Discussion
DISCUSSION

Cancer is a fatal disease which occurs due to uncontrolled proliferation of cells with abnormal characteristics. In cancer, genetic alterations occur affecting the signalling pathways and allowing the unrestricted cell growth. There are many facets to cancer treatment and prevention. Chemotherapy attempts directly to inhibit proliferation of cancer cells or to selectively remove transformed cells by inducing apoptosis and eliminating the cause of the growth advantage [267]. Chemopreventive agents restore normal growth control to cancerous cells by amending abnormal signalling pathways or by inducing cell death in cells beyond repair. Their features include selectivity for transformed cells and many mechanisms of action to stop the redundancy in signalling pathways [268]. One of the chemopreventive measures is using natural or synthetic compounds to suppress, retard or inverse carcinogenesis [269].

Phytochemicals exhibit chemopreventive properties against various cancers [270]. Curcumin is one of the most widely characterized phytochemicals. Several studies demonstrate the inhibitory effect of curcumin on tumourigenesis and tumour growth in vitro and in vivo [271, 272]. Due to its anticancer properties, absence of deleterious effects and lack of systemic toxicity [240], curcumin is considered as an ideal candidate for chemotherapy. On the other hand, the limited in vivo application of curcumin, due to its low potency, less aqueous solubility, poor selectivity, intense staining colour, poor absorption, limited tissue distribution, rapid metabolism and subsequent elimination from the body [273-275], necessitates the search for new curcumin analogs.

BDMC-A is one such analog with ortho hydroxyl group in its structure. There have been contradictory reports supporting the two different sites in the structure of curcumin for the attack of free radicals and hence for therapeutic effects. Initial reports attributed the antioxidant activity to the phenolic OH group [276]. Jovanovic et al. have indicated that hydrogen abstraction from methylene CH₂ group is responsible for the antioxidant activity of curcumin [277]. Later, Basclay et al. by following the inhibition of styrene oxidation by a number of curcumin derivatives confirmed that the H atom from the phenolic OH is responsible for the antioxidant activity and not the methoxy group
Hence we propose that the absence of methoxy group and the presence of ‘OH’ group in $o$-position in our synthetic curcuminoid (BDMC-A) can add to its antioxidant character and thus therapeutic potential. The $o$-hydroxyl group, because of its resonance property, easily donates electrons to free radicals and effectively neutralizes them.

*In vitro* models for cancer research involves the usage of cancer cell lines. Cell lines are broadly used in many aspects of laboratory research as these models have a number of advantages. Some of the advantages are: they are easy to handle, they represent a limitless self-replicating source that can be easily retrieved from frozen stocks and they exhibit high degree of homogeneity [279]. Cancer cell line models have emerged as valuable tools to evaluate gene expression, define the molecular pathways [267] in carcinogenesis and to identify potential chemotherapeutic and chemopreventive agents. Cancer cell line selection is an innovative step for anticancer drug discovery [280].

The MCF-7, a model for human breast cancer cells, are metabolically active and are commonly used cell lines in toxicological investigations [281]. Hep-2 (human laryngeal carcinoma cells) is used in several anticancer drug evaluation and mutagenic studies [282]. Therefore, these cells were employed in this study as suitable *in vitro* model system to evaluate the anticarcinogenic efficacy of BDMC-A and compare it with parent compound curcumin.

**BDMC-A inhibits cancer cell proliferation**

Cytotoxicity of chemotherapeutic agents are analysed by MTT assay which measures the mitochondrial activity of viable cells. The tetrazolium yellow MTT dye, under defined conditions, is reduced by the enzyme NAD(P)H dependent cellular oxidoreductases into insoluble purple formazan crystals. These crystals are dissolved by adding DMSO and the absorbance of the resulting coloured solution is used as a measure for cytotoxicity.
Curcumin has antiproliferative activity against a range of human cancers such as ovarian, osteosarcoma, lung, breast, head and neck [283-287]. A number of mechanisms by which curcumin can inhibit the cell proliferation has been described previously [288]. In our work, MTT assay of curcumin and its analog BDMC-A revealed that BDMC-A inhibits cell proliferation at an equal concentration (IC50 at 30 μM) to that of curcumin (IC50 at 30 μM) in MCF-7 cells and about two times more potently than curcumin in Hep-2 cells where the IC50 of curcumin was 50 μM and that of BDMC-A was 20 μM. The observed effects of curcumin on MCF-7 and Hep-2 cells proliferation in our study are consistent with previous reports [289, 290].

In LDH release assay, the cytosolic LDH enzyme leaked from the dead cell to the cell culture media was measured colorimetrically. The enhanced LDH release in curcumin and BDMC-A treated cells indicate the efficacy of these drugs as potent cytotoxic inducers in MCF-7 and Hep-2 cancer cells.

Cell count assay uses the diazo dye trypan blue to stain the dead tissues or cells. Live cells or tissues with intact cell membranes allow only selective compounds to pass through the membrane. Therefore viable cells do not absorb trypan blue and appear colourless whereas the dye traverses the membrane of dead cells staining them blue which could be viewed under microscope. Cell count assay analysis confirmed the presence of dead MCF-7 and Hep-2 cells treated with curcumin and BDMC-A and that BDMC-A exhibited good cytotoxicity.
BDMC-A arrests cell cycle in cancer cells

Advancement in the knowledge about the mammalian cell cycle molecular mechanisms helps us to understand premalignant lesions, diagnostics, and promising therapeutic procedures [291]. Cells have checkpoints in G1-S and G2-M transitions of normal cell cycle to ensure proper performance of cell cycle events [9, 292]. Cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs) have an important role in these processes [293]. One of the subgroup of cyclins, cyclin D1, acts as a rate-limiting factor in progression of cells through the G1 phase of the cell cycle. Cyclin D1 is a proto-oncogene and is overexpressed in many cancers, including breast, oesophagus, head and neck, prostate and mantle cell lymphoma [294] as a result of gene amplification or translocation. The cyclin D1 gene is amplified in 20-50% and its protein is overexpressed in 80% of SCCs. The cyclin D1 gene is also amplified in up to 20% of human breast cancers [37]. The cyclin B1/CDK1 complex is crucial for advancement of the cells through mitosis and a decrease in cyclin B1 proteins can result in G2/M arrest [295].

The flow cytometric studies in the present work confirmed an accumulation of curcumin or BDMC-A treated MCF-7 cells in the G2/M phase. Curcumin has previously been reported to arrest cancer cells including colon [296], pancreatic [297] and breast carcinoma cells [298] in G2/M or S(G0/G1) phases of cell cycle by up-regulating p21/Cip1/waf1, p27 Kip1 and down-regulating cyclin B1 and Cdc2 [295]. Another study has shown that curcumin induces carcinoma cell apoptosis in G2 phase only, in MCF-7 cells and it occurs via p53-mediated pathway where cytochrome c release plays an important role [299]. In the present study BDMC-A treated MCF-7 cells accumulated at G2/M cell cycle phase. This could be correlated to the observed results of downregulation of cyclin B1 expression and upregulation of p53 expression by BDMC-A.

In HNSCC cell lines, curcumin was reported to inhibit the growth by reducing the expression of cyclin D1 and arresting the cell cycle in the G1/S phase [300]. Previous reports suggest that the cells are held back in G1 and prevention of entry to S phase by curcumin is through the mechanism of cell cavitation and chromatin agglutination [301]. Similarly in our study, there was more accumulation of Hep-2 cells in G1 phase and
downregulation of cyclin D1 expression on BDMC-A treatment, indicating its potent cell cycle arresting property than curcumin.

Mechanism of apoptotic induction in cancer cells by BDMC-A

Apoptosis or programmed cell death is essential to maintain the homeostasis in multicellular organisms by destroying excess, damaged, or abnormal cells. Apoptotic cells display morphological changes like membrane blebbing, chromatin and cytoplasmic condensation, nuclear breakdown, DNA fragmentation and assembly of membrane-enclosed vesicles called apoptotic bodies and are finally subjected to phagocytosis [269]. At molecular level, it denotes a collection of intricate pathways where more than 100 proteins actively participate in signal transduction, zymogen-type cascade and destruction of cytoskeletal structures and DNA in the marked cell. A delicate fine tuning of pro and anti apoptotic factor ratio decides whether a cell death signal can execute apoptosis.

In cancer, the apoptosis/cell-division ratio is altered and a compromised apoptosis is, therefore, considered as one of the hallmarks of cancer. Virtually all cancer cells are prone to resisting apoptosis. Understanding the mechanisms of resistance to apoptosis may
lead to the development of anticancer drug that can reprogram cell death. Resistance to apoptosis is acquired by cancer cells through overexpressing antiapoptotic proteins and downregulating proapoptotic proteins. This knowledge provides new ways to diagnose, prognose and treat cancer [302]. Chemotherapy, radiation, and immunotherapy rely on apoptosis to kill breast cancer and HNSCC cells [303, 304]. It has been reported that the prime method by which chemotherapeutic agents such as cisplatin, camptothecin, etoposide etc. induces cancer cell death is through apoptosis. The efficiency of anti-tumour agents has been related to the intrinsic propensity of target tumour cells to respond to these agents through apoptosis [305].

Apoptosis has been scrutinised for cancer chemoprevention targets. Dietary chemopreventive compounds at human acceptable doses are considered as valuable resources to inhibit carcinogenesis and to induce apoptosis or cell-cycle arrest in genetically damaged neoplastic cells. Several studies support this concept of apoptotic pathway being a critical target for dietary bioactive agents in chemoprevention of cancer. Additionally, some of the agents exhibit specificity for neoplastic cells and spares normal cells. One such well known dietary agent that acts as good chemopreventive agent is curcumin [306].

Curcumin can trigger apoptosis in various cancers in vivo and in vitro, and induce cell death by affecting the expression of apoptosis-associated genes [307]. However, whether curcumin can induce apoptosis, depends on its concentration, cell type, and environmental factors. Curcumin promotes apoptosis in lung [271], breast [272], colon carcinoma cells [308], leukemia [309], bladder [310], prostate [311], and hepatocellular [312] including apoptotic resistant multidrug resistance phenotype [313]. MCF-7 cells are more sensitive to curcumin and several reports indicate that curcumin induces apoptosis in this cell line [314, 315]. Curcumin has been shown to induce apoptosis in Hep-2 cells [316]. Here, we have investigated the mechanisms underlying BDMC-A induced apoptosis in MCF-7 and Hep-2 cells and compared them with those induced by curcumin by studying the putative markers for both intrinsic and extrinsic apoptotic pathways.

**Intrinsic apoptotic pathway:** In our present study, we analyzed the intrinsic apoptotic markers viz. p53, Bcl-2, Bax, cytochrome c, Apaf-1, caspase-9, caspase-3 and...
PARP. Previous report has suggested that p53 was overexpressed by curcumin in MCF-7 cells [317]. In normal cells, the intrinsic apoptotic pathway involves p53, a ‘master regulator’ and a molecular ‘guardian of the genome’. p53 acts as a transcription factor and binds in particular with apoptosis responsive genes that increase the synthesis of p21cip1 or Bax [123, 318]. p53 also inhibits Bcl-2 gene expression [319]. Bcl-2 family members are critical determinants of cellular homeostasis. They are structurally related proteins and positively or negatively influence apoptosis. Bax, a member of Bcl-2 family, is a proapoptotic signalling protein, which antagonizes Bcl-2, an antiapoptotic signalling protein, and induces apoptosis in many types of cancers [320, 321]. Balance between the Bcl-2/Bax ratio confirms the apoptotic status of the cell. Wild-type p53 thus can alter the Bcl-2/Bax ratio by downregulating Bcl-2 and upregulating Bax and disposing to programmed cell death [322]. Previous studies indicate that in cancer cells, p53 is inactivated and this results in increase of Bcl-2 and suppression of Bax [323, 324].

It has previously been reported that curcumin upregulates pro-apoptotic factors (like p53, Bax) and downregulates the anti-apoptotic factors (Bcl-2) in ovarian cancer cell [325].

Here we show that BDMC-A upregulated p53 expression in both MCF-7 and Hep-2 cells. Concordantly, we found a stronger upregulation of Bax expression when the cells were treated with BDMC-A compared to curcumin. Since p53 is a well known direct activator of Bax, and since Bax is a well known inhibitor of Bcl-2, the down-regulation of Bcl-2 that we observed in this study can be attributed to the upstream distortion of this pathway. mRNA expression analysis also confirmed Bcl-2 downregulation and Bax upregulation in BDMC-A treatment.

Increased Bax/Bcl-2 ratio results in disruption of mitochondrial membrane. This increases the mitochondrial permeability resulting in cytochrome c leakage into cytosol [21]. Thus in our study, on treatment with BDMC-A, activated p53 upregulated cytochrome c through Bax and acted as a master switch for inducing apoptosis in a time dependent fashion.
Cytochrome c is an essential factor in the electron transport chain in mitochondria. It is involved in initiation of apoptosis [326]. Several reports have shown that the leakage of cytochrome c (hallmark for intrinsic apoptotic pathway) activates caspase-3 and induces apoptosis in wide range of cancers with curcumin treatment [327, 328]. In our study, the expression of cytochrome c was comparatively higher in BDMC-A treatment compared to curcumin treatment. Cytochrome c binds to Apaf-1 and caspase-9 and forms apoptososome complex, which initiates the caspase cascade via activation of caspase-3 and induces apoptosis in many cancer cells [329].

Caspases belonging to aspartic acid-specific cysteine proteases group participate in the initiation and execution of apoptosis. Caspase-3 is known to act as an effector caspase that precedes PARP cleavage and DNA fragmentation [330, 331]. Accumulating evidences confirm that curcumin induces apoptosis in several cancer cell lines by inducing cytochrome c release, caspase-3 activation and PARP cleavage [332-334]. In our study BDMC-A proved to be more effective in upregulating Apaf-1 expression and activation of initiator caspase, caspase-9 than curcumin. Along with cytochrome c, Apaf-1, activated caspase-9 might have formed the apoptososome complex and activated the effector caspase, caspase-3. The activated caspase-3 in turn might have cleaved PARP. The cleaved PARP might have caused the oligonucleosomal fragmentation of DNA. The PARP cleavage and caspase-3 induction were found to be more pronounced in BDMC-A treated MCF-7 and Hep-2 cells compared to curcumin treated cells. Moreover, BDMC-A induced the formation of typical apoptosis-related DNA laddering patterns. These data clearly indicate that BDMC-A acts as a potent inducer of apoptosis by upregulating caspase-3, PARP cleavage and DNA fragmentation. Thus our results with the intrinsic pathway provide the evidence that apoptosis happened via mitochondrial pathway and proved that BDMC-A was more potent than curcumin in inducing apoptosis.

**Extrinsic apoptotic pathway:** Several reports have shown the stimulatory effect of curcumin on extrinsic apoptotic pathway [335]. Extrinsic apoptotic pathway is triggered by the binding of “Death activators” such as TNF-α and Fas ligand with their corresponding cell surface receptors. Activation of death receptor pathway initiates caspase cascade by activating the initiator caspase, caspase-8 which results in the
activation of the effector caspase, caspase-3. The activated effector caspase leads to apoptosis.

Upregulation of death receptors by chemopreventive agents like curcumin has been shown to sensitize cancer cells and aggregate Fas receptors thereby increasing the levels of caspase-8 and 3 in many cancer cells [127]. In the present study, a significant increase in FasL and activated caspase-8 expression in BDMC-A treated cells compared to curcumin treated cells was observed. This is concomitant with the upregulation of cleaved caspase-3 observed previously. Thus BDMC-A possesses more ability to activate the extrinsic apoptotic pathway compared to curcumin.

Previous studies have reported that in most cells curcumin sequentially induces activation of caspase-8, cleavage of BID, loss of mitochondrial membrane potential, opening of transition pores, release of cytochrome c and induces apoptosis mediated cell death [37, 294].

A clear evident of cellular morphological changes pertained to apoptosis such as chromatin condensation and nuclear fragmentation were observed in BDMC-A treated MCF-7 and Hep-2 cells. These changes were significant compared to curcumin.

Hoechst, the family of bis-benzimides stains, are blue fluorescent dyes that stain DNA [336]. They bind to the minor groove of DNA preferably at adenine and thymine rich sequences [337]. In contrast to PI, these dyes are cell-permeable. Hence they are called supravital. Hoechst dyes are preferred over DAPI as they are less toxic and are more cell-permeable. They are used in fluorescence microscopy [338] and flow cytometry [339].

PI is an intercalating fluorescent molecule. A 20-30 fold increase in its fluorescence is noticed when the dye binds to nucleic acids. PI is generally membrane impermeant. Once inside cells, it intercalates among bases of DNA, in a proportion of 1 dye per 4–5 bp. It is used as counterstain [340] and in differentiating necrotic, apoptotic and normal cells [341]. It is also utilized in flow cytometry and in microscopy.
In the present study, BDMC-A induced morphological changes such as DNA damage, chromatin condensation and nuclear fragmentation in MCF-7 and Hep-2 cells indicate the induction of apoptotic death, in these cells. Previous study has shown that curcumin could induce apoptotic body formation in various cancer cells [315, 342]. The apoptotic morphological changes were more with BDMC-A treatment than with curcumin treatment in PI or Hoechst stained MCF-7 and Hep-2 cells.

Early apoptosis is marked by annexin proteins. Annexins, the calcium-dependent phospholipid-binding proteins, binds to PS. PS is chiefly accumulated in the inner leaflet of plasma membrane of living cells. Upon initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and gets translocated from inner to outer leaflet of plasma membrane and will be available for binding. Cell surface PS is detected by a preferential binding of PS with protein AnnCy3, using annexinV-Cy3 detection kit. The live cells will be labelled only with 6-CF (green), while necrotic cells only with AnnCy3 (red), however early apoptotic cells will be labelled with both AnnCy3 (red) and 6-CF (green).

Evidences show that early apoptosis were induced by many chemopreventive agents [343]. Our results correlate well with the externalization of PS as the treated cells were labelled in both red and 6-CF green. This indicates that with BDMC-A treatment early onset of apoptosis is induced in cells more potently than with curcumin treatment.

Dual staining with AO/EB is used to evaluate the nuclear morphology of apoptotic cells [344, 345]. Live cells will appear uniformly green. Early apoptotic cells will have bright green dots in their nuclei as a result of chromatin condensation and nuclear
fragmentation. Late apoptotic cells will incorporate ethidium bromide and therefore stain orange, and possess condensed and often fragmented nuclei. Necrotic cells too stain orange, but have a nuclear morphology similar to that of viable cells. In our study, in control, uniformly green live cells were observed with normal and large nucleus. Whereas in BDMC-A treated cells, a comparative bright green and orange with fragmented nuclei staining were observed. These results confirmed that BDMC-A significantly induced early apoptosis in breast and laryngeal cancer cells than curcumin.

Oxidative stress may generate ROS in tumour cells, which may increase the cytotoxic activity of therapeutic drugs [346]. Though curcumin acts as a free radical scavenger, an effect on ROS generation has been reported in many cancer types [347]. Curcumin’s apoptotic triggering effect was reported to involve generation of reactive oxygen intermediates [348]. The formation of ROS is important for cytochrome c release from mitochondria and, thus, for triggering caspase-mediated apoptosis. In the present study, formation of ROS is followed by staining the cells with the cell-permeant DCFH-DA. DCFH-DA is a chemically reduced form of fluorescein that is used as an indicator for ROS in cells. The nonfluorescent DCFH-DA upon cleavage of the acetate groups by intracellular esterases is converted to the highly fluorescent 2’,7’-dichlorofluorescein (DCF). The fluorescence emitted can be monitored using a flow cytometer, microplate reader, or fluorescence microscope, using suitable excitation sources and filters. BDMC-A treatment in MCF-7 and Hep-2 cells resulted in increased fluorescence than curcumin treatment. This indicates that with BDMC-A treatment higher ROS production occurs, which in-turn might be one of the reasons of eliciting intrinsic apoptotic pathway.

Mitochondria, “the powerhouse of the cell” are intracellular organelles that produce energy. They take part in diverse cellular events like release of caspase activators, electron transport etc. Loss of mitochondrial membrane potential (ΔΨm) occurs with the participation of both pro- and anti-apoptotic Bcl-2 family proteins [349, 350]. Alterations in mitochondrial structure and function play an important role in caspase-9-dependent apoptosis [351]. Normally there is electrochemical gradient across inner membrane of mitochondria. This is important for the accurate function of electron transport chain. Degeneration of the electrochemical gradient or membrane potential results in opening of
MPTP. This triggers cell death by releasing apoptogenic factors from mitochondria to cytosol. Some of the released mitochondrial proteins are cytochrome c, endonuclease G, Smac/DIABLO, Omi/HtrA2 and apoptosis-inducing factor (AIF) \[352\].

The opening of MPTP is considered as the direct reason of cell apoptosis. $\Delta\Psi_m$ was monitored by the fluorescent cell permeable cationic dye, Rhodamine 123. This dye enters into mitochondria based on highly negative $\Delta\Psi_m$. Depolarization of $\Delta\Psi_m$ during apoptosis leads to the loss of dye from mitochondria and a clear shift from orange/red fluorescence to green fluorescence will be observed. It was reported earlier that some of the dietary bioactive agents that can alter mitochondrial membrane function and/or dissipate $\Delta\Psi_m$ can induce apoptosis. Curcumin induces mitochondrial swelling and collapses the $\Delta\Psi_m$, resulting in apoptosis of many cells \[353, 354\]. Our study indicates the occurrence of mitochondrial membrane depolarization. This result supports the notion that BDMC-A can induce apoptosis in MCF-7 and Hep-2 cells through signalling at mitochondrial level.

The final stage of apoptosis is indicated by oligonucleosomal DNA fragmentation. The typical DNA laddering pattern of inter-nucleosomal fragmentation has been observed previously with curcumin treatment in cancer cells \[355, 356\]. In our study DNA fragmentation was induced significantly by BDMC-A.

Thus in our study, BDMC-A induced apoptosis essentially by engaging in key mitochondrial events peculiar to intrinsic pathway such as translocation of Bax to mitochondria, alteration in Bcl-2/Bax ratio, generation of ROS, drop in $\Delta\Psi_m$, release of cytochrome c and activation of caspase-3. The disruption of mitochondrial homeostasis by BDMC-A suggests that it can engage cell-intrinsic pathway but is not limited to intrinsic pathway. In the extrinsic pathway, BDMC-A induces apoptosis through caspase-8, an initiator caspase that directly activates effector caspase importantly caspase-3. Hence it can be put forth that BDMC-A induced activation of caspase-3 is mediated by initiation of both intrinsic and extrinsic apoptotic pathways and it is more potent than curcumin in inducing apoptosis.
**BDMC-A regulates the cell survival through src and PI3k/Akt**

Src, a non-receptor protein tyrosine kinase in its activated form promotes survival, angiogenesis, proliferation, decreased adhesion, increased motility and invasion. Thus src promotes the neoplastic phenotype. Src is overexpressed in HNSCC as well as breast cancers. Some of the important pathways with which src interacts are PI3K/Akt, NF-κB, STAT3, VEGF, IL-8, c-Jun [357-359]. Thus src can be regarded as an encouraging target for cancer treatment. A number of tyrosine kinase inhibitors that target src tyrosine kinase have been developed for therapeutic use. Inhibition of src expression by curcumin was reported in many cancer cells. BDMC-A also down-regulated src expression to a greater extent than curcumin. Furthermore, curcumin has been proved to suppress EGFR activation [360] and src activity [361].

PI3K/Akt pathway has significant role in cell growth and proliferation. Several cellular kinases implement their survival effect through PI3K/Akt pathway. PI3K catalyzes PIP3 formation and activates the downstream signalling Akt/mTOR. PI3K activity is regulated by the binding of regulatory subunits (p85/p55/p101) to catalytic subunits (p110) and a series of phosphorylation events (68). Numerous researches in breast cancer have shown that deregulation of this pathway is implicated in tumourigenesis and hence this has become an important target for breast cancer treatment [362, 363]. Particularly luminal A breast cancers were characterized by a high frequency of mutation in the PI3K catalytic subunit α gene. This pathway is activated through the EGFR often in tumours, particularly in laryngeal squamous cell carcinomas in which the EGFR is over-expressed. Activated PI3K/Akt signalling can promote tumour growth.

Akt is an attractive target for chemoprevention and therapy. Akt plays significant role in signalling cancer cell survival [364] through activating the NF-κB signalling pathway [365]. Based on these functional relationships, it is thought that for the control of tumour growth, PI3K inhibitors will be most effective in combination with EGFR inhibitors [366]. Previous studies imply that curcumin targets many components of Akt signaling pathway and thus inhibits proliferation and induces apoptosis [367]. PI3K/Akt/mTOR signalling pathway suppression by curcumin is implicated in cell lines derived from liver, breast, thyroid and renal cancers, melanoma and burkitt’s lymphoma.
[286, 368-372]. Here, we found that both curcumin and BDMC-A downregulated PI3K and pAkt, but the effect of BDMC-A was more pronounced. Previously, it has also been found that pAkt can activate Mdm2 which, in turn, can inhibit p53 [373]. The BDMC-A induced upregulation of p53 that we observed in our study can thus be attributed to the inhibition of PI3K and pAkt.

**Transcription factors regulated by BDMC-A**

Many of the oncogenes and tumour suppressor genes have been shown to encode transcription factors. Deregulated expression of transcription factors plays crucial roles in all the steps of carcinogenesis. Transcription factors can be regulated through various mechanisms. Many of these factors are inactive under normal physiological conditions. They represent highly desirable and logical targets for chemotherapy [374].

One important intracellular target in chemotherapy for cancer is NF-κB, a “rapid-acting” primary transcription factor. It is the 1st responder to different cellular stimuli. It regulates expression of over 200 genes that influence inflammation, cell growth, invasion, angiogenesis and metastasis. NF-κB plays key role in the progression of breast, colon and pancreatic cancers.

Proteins of the NF-κB family share a Rel homology domain and are synthesized as large precursors, p105 and p100, which are processed into mature NF-κB subunits, p50 and p52. Both subunits participate in target gene transactivation by forming heterodimers with other NF-κBs such as RelA (NF-κB p65), RelB, or c-Rel [375].

Curcumin has been shown to inhibit NF-κB activation and thereby suppress cancer cell proliferation in breast cancer [376], ovarian cancer [377], pancreatic cancer [378], oral cancer [379], bladder cancer [380], head and neck cancers [381]. Anand et al have reported that curcumin reduced breast cancer metastasis by suppressing NF-κB, COX-2 and MMP-9. Curcumin has been proved to downregulate the expression of NF-κB regulated gene products involved in

a. cellular proliferation (COX-2, cyclin D1, and c-myc) [382]

b. anti-apoptosis (IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, Bfl-1/A1, TNF receptor-associated factor 1) [224]

c. metastasis (MMP-2, MMP-9, and ICAM-1) [383, 384]
d. angiogenesis VEGF and IL-8 [385]
e. proinflammation (IL-6, TNF-α) [386]

Curcumin inhibits the stimulation of upstream signal of NF-κB i.e. Akt. It downregulates NF-κB targets COX-2 and MMP-9 [382, 383]. We observed that BDMC-A downregulated RelA and its related oncogene c-Rel more efficiently which proved that BDMC-A, was more effective than curcumin in terms of effects on NF-κB and related markers in MCF-7 and Hep-2 cell lines. The observed effect of BDMC-A on NF-κB might be correlated to those on Bcl-2, cyclin D1, MMPs, VEGF discussed previously in this study.

AP-1 has been reported to get transactivated by numerous tumour-promoting agents. c-Jun and c-Fos form the AP-1 early response transcription factors. Agents that activate NF-κB also activate 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 transformation from preneoplastic to neoplastic state of cancer cells in ex vivo and in vivo models [37]. AP-1 also participates in tumour progression and metastasis. Curcumin has been reported to suppress AP-1 activation [387]. It interacts with AP-1 DNA binding motif and inhibits AP-1 activation.

We investigated the effect of BDMC-A on the c-Jun and c-Fos expression levels and found that these levels were more significantly downregulated with BDMC-A treatment compared to curcumin treatment in MCF-7 and Hep-2 cells. This effect of BDMC-A may in part be due to the suppression of NF-κB as previous study has reported a distinct and essential role of NF-κB in regulating AP-1 transcription factor.

STAT3 converges with NF-κB in various oncogenic signalling pathways [388]. Lee et al reported that for maintaining activated NF-κB, cancer cells require STAT3 [183]. Constitutive activation of STAT3 has been observed in cancer and immune cells. It takes part in carcinogenesis, as well as in tumour 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 [389]. Mutation in EGFR has been attributed for constitutive STAT3 phosphorylation in lung adenocarcinomas [390]. Suppression of STAT3 activation by curcumin has been observed in T-cell leukemia [391], lung [392], HNSCC [393], ovarian cancer [394]. Curcumin has been reported previously to downregulate the
activation of STAT5 in K562 leukemia cells [395]. We examined the effect of BDMC-A on the STAT3 and STAT5 expression levels. The levels were more significantly downregulated in BDMC-A treated cells compared to curcumin treated cells. Thus decreased level of NF-κB in BDMC-A treated MCF-7 and Hep-2 cells may be partially due to the effect of the drug on STATs.

β-catenin is the important transcription factor which is activated by WNT signalling pathway. Impact of curcumin on WNT signalling pathway especially through modulation of β-catenin/Tcf/LEF has been observed in osteosarcoma, colon cancer and breast cancer cells [117,396, 397]. Curcumin stimulates caspase-3-mediated β-catenin cleavage as well as suppress β-catenin/Tcf/LEF transactivation by c-myc and cyclin D1 [108]. We investigated the effect of BDMC-A on the β-catenin levels in MCF-7 and Hep-2 cells treated with curcumin or BDMC-A and found that the β-catenin level was more significantly reduced in BDMC-A treated cells.

Another transcription factor that is emerging as a potential target for cancer therapy is PPAR-γ. Anti-inflammatory role of curcumin via PPAR-γ has been shown previously [398] and activation of PPAR-γ by curcumin inhibits moser cell growth by suppressing cyclin D1 and EGFR [399]. Recent report proposes that PPAR-γ ligands exert their effects in HT-29 colon cancer by interacting with p65 subunit of NF-κB. This prevents NF-κB pathway activation [400]. In our study BDMC-A significantly upregulated PPAR-γ level in both MCF-7 and Hep-2 cell lines. This could have also aided the previously observed downregulation of NF-κB in BDMC-A treated cells.

**Mechanism of BDMC-A mediated inhibition of cancer invasion and metastasis**

Multiple cellular pathways influence the growth and metastatic potential of tumours. Two major factors decide the distribution of cancers to distant organs. They are the biological properties of cancer cells and the environment at the metastatic site. The process of primary tumour growth, invasion and metastasis to distant organs are dependent on a highly orchestrated series of events including cellular transformation, angiogenic environment, local tumour cell growth, invasion through the ECM and basement membrane, entry into the circulation and eventually non-random tumour-cell
metastasis to distant organs. These processes provide various targets for therapeutic agent development [401]. 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 initial and critical step in promoting cell invasion and metastasis [402]. Tumour cell invasion through the ECM and extravasation involve cellular detachment, motility through ECM and basement membrane degradation. These are steps depend on a range of enzymes that include metalloproteinases (MMPs) and serine and cysteine proteinases. The members of the Zn-dependent matrix metalloproteinases (MMPs) remodel the ECM [403]. ECM degradation is one of the hallmarks of tumour invasion and migration [404]. Under physiological conditions, low level of MMP is produced in most cells. However many tumours exhibit dramatic overexpression of these enzymes. This, leads to an intense proteolysis of the ECM and basement membrane, promoting the cancer cell invasion.

Overexpression of various MMPs is proved to markedly increase the invasive behaviour of tumour cells and their ability to metastasize in experimental animal models. These proteolytic enzymes are produced directly by carcinoma cells or the carcinoma cells recruit them from host cells. Increased expression of MMPs correlates with the invasion and metastasis in human pancreatic, lung, breast, colon and head and neck [405-409]. The MMP family has since emerged as an attractive pharmaceutical target. Blocking MMP gene transcription, proenzyme activation or active site-directed inhibitions are considered as possible approaches for therapeutic intervention.

Liu et al have reported the higher levels of MMP-2 and MMP-9 in breast tumour tissues when compared to corresponding normal tissue [410]. MMP-2 produced by mesenchymal cells and MMP9 secreted by inflammatory cells (macrophages and neutrophils) are now viewed as key regulators of pathological angiogenesis. Elevated levels of MMP-2 and MMP-9 are linked to aggressive, invasive or metastatic tumours [411]

Curcumin has been proved to inhibit MMP-2, which is implicated in aggressive melanoma and prostate cancers. Curcumin has been shown to affect both the transcriptional and posttranscriptional levels of MMPs [294]. Curcumin has also been reported to inhibit MMP-9 expression in pancreatic tumours, in ovarian tumours and in in
vitro cancer cells [241]. MMP-9 expression is induced by AP-1, and this expression is inhibited by curcumin in human breast cancer MCF-7 cells [412].

In our study, BDMC-A or curcumin treatment reduced the levels of MMP-9 in both MCF-7 and Hep-2 cells. This may be due to the effect of BDMC-A on the upstream targets of MMPs such as NF-κB and AP-1 as the mRNA levels of MMP-9 in BDMC-A treated cells was also found to be reduced.

TIMPs are natural inhibitors of MMPs: TIMP-2 inhibits MMP-2 and TIMP-1 inhibits MMP-9 [413]. TIMP-2 has been found to block tumour cell invasion both in vitro and in vivo and may act as metastasis suppressor gene. TIMP overexpression results in decreased invasion of endothelial and tumour cells both in vitro and in vivo. TIMP is reduced in many cancer cells [414].

Previous studies have shown that curcumin enhances the expression of anti-metastatic protein, TIMP-2 in melanoma cells [415]. In breast cancer, it was proved to downregulate MMP-2 and upregulate TIMP-1 [416] BDMC-A has been proved to regulate aberrant levels of MMPs and TIMPs in other diseases [257].

In our study, MCF-7 and Hep-2 cells treated with BDMC-A showed higher levels of TIMP-2 compared to curcumin, highlighting the potential of BDMC-A as an anti-metastatic agent.

**BDMC-A regulates angiogenic factors and products of angiogenic pathways**

Tumour angiogenesis is an essential process for incessant growth and spreading of solid tumours. The angiogenic signalling pathway is a complex, interconnected web. Inhibition of one part of the web may result in compensation through another pathway. Angiogenesis occurs by the secretion of ‘angiogenic factors’ from cancer cells. The angiogenic factors include growth factors like VEGF, cytokines like IL-6, IL-8, and a number of small molecules [417]. Angiogenesis based therapy has been developed as one of the main therapeutic methods to cancer [418]. Thus, identifying novel therapeutic compounds targeting the angiogenic pathways could be favourable for cancer therapy. Of the angiogenic factors, VEGF has been known as the important target [419]. Highly malignant tumours 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 [420]. An effective oncotherapy should address angiogenesis as well. In this respect too, BDMC-A hold potential as it downregulated the expression of VEGF. Curcumin has been shown to downregulate VEGF in breast cancer and HNSCC [421]. This correlates with the present study where both curcumin and BDMC-A downregulated VEGF expression and BDMC-A was more potent.

Tissue microenvironment has been identified to exert an intense effect on cell proliferation and differentiation [422]. IL-6, the pleiotropic cytokine, exerts pro-angiogenic activities in tumour microenvironment. There are growing evidences that reveal significant relationships between IL-6 levels and failure of treatment directed against VEGF [423]. In the present study the increased levels of IL-6 were reduced upon treatment with BDMC-A in MCF-7 and Hep-2 cells. Thus BDMC-A reduces the VEGF expression through IL-6.

The angiogenic pathway produces TGF-β, COX-2 and EGFR. COX-2 is downstream to NF-κB in the signalling pathway. COX-2 expression is also correlated to angiogenesis [424]. Accumulating evidences indicate that COX-2 is implicated in the development of tumours [425]. COX-2 overexpression by malignant cells enhances cellular invasion, angiogenesis, antiapoptotic cellular defenses, and immunologic resistance through production of prostaglandin E2 [426]. A number of studies have reported the presence of increased levels of COX-2 in solid tumours [427]. COX-2 increases the invasion of breast cancer cells in vitro and in vivo and its overexpression has been associated as a marker of high metastatic potential. High level of COX-2 is coupled with lymph node and distant metastasis [428]. Angiogenesis mediated by COX-2 also has a role in the advancement of pre-neoplastic lesions to the invasive phenotype [429]. High levels of COX-2 have also been correlated with increased expression of VEGF, IL-11, and IL-8 in lung cancer cells [430]. COX-2 overexpression correlates with the aggressiveness of breast cancers [431].

NF-κB positively regulates COX-2 in varied cell types [88]. The 5’- promoter region of COX-2 contains 2 putative NF-κB binding sites. Activation of AP-1 is also implicated in the transcription of COX-2 [432]. Besides these transcription factors, IL-6
has been shown to stimulate COX-2 transcription. Inhibitors of COX-2 have exhibited effective anticancer properties in breast cancer patients [424]. Previous report showed that curcumin inhibited COX-2 and MMP-9 expressions and reduced the invasive and metastatic properties of cells [294]. In our study, we found that BDMC-A was more effective in reducing the COX-2 level compared to curcumin in both MCF-7 and 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-κB, AP-1, VEGF and IL-6 levels.

One event associated with progression of cancer is the EMT. EMT impacts cell motility and invasiveness. EMT is mediated and maintained by chemokines/cytokines such as IL-8 and TGF. IL-8 increases invasiveness of MCF-7 by 2 folds [433]. Since IL-8 expression is also regulated by NF-κB and AP-1, as expected, BDMC-A decreased the IL-8 expression in MCF-7 cells and Hep-2 cells. Inhibiting TGF-β induced EMT is an ideal strategy for treatment of invasion and metastasis of cancer [434]. Exposure to BDMC-A produced a significant downregulation of TGF-β in both cells.

Another molecule of interest is IL-1β, which induces NF-κB and induces angiogenesis through VEGF and TNF-α, and plays a key role in inflammatory response [435]. IL-1 is also required for tumour cell invasion and angiogenesis. We observed that in MCF-7 and Hep-2 cells, BDMC-A treatment not only significantly lowered the levels of both IL-1β and TNF-α, but also reduced the invasiveness of these cells. Curcumin has been reported to downregulate the expression of various pro-inflammatory cytokines including TNF, IL-1, IL-2, IL-6, IL-8, IL-12, and chemokines, mainly through inactivation of the transcription factor NF-κB. Curcumin was found to prevent tumour induction via down-regulation of TGF-β in cancer cells [108]. In hepatoma cells, curcumin has been reported to inhibit IL-6 production and AP-1 activation [436]. The observed effect of BDMC-A on IL-6, IL-8, TGF-β, IL-1β and TNF-α levels can be corroborated with these studies of curcumin. Nevertheless, BDMC-A was more potent than curcumin in bringing out these effects.

Thus BDMC-A opens up a therapeutic window for the treatment of breast and laryngeal cancer. Further in vivo studies and clinical trials are required to use this molecule as a promising chemopreventive/therapeutic agent.