

Synthetic Curcumin Analog: Inhibiting the Invasion, Angiogenesis, and Metastasis in Human Laryngeal Carcinoma Cells via NF- κ B Pathway

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Abstract

Laryngeal carcinoma, the most common among head and neck squamous cell carcinoma (HNSCC) induces 1% of all cancer deaths. Curcumin, the active constituent of turmeric is more effective in the treatment of various cancer. In the present study, with an aim to explore the mechanistic role of BDMC-A as a chemotherapeutic agent, we have investigated the inhibitory effect of BDMC-A on invasion, angiogenesis, and metastasis in Hep-2 cells and compared it with curcumin. Curcumin and BDMC-A treated Hep-2 cells were quantified using western blotting and RT-PCR technique to investigate the effect of Curcumin and BDMC-A on transcription factors involved in signal transduction cascade, invasion and angiogenesis associated markers. Pro-inflammatory markers of curcumin and BDMC-A treated Hep-2 cells were estimated using the ELISA kit. The results showed that BDMC-A might exhibit the anti-cancer activity by inhibiting transcription factors mainly NF- κ B, p65, c-Jun, c-Fos, STAT3, 5, PPAR- γ and β -catenin, which are responsible for tumor progression and malignancy. Downregulation of MMP-9, VEGF, IL-6 and IL-8 and upregulation of TIMP-2 levels further supports the anti-tumor potential of BDMC-A. Our overall results revealed that BDMC-A more effectively inhibited the markers of invasion, angiogenesis and metastasis in comparison with curcumin.

1. Introduction

Laryngeal carcinoma, the most common type of head and neck squamous cell carcinoma (HNSCC), comprises 1% of all the cancers and reported to be responsible for 1% of all cancer-induced deaths [1, 2]. Laryngeal cancer mainly results from the usage of Tobacco. In addition to this, alcohol use, nutritional deficiencies, and genetic predisposition may also play a role. Besides surgery, laryngeal carcinoma could be most effectively treated by chemotherapy, and the widely used drug cisplatin prominently improves the survival rate [3, 4]. However, the side effects such as leukopenia and kidney failure, are reported due to chemotherapy [5]. Identification of new drugs with low toxicity showing improved therapeutic potential is of great interest among the researchers.

Curcumin, a principle ingredient isolated from the rhizomes of *Curcuma longa*, is reported for its anti-inflammatory, antiviral, antibacterial, antifungal, anti-diabetic, anti-carcinogenic, and pharmacological properties [6]. In previous studies, curcumin was reported for its inhibitory effect on tumor proliferation, metastasis, invasion and angiogenesis [7]. Curcumin inhibits the proliferation of various cancer cells through the down-regulation of cell-signaling targets like Vascular Endothelial Growth Factor (VEGF), Matrix metalloproteinase-9 (MMP-9), AKT, AP-1, Cyclin D1, and STAT-3 [8]. NF- κ B activation suppresses apoptosis and induces proliferation, invasion, metastasis and inflammation [9]. Curcumin possesses anticancer effects on pancreatic cancer [10], and breast cancer [11] due to its ability to suppress NF- κ B activity. However, due to its low stability and poor bioavailability researchers are interested to synthesize analogs of curcumin to acquire improved potency.

The anti-tumor and anti-mutagenic as well as anti-oxidant [12] activities of the bis-demethoxy curcumin analog (BDMC-A), a synthetic analog of curcumin, was reported. We have investigated and reported the

therapeutic potential of BDMC-A on alcohol and Δ PUFA-induced oxidative stress [13], hyperlipidemia [14] and matrix metalloproteinase [15]. Previously BDMC-A was reported for its anticarcinogenic effects on DMH induced-colon cancer rats [16]. Moreover, the anti-proliferative, cell cycle arrest potential and the molecular mechanisms of BDMC-A inducing apoptosis in human laryngeal cancer (Hep-2) cells have been compared with curcumin [17, 18]. In continuation of this, the present study is focused on the investigation of the anti-invasive, anti-angiogenic, and anti-metastatic potential of BDMC-A and curcumin using various markers.

2. Materials And Methods

2.1. Materials

BDMC-A was synthesized as per the method reported earlier [19]. Curcumin, Dulbecco's Modified Eagle Medium (DMEM), antibiotics and fetal bovine serum (FBS) were purchased from Sigma–Aldrich, Bangalore, India. Primary antibodies against NF-kB p65, c-Rel, AP-1, COX-2, MMP-9, TIMP-2 were procured from Santa Cruz Biotechnology and Cell Signaling Technology, USA. mRNA primers for COX-2, MMP-9, VEGF were obtained from Sigma–Aldrich, Bangalore, India. Hep-2 cell line was obtained from NCCS, Pune, India and maintained in 1× Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.

2.2. Methodology

2.2.1. Western blotting

Western blotting was done using the method described by Fido et al. [20]. Briefly, Hep-2 cells were grown in 6-well plates and treated with selected doses of curcumin and BDMC-A. After 24h of incubation with curcumin or BDMC-A, total cell proteins were extracted and estimated by Bio-Rad Bradford protein assay. Lysate samples were resolved on 10% SDS-PAGE and electro-transferred to nitrocellulose membrane. The membranes were then blocked using blocking buffer and probed with primary antibody followed by appropriate secondary antibodies. β -actin was used as a control for protein loading. Protein bands were discovered by enhanced chemiluminescence method and the signals were detected by ECL, (Pierce, Rockford, IL, USA) and Chemi Doc Imaging System (Bio-Rad, Hercules, CA, USA).

2.2.2. Total RNA extraction and RT PCR

Total RNA was extracted from curcumin or BDMC-A treated Hep-2 cells followed by 24h incubation using TRIzol Reagent (Sigma–Aldrich, Bangalore, India) as per manufacturer's protocol. cDNA was synthesized by reverse transcription of total RNA (2 μ g) using random hexamer primers and MMLV RT enzyme. Real-Time PCR was performed on a CFX 96 Touch Real-Time PCR (Bio-Rad, USA) using the HOT FIRE Pol EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia). Cycle conditions were: 95°C for 5 m, followed by 45 cycles (95°C for 30 s, 58°C for 30 s). Sequences of gene-specific forward and reverse primers are: COX-2 forward 5'-TGTATGCTACCATCTGGCTTCGG-3' and reverse 5'-GTTTGGAAACAGTCGCTCGTCATC-3'; MMP-9

forward 5'-CGGAGTGAGTTGAACCAG-3' and reverse 5'-GTCCCAGTGGGGATTTAC-3'; VEGF forward 5'-GAAGTGGTGAAGTTCATGGATGTC-3' and reverse 5'-CGATCATTCTGTATCAGTCTTTCC-3'. The amplification specificity of the product was determined by the melting curve analysis for each primer pairs. Data were analyzed by the comparative CT method and the fold-change was calculated by the $2^{-\Delta\Delta CT}$ method using CFX Manager Version 2.1 (Bio-Rad, USA) [11].

2.2.3. Measurement of proinflammatory cytokines

Amounts of proinflammatory cytokines TGF- β , IL-6 and IL-8 in the supernatant of curcumin or BDMC-A treated Hep-2 cells were determined using commercial ELISA assay kits (eBiosciences, USA) following the manufacturer's instructions. The samples were run in triplicate and the data were averaged.

2.2.4. Invasion assay

Matrigel-coated transwell cell culture chambers (8 mm pore size) (BD Biosciences, USA) were used for cell invasion assay. Hep-2 cells were placed in the upper chamber of the transwell insert (5×10^4 cells/well) followed by the incubation in serum-free medium for 24h and then treated with the selected concentration of curcumin or BDMC-A. The lower chamber was filled with medium with serum. Plates were then incubated for 24h or 48h. Non-invasive cells were removed from the upper chamber by wiping with a cotton swab. The invasive cells were stained with 0.4% Trypan Blue. Cells that penetrated through the Matrigel appeared in the lower surface of the filter and were counted under a light microscope.

2.3. Statistical analysis

SPSS 7.5-Windows Students version software (SPSS Inc., Chicago, IL, USA) was used for data analysis. One-way ANOVA followed by Tukey's test was used to assess the statistical significance between groups at the level of $p \leq 0.05$.

3. Results

In a previous study, we have determined the cell viability of Hep-2 cells treated with BDMC-A and the IC_{50} at 48h was found to be 20 μM , which is lesser in comparison with curcumin (IC_{50} at 48h is 50 μM) [17]. The present study compares the BDMC-A-induced inhibitory effect by modulating the NF- κB signaling pathway and related markers in Hep-2 cells using the sub-optimal cytostatic dose of curcumin (25 μM) and BDMC-A (10 μM).

3.1. Effect of BDMC-A towards transcription factors involved in signal transduction cascade

The effects of curcumin and BDMC-A on proteins of signaling pathways like NF- κB -p65, AP-1, STAT3, STAT5, β -catenin and PPAR- γ were quantified in Hep-2 cells using the western blotting technique. Total protein lysate from the control cells and treated cells were extracted and analyzed for NF- κB -p65, c-Rel, AP-1, STAT3, STAT5, β -catenin and PPAR- γ by western blot. The results are shown in Fig. 1 and Fig. 2. As

expected, a significant downregulation was observed in the expression levels of NF- κ B p65 and c-Rel in cells treated with BDMC-A than curcumin. Our western blot results showed that there was a considerable decrease in AP-1 complex i.e. c-Jun and c-Fos expression on treatment with BDMC-A than with curcumin-treated cells. In comparison with curcumin, BDMC-A showed an equipotent decrease in the level of STAT3 and STAT5 expression in Hep-2 cells as compared to curcumin. The expression of β -Catenin significantly downregulated in Hep-2 cells upon BDMC-A treatment than curcumin. BDMC-A was also observed to upregulate the PPAR- γ expression more effectively than curcumin in Hep-2 cells.

3.2. Effects of BDMC-A on putative invasion and angiogenesis -associated markers

Hep-2 cells treated with curcumin and BDMC-A were lysed and analyzed by western blot and RT-PCR. RNA extracted from cells that were used for RT-PCR to assess the markers of invasiveness and angiogenesis. The western blot results in Hep-2 cells, showed significant down regulation of COX-2 when treated with BDMC-A in comparison with curcumin. The Hep-2 cells' western blots showed that there was a significant down regulation of MMP-9 levels and significant upregulation of TIMP-2 levels when compared to that of curcumin treatment (Fig. 3a & 3b).

Q-PCR analysis of Hep-2 cells revealed a significant downregulation of mRNA levels of COX-2, MMP-9 and VEGF (Fig. 4) in BDMC-A treated cells when compared to curcumin-treated cells. These observations indicate that BDMC-A has a stronger anti-invasive, anti-metastatic and anti-angiogenic effects compared to curcumin.

3.3. Effects of BDMC-A on the invasion of Hep-2 cells

The effect of curcumin and BDMC-A on the invasive potential of Hep-2 cells was tested by its ability to migrate across a membrane. Compared to control only fewer cells treated with curcumin and BDMC-A entered into the lower chamber from the upper separated by EHS-coated filter. The migration of cells across the filter was inhibited more effectively by BDMC-A in Hep-2 cells (Fig. 5) than curcumin.

3.4. Effects of BDMC-A on cancer-related inflammatory markers

Curcumin and BDMC-A treated Hep-2 cells were tested using ELISA kit for pro-inflammatory markers such as TGF- β , IL-6, and IL-8 as they are considered the hallmark of cancer. Compared with control cells, BDMC-A treated Hep-2 cells had significantly downregulated expression of TGF- β , IL-6, IL-8 (Fig. 6). Overall, these are the common pathways of tumor development that were observed with some bladder, breast, head and neck and lung cancers, despite their diverse tissue origins

4. Discussion

Therapeutic agents with multiple gene targeting potentials could be the right choice for the treatment of cancer as various genes are known to be dysregulated in cancers. NF- κ B, a “rapid-acting” primary transcription factor is the important intracellular target in chemotherapy for cancer as it responds to various cellular stimuli that regulate the expression of various genes resulting in inflammation, cell survival, invasion, angiogenesis and metastasis [21, 22]. Our previous reports have shown that BDMC-A is a more effective inhibitor of NF- κ B and related markers in a breast cancer cell line in comparison with curcumin [11]. We have also reported that BDMC-A inhibits Hep-2 cells more efficiently than curcumin through apoptosis rather than necrosis. In the present study we showed that BDMC-A affects the NF- κ B signaling pathway in Hep-2 cells.

We observed that BDMC-A downregulated NF- κ B, p65 and its related oncogene c-Rel more efficiently than curcumin in Hep-2 cell lines. Curcumin was reported to inhibit NF- κ B activation and thereby suppress cancer cell proliferation in head and neck cancers [23]. Curcumin inhibits the stimulation of the upstream signal of NF- κ B i.e. Akt. It downregulates NF- κ B targets COX-2 and MMP-9 [24, 25]. Agents that activate NF- κ B also stimulate the transcription factor AP-1. Activated AP-1 has been associated with cell proliferation and chemical carcinogenesis. Expression of genes regulated by AP-1 has been related to the transformation from preneoplastic to the neoplastic state of cancer cells in ex vivo and in vivo models [26] AP-1 also participates in tumor progression and metastasis. The results of our present study showed that treatment of Hep-2 cells with BDMC-A significantly downregulated the c-Jun and c-Fos expression in comparison with curcumin treatment. This effect of BDMC-A may in part be due to the suppression of NF- κ B as the previous studies have reported a distinct and essential role of NF- κ B in regulating the AP-1 transcription factor. Curcumin has been reported to suppress AP-1 activation [27]. It interacts with AP-1 DNA binding motif and inhibits AP-1 activation.

STAT3 converges with NF- κ B in various oncogenic signaling pathways [28]. Constitutive activation of STAT3 has been observed in cancer and immune cells. It takes part in carcinogenesis, as well as in tumor immune evasion. STAT3 has been proved to control cell proliferation, survival and regulate the expression of c-myc, cyclin D and Bcl-2 in colon cancer [29]. Mutation in EGFR has been attributed for constitutive STAT3 phosphorylation in lung adenocarcinomas [30]. In our study the STAT3 and STAT5 expression levels were more significantly downregulated in BDMC-A treated cells compared to curcumin treated cells. Thus, decreased level of NF- κ B in BDMC-A treated Hep-2 cells may be partially due to the effect of the drug on STATs. Suppression of STAT3 activation by curcumin has been observed in T-cell leukemia [31], lung [32], HNSCC [33], ovarian cancer [34]. Curcumin has been reported previously to downregulate the activation of STAT5 in K562 leukemia cells [35].

β -catenin is an important transcription factor that is activated by the WNT signaling pathway. We investigated the effect of BDMC-A on the β -catenin levels in Hep-2 cells and found that the β -catenin level was more significantly reduced in BDMC-A than curcumin-treated cells. Curcumin stimulates caspase-3-mediated β -catenin cleavage as well as suppress β -catenin/Tcf/LEF transactivation by c-myc and cyclin D1 [36]. Another transcription factor that is emerging as a potential target for cancer therapy is PPAR- γ . In our study, BDMC-A significantly upregulated the PPAR- γ level in Hep-2 cell lines. This could have also

aided the previously observed downregulation of NF- κ B in BDMC-A treated cells. The anti-inflammatory role of curcumin via PPAR- γ has been shown previously and activation of PPAR- γ by curcumin inhibits MOSER cell growth by suppressing cyclin D1 and EGFR [37]. The recent report proposes that PPAR- γ ligands exert their effects in HT-29 colon cancer by interacting with the p65 subunit of NF- κ B. This prevents NF- κ B pathway activation [38].

Multiple cellular pathways influence the growth and metastatic potential of tumors. In view of their high metastatic potential, chemotherapy has become one of the main treatments for HNSCC in recent years. It has been reported that cancer cell-matrix interaction will play an important role in promoting cell invasion and metastasis [39]. Overexpression of various metalloproteinases (MMPs) is proved to markedly increase the invasive behavior of tumor cells and their ability to metastasize in experimental animal models. Increased expression of MMPs correlates with the invasion and metastasis in head and neck cancer [40]. In our study, both BDMC-A and curcumin treatment reduced the levels of MMP-9 in Hep-2 cells. This may be due to the effect of BDMC-A on the upstream targets of MMPs such as NF- κ B and AP-1 as the mRNA levels of MMP-9 in BDMC-A treated cells was also found to be reduced. Curcumin has been shown to affect both the transcriptional and post transcriptional levels of MMPs [41].

TIMP-2 has been found to block tumor cell invasion both *in vitro* and *in vivo* and may act as a metastasis suppressor gene. TIMP overexpression results in the decreased invasion of endothelial and tumor cells both *in vitro* and *in vivo*. TIMP is reduced in many cancer cells [42]. In our study, Hep-2 cells treated with BDMC-A showed higher levels of TIMP-2 than curcumin. Previous studies have shown that curcumin enhances the expression of the anti-metastatic protein, TIMP-2 in melanoma cells [43]. Curcumin was reported for its potential to reduce tumor cell invasion and metastasis in Hep2 cells [44]. BDMC-A has been proved to regulate aberrant levels of MMPs and TIMPs in other diseases [15], highlighting the potential of BDMC-A as an antimetastatic agent.

Tumour angiogenesis is an essential process for incessant growth and the spreading of solid tumors. Angiogenesis occurs by the angiogenic factors including the growth factors VEGF, cytokines like IL-6, IL-8, and a number of small molecules secreted by the cancer cells [45]. Highly malignant tumors are characterized by enriched vascularization, which is further correlated with increased VEGF expression. ECM is considered as a reservoir for VEGF. Degradation of the ECM releases VEGF which is a pro-angiogenic factor [46]. An effective oncotherapy should address angiogenesis as well. In this respect too, BDMC-A holds potential as it downregulated the expression of VEGF. Curcumin has been shown to downregulate VEGF in HNSCC [47]. This correlates with the present study where both curcumin and BDMC-A downregulated VEGF expression and BDMCA was more potent.

Tissue microenvironment has been identified to exert an intense effect on cell proliferation and differentiation. IL-6, the pleiotropic cytokine, exerts proangiogenic activities in the tumor microenvironment. There is growing evidence that reveals significant relationships between IL-6 levels and failure of treatment directed against VEGF [48]. In the present study, the increased levels of IL-6 were

reduced upon treatment with BDMC-A in Hep-2 cells. Thus BDMC-A reduces the VEGF expression through IL-6.

NF- κ B positively regulates COX-2 in varied cell types. The 5'- promoter region of COX-2 contains 2 putative NF- κ B binding sites. Activation of AP-1 is also implicated in the transcription of COX-2 [49]. The previous report showed that curcumin reduced the invasive and metastatic properties of cells through the inhibition of COX-2 and MMP-9 expressions [41]. In our study, we found that BDMC-A was more effective in reducing the COX-2 level compared to that of curcumin in Hep-2 cells. Thus, the observed reduction in the mRNA levels of COX-2 with BDMC-A treatment in the present study can be attributed to the suppressed levels of NF- κ B, AP-1, VEGF and IL-6 levels.

Epithelial to mesenchymal transition (EMT) is known to be associated with the progression of cancer and impacts cell motility and invasiveness. EMT is mediated and maintained by chemokines/cytokines such as IL-8 and TGF- β . Since IL-8 expression is also regulated by NF- κ B and AP-1, as expected, BDMC-A decreased the IL-8 expression in Hep-2 cells. Inhibiting TGF- β induced EMT is an ideal strategy for the treatment of invasion and metastasis of cancer [50]. Treatment with BDMC-A produced a significant downregulation of TGF- β in Hep2 cells. We observed that in Hep-2 cells, BDMC-A treatment not only significantly lowered the levels of TGF- β but also reduced the invasiveness of these cells. Curcumin has been reported to downregulate the expression of various pro-inflammatory cytokines including TNF- α , IL-1, IL-2, IL-6, IL-8, IL-12, and chemokines, mainly through inactivation of the transcription factor NF- κ B. Curcumin was found to prevent tumor induction via down-regulation of TGF- β in cancer cells [36]. In hepatoma cells, curcumin has been reported to inhibit IL-6 production and AP-1 activation [51]. The observed effect of BDMC-A on TGF- β , IL-6 and, IL-8 levels can be corroborated with these studies of curcumin. Nevertheless, BDMC-A was more effective than curcumin in bringing out these changes.

5. Conclusions

We conclude that BDMC-A is a more potent cytotoxic agent against Hep-2 cells in comparison with curcumin. This was further confirmed by the significant downregulation of metastasis, invasiveness, and angiogenesis markers in Hep-2 cells treated with BDMC-A compared to curcumin. Overall, BDMC-A could be promoted as a potential therapeutic agent for head and neck cancer in the future.

Declarations

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Conflict of interest

The authors declare that there are no conflicts of interest.

Availability of data and material - Not applicable

Code availability- Not applicable

Authors' contributions

RR*and KM designed the experiments. KM and SP performed the experiments. RR*and KM analysed the data. APF wrote the paper. RR* edited the article.

Ethics approval - Not applicable

Consent to participate - Not applicable

Consent for publication - Not applicable

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Figures

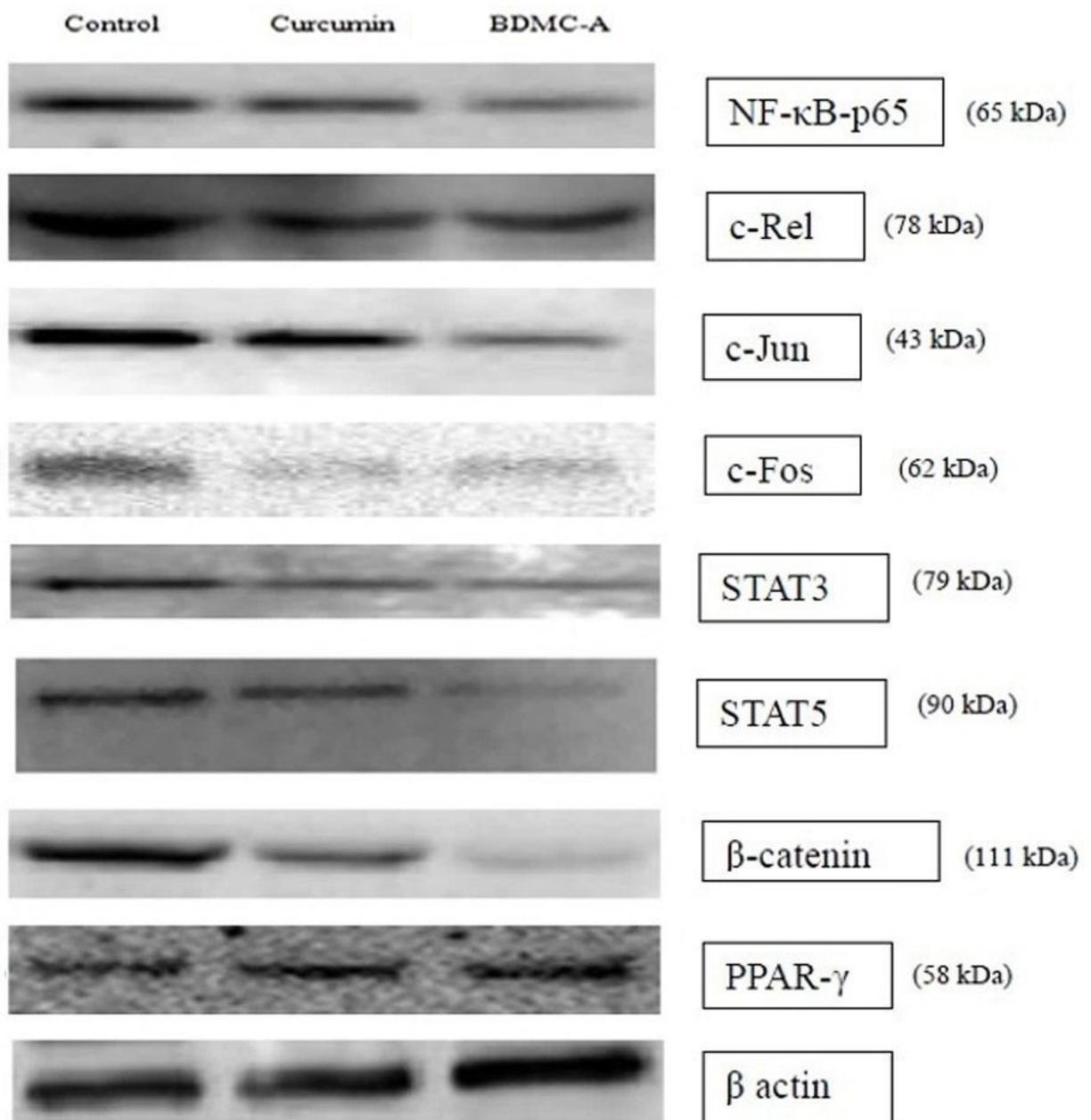


Figure 1

Effect of curcumin and BDMC-A on the expression of transcription factors. β -actin was used as the internal loading control.

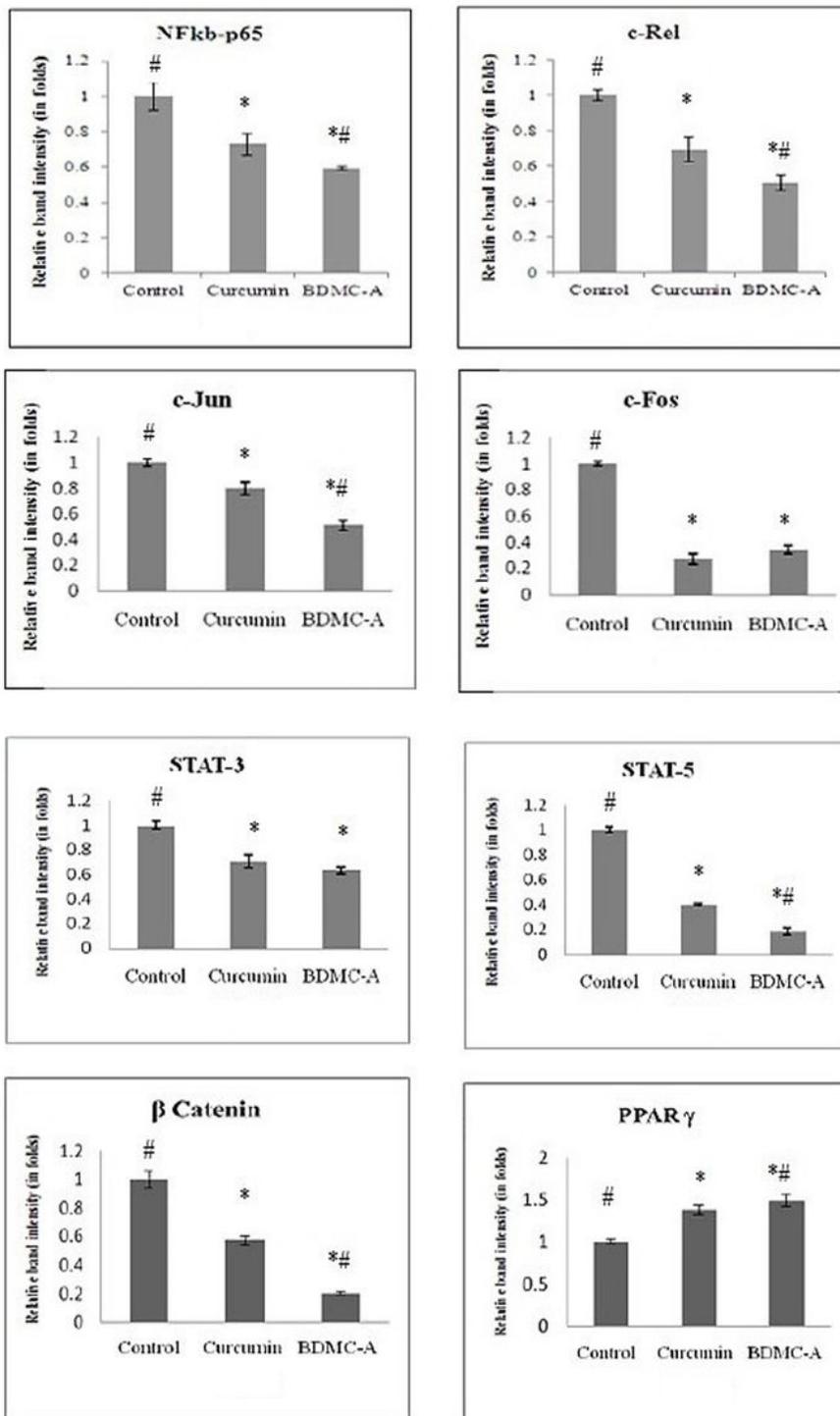


Figure 2

Densitometry analysis of protein levels expressed in relative band intensity. Values represent the mean \pm SD of three independent experiments. * $p \leq 0.05$, significance compared to control. # $p \leq 0.05$, significance compared to curcumin.

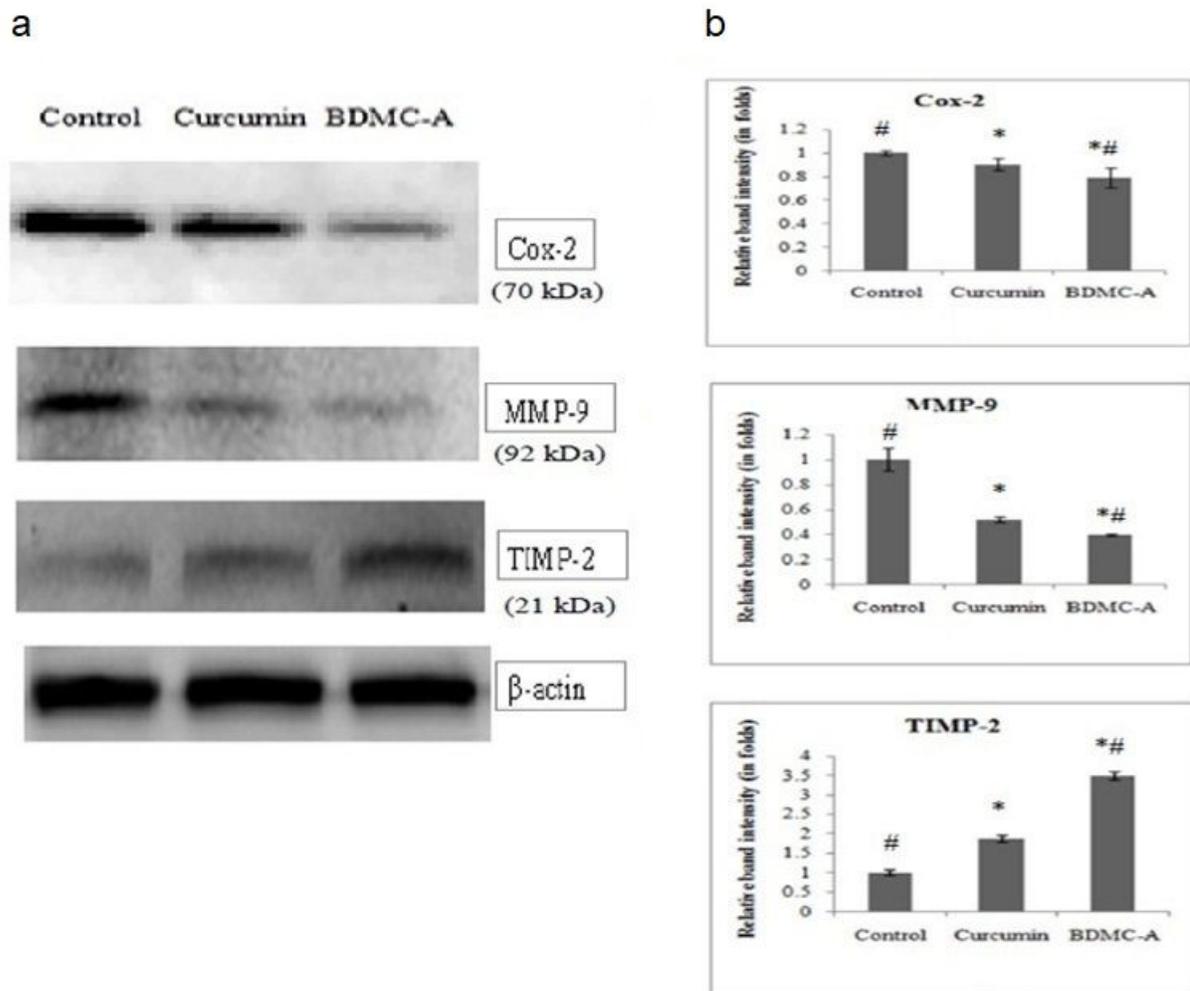


Figure 3

Western Blot for metastatic markers a. Effect of curcumin and BDMC-A on the expression of metastatic proteins. β -actin was used as the internal loading control. b. Densitometry analysis of protein levels expressed in relative band intensity. Values represent the mean \pm SD of three independent experiments. * $p \leq 0.05$, significance compared to control. # $p \leq 0.05$, significance compared to curcumin.

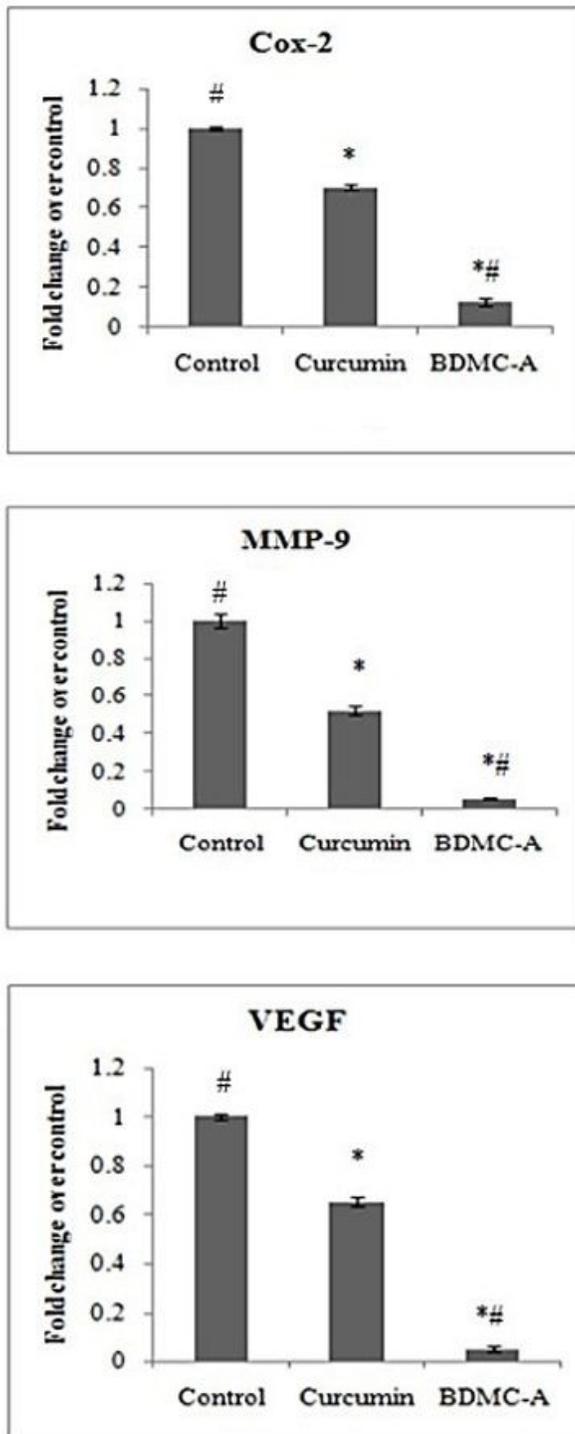


Figure 4

Effect of curcumin and BDMC-A on mRNA expression of metastatic proteins. GAPDH was used as the internal loading control. Values represent the mean \pm SD of three independent experiments. * $p \leq 0.05$, significance compared to control. # $p \leq 0.05$, significance compared to curcumin.

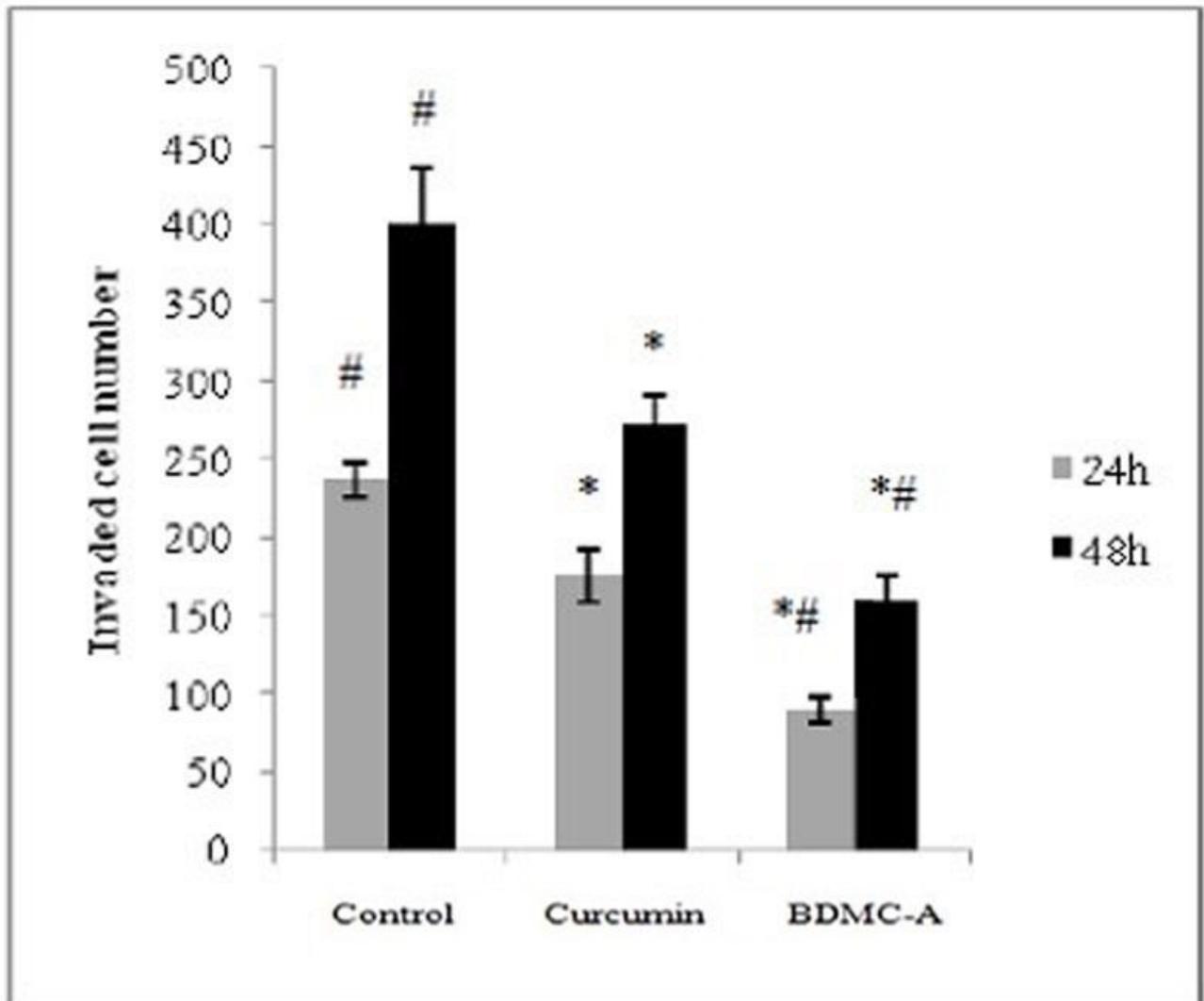


Figure 5

Effect of BDMC-A and curcumin on the invasion of Hep-2 cells in vitro. Values represent the mean \pm SD of three independent experiments. * $p \leq 0.05$, significance compared to control. # $p \leq 0.05$, significance compared to curcumin.

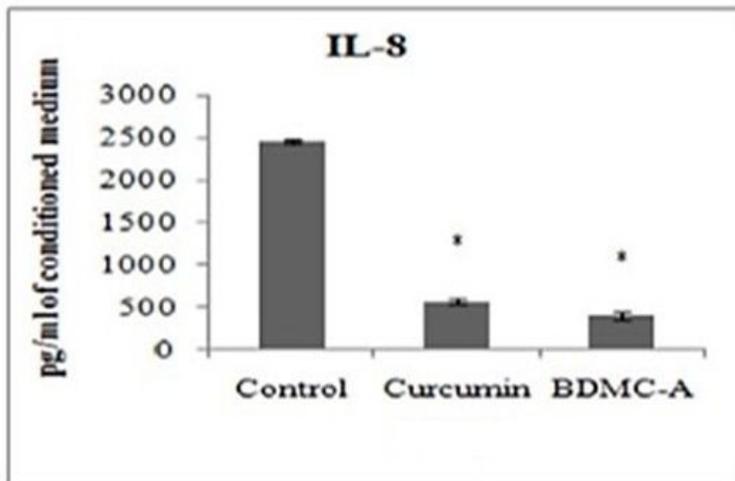
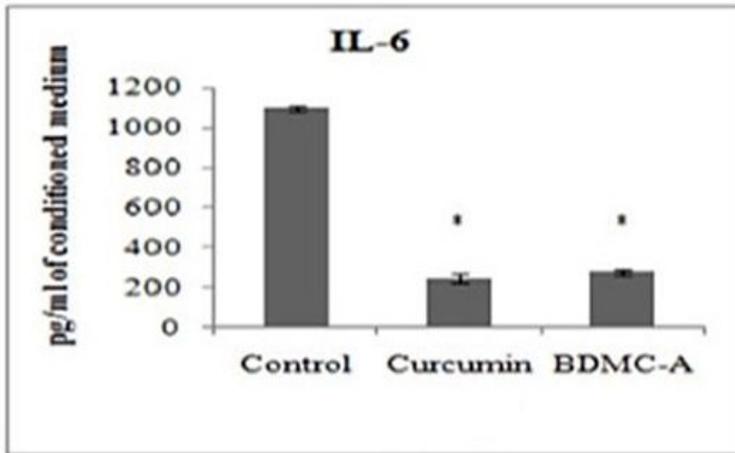
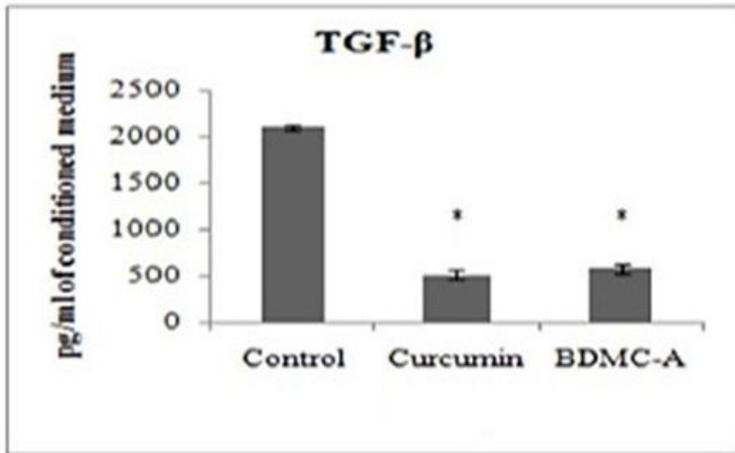


Figure 6

Effect of curcumin and BDMC-A on the expression of inflammatory markers. Values represent the mean \pm SD of three independent experiments. * $p \leq 0.05$, significance compared to control