Results
RESULTS

Cytotoxicity by MTT Assay

Human breast cancer cells (MCF-7) and laryngeal carcinoma cells (Hep-2) were grown in the presence of varying concentrations of curcumin (10-50 µM for MCF-7 and 15-75 µM for Hep-2) and BDMC-A (15-75 µM for MCF-7 and 10-50 µM for Hep-2) for 24 and 48h and cell viability was measured by MTT assay. The half maximal inhibitory concentration (IC50) at 48h for curcumin was attained at 30 µM in MCF-7 and at 50 µM in Hep-2 cells. As compared to curcumin, the IC50 at 48h for BDMC-A was obtained at a similar level of 30 µM for MCF-7 cells but at a lesser level of 20 µM for Hep-2 cells (Fig. 23, 24).

For further experiments, a sub-optimum cytostatic dose of 15 µM for BDMC-A and 15 µM for curcumin was used for MCF-7 cells and a cytostatic dose of 10 µM for BDMC-A and 25 µM for curcumin was used for Hep-2 cells.

Cytotoxicity by LDH Assay

MCF-7 and Hep-2 cells were cultured for 24 and 48h with different concentrations of curcumin (10-50 µM for MCF-7 and 15-75 µM for Hep-2) and BDMC-A (15-75 µM for MCF-7 and 10-50 µM for Hep-2). There was an increase in LDH leakage in both the cell lines when compared to control. The cytotoxicity exhibited by BDMC-A and curcumin was similar in MCF-7 cells. However in Hep-2 cells BDMC-A exhibited more LDH leakage at lesser concentration compared to that of curcumin (Fig. 25, 26).

Cell Count Assay

MCF-7 and Hep-2 cells were cultured for 24 and 48h with different concentrations of curcumin and BDMC-A. The results showed that for MCF-7 cells, the cell viability declined to about 50% at a concentration of 30 µM in both curcumin and BDMC-A treated cells. Whereas in Hep-2 cells, the viability decreased to 50% at a concentration of 20 µM of BDMC-A and 50 µM of curcumin (Fig. 27, 28).
Fig. 23. Cytotoxic effects of BDMC-A and curcumin on MCF-7 cells - MTT assay

The data represents the mean ± standard deviation (SD) of three independent experiments. *p ≤ 0.05, significance compared to control.
Fig. 24. Cytotoxic effects of BDMC-A and curcumin on Hep-2 cells - MTT assay

The data represents the mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
Fig. 25. Cytotoxic effects of BDMC-A and curcumin on MCF-7 cells - LDH Assay

The data represents the mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
The data represents the mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
The data represents mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
The data represents the mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
Cell cycle distribution through Flow cytometry

The cell cycle analysis was performed by flow cytometry. MCF-7 cells were treated with 15 μM of either curcumin or BDMC-A and the cell cycle distribution was examined after 12h of drug treatment. Cell cycle analysis revealed a progressive accumulation of cells in the G2/M phase of the cell cycle (Figure 29a). Flow cytometric analysis of the curcumin treated cells showed a decrease in the percentage of cells in the G1 phase from 67 to 58% and an increase in the percentage of cells from 6 to 13% in the G2-M phase. Whereas, in BDMC-A treated cells, it showed a decrease in the percentage of cells in the G1 phase from 67 to 62% and an increase in the percentage of cells in the G2-M phase, from 6 to 11%.

Hep-2 cells were treated with 10 μM BDMC-A and 25 μM curcumin, and the cell cycle distribution was examined after 24h of drug treatment. The flow cytometric studies showed accumulation of cells in the G0/G1 phase (Figure 30a). BDMC-A showed a 7% increase at 10 μM and curcumin an 11% increase at 25 μM concentration.

Western blot of cyclins

Western blot analysis in MCF-7 cells treated with curcumin and BDMC-A for cyclin B1 (Fig. 29b) indicated that there was a significant downregulation in the expression of cyclin B1 in BDMC-A treatment compared to curcumin treatment. In Hep-2 cells, western blot analysis for the expression of cyclin D1 showed that there was a significant decrease in the expression of cyclin D1 in both curcumin and BDMC-A treated cells (Fig. 30b) when compared to control.
Fig. 29a. Cell cycle distribution through Flow cytometry in MCF-7 cells

![Cell cycle distribution](image)

**Fig. 29a.** Cell cycle distribution through flow cytometry. MCF-7 cells exposed to 15 μM of BDMC-A and 15 μM of curcumin for 24h. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.

**Fig. 29b.** Western blot for cyclin B1 expression in MCF-7 cells

![Western blot](image)

**Fig. 29b.** (A) Effect of BDMC-A and curcumin on the expression of cyclin B1 protein. MCF-7 cells were plated and incubated with 15 μM curcumin and 15 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 12% SDS–PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control. (B) Densitometry
**Fig. 30a.** Cell cycle distribution through flow cytometry in Hep-2 cells

![Cell cycle distribution through flow cytometry](image)

Fig. 30a. Cell cycle distribution through flow cytometry. Hep-2 cells exposed to 10 μM of BDMC-A and 25 μM of curcumin for 24h. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.

**Fig. 30b.** Western blot for cyclin D1 expression in Hep-2 cells

![Western blot](image)

**Fig. 30b.** (A) Effect of BDMC-A and curcumin on the expression of cyclin D1 protein. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 12% SDS–PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control. (B) Densitometry
Effect of curcumin and BDMC-A on apoptosis

We investigated the putative markers of apoptosis induced cell death - both the intrinsic (mitochondrial) and the extrinsic (death receptor) pathway.

BDMC-A regulates intrinsic apoptotic signalling molecules

To investigate intracellular signalling involved in apoptosis in MCF-7 and Hep-2 cells after treatment with BDMC-A or curcumin, we analysed the protein levels of intrinsic apoptotic markers: p53, Bcl-2, Bax, cytochrome c, Apaf-1, caspase-9, caspase-3 and cleaved PARP by western blotting.

In MCF-7 cells, with BDMC-A treatment, the levels of p53 and cytochrome c were significantly upregulated in a time and dose dependent manner compared to that of curcumin (Fig. 31 A,B,C (i & ii)). Treatment with BDMC-A elicited a significant upregulation of p53 and cytochrome c expression in a time and dose dependent manner in case of Hep-2 cells (Fig. 32 A, B, C (i & ii)).

The intrinsic apoptotic marker Bcl-2 was significantly downregulated (Fig. 33A & 33B (i)) whereas Bax, (Fig. 33A & 33B (ii)) Apaf-1(Fig. 33A & 33B (iii)), activated caspase-9 (Fig. 33A & 33B (iv)) and cleaved PARP (Fig. 33A & 33B (vi)) were significantly upregulated by BDMC-A than curcumin in MCF-7 cells. The activated effector caspase-3 (Fig. 33A & 33B (v)) level was significantly upregulated in both curcumin and BDMC-A treated cells compared with control.

In Hep-2 cells, BDMC-A treatment exhibited a significant downregulation of Bcl-2 (Fig. 34A & 34B (i)), significant upregulation of Bax (Fig. 34A & 34B (ii)), Apaf-1 (Fig. 34A & 34B (iii)) and activated caspase-9 (Fig. 34A & 34B (iv)) more effectively than curcumin treated cells. Also, BDMC-A treatment significantly upregulated the effector caspase-3 (Fig. 34A & 34B (v)), and cleaved PARP (Fig. 34A & 34B (vi)) more effectively than curcumin treatment.

From the mRNA expression analysis, we found that the Bcl-2 mRNA expression level (Fig. 35A & 35C (i)) was downregulated, whereas the Bax mRNA expression level (Fig. 35B & 35C (ii)) was significantly upregulated, after BDMC-A treatment compared to curcumin treatment in MCF-7 cells. In Hep-2 cells also, the Bcl-2 mRNA expression level (Fig. 36A & 36C (i)) was downregulated, whereas the
Bax mRNA expression level (Fig. 36B & 36C (ii)) was significantly upregulated, after BDMC-A treatment compared to curcumin treatment.

**BDMC-A affects the regulation of extrinsic apoptotic signalling molecules**

While analyzing the extrinsic apoptotic markers, we observed a significant increase in FasL (Fig.37A & 37B (i)) as well as the initiator caspase-8 (Fig.37A & 37B (ii)) protein expression in BDMC-A treated MCF-7 cells compared to curcumin treated cells. We observed an increase in FasL (Fig. 38A & 38B (i)) and significant increase in initiator caspase-8 (Fig. 38A & 38B (ii)) protein expressions in BDMC-A treated Hep-2 cells compared to control and curcumin treated cells.
Fig. 31. Intrinsic apoptotic markers in MCF-7 cells

Time and Dose dependent expression in p53 and Cytochrome c

(A) With 12h treatment

(B) With 24h treatment

15µM 30µM 15µM 30µM

15µM 30µM 15µM 30µM

Fig. 31. (A and B) Time and dose dependent expression of p53 and cytochrome c. MCF-7 cells were plated and incubated with 15 & 30 µM of curcumin and 15 & 30 µM of BDMC-A for 12 (A) and 24 h (B). After incubation, whole cell extracts were prepared and resolved on 12% SDS–PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control. (C (i & ii)). Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 32. Intrinsic apoptotic markers in Hep-2 cells

**Time and Dose dependent expression in p53 and Cytochrome c**

(A) With 12h treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Curcumin</th>
<th>BDMC-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>25μM</td>
<td>50μM</td>
<td>10μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(B) With 24h treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Curcumin</th>
<th>BDMC-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>25μM</td>
<td>50μM</td>
<td>10μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **p53** (53 kDa)
- **Cytochrome c** (12 kDa)
- **β-actin**

(C) Densitometry

(i) With 12h treatment

(ii) With 24h treatment

![Graphs showing relative band intensities for p53 and Cytochrome c](image)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Curcumin</th>
<th>BDMC-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>25μM curcumin or 10μM BDMC-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50μM curcumin or 20μM BDMC-A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 32.** (A & B). Time and dose dependent expression of p53 and cytochrome c. Hep-2 cells was incubated with 25 and 50 μM curcumin and 10 and 20 μM BDMC-A for 12 (A) and 24 h (B). Western blots were prepared and probed with antibodies. β-actin was used as internal loading control. C (i) and (ii): Protein levels were quantified using densitometry analysis and expressed by relative band intensities. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 33. A. Western blot for intrinsic apoptotic markers in MCF-7 cells

Fig. 33. (A) Effect of BDMC-A and curcumin on expression of intrinsic apoptotic proteins. MCF-7 cells were plated and incubated with 15 µM curcumin and 15 µM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 12% SDS–PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control.
Fig. 33. B  

Densitometry

Fig. 33. (B) Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. †p ≤ 0.05, significance compared to curcumin.
Fig. 34. A. Western blot for intrinsic apoptotic markers in Hep-2 cells

(a) Effect of BDMC-A and curcumin on the expression of intrinsic apoptotic proteins. Hep-2 cells were incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h Western blots were prepared and probed with antibodies. β-actin was used as internal loading control.
Fig. 34. B. Densitometry

Fig. 34. B. Protein levels were quantified using densitometric analysis and expressed in relative band intensities. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 35. Reverse Transcriptase-PCR in MCF-7 cells

Fig. 35. Reverse Transcriptase-PCR (A&B). Effect of BDMC-A and curcumin on mRNA expression of Bcl-2 and Bax. MCF-7 cells were plated and incubated with 15 µM curcumin and 15 µM BDMC-A for 24h. After 24h, whole cell extracts were prepared using TRI solution. The mRNA expression for antiapoptotic Bcl-2 and pro-apoptotic Bax were analysed by Reverse transcriptase PCR (RT-PCR) with agarose gel electrophoresis. GAPDH was used as internal loading control. (C) Densitometry. mRNA levels were quantified using densitometry analysis and expressed as relative intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. *#p ≤ 0.05, significance compared to curcumin.
Fig. 36. Reverse Transcriptase-PCR in Hep-2 cells

**Reverse Transcriptase-PCR**

(A) 500 400 300 200 100

- a: GAPDH 240bp
- b: Bcl-2 176bp

(B) 500 400 300 200 100

- a: GAPDH 240bp
- b: Bax 168bp

a-100bp ladder, b-Control, c-Curcumin, d-BDMC-A

**Densitometry**

(i) **Bcl-2**

- Control
- Curcumin Groups
- BDMC-A

(ii) **Bax**

- Control
- Curcumin Groups
- BDMC-A

**Fig. 36.** Reverse Transcriptase-PCR (A&B). Effect of BDMC-A and curcumin on mRNA expression of Bcl-2 and Bax. Hep-2 cells were plated and incubated with 25 µM curcumin and 10 µM BDMC-A for 24h. After 24h, whole cell extracts were prepared using TRI solution. The mRNA expression for antiapoptotic Bcl-2 and pro-apoptotic Bax were analysed by Reverse transcriptase PCR (RT-PCR) with agarose gel electrophoresis. GAPDH was used as internal loading control. (C) Densitometry. mRNA levels were quantified using densitometry analysis and expressed as relative intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 37. Extrinsic apoptotic markers in MCF-7 cells

(A) Effect of BDMC-A and curcumin on expression of extrinsic apoptotic proteins. MCF-7 cells were plated and incubated with 15 μM curcumin and 15 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 12% SDS–PAGE, electrotransferred and probed with antibody. β-actin was used as internal loading control. (B) Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 38. Extrinsic apoptotic markers in Hep-2 cells

(A) Effect of BDMC-A and curcumin on expression of extrinsic apoptotic proteins. Hep-2 cells were plated and incubated with 25 µM curcumin and 10 µM BDMC-A for 24h. After 24h, whole cell extracts were prepared and resolved on 12% SDS–PAGE, electrotransferred and probed with antibody. β-actin was used as internal loading control. (B) Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.

a-Control, b-Curcumin , c-BDMC-A
Morphological changes indicating apoptosis in MCF-7 and Hep-2 cells

In order to visualise apoptosis, MCF-7 and Hep-2 cells were treated with curcumin and BDMC-A for 24h. After this treatment, the cells were stained with Hoechst 33258 and PI individually and observed under fluorescent microscope using appropriate filters. A clear evidence in the morphological changes such as chromatin condensation and nuclear fragmentation were detected in BDMC-A treated MCF-7 cells than in curcumin treated cells (Fig. 39A & 39B). In Hep-2 cells also good morphological changes such as chromatin condensation and nuclear fragmentation were observed with BDMC-A treatment compared to that of curcumin treatment (Fig. 40A & 40B).

Early onset apoptosis revealed by phosphatidylserine (PS) externalization

In order to assess the onset of apoptosis, externalization of PS from the inner leaflet of plasmamembrane to its outer leaflet was identified by using an Annexin V-Cy3 detection kit. In MCF-7, the live untreated control cells were stained green by 6-carboxyfluorescein (6-CF). Treatment of MCF-7 cells with BDMC-A indicated an early onset of apoptosis and clearly showed an increased externalization of phosphatidylserine in comparison to curcumin treated cells which were double stained with fluorescently labelled AnnexinV-Cy3 and 6-CF, emitting red and green fluorescence (Fig. 39C). Evaluation of Hep-2 cells treated with BDMC-A indicated an early onset of apoptosis. The cells showed a double stain of Cy3 labeled AnnexinV and 6-CF, emitting red and green fluorescence signals, respectively. Early apoptosis was more prominent in BDMC-A treated cells than in curcumin treated cells (Fig. 40C).
Fig. 39. A & B: A. **Hoechst 33258 staining:** Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a-control, b-curcumin and c-BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis and fragmented apoptotic bodies were examined by Hoechst 33258 staining. Condensed or fragmented nuclei were observed under a fluorescence microscope.  
**B. Propidium Iodide staining:** Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Morphological analysis of nuclear chromatin condensation of cell undergoing apoptosis and fragmented apoptotic bodies were examined by Propidium Iodide staining. Condensed or fragmented nuclei were observed under a fluorescence microscope.
Fig. 40. A & B Hoechst 33258 and Propidium Iodide staining in Hep-2 cells

Hoechst 33258 staining

a. b. c.

Propidium Iodide staining

a. b. c.

**Fig. 40. A & B: A. Hoechst 33258 staining:** Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a-control, b-curcumin and c-BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis and fragmented apoptotic bodies were examined by Hoechst 33258 staining. Condensed or fragmented nuclei were observed under a fluorescence microscope. **B. Propidium Iodide staining:** Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a-control, b-curcumin and c-BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis and fragmented apoptotic bodies were examined by Propidium Iodide staining. Condensed or fragmented nuclei were observed under a fluorescence microscope.
Fig. 39. C: Annexin V staining: Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Phosphatidyl externalization was observed with Annexin V. 6-Carboxyfluorescein stains green, cy-3 stains red and merged stain orange red denotes early apoptotic stage.
Fig. 40.C: Annexin V staining: Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Phosphatidyl externalization was observed with Annexin V. 6-Carboxyfluorescein stains green, cy-3 stains red and merged stain orange red denotes early apoptotic stage.
**Early and late onset of apoptosis revealed by AO/EB staining**

Dual staining with AO/EB was used to evaluate the nuclear morphology of apoptotic cells. AO is a vital dye that stains both living and dead cells, whereas EB stains cells when membrane damage occurs. In this dual staining approach, green indicates viable cell, yellow indicates early apoptotic cells and orange/red indicates late apoptotic cells. In MCF-7, uniformly green live cells were observed with normal and large nucleus in control. Whereas in BDMC-A treated cells, a comparative yellow (early apoptotic), orange/red (late apoptotic) staining were observed (Fig. 39D). These results confirmed that BDMC-A significantly induced apoptosis in breast cancer cells than curcumin. As expected in Hep-2 cells, the untreated control cells were uniformly green with normal and large nuclei and in BDMC-A and curcumin treatment, yellow (early apoptotic) and orange/red (late apoptotic) cells were observed (Fig. 40D).

**BDMC-A induces apoptosis through ROS**

In order to assess whether ROS are involved in BDMC-A mediated cytotoxicity, DCFH-DA was used. Non-fluorescent DCFH is converted into highly fluorescent DCF intracellularly through the action of hydrogen peroxide generated in the presence of peroxidase. In MCF-7 cells, BDMC-A treatment resulted in cells with green fluorescence indicating more ROS production than curcumin treatment (Fig. 39E). BDMC-A treated Hep-2 cells also showed more ROS production than curcumin treated cells (Fig. 40E).

**BDMC-A induces loss of mitochondrial membrane potential**

Rhodamine 123, a lipophilic cationic dye, was used as an indicator for mitochondrial membrane potential. Through this dye, depolarized mitochondria are marked by a green fluorescence and polarized mitochondria by an orange-red fluorescence. The BDMC-A and curcumin treatment to MCF-7 cells resulted in loss of mitochondrial membrane potential, in which BDMC-A was significant in inducing membrane depolarization than curcumin (Fig. 39F). In Hep-2 cells, we found that both BDMC-A and curcumin treated cells emitted green fluorescence, indicating loss of mitochondrial membrane potential. We also found that BDMC-A was more potent in inducing membrane depolarization than curcumin (Fig. 40F).
**Fig. 39. D & E Acridine Orange/ Ethidium Bromide (AO/EB) and Reactive Oxygen Species (ROS) staining in MCF-7 cells**

**Acridine Orange / Ethidium Bromide Staining**

- **a.**
- **b.**
- **c.**

**Production of Reactive oxygen species (ROS)**

- **a.**
- **b.**
- **c.**

**Fig. 39. D & E: D.** Acridine Orange/Ethidium Bromide Staining: Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis was examined by AO/EB staining. Live cells stain green, yellow – the early apoptotic and orange – the late apoptotic stages were examined by AO/EB staining. E. Production of Reactive oxygen species (ROS): Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Production of reaction oxygen species were examined by DCFH-DA stain.
Fig. 40. D & E Acridine Orange/Ethidium Bromide (AO/EB) and Reactive Oxygen Species (ROS) staining in Hep-2 cells

**Acridine Orange / Ethidium Bromide Staining**

a. control, b. curcumin and c. BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis was examined by AO/EB staining. Live cells stain green, yellow – the early apoptotic and orange – the late apoptotic stages were examined by AO/EB staining.

**Production of Reactive oxygen species (ROS)**

a. control, b. curcumin and c. BDMC-A. Production of reactive oxygen species were examined by DCFH-DA stain.

**Fig. 40. D & E: D.** Acridine Orange/Ethidium Bromide Staining: Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Morphological analysis of nuclear chromatin condensation of cells undergoing apoptosis was examined by AO/EB staining. Live cells stain green, yellow – the early apoptotic and orange – the late apoptotic stages were examined by AO/EB staining. **E.** Production of Reactive oxygen species (ROS): Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Production of reaction oxygen species were examined by DCFH-DA stain.
Fig. 39. F. Rhodamine 123 staining for mitochondrial membrane potential in MCF-7 cells

Fig. 39. F. Rhodamine 123 for mitochondrial membrane potential: Induction of apoptosis by BDMC-A and curcumin in MCF-7 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Depolarization of mitochondrial membrane potential was observed as green fluorescence and polarized mitochondria as orange/red fluorescence.

Fig. 40. F. Rhodamine 123 staining for mitochondrial membrane potential in Hep-2 cells

Fig. 40. F. Rhodamine 123 for mitochondrial membrane potential: Induction of apoptosis by BDMC-A and curcumin in Hep-2 cells at 24h in fixed concentration. a. control, b. curcumin and c. BDMC-A. Depolarization of mitochondrial membrane potential was observed as green fluorescence and polarized mitochondria as orange/red fluorescence.
BDMC-A induces DNA fragmentation

Cleavage of DNA at the inter-nucleosomal linker sites, yielding DNA fragments of 180 bp, is regarded as a biochemical hallmark of apoptosis. Genomic DNAs extracted from untreated and curcumin and BDMC-A treated MCF-7 (Fig. 41.) and Hep-2 (Fig. 42.) cells were subjected to agarose gel electrophoresis. In contrast to the untreated control cells, we readily observed ladder formation in both BDMC-A and curcumin treated cells, with a considerable higher level of DNA fragmentation in the BDMC-A treated cells.

Fig. 41. DNA Fragmentation in MCF-7 cells

Fig. 42. DNA Fragmentation in Hep-2 cells

a-100bp ladder, b-Control, c-Curcumin, d-BDMC-A
BDMC-A and curcumin affects cell survival through src and PI3K/Akt pathway

Src is tethered to EGFR that was in cell membrane and mediate several processes such as growth, proliferation and survival through communicating with PI3K/Akt pathway. Alterations in this pathway through chemopreventive agents are known to inhibit cell survival. BDMC-A exhibited potent inhibitory effect by modulating this pathway in MCF-7 cells compared to curcumin. Level of src, a proto-oncogene was significantly reduced in BDMC-A-treated MCF-7 cells compared to curcumin-treated cells (Fig. 43A & 43B (i)). PI3K is an important signal transducer protein which in turn activates Akt. Significant downregulation of PI3K (Fig. 43A & 43B (ii)) and pAKT, (Fig. 43A & 43B (iii)) a downstream target of PI3K were observed on treatment with BDMC-A compared to curcumin in MCF-7 cells. In BDMC-A treated Hep-2 cells also, we observed a significant downregulation in src (Fig. 44A & 44B (i)). We observed that PI3K (Fig. 44A & 44B (ii)) and pAKT (Fig. 44A & 44B (iii)) protein expression were significantly downregulated in BDMC-A treated Hep-2 cells compared to that of curcumin treated cells.

BDMC-A targets key transcription factors involved in signal transduction cascade

The effects of curcumin and BDMC-A on proteins of signalling pathways like NF-κB, AP-1, STAT3, STAT5, β-catenin and PPAR-γ were blotted and quantified in both MCF-7 and Hep-2 cells. Total protein lysate were prepared from the control cells and cells exposed to either curcumin or BDMC-A from both MCF-7 and Hep-2. They were analysed for NF-κB, c-Rel, AP-1, STAT3, STAT5, β-catenin and PPAR-γ by western blot. Relative to curcumin, treatment with BDMC-A significantly downregulated the expression levels of NF-κB p65 (Fig. 45A & 45B (i)) and c-Rel, (Fig. 45A & 45B (ii)), the subfamily of NF-κB proteins in MCF-7 cells. As expected, we also observed significant downregulation of the expression levels of NF-κB p65 (Fig. 46A & 46B (i)) and c-Rel (Fig. 46A & 46B (ii)) in BDMC-A treatment than curcumin treatment in Hep-2 cells.
Our western blot results showed that there was a considerable decrease in AP-1 complex i.e. c-Jun (Fig. 45A & 45B (iii)) and c-Fos (Fig. 45A & 45B (iv)) expression on treatment with BDMC-A than with curcumin in MCF-7 cells and same pattern was observed in Hep-2 cells in case of c-Jun (Fig. 46A & 46B (iii)) and c-Fos (Fig. 46A & 46B(iv))

In comparison with curcumin, BDMC-A showed a significant decrease in the level of STAT3 (Fig. 45A & 45B (v)) protein expression in MCF-7 cells, whereas in Hep-2 cells (Fig. 46A & 46B (v)) it displayed an equipotent decrease. BDMC-A treatment also exhibited a moderate decrease in STAT5 expression in MCF-7 cells ((Fig. 45A & 45B (vi)) compared to control and a significant decrease in STAT5 expression in Hep-2 cells (Fig. 46A & 46B (vi)) as compared to curcumin.

BDMC-A significantly downregulated the expression of β-Catenin in both MCF-7 cells (Fig. 45A & 45B (vii)) as well as Hep-2 cells (Fig. 46A & 46B (vii)) than curcumin. BDMC-A was also observed to upregulate the PPAR-γ expression more effectively than curcumin in both MCF-7 cells (Fig. 45A & 45B (viii)) and Hep-2 cells (Fig. 46A & 46B (vii)).
Fig. 43. A & B. Western Blot for Cell survival markers. A. Effect of BDMC-A and curcumin on expression of cell survival proteins. MCF-7 cells were plated and incubated with 15 μM curcumin and 15 μM BDMC-A for 24h. After 24h, whole cell extracts were prepared and resolved on 10% SDS-PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control. B. Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
**Fig. 44. A & B. Western Blot for cell survival markers.**

**A.** Effect of BDMC-A and curcumin on expression of cell survival proteins. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 10% SDS-PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control. **B. Densitometry.** Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 45. A. Transcription factors in MCF-7 cells

(a) NF-κB p65 (65 kDa)
(b) c-Rel (78 kDa)
(c) c-Jun (43 kDa)
(d) c-Fos (62 kDa)
(e) STAT3 (79 kDa)
(f) STAT5 (90 kDa)
(g) β-catenin (111 kDa)
(h) PPAR-γ (58 kDa)

a-Control, b-Curcumin, c-BDMC-A

Fig. 45. A. Western Blot for Transcription factors Effect of BDMC-A and curcumin on expression of transcription factors. MCF-7 cells were plated and incubated with 15 μM curcumin and 15 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 10% SDS-PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control.
Fig. 45. B. Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 46. A. Nuclear Transcription factors in Hep-2 cells.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td></td>
<td>NF-κB-p65 (65 kDa)</td>
</tr>
<tr>
<td>(ii)</td>
<td></td>
<td>c-Rel (78 kDa)</td>
</tr>
<tr>
<td>(iii)</td>
<td></td>
<td>c-Jun (43 kDa)</td>
</tr>
<tr>
<td>(iv)</td>
<td></td>
<td>c-Fos (62 kDa)</td>
</tr>
<tr>
<td>(v)</td>
<td></td>
<td>STAT3 (79 kDa)</td>
</tr>
<tr>
<td>(vi)</td>
<td></td>
<td>STAT5 (90 kDa)</td>
</tr>
<tr>
<td>(vii)</td>
<td></td>
<td>β-catenin (111 kDa)</td>
</tr>
<tr>
<td>(viii)</td>
<td></td>
<td>PPAR-γ (58 kDa)</td>
</tr>
</tbody>
</table>

β actin

Fig. 46. A. Western Blot for Transcription factors. Effect of BDMC-A and curcumin on expression of transcription factors. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h, whole cell extracts were prepared and resolved on 10% SDS-PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control.
Fig. 46.B. Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
**Effects of BDMC-A on putative invasion and angiogenesis - associated markers**

To characterize the effect of BDMC-A and curcumin on markers related to invasion and angiogenesis, MCF-7 and Hep-2 cells grown in presence of these drugs were either lysed and lysates were analysed by western blot or RNA extracted from cells were used for RT-PCR to assess the markers of invasiveness and angiogenesis. The western blot results showed that the expression of COX-2, a downstream target of NF-κB, was significantly lower in BDMC-A and curcumin treated MCF-7 cells (Fig. 47A & 47B (i)) compared to control. In Hep-2 cells, a significant downregulation of COX-2 was observed when treated with BDMC-A compared with curcumin (Fig. 48A & 48B (i)).

The MCF-7 western blots showed that the levels of MMP-9 were reduced significantly in BDMC-A treated cells compared to curcumin-treated cells (Fig. 47A & 47B (ii)). On the other hand, levels of the MMP inhibitor TIMP-2 protein levels were significantly higher in BDMC-A-treated cells relative to curcumin-treated cells ((Fig. 47A & 47B (iii)). In Hep-2 cells also a similar effect was exerted by BDMC-A and there was significant downregulation of MMP-9 (Fig. 48A & 48B (ii)) levels and significant upregulation of TIMP-2 (Fig. 48A & 48B (iii)) levels when compared to that of curcumin treatment.

Q-PCR analysis of MCF-7 cells showed that mRNA levels of COX-2 (Fig. 49 (i)), MMP-9 (Fig. 49 (ii)) and VEGF (Fig. 49 (iii)) were significantly lower in BDMC-A-treated cells compared to curcumin-treated cells. In case of Hep-2 cells, the same pattern of significant downregulation of mRNA levels of COX-2 (Fig. 50 (i)), MMP-9 (Fig. 50 (ii)) and VEGF (Fig. 50 (iii)) were observed in BDMC-A treated cells than curcumin treated cells. These observations indicate that BDMC-A has stronger anti-invasive, anti-metastatic and anti-angiogenic effects compared with curcumin.

**Effects of BDMC-A on invasion of MCF-7 and Hep-2 cells**

The ability of MCF-7 and Hep-2 cells to migrate across a membrane was used as a test for the effect of curcumin and BDMC-A on the potential of cells to be invasive. Compared to control only fewer cells treated with curcumin and BDMC-A invaded from the upper to the lower chamber separated by EHS-coated filter.
Migration of cells across the filter was inhibited more effectively by BDMC-A in MCF-7 (Fig. 51) and Hep-2 cells (Fig. 52) than curcumin.

**Effects of BDMC-A on cancer related inflammatory markers**

Expression of pro-inflammatory markers was considered as the seventh hallmark of cancer. To test the effect of exposure of MCF-7 and Hep-2 cells to BDMC-A and curcumin, treated cells were tested by ELISA for TNF-α, IL-1β, TGF-β, IL-6 and IL-8. Compared with control cells, BDMC-A treated MCF-7 cells showed significantly decreased expression levels of TNF-α (Fig. 53 (i)), IL-1β (Fig. 53 (ii)), TGF-β (Fig. 53 (iii)), and IL-8 (Fig. 53 (iv)). In case of Hep-2 cells, TGF-β (Fig. 54 (i)), IL-6 (Fig. 54 (ii)), IL-8 (Fig. 54 (iii)), were significantly downregulated with both BDMC-A and curcumin treatment.

Taken together, these observations indicate that, despite their diverse tissue origins, some bladder, breast, head and neck and lung cancers share common pathways of tumour development.
Fig. 47. A. Metastatic markers in MCF-7 cells

Fig. 47. A & B. Western Blot for metastatic markers

A. Effect of BDMC-A and curcumin on expression of metastatic proteins. MCF-7 cells were plated and incubated with 15 μM curcumin and 15 μM BDMC-A for 24h. After 24h whole cell extracts were prepared and resolved on 10% SDS-PAGE, electrotransferred and probed with antibody. β-actin was used as internal loading control.

B. Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. *p ≤ 0.05, significance compared to curcumin.
**Fig. 48. A. Metastatic markers in Hep-2 cells**

- **A.** Western Blot for metastatic markers
  - (i) **Cox-2** (70 kDa)
  - (ii) **MMP-9** (92 kDa)
  - (iii) **TIMP-2** (21 kDa)

- **B.** Densitometry

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Curcumin</th>
<th>BDMC-A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cox-2</strong></td>
<td> </td>
<td> </td>
<td> </td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td> </td>
<td>*</td>
<td>#</td>
</tr>
<tr>
<td><strong>TIMP-2</strong></td>
<td> </td>
<td>*</td>
<td>#</td>
</tr>
</tbody>
</table>

**Fig. 48. A & B. Western Blot for metastatic markers**

A. Effect of BDMC-A and curcumin on expression of metastatic proteins. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h, whole cell extracts were prepared and resolved on 10% SDS-PAGE, electro transferred and probed with antibody. β-actin was used as internal loading control.

B. Densitometry. Protein levels were quantified using densitometry analysis and expressed in relative band intensity. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 49. mRNA expression by Real Time PCR in MCF-7 cells

(i) **Cox-2**

(ii) **MMP-9**

(iii) **VEGF**

Fig. 49. mRNA expression by Real Time PCR. Effect of BDMC-A and curcumin on mRNA expression of metastatic proteins. MCF-7 cells were plated and incubated with 15 μM BDMC-A and 15 μM curcumin for 24h. After 24h, whole cell extracts were prepared using TRIzol solution. The quantitative measurement of mRNA expression for COX-2, MMP-9 and VEGF were analysed by Real Time PCR (RT-PCR). GAPDH was used as internal loading control. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
**Fig. 50. mRNA expression by Real Time PCR in Hep-2 cells**

(i) **Cox-2**

(ii) **MMP-9**

(iii) **VEGF**

**Fig. 50. mRNA expression by Real Time PCR.** Effect of BDMC-A and curcumin on mRNA expression of metastatic proteins. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. After 24h, whole cell extracts were prepared using TRIzol solution. The quantitative measurement of mRNA expression for COX-2, MMP-9 and VEGF were analysed by Real Time PCR (RT-PCR). GAPDH was used as internal loading control. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 51. Invasion assay in MCF-7 cells

**Fig. 51. Invasion Assay.** BDMC-A and curcumin inhibited the invasion of MCF-7 cells in *vitro*. Cells were plated and treated with 15 μM BDMC-A and 15 μM curcumin for 24h and 48h and cells that penetrated through the matrigel to the lower chamber of the filter was quantified by counting cells at 200X. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.

Fig. 52. Invasion assay in Hep-2 cells

**Fig. 52. Invasion Assay.** BDMC-A and curcumin inhibited the invasion of Hep-2 cells in *vitro*. Cells were plated and treated with 10 μM BDMC-A and 25 μM curcumin for 24h and 48h and cells that penetrated through the matrigel to the lower chamber of the filter was quantified by counting cells at 200X. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control. #p ≤ 0.05, significance compared to curcumin.
Fig. 53. Cancer related inflammatory (CRI) markers in MCF-7 cells

Effect of curcumin and BDMC-A on expression of inflammatory markers. MCF-7 cells were plated and incubated with 15 μM BDMC-A and 15 μM curcumin for 24h. The quantitative measurement of inflammatory cytokines TNF-α, IL-1β, TGF-β, and IL-8 was done in supernatant of both curcumin and BDMC-A treated MCF-7 cells. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.
Fig. 54. Cancer related inflammatory (CRI) markers in Hep-2 cells

(i) TGF-β

(ii) IL-6

(iii) IL-8

Fig. 54. Cancer related inflammatory markers. Effect of curcumin and BDMC-A on expression of inflammatory markers. Hep-2 cells were plated and incubated with 25 μM curcumin and 10 μM BDMC-A for 24h. The quantitative measurement of inflammatory cytokines TNF-α, IL-6, and IL-8 was done in supernatant of both curcumin and BDMC-A treated Hep-2 cells. Values represent mean ± SD of three independent experiments. *p ≤ 0.05, significance compared to control.