Inhibition of miR-19a-3p Increases Bortezomib-Induced Apoptosis in Myeloma Cell Lines by Targeting SOCS3

Azam KAZEMI, Saeid ABROUN, Masoud SOLEIMANI

Department of Hematology and Blood Banking, Faculty of Medical Sciences, Tarbiat Modares University, Tehran-Iran

OBJECTIVE
Apoptosis is considered the main factor inducing by bortezomib in myeloma cells. Although bortezomib (BTZ) is a crucial drug for the treatment of MM, chemoresistance is a major problem. OncomiR-19a plays an oncogenic role in many types of cancer such as MM; however, the function of miR-19a in the pathogenesis of MM and drug resistance has not been identified well. The present research aims at investigating the inhibition of miR-19a by antagomir to conclude BTZ responsiveness and suggests that miR-19a may be a biomarker for the prognosis of the patient.

METHODS
In the present research, the viability and apoptosis of myeloma cells were analyzed by colorimetric MTT and Annexin-PI flow cytometric assays. QRT-PCR was implemented to evaluate the expression level of miR-19a, its targets SOCS3 and STAT3, at the mRNA level.

RESULTS
Following treating transfected cells with bortezomib we found out that miR-19a was downregulated, and the rate of apoptosis of myeloma cells after bortezomib treatment considerably increased. It indicated that mRNA of SOCS3 increased and STAT3 decreased.

CONCLUSION
The research results indicate that oncomiR-19a as a biomarker may induce better responsiveness to bortezomib in myeloma cell lines through its targets SOCS3, STAT3, and may provide new therapeutic targets in the future for myeloma. The research results indicate that oncomiR-19a as a biomarker may induce better responsiveness to bortezomib in myeloma cell lines through its targets SOCS3, STAT3 and may provide new therapeutic targets in the future for myeloma.

Keywords: Antagomir-19a; bortezomib; multiple myeloma; SOCS3.

Introduction
Multiple Myeloma (MM) is some sort of tumor of differentiated B cells from the germinal center, plasma cells, within 10% of all hematologic neoplasms, and is considered the second most commonly occurring non-Hodgkin lymphoma.[1,2] Over the last two decades, due to advancements in available treatments, the median survival time of patients with MM has increased from 3 to 6 years and is accounting for 1.1% of total cancer deaths and the age-standardized risk of MM is about 2%.[3] Although great discoveries have been made in the growing evolution of new healthcare strategies in the past decade, to a great extent the tu-
mor is still incurable and new therapies are required to overcome it. [4,5] Despite the innovation and benefitting from a new therapeutic strategy such as proteasome inhibitors, the clinical outcome of the patients aggravates, and in most patients finally, MM relapses and engenders drug resistance.[1]

The proteasome inhibitor, bortezomib (BTZ), is a crucial and an FDA-approved drug for the treatment of MM, especially relapsed & refractory MM.[6] Although BTZ has a significant impact on the treatment of MM,[5] drug resistance or relapse are still two major challenges, and the prognosis of patients with BTZ resistance is highly undesirable.[7,8] Therefore, to prevent BTZ resistance, new therapeutic methods are desperately required. On the other hand, a more profound molecular grasp of cancer's pathogenesis is, thus, urgently required to recognize new molecular targets and to bring forth therapeutic agents that are suitable for the patient.[9] In this regard, there is developing evidence indicating that MM stems from the deregulation of noncoding RNAs, including miRNAs.[1] Recent studies show that MM is caused by interruptions in many different signaling pathways, which are driven by the microRNAs and are a class of non-coding RNAs (ncRNAs) of about 18–22 nucleotides in length. These microRNAs act as master regulators of gene expression at the post-transcriptional level via RNA interference pathways.[6] miRNAs are involved in many biological processes, including differentiation, senescence, survival, and, apoptosis.[3,6] It is well established that the disturbance in the regulation of miRNAs is accompanied by the pathogenesis of diseases such as cancer, and miRNA expression profiles have prognostic implications in numerous types of cancer.[6] Altogether, miRNAs play a fundamental role as an oncogene. In other words, miRNAs operate as 'oncomiRs' if their targets are the tumor suppressor genes.[1] Therefore, controlling oncomiRs may be an effective strategy for treatment.

Chief among oncomiRs, miR-17-92 clusters that are placed in chromosome 13q31.3, including miR-19a are the very first oncomiRs that were discovered. It is proved that any disturbance in the expression level of miR-17-92 clusters results in the malignant progress of MM.[4,5] MiR-19a, a crucial component of the miR-17-92 cluster, is directly involved in myeloma pathogenesis and progression of multiple myeloma.[9] Also, besides target genes of miR-19a are considered as potential biomarkers of the disease.[10] Moreover, compared with normal plasma cells, miR-19a has been revealed to be up-regulated in patients with MM, as well as in MM cell lines.

Furthermore, miR-19a can adjust the expression of proteins that are essential for myeloma pathogenesis including suppressors of cytokine signaling (SOCSs).[9] It is reported that miR-19a targets SOCS3, a potent regulator of the JAK-STAT pathway, as is followed by a considerable reduction of SOCS3 mRNA together with enhanced activation of SOCS3 target, STAT3.[9] Based on these findings, a strategy that can be developed to regulate miRNAs' aberrant expression in cancer is the inhibition of up-regulated miRNAs.[1] Therefore, we have assumed that the use of mir-19a inhibitors (Antago-miR) could be a new approach for the treatment of multiple myeloma. On the other hand, based on the use of BTZ in the treatment of patients with MM and the resistance in 60% of them, the present study investigates the impact of antagomiR-19a on better responsiveness to BTZ and may present miR-19a as an effective biomarker for response to treatment.[9,11]

Materials and Methods

Cell Lines & Cell Culture

In the present research, the RPMI8226 and U266 cell lines were purchased from the Pasteur Institute of Iran (PI). The cells were grown in suspension in RPMI1640 medium (BIO-IDEA from Bio Idea Group) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL), penicillin (100 mg/mL), streptomycin (100 mg/mL), and 2mM l-glutamine (BIO-IDEA from Bio Idea Group). Cells were maintained at 37°C in an environment of 5% carbon dioxide and 95% air and underwent passage twice a week.

Reagents

Bortezomib (PS-341) (Selleckchem.com Cat. No.S1013) was dissolved in 0.2603 mL DMSO to prepare a stock solution with a concentration of 50 mM for storage at -20°C. LentimiRa-off-has-miR-19a-3p vector (Applied Biological Materials (ABM) Inc. with Cat. No. mh30299) with GFP promoter, miRNA insert, and kanamycin resistance gene that was transformed in DH5α E.coli strain and isolated by Qiagen plus Midi plasmid purification kit and stored at -20°C.

In Vitro Cell Culture and Drug Treatment

Human myeloma cell lines RPMI8226 and U266 were cultured and 50 mM Stock solution of Bortezomib was further diluted to working concentrations in RPMI1640 medium before use. To determine the concentration of drug that inhibited cell proliferation by 50% (IC50),
5×10^3 cells were seeded in 96well plate, then treated with different concentration of working solutions of BTZ based on the approximate concentration that was noted in cell assay part of BTZ data-sheet in ranges of 0.5, 5, 50 µM for U266 and 150, 450, 750, 1050 nM for RPMI8226, mixed well in RPMI1640 media and 10% FBS and finally incubated for 48 hours.

**MTT Colorimetric Assay**
Using a standard protocol, the inhibitory impact of BTZ on cell growth was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. Briefly, cells from 48-hour cultures were pulsed with 10 µL of 5 mg/mL MTT to each well for at least 4 hours of 48-hour cultures, followed by 100 µL of isopropanol containing 0.04 N HCl, absorbance was measured at 570 nm using a spectrophotometer and results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the number of viable cells compared to cell control (100%). To decide the optimum dosage of the drugs for further studies, the cell viability was plotted in a graph and the IC 50 was calculated accordingly.

**Cell Viability Analysis**
The effect of transfection on cellular viability was assessed by flow cytometry using propidium iodide (PI). PI can only pass through disordered areas of the dead membrane cells and intercalated with the DNA of the nuclei, emitting red fluorescence light. It was used with 1mg/mL concentration by dissolving PI (Sigma, P 4170) in dH₂O. PI solution was added in a final concentration of 2µg/mL to 1×10⁶ cells in suspension, incubated at a dark place and after 5 minutes, analyzed by flow cytometry using Attune NXT flow cytometer.

**Analysis of Apoptosis**
The apoptosis of cell lines after transfection with Plenti-III-miR-Off-has-miR-19a -3p vector, after treatment with bortezomib and after treatment of transfected cells with bortezomib compared with untreated cells were analyzed with Annexin V/PI (eBioscience Cat. No. 88-8005 from Thermo Fisher Scientific) by flow cytometry using Attune NXT flow cytometer. Cells were washed in PBS and then they were washed in 1X bindings buffer once, after that we resuspended cells in 1X bindings buffer at 1×10⁶/mL, then we added 5 µL of FITC-conjugated Annexin V to 100 µL of the cell suspension, incubated for 10-15 minutes in a dark place at room temperature, then they were washed with binding buffer, resuspended in it afterward we added 5 µL of Propidium Iodide Staining Solution (Sigma, P 4170), analyzed by flow cytometry.

**Prediction of SOCS3 as a Target of miR-19a**
Target Scan (Version 5) and pic tar were used to confirm the SOCS3 molecule as a target of miR-19a in its 3’ UTR. Target Scan predicted biological targets of miRNA by searching for the presence of conserved 8 and 7 base sites that match its seed region.

**Cell Transfection**
Cells were grown in RPMI1640 medium containing no antibiotics before transfection. U266 and RPMI8226 were transfected by final concentration 2µg of Plenti-III-miR-Off-has-miR-19a -3p vector containing GFP purchased from Applied Biological Materials Inc. (ABM). Transfection of cells was performed using UltraCruz Transfection Reagent (SCBT, Santa Cruz Biotechnology, Inc.). Briefly, before transfection, we prepared a transfection reagent and vector in Opti-MEM I reduced serum medium (Gibco) following the manufacturer’s protocol. After preparing the transfection reagent and plasmid in OPTI-MEM I medium and incubation at room temperature, plasmid reagent was added to transfection reagent, vortex vigorously incubates for 20 minutes. 6×10³ cells were added to the Eppendorf tube and were poured above mix dropwise to the cells, incubated in the incubator for 2 hours, every half an hour flicked with a fingertip, finally were transferred to 6-well plate and after 24-72 hours GFP expression was evaluated by Attune NXT flow cytometer.

**Q-RT-PCR Assessment of miR-19a Expression**
Total RNA was isolated from un-transfected, transfected and BTZ treated cells: RPMI8226 and U266 while following the Trizol manufacturer’s protocol (InvitrogenTM, USA). 2000ng of RNA was reverse transcribed using specific microRNA stem-loop primers from Qia-gen for miR-19a, to generate cDNA using Hyperscript reverse transcriptase first-strand synthesis kit (Gene-All Biotechnology Co., Ltd, Korea). The expression of Snord47 was used as the internal control for miRNA expression normalization. The quantitative real-time PCR was performed with the SYBR® Premix Ex Taq™ miRNA RT-qPCR Detection Kit (Takara, Cat.No. RR820Q) is using the Qiagen Rotor-Gene Q 5PLEX HRM Real-Time PCR and primers with sequences
Inhibition of miR-19a-3p Increases Bortezomib-Induced Apoptosis in Myeloma Cell Lines by Targeting SOCS3

**Results**

**IC50 of BTZ in (U266 and RPMI 8226) MM Cell Lines was 5 µM and 150 nM, Respectively**

We treated RPMI 8226 and U266 cell lines with different concentrations of BTZ to optimize its IC50 for the treatment of cell lines with proper concentrations throughout the analysis. By using the MTT assay and viability test with PI through flow cytometry, we found out that the optimized concentration of BTZ for the U266 cell line was 5 µM and 150 nM for RPMI8226 (Fig. 1a, c, and b, d).

**Downregulation of miR-19a Decreased Cell Viability and Prevented the Proliferation of Myeloma Cell Lines**

We first evaluated the expression level of miR-19a in non-transfected (control) myeloma cell lines (RPMI 8226 and U266). After the transfection of myeloma cell lines with the LentimiRa-off-has-mir-19a-3p vector, the efficiency of transfection was monitored by GFP evaluation using both fluorescent microscopy (Fig. 2a and 3a) and flow cytometry (Fig. 2b and 3b). The expression level of miR-19a was then determined in

---

**Table 1** Primer sequences, product length, and annealing temperature used in the qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-19a</td>
<td>F: CTC CGT TGT CCA AAT CTA TG R: GAG CAG GGT CCG AGG T</td>
<td>72</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>RT: GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACT CAG TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snord 47</td>
<td>F: ACT ACT GGA AAA CCG TTC CA R: GAG CAG GGT CCG AGG T RT: GTC GTA TGC AGA GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACA ACC TC</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>SOCS3</td>
<td>F: CCAAAGCAGGAGACTTCG R: GAAACTTGCTGTGGGTGAC</td>
<td>140</td>
<td>60</td>
</tr>
<tr>
<td>STAT3</td>
<td>F: CGG CGT CCA GTT CAC TAC R: CCC AGA GTC TTT GTC AAT GC RT: CAG GAG AAA TCA ACA GAGGC</td>
<td>97</td>
<td>60</td>
</tr>
</tbody>
</table>
suggests that the miR19a suppression decreased the proliferation of the cells.

The Expression Level of miR-19a in Myeloma Cell Lines was Reduced Upon BTZ Treatment

The expression level of miR-19a was evaluated after BTZ treatment by qRT-PCR analysis, and our data showed that its expression level was compared with non-treated cell lines, reduced after treatment. In the meantime, this expression was also evaluated in cells transfected by the LentimRa-off-has-mir-19a-3p vector and the data showed that miR-19a expression level was highly reduced after BTZ treatment, compared to non-treated and non-transfected controls (Fig. 2c and 3c). Cell viability evaluation in 72 hours with Propidium iodide (PI) using flow cytometry showed that it is

transfected myeloma cell lines (RPMI 8226 and U266) by qRT-PCR. As shown in fig. 2C and 3C, compared with the un-transfected myeloma cell line, the expression level of miR-19a significantly decreased in the transfected group. The data showed that antago-miR-19a downregulated expression of miR-19a which is a previously-known oncomiR. After the transfection of myeloma cell lines (RPMI 8226, U266) with LentimRa-off-has-mir-19a-3p vector, cell viability was evaluated in 72 hours with PI (Propidium Iodide) and the rate of viability was detected by flow cytometry (Fig. 2d and 3d). The data confirms the possibility of the analyses of transfected cell lines within 72 hours after transfection, while indicating that the viability of cell lines decreased because of the downregulation of miR-19a. A comparison with the negative control group,
possible to evaluate every analysis on transfected cell lines in 72 hours after transfection and on the other hand, the viability of cell lines decreased due to the downregulation of miR-19a (Fig. 2d and 3d).

**Anti-miR-19a Increased Susceptibility to Bortezomib-Induced Apoptosis**

In order to investigate that antago-miR-19a affects drug-induced apoptosis of myeloma cells, the percentage of apoptotic cells by Annexin -V, PI through flow cytometry after transfection of myeloma cell lines by lentimiRa-off-has-mir-19a-3p and incubation with bortezomib (BTZ, final concentration 5 µM for U266 and 150 nM for RPMI8226) for 48 hours were analyzed. Analysis of flow cytometry data showed that the percentage of cells that underwent apoptosis increased after transfection, compared with non-transfected cell lines and the negative control (Fig. 4a, b, c and d) (RPMI8226: 23.5% Vs. 68.2 p=0.0038 U266: 25.2 vs. 96.4 p=0.0006). The data showed that antago-miR-19a could increase myeloma cell susceptibility to drug-induced apoptosis.

SOCS3 and STAT3 mRNA expression level upregulated and downregulated, respectively after transfection of myeloma cell lines with LentimiRa-off-has-mir-19a-3p vector.

After recognizing SOCS3 as the target of miR-19a, SOCS3 and its target STAT3 mRNA level in myeloma cell lines after transfection with Lentini-off-has-mir-19a-3p vector was evaluated. The data showed that the mRNA expression level of SOCS3 increased, while STAT3 mRNA expression level decreased, compared with non-transfected cell lines as the negative control (Fig. 5a, b, 5c, and d).
critical role for it in the pathogenesis of myeloma.[10] Considering that better response in patients with the low level of miR-19a in serum and shortened progression-free with downregulation of it obtained from others studies,[15] we tried to evaluate the responsiveness of MM cell lines to BTZ in the presence of antagonomiR-19a to find a way to evade from resistance to it through targets of miR-19a. Recent studies figured out that SOCS3 is a target of miR-19a and indicated it is a negative regulator of SOCS3[16,17] and showed that the molecule beneath it, i.e. STAT3, is a significant promoter of cancers such as MM when activated.[5,18,19] On the other hand STAT3, one of the components of the JAK/STAT pathway acts as an onco-gene in human cancers.[19,20] Some miRNAs have close relativity with drug resistance, for example, past studies have shown that miR-181a expression is consistent with MM tumor load and could be a biomarker...
indicated in our results, the expression level of STAT3 in BTZ treatment highly decreased using it in parallel with anatago-miR-19a. So, it can be argued that miR-19a inhibition approach could be used to improve BTZ responsiveness in myeloma cells.

The results are honorable & supplement the findings that microRNAs are differentially expressed in BTZ-resistant myeloma cells. So miR-19a could be proposed as a prognostic biomarker for responsiveness to BTZ in MM patients. To overcome the resistance and improve the level of responsiveness to BTZ, miR-19a targets such as SOCS3 and STAT3 could be tracked. We have faced some restrictions in our studies that must be adverted. They include the use of other MM cell lines and myeloma cells obtained from MM patients, and the evaluation of targets in protein level expression and downstream molecules, that could have helped us to generalize our results.

**Conclusion**

Overall, our data propose that induction of antago-miR-19a in myeloma cell lines resulted in a down-regulation of miR-19a and enhancement of the level of treatment monitoring as much as miR-20a that showed the potential of a diagnostic biomarker.[4,6] BTZ as a proteasome inhibitor is an effective drug in the treatment of MM; however, drug resistance is still a major problem in 60% of patients.[8,21] So our data primarily showed that antagonmir-19a down-regulated the miR-19a expression, and BTZ caused inhibition of miR-19a in a concentration-dependent manner and thereby in the presence of antago-miR-19a and BTZ, miR-19a efficiently downregulated in parallel with an increase in the proportion of apoptotic cells following treatment with BTZ. On the other hand, our viability studies showed that the ratio of the proliferation of myeloma cell lines decreased after transfection with antagonmiR-19a vector. The data are consistent with other studies that proved miR-181a and miR-20a inhibited by BTZ.[6] In another part of our studies, we found out that downregulation of STAT3 and upregulation of SOCS3 at mRNA level which confirmed the reality of miR-19a function on SOCS3 is a negative regulator, the same as previous data.[16,17] As much as, the oncogenic function of STAT3 has been previously proved,[18,19] we could conclude that inhibition of miR-19a causes suppression of STAT3; and as

![Fig. 4. Evaluation of apoptosis by Annexin V-FITC/PI staining and analysis by flow cytometry in RPMI 8226 & U266 Myeloma cell lines. (a) Untreated & nontransfected cell line as negative Control (b) After BTZ treatment. (c) after transfection by plenti-anti-miR-19a vector. (d) After treatment of transfected RPMI cell line with bortezomib.](image-url)
responsiveness to BTZ treatment. On the other hand, the ratio of apoptosis in BTZ-treated cell lines was drastically more effective in the presence of miR-19a inhibition, so miR-19a could be suggested as a prognostic biomarker in MM treatment.

Peer-review: Externally peer-reviewed.

References


