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Original Article

Scutellarin circumvents chemoresistance, promotes apoptosis, and represses tumor growth by HDAC/miR-34a-mediated down-modulation of Akt/mTOR and NF-κB-orchestrated signaling pathways in multiple myeloma

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Abstract: Multiple myeloma (MM) is a neoplastic dyscrasia of monoclonal immunoglobulin-secreting plasma cells culminating in multi-organ dysfunction. In this study, we sought to investigate whether scutellarin (STN), a flavonoid, could reduce MM progression, mitigate chemoresistance of MM cells to bortezomib (BTB), and cause MM cell apoptosis in a xenograft mouse model of MM. Epigenetic signalling plays a main role in the modulation of various pathways involved in multiple myeloma progression. At the outset, mechanistic analyses of the MM pathways indicated that key epigenetic molecules including HDAC1/3 and miR-34a were up-modulated and down-modulated respectively, in the MM mice. Besides, the downstream signalling analysis of miR-34a depicted that the c-Met/AKT/mTOR pathway was activated in the MM mice. We also investigated the expression of NF-κB, one of the major chemoresistance inducers in cancer treatment, in the MM mice. As anticipated, the tumor-bearing mice expressed more NF-κB along with elevated anti-apoptotic Bcl-xL protein, as well as reduced pro-apoptotic Bim protein. On the other hand, STN+BTB co-treatment effectively combated the MM tumor progression, and STN circumvented the MM tumor resistance to BTB and provoked apoptotic cell death in MM. Based on our study data, we deduce that STN, in combination with BTB, appears to be a reliable tumoricidal strategy.

Keywords: Histone deacetylases, miR-34a, multiple myeloma, scutellarin

Introduction

Multiple myeloma (MM) is a neoplastic dyscrasia of monoclonal immunoglobulin-secreting plasma cells, clinically manifested by multi-organ impairment-anemia, bone pain, renal dysfunction, neuropathy and immunological malprogramming. MM is the second most prevalent hematological malignancy next to non-Hodgkin's lymphoma. Globally, with about 126% increase in incidence rate in MM cases, the death toll has increased by 94%. Despite the discovery and approval of an array of oncotherapeutics, and appreciable improvement in the overall survival rate, relapsed refractory MM cases still pose treatment challenges. This underscores the unmet need in the advent of an armamentarium of novel chemo/immunotherapies [1] in relation to the potential to subdue drug resistance, reduced adverse effects, and improved clinical outcome.

Most of the current oncotherapeutics including melphalan, adriamycin, dexamethasone, thalidomide, lenalidomide, pomalidomide and bortezomib, used alone or in combination, are associated with substantial chemoresistance and non-cancer cell toxicity, leading to a spectrum of adverse effects. Bortezomib (BTB), the first-in-class proteasome inhibitor and a first-line MM therapy, is associated with drug resistance, hematological (thrombocytopenia, neutropenia, etc.) neurological (e.g., hypoesthesia) and cardiological (e.g., ventricular insufficiency, cardiac arrest, etc.) adverse effects [2, 3]. Furthermore, enhancing the tumor cell longevity and development of resistance to BTB therapy is reported to be due to stromal cell-MM cell

micro-interaction by soluble IL-8-mediated NF-κB activation [4]. Hence, targeting NF-κB repression might reinforce MM cell sensitivity to BTB and trigger the tumor cell death pathway.

Recently, epigenetic dysregulation-associated with aberrant DNA methylation, histone (de) acetylation and other modifications, deregulated non-coding/microRNAs-is burgeoning as a critical pathologic phenomenon in the development and metastasis of MM. Various seminal studies demonstrated that blockage of histone deacetylases (HDACs) and the inhibition of lysine residue deacetylation in the histones/non-histone proteins are growing as novel oncotherapeutic strategies for MM [5]. Of note, targeting class I HDACs (HDAC1, HDAC2 and HDAC3) as an inhibition-based approach, illustrated the beneficial effects in the context of chemosensitivity enhancement and direct antagonistic activity in hematologic malignancies including MM and acute myeloid leukemia [6, 7]. Apart from the HDAC blockage strategies, regulation of microRNA strategies including long non-coding RNA ANGPTL1-3/miR-30a-3p [8], miR-155 or miR-520g/miR-520h [9] and snail1/hsa-miRNA-22-3p/P53 signalling are implicated in the regulation of tumor and the tumor microenvironment, and alleviation of BTB resistance in MM [10]. Also, there are reports highlighting the connection between NF-κB and microRNAs including miR-21 and miR-29b [11, 12] in the modulation of multiple myeloma cell growth. Hence, oncotherapeutics with multi-modal targeting potential as well as safe and holistic facets is a dire need for effective anti-MM treatment outcomes.

Scutellarin (STN), a flavonoid glycoside and a proteasome inhibitor is known to exert anti-tumor activity across a band of cancers including lung adenocarcinoma, hepatocarcinoma, lymphoma, breast and colorectal carcinomas [13]. Notably, STN offers anti-tumor immunity and mitigate chemoresistance in neoplastic diseases through multiple signal transduction pathways: ERK/p53, c-met/AKT, AKT/mTOR/4EBP1 and STAT3 [14, 15]. Interestingly, targeted delivery of miR-34a mimics sensitized MM cells to BTB by repression of ERK and AKT signaling pathways [16, 17]. Another report delineated the role of miR-125b/IL-6R/STAT3 feedback loop in modulating miR-34a in multiple myeloma [18]. Based on the literature, we surmised that STN might exhibit oncosuppressive role, enhance the chemosensitivity of MM cells to BTB and confer durable therapeutic outcomes in MM through multiple signalling mechanisms.

Materials and methods

Reagents and cells

Scutellarin was obtained with a purity greater than 98% from AbMole BioScience, U.S.A. Bortezomib was procured from Abcam, U.S.A. Primary antibodies against HDAC1, HDAC3, c-Met, AKT, P-AKT, mTOR, NF-κB, XIAP, and β-actin and the secondary HRP-conjugated antibodies were procured from Cell Signalling Technology, U.S.A. The multiple myeloma MM.1S cells were obtained from American Type Culture Collection, U.S.A.

In vivo xenograft MM treatment regimen

All the animal handling procedures were done in accordance with the guidelines for animal care approved by our institution. To induce MM, 2×10^6 of MM.1S cells were injected subcutaneously on the left side of the BALB/C nude mice and the tumor volume was allowed to reach up over 150 mm^3. Among the randomly segregated cohorts, STN or BTB were injected at 60 mg/kg dosage through the tail vein, while STN/BTB co-injection was done with 30 mg/kg of each drug; alternate day treatment was given in each cohort for six times during the study. After the study period, all the mice were euthanized and the MM xenograft tumor was resected for further molecular studies.

Isolation of RNA and RT-PCR analysis

The tumor tissues were resected out on ice from all the mice. To perform the RT-PCR analysis, total RNA was extracted by using total RNA isolation kit-Rneasy (Qiagen, Valencia, CA). The target gene expression was normalized using β-actin gene as the control in each sample. Following are the PCR primers used for the analysis: β-actin: F: TCTTGGGTATGGAATCCTGTG and R: ATCTCCTTCTGCATCCTGTCA; miR-34a: F: ACGCTTGTGTTTCTCAGTCCG and R: TGGTCTAGTTCCCGCCTCCT.

Immunohistochemical analysis

Based on the manufacturer instructions, pretreated tumor sections were treated with 3% H_2O_2 and then subjected to microwave oven

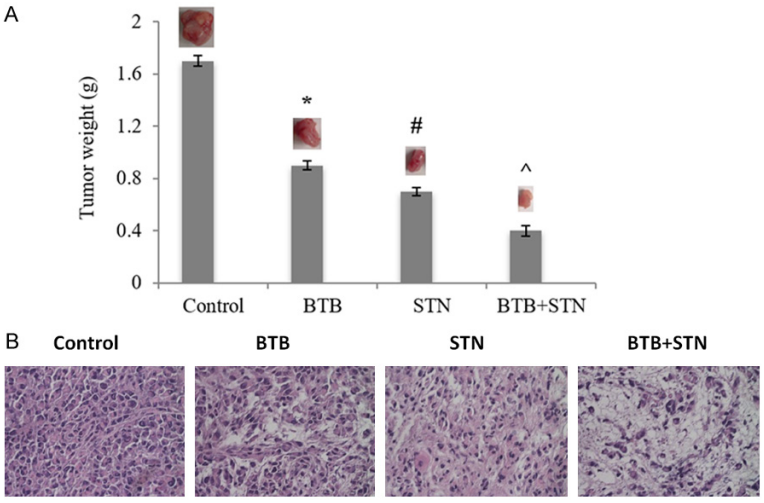

Figure 1. STN potentiated the anti-MM activity of BTB in a xenograft mouse model. A. Representative tumors and tumor weight across the animal groups. B. Representative histologic sections of tumor tissue across the animal groups. Data are shown as mean ± S.D ($P < 0.05$). *BTB vs. control; #STN vs. control; ^BTB+STN vs. control.

heating with 10-mM citrate buffer for 10 min, followed by serum blocking procedures. The corresponding primary antibody for HDAC1 and HDAC3 (Beijing Biosynthesis Biotechnology Co., Ltd., China), were added and the tissue sections were incubated overnight at 4°C. The secondary antibody was then added and incubated for 30 min and subjected to 3,3'-diaminobenzidine, a chromogen for brown staining. The stained slides were analysed using Image-Pro Plus image analysis system (Media Cybernetics, USA).

Western blot analysis

The resected tumor sections were cut and homogenized in ice-cold RIPA using an electric homogenizer. The tissue homogenate was centrifuged at 16,000 × g at 4°C for 20 minutes to obtain the supernatant containing the proteins. The SDS-polyacrylamide gel was used along with the Tris blocking buffer system to separate the proteins by overnight incubation with specific primary antibodies. The blotted membranes were rinsed and subsequently incubated with secondary HRP (horseradish peroxidase)-conjugated antibodies. Ultimately, quantitative analysis of the antibodies was done by using the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA).

Statistical analysis

SPSS software (V13.0; SPSS, Inc., USA) was used for statistical evaluation, and one-way analysis of variance (ANOVA) was applied using Tukey's post-hoc test for comparisons among different animal groups. Significance level was set at $P < 0.05$.

Results

STN potentiates the anti-MM activity of BTB in a xenograft mouse model

BTB, a proteasome inhibitor, is an FDA-approved single agent for the clinical treatment of MM. However, circumvention of resistance to BB in relapsed and refractory MM cases is an unmet need in the clinical milieu. Hence, we investigated the potential of STN to abrogate chemoresistance of MM cells to BTB using a xenograft MM mouse model. In our study, BTB treatment significantly ($P < 0.05$) mitigated the tumor growth against that of control, in the context of tumor weight (0.9 g in BTB vs. 1.7 g in control). On the other side, STN alone treated MM mice depicted incremental reduction in tumor weight (0.7 g). In fact, co-treatment of BTB and STN portrayed dramatically ($P < 0.05$) reduced tumor weight (0.4 g) as shown in **Figure 1A**. This indicates that STN has the ability to outperform and amplify the anti-MM effects of BTB. Staining of the tumor sections with H&E revealed loss of cellular architecture with hypercellularity in the controls, while BTB+STN treatment depicted significant pyknosis, compared to BTB or STN treated mice (**Figure 1B**).

Effect of BTB and STN on the epigenetic modulators HDAC1/3 and miR-34a

Probing of the epigenetic modulators including HDAC1 and HDAC3 disclosed that these molecules were effectively down-modulated in the BTB and STN treated mice, counting to 33% and 58% reduction ($P < 0.05$) of HDAC1 and 29% and 59% reduction ($P < 0.05$) of HDAC3 respectively. As anticipated, STN potentiated the anti-MM activity of BTB leading to > 75%

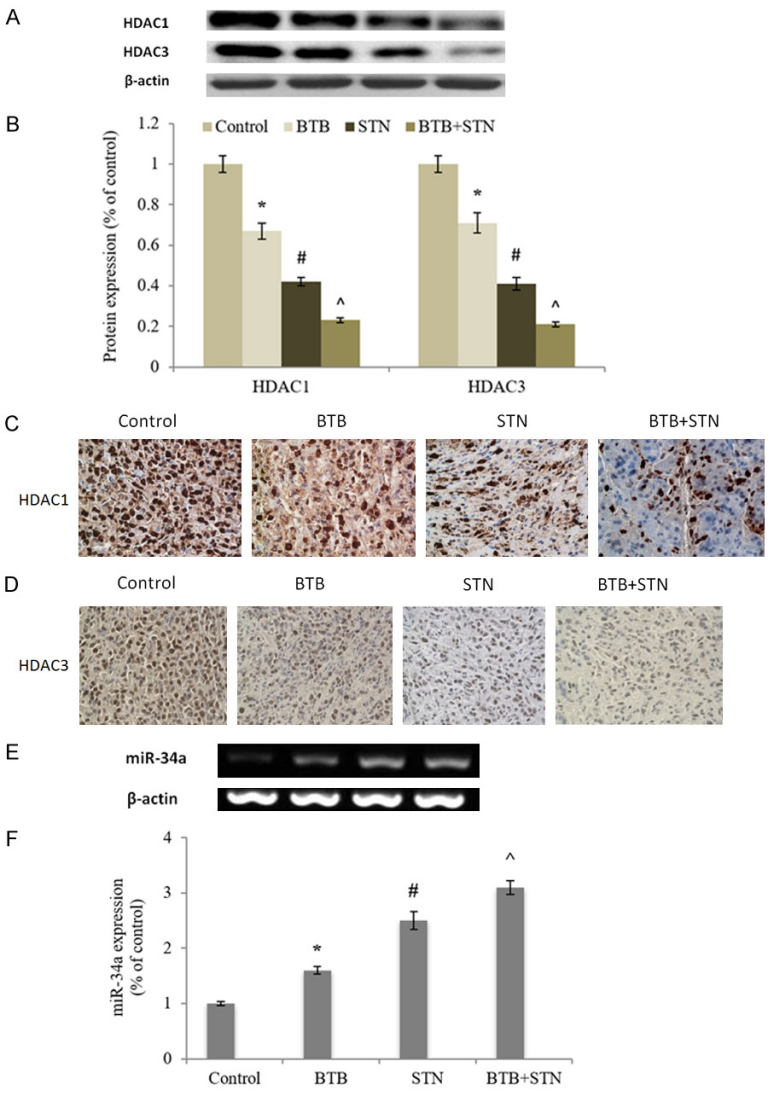

Figure 2. Effect of BTB and STN on the epigenetic modulators HDAC1/3 and miR-34a. A. Shown are representative bands of HDAC1 and HDAC3 expressions with β-actin control (internal). B. Assessment of HDAC1 and HDAC3 expressions represented as % of control (internal). C. Representative immunohistochemical sections of tumor tissues showing HDAC1 expression across the animal groups. D. Representative immunohistochemical sections of tumor tissues showing HDAC3 expression across the animal groups. E. Shown are representative bands of miR-34a expression with β-actin control (internal). F. Assessment of miR-34a represented as % of control (internal). Data are shownas mean ± S.D ($P < 0.05$). *BTB vs. control; #STN vs. control; ^BTB+STN vs. control.

drop in both HDACs (**Figure 2A**, **2B**). Inhibition of HDACs could be ascribed to the tumoricidal activity of BTB/STN combination strategy in MM. Validation of the expression of HDACs in all the animal groups using immunohistochemical staining (**Figure 2C**, **2D**) also reflected the findings of western blot analysis.

On the other hand, we observed that miR-34a, a key oncosuppressor, was less expressed in the control mice, whereas BTB and STN significantly ($P < 0.05$) amplified the expression of miR-34a to 1.6-fold and 2.5-fold respectively. In harmony, co-injection of BTB and STN depicted remarkable hike in the expression (**Figure 2E**, **2F**), underscoring the potentiating effect of STN in BTB-induced MM tumor suppression.

Effect of BTB and STN on the c-Met/Akt/mTOR signaling axis

c-Met/Akt/mTOR signalling axis is a critical pathway in the cancer cell survival, proliferation and invasion in various malignant conditions including MM. In this view, when we analysed these oncogenic molecular signals, we observed that c-Met, p-Akt, and mTOR protein expressions were significantly ($P < 0.05$) declined in both STN and BTB treatment groups (28%, 29% and 45% reduction in c-Met, p-Akt and mTOR respectively in BTB; 51%, 53% and 62% reduction in c-Met, p-Akt and mTOR in STN respectively). In congruence with other observations of this study, STN enhanced the inhibitory effects of BTB against MM (**Figure 3**).

Effect of BTB and STN on the NF-κB and anti-apoptotic XIAP signalling

NF-κB, the master transcription factor regulates the activation of various apoptosis-related molecules including the anti-apoptotic protein, X-linked inhibitor of apoptosis protein (XIAP). In fact, XIAP is the widely researched apoptosis inhibiting protein that negatively regulates caspases in MM. In our current work, STN and BTB caused a substantial ($P < 0.05$) decline (36% and 61%) in the NF-κB as well as (39% and 62%) in the XIAP protein expressions. Further, combined treatment of BTB and STN caused an extensive

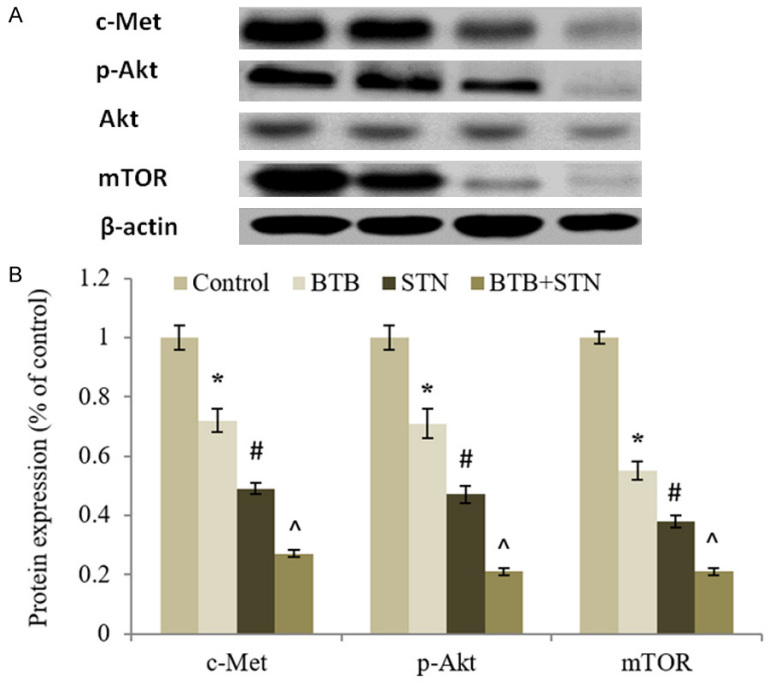

Figure 3. Effects of BTB and STN on the c-Met/Akt/mTOR signalling axis. A. Shown are representative bands of c-Met, Akt and mTOR expressions with β-actin control (internal). B. Assessment of c-Met, Akt and mTOR expressions represented as % of control (internal). Data are shown as mean ± S.D ($P < 0.05$). *BTB vs. control; #STN vs. control; ^BTB+STN vs. control.

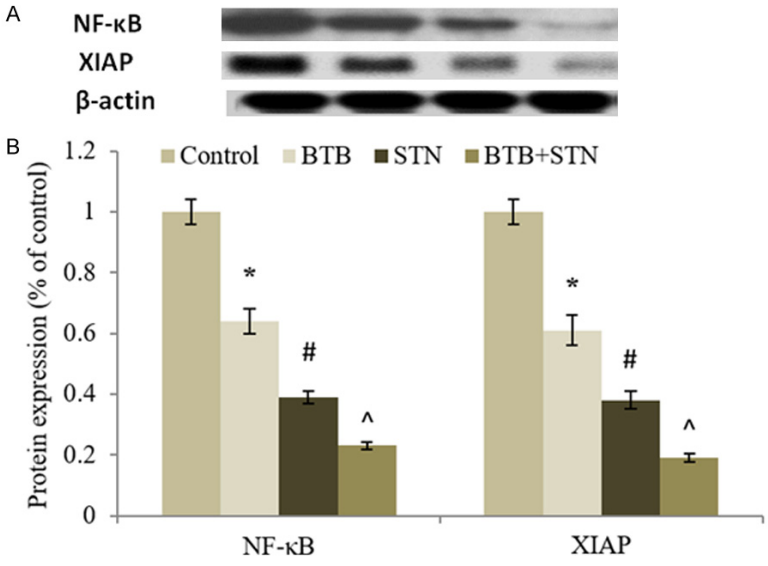

Figure 4. Effect of BTB and STN on the NF-κB and anti-apoptotic XIAP signalling. A. Shown are representative bands of NF-κB and XIAP expressions with β-actin control (internal). B. Assessment of NF-κB and XIAP expressions represented as % of control (internal). Data are shown as mean ± S.D ($P < 0.05$). *BTB vs. control; #STN vs. control; ^BTB+STN vs. control.

77% and 81% drop in the expressions of NF-κB and XIAP respectively (**Figure 4**).

Discussion

The anti-myeloma potential of STN was gauged in this study by using a mouse xenograft model of MM. We investigated how STN modulates the survival/death signalling pathways and the linkage with epigenetic changes mediated by microRNAs and HDACs. In recent years, HDAC inhibitors (HDACi) are receiving attention due to their anti-tumor potential in regulating the oncogenes in MM. In this line, there are several studies [6, 7, 19-21] which illustrated that class I HDACs (HDAC1, HDAC2 and HDAC3) could be promising molecular targets in the treatment of hematological malignancies like MM. It is evident that BTB exerts appreciable anti-MM activity by inhibition of class I HDACs; however, combination of BTB with other drugs like HDACi is recommended to treat the resistance-prone MM cases [21]. In our study, we noticed that HDACs were up-modulated in the MM mice, while co-treatment with BTB and STN effectively inhibited the HDAC expressions compared to BTB/STN alone. Nevertheless, STN treatment has a better impact on the blockage of HDACs than BTB. In fact, the efficacy of STN in antagonizing the HDAC1 and HDAC3 activity was already proven in a study by Vidakovic et al. [22].

To further drill down the molecular connections pertinent to class I HDACs, we further assessed the role of miR-34a in STN/BTB treated and MM mice. The idea behind the selection of miR-34a for further probing is based on the following traits: i) miR-34a affords onco-suppressive/

anti-tumor effects in the context of regulating an array of downstream proteins involved in cell cycle, proliferation/differentiation and apoptosis [16, 17]; ii) miR-34a activation/overexpression has been proven beneficial against various *in vivo* and *in vitro* models of MM and other cancers [18, 23]. iii) successful research outcomes of various miR-34a mimetics using novel delivery formulations (e.g., stable nucleic acid lipid particles) against MM [16, 24]; iv) plausible HDAC blockage-mediated up-modulation of miR-34a and consequent mitigation of cancer cell proliferation [25]. A major finding of our study was that miR-34a was down-modulated in the MM mice, whereas BTB/STN co-treatment regimen restored the miR-34a level. In this regard, a preliminary report indicated that miR-34a improves the sensitivity of MM cells to BTB [17], while another report indicated that genetic knockdown or drug-based inhibition of HDAC1 upregulated miR-34a expression [25]. Together, these results underscore that HDAC1-mediated miR-34a upregulation underlies the anti-myeloma effects of BTB/STN.

p53, a tumor suppressor, is a molecular connector underlying HDAC-mediated modulation of miR-34a in MM. Notably, HDAC1/3 inhibition up-modulates the expression of p53 through acetylation of p53 and modifying its transcriptional effect, thereby enhancing the apoptotic activities in MM [26-28]. A landmark study by Li et al. [29] reported that miR-34a blocks cancer cell proliferation by directly targeting and down-modulating c-Met expression. Genetic ablation of c-Met, an oncogenic protein, obstructs Akt/mTOR activities and thus, sensitizes multiple myeloma cells to bortezomib-provoked apoptosis [30]. In our study, c-Met expression was decreased in the BTB/STN co-treated mice against MM; thus, it down-modulated the expressions of Akt and mTOR in the BTB/STN co-treatment group and promoted the cell cycle arrest and apoptosis of cancer cells in the MM mice. This outcome is in harmony with an earlier study, which demonstrated that STN enhances chemosensitivity and promotes cancer cell death partly by the Akt/mTOR pathway [14].

Nuclear factor kappa B (NF-κB) is a key factor in the proliferation and survival of MM cells; thus, endorsing development of chemoresistance in MM [26]. NF-κB shifts the survival-death equilibrium in MM towards survival mode through the modulation of a gamut of anti-apoptotic (XIAP, survivin, Bcl-2, Bcl-xL, etc.) and pro-apoptotic (Bax, Bim, etc.) proteins. Hence, suppression of anti-apoptotic proteins and activation of pro-apoptotic proteins imparts anti-cancer and chemosensitivity effects against MM. In an earlier study, Shi et al. [27] reported that STN activates apoptosis in MM cells by down-modulation of Bcl-2 and up-modulation of Bax. In our study, we observed that anti-apoptotic XIAP protein was up-modulated in the MM group; however, STN/BTB co-treatment reversed this equilibrium.

We found that scutellarin circumvented the resistance of MM cells to BTB by multiple mechanistic pathways involving epigenetic regulation of the c-Met/Akt/mTOR pathway by HDAC/miR-34a as well as NF-κB-mediated activation of the apoptotic cascade. This creates a robust argument for the scutellarin to be considered an effective anti-neoplastic agent alone or in combination with other anticancer drugs. However, there are certain limitations in our study: effect of BTB/STN on the inhibition on other HDACs and their interplay relevant miRNA panel have not been assessed; executionary and effector apoptotic markers and their validity in MM diagnosis has not been investigated; whether these biofactors would help in assessing the grade or severity of MM needs to be assessed. Hence, further studies are warranted in this milieu to overcome the limitations, reinforce our findings, and answer pertinent research questions.

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Disclosure of conflict of interest

None.

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