression was significantly upregulated in the tumor samples compared to benign [Figure 5b]. While not significant, a trend toward upregulation of BRD4 short transcript was observed in all three histological subtypes of MPM compared to benign [Figure 5c].

Antiproliferative Activity

The BrdU assay detects incorporated BrdU into the cellular DNA of actively proliferating cells. This assay was performed to measure changes in proliferating cells after 48 h following treatment with JQ1 drug. REN-treated cells appear to show a significant decrease in proliferation activity with 5, 10, 15, 20, and 25 μM drug concentrations, while NCI-H226 treated cells appear to show significant decline in proliferation with 0.25, 0.5, 0.75, 1, 15, and 25 μM drug concentrations [Figure 6]. The assay was accomplished in triplicate and the statistical analyses expressed as mean of treatments using one-way ANOVA and Dunnett's multiple comparison test.

DISCUSSION

MPM is a rare and aggressive form of cancer with limited treatment chances that have inconsiderable impact on survival improvement. Asbestos has been known as a non-mutagenic carcinogen that induces mesothelioma and in such case, cancer may be developed as an outcome of somatic epigenome dysregulation.[10] And so, aberrant epigenetic events have been spotted in MPM. Epigenome regulators include DNA methylation, histone modifications, chromatin remodeling, and regulation of non-coding RNAs. It is not clear why precisely epigenetic modifications are a notable feature of pleural mesothelioma,

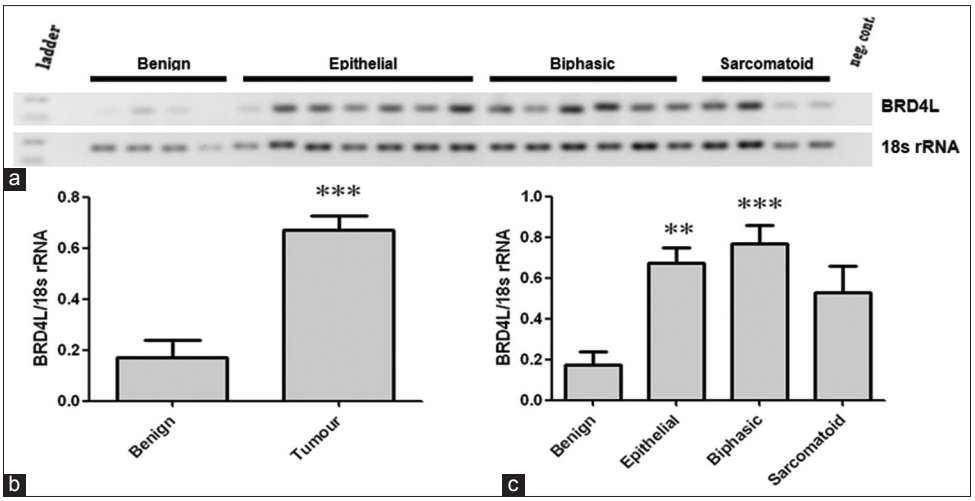

Figure 4: BRD4 long transcript expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD4L levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD4L expression level in tumor patients is significantly different compared to benign (***$P < 0.001$, two-tailed *t*-test). (c) Levels of BRD4L expression epithelial and biphasic subtypes are significantly different compared to benign, while not significantly different in sarcomatoid subtype (**$P < 0.01$, ***$P < 0.001$, one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

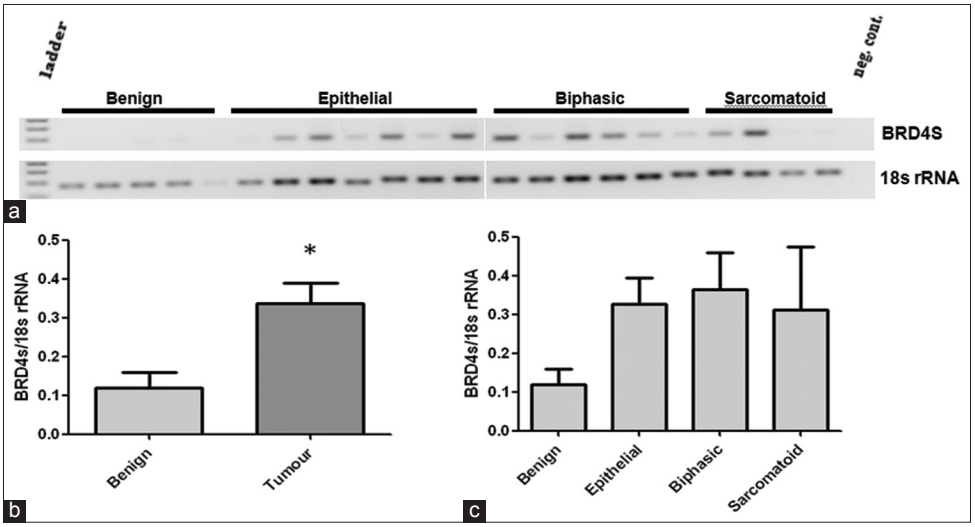

Figure 5: BRD4 short transcript expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD4S levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD4S expression level in tumor patients is significantly different compared to benign (*$P < 0.05$, two-tailed *t*-test). (c) Levels of BRD4S expression in all three histological subtypes show no significance compared to benign (one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

but histone modifications have emerged as remarkable modifications in the carcinogenesis of MPM.[11] Aberrant regulation of histone modification plays a role in oncogenesis by affecting gene activity. The acetylation of histones is mediated by lysine acetyltransferases. Lysine acetylation is mainly recognized by bromodomains and tandem PHD domains.[12] BET proteins are readers of acetyl-lysine signs. In some cancer types, bromodomain-containing proteins which identify histone acetylation signs are mistargeted or overexpressed.[13]

This study focuses precisely on the role of two BET proteins, BRD2 and BRD4, in MPM and the effect of JQ1 compound as an inhibitor for BRDs. The expression of these BET proteins in MPM has been evaluated in a panel of mesothelioma cell lines and in primary MPM patient cells. A panel of cell lines [Figures 1 and 2] was screened to evaluate whether there are any prominent patterns of expression which need to be identified before screening of primary MPM patient cells. Slight changes in expression were noted. BRD4 short transcript variant is expressed weakly in some of the cell lines. Among the analyzed cell lines are a cisplatin resistance cell line. Cisplatin is a chemotherapeutic agent used in cancer treatment. A specific feature of MPM is that it is a cisplatin resistance tumor.[14] Although there were no remarkable variations in the expression level of any of the evaluated BRDs, additional MPM chemoresistant cell lines need to be included so that it would be a conclusive analysis.

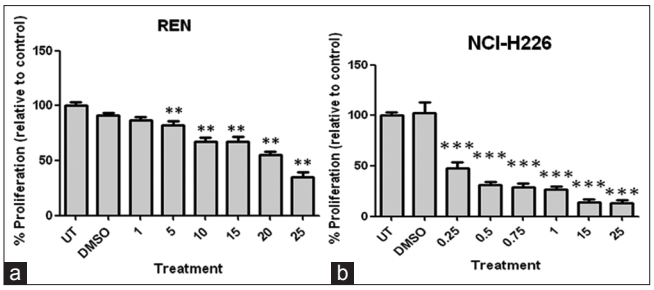

Figure 6: 5-bromo-2-deoxyuridine proliferation assay following 48 h treatment with various concentrations of JQ1. (a) REN cells show significant decline in cell proliferation at 5, 10, 15, 20, and 25 μM drug concentrations ($P < 0.01$, one-way ANOVA). (b) NCI-H226 cells show significant decline in cell proliferation at 0.25, 0.5, 0.75, 1, 15, and 25 μM drug concentrations ($P < 0.001$, one-way ANOVA)

Subsequently, a panel of primary MPM patient cells including benign, epithelioid, sarcomatoid, and biphasic histological subtypes was screened [Figures 3-5]. Expression of the three evaluated BRDs varies in malignant compared to benign cells. Expression of BRD2 was significantly different in malignant cells compared to benign cells. In addition, all three histological subtypes showed significantly lower levels of BRD2 expression compared to benign even though BRD2 was expressed in benign. On contrary, BRD4 long transcript expression levels are significantly higher in malignant cells compared to benign. In consistent with the results of BRD4 long transcript expression levels, the expression of BRD4 short transcript confirmed significantly elevated levels in malignant cells compared to benign, and this indicates that BRD4 long and short transcripts are overexpressed in malignant cell. Our findings have proven that BRD2 is downregulated in MPM tumors, while BRD4 long and short transcripts are significantly upregulated in MPM tumors. Moreover, since BRD4 is the main target for JQ1, consequently, this makes targeting MPM with JQ1 and excellent strategy.

To further support our findings, the antiproliferative activity of JQ1 was evaluated *in vitro* using two specific MPM cell lines (REN and NCI-H226). These two cell lines were chosen according to their common use in human tumor xenografts, as they perform well when xenograft in mouse models and so, the evaluation of JQ1 drug can be smoothly carried out in such *in vivo* models. JQ1 is a BET inhibitor that has been selected for this study. Scientists have been competing to develop novel BET protein inhibitors. A recently developed dihydroquinazoline-2-one inhibitor has been found to target BET.[15] BrdU assay was the first move in analyzing the proliferative activity of JQ1 compound. This assay detects BrdU incorporated into the newly synthesized DNA of actively proliferating cells. BrdU assay was chosen to measure the proliferating activity of cells. REN and NCI-H226 cells were treated with a range of JQ1 concentrations [Figure 6]. Response of NCI-H226 cells to JQ1 was clearly observed, with a significant decrease in cell proliferation starting from 0.25 μM up to 25 μm concentrations compared to untreated cells. While REN cells showed a significant decrease in proliferative activity with concentrations from 5 μM up to 25 μM. DMSO was used as a control to ensure that any change in proliferative activity was caused by JQ1 and not DMSO. DMSO induced no change in the cellular activity of both cell lines and so, noted results are accurate. JQ1 inhibits the proliferative activity of REN and NCI-H226 cells with concentrations of 5 μM and 0.25 μM, respectively. JQ1 has shown to have antiproliferative effects on mesothelioma cell lines and efficiently abrogates their clonogenic growth. Exposure of sensitive cell lines with JQ1 results in decreased proliferative cell activity and induction of apoptosis. In addition to confirming the effect of JQ1, the BrdU also provided a guideline for treatment concentrations to be used in a further study.

CONCLUSION

This study has shown that BET domain proteins BRD2 and BRD4 are significantly altered in the tumors of patients suffering from MPM. Critically levels of BRD4 are significantly elevated in the tumors. JQ1 is a small molecule inhibitor of BRD4 and BRD2. This study has shown that JQ1 may be a potentially effective therapy for MPM *in vitro*.

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