Oxidative stress induced by curcumin promotes the death of Cutaneous T cell lymphoma (HuT-78) by disrupting the function of several molecular targets

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Abstract

Curcumin is known to exert its anticancer effect either by scavenging or by generating reactive oxygen species (ROS). In this study, we report that curcumin mediated rapid generation of ROS induce apoptosis by modulating different cell survival and cell death pathways in HuT-78 cells. Curcumin induces the activation of caspase-8, caspase-2 and caspase-9, alteration of mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-3 and concomitant poly (ADP-ribose) polymerase (PARP) cleavage. But addition of caspase inhibitors only partially blocked the curcumin mediated apoptosis. Curcumin also down regulates the expression of antiapoptotic proteins c-FLIP, Bcl-xL, cIAP and XIAP in a ROS dependent manner. Curcumin disrupts the integrity of IKK and Beclin-1 by degrading Hsp90. Degradation of IKK leads to the inhibition of constitutive NF-κB. Degradation of Beclin-1 by curcumin leads to the accumulation of autophagy specific marker, microtubule-associated protein1 light chain 3 (LC3), LC3-I. Our findings indicate that HuT-78 cells are vulnerable to oxidative stress induced by curcumin and as a result eventually undergo cell death.

Introduction

Cutaneous T cell lymphomas (CTCL) are a class of non-Hodgkin's lymphomas characterized by the uncontrolled growth of mature CD4+ T cells with distinct T cell markers. The two main forms are Mycosis fungoides (MF) and its leukemic counterpart Sézary syndrome, SS (1, 2). Defective T cell apoptosis and constitutive activation of NF-κB contribute to the pathogenesis of CTCL (2, 3). NF-κB is a regulator of many genes involved in inflammation, cell proliferation and apoptosis. Activation of NF-κB enhances the expression of antiapoptotic proteins like inhibitor of apoptosis proteins (IAP), X-linked inhibitor of apoptosis (XIAP), Bcl-2, Bcl-xL and c-FLIP *etc*. (4). Human IAP proteins, including cIAP-1, cIAP-2, XIAP *etc*. are known to inhibit apoptosis by inhibiting effector caspases, caspase-7 and caspase-3 (5).

Apoptotic stimulation activates at least one of the two major apoptotic pathways, the intrinsic or mitochondrial cell death pathway and the extrinsic receptor mediated cell death pathway (6). The mitochondrial pathway leads to disruption of mitochondrial membrane, release of cytochrome *c* into the cytosol, which then binds to the apoptotic protease activating factor (Apaf) complex and triggers the activation of procaspase-9 to active caspase-9 (7). Heat shock protein 90 (Hsp90) is a molecular chaperone which is crucial for the stability of many client proteins involved in signaling pathways for cell survival (8, 9). It has been demonstrated that Hsp90 function is required for constitutive IKK/NF-κB activity in lymphoma cells (10). It is also reported recently that Hsp90 protect Beclin-1 (Atg6), a key autophagy promoting protein that regulates the formation of autophagosomes from ubiquitination associated proteolytic degradation (11).

Curcumin, a yellow pigment derived from rhizome of *Curcuma longa* has been a subject of extensive investigations over the last five decades and has confirmed its antiapoptotic activity in a panel of tumor cells (12). The potent anticancer property of curcumin is credited to its

antioxidant effect that restrains free radical mediated lipid peroxidation and DNA damage (13). However, a rising number of recent reports demonstrated that curcumin exerts its anticancer effect by acting as a pro-oxidant, by inducing ROS generation. Interestingly, Kang et al. found that the anticarcinogenic mechanism exerted by curcumin differs depending on its concentration (12, 14).

Recently, it has been reported that curcumin selectively induces apoptosis in cutaneous T cell lymphoma cell lines by inhibiting STAT-3 and NF-κB (15). But this study did not address the antioxidative or pro-oxidative effect of curcumin on CTCL cells. Thus, the present study was designed to find out the effect of curcumin on intracellular ROS formation and its potential link with curcumin induced cytotoxicity in HuT-78 cells.

Here, we report that curcumin through generation of ROS disrupt the function of several important molecules involved in cell survival pathways and as a consequence, HuT-78 cells undergo apoptosis.

Materials and Methods

Cell culture and reagents

Human CTCL cell line, HuT-78 was procured from National Centre for Cell Science (Pune, India), a cell banking repository which supplies cell lines for research purposes. MyLa was obtained from and authenticated by European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Both the cell lines were passaged for less than 3 months before use in this study. These cell lines were cultured in RPMI-1640 medium supplemented with 10% heat inactivated Foetal Bovine Serum, GIBCO (16). Curcumin, N-acetyl-L-cysteine (NAC), rapamycin, 3-methyladenine (3-MA), wortmannin, propidium iodide (PI), 3,3'- dihexyloxacarbocyanine iodide ($DiOC_6$), anti-actin antibody, anti-mouse HRP antibody and anti-rabbit HRP antibody were purchased from Sigma. Dihydroethidium (DHE) and Amplex ® Red Hydrogen Peroxide/Peroxidase Assay Kit were purchased from Molecular Probes (Invitrogen). Annexin V-FITC apoptosis detection kit, APO-BRDU^{TM} kit and antibodies against cytochrome *c*, PARP, Bcl-xL, cIAP-1, cIAP-2, XIAP, Hsp90, Hsp70, IKK-α , IKK-β, Beclin-1, caspase-2, were purchased from BD PharMingen. LC3, Atg5 and Atg7 antibodies were purchased from Cell Signaling. 17- allylamino- 17-demethoxygeldanamycin (17-AAG) was purchased from Calbiochem.

Measurement of ROS

Dihydroethidium (DHE) fluorescent probe was used to measure the intracellular generation of superoxide anion radical, $O_2^{\cdot-}$ (17). Briefly, 5 x 10^5 cells (HuT-78 or MyLa) were seeded and treated with curcumin (25 μM) for different time points. Cells were exposed to 5 μM DHE for 30

min at 37 °C and the fluorescence intensity in cells was determined by using flow cytometer (Becton Dickinson).

H_2O_2 release in the culture supernatant of HuT-78 or MyLa cells were measured by Amplex ® Red Hydrogen Peroxide/Peroxidase Assay Kit according to manufacturer's instructions. Fluorescence was determined using a Synergy 4 multidetection microplate reader (Bio-Tek, USA) with an excitation of 530 nm and emission at 590 nm.

PI exclusion assay for cell viability

Cell viability was determined by PI exclusion assay (16). Briefly, 2x 10^5 cells (HuT-78 or MyLa) were treated with different concentration of curcumin (0-25 μM) for 24 h (18) .The level of PI incorporation was quantitated by flow cytometry on a FACSCalibur (Becton Dickinson).

Terminal deoxynucleotidyltransferase dUTP nick end labelling (TUNEL)

To measure the DNA strand breaks during apoptosis, TUNEL assay was performed using APO-BRDU™ kit (BD Pharmingen) according to manufacturer's protocol as described by Gahlot et al. (16). Stained cells were analyzed with flow cytometry.

Annexin V staining

Annexin V staining kit (BD Pharmingen) as described previously (16). Stained cells were analyzed with a FACSCalibur (Becton Dickinson).

Mitochondrial membrane potential

The change of mitochondrial membrane potential was determined by the retention of the dye $DiOC_6$ as described by Rishi et al. (19).

Caspase-8, -9 and -3 activity assay

Caspase-8 (Sigma), caspase-9 (Caspase-Glo® 9 assay system) and caspase-3 (Promega) activities were determined according to manufacturer's protocol.

Measurement of cytochrome *c* release

Cytosolic extracts were prepared according to the method as described by Miyoshi et al. (20). The release of cytochrome *c* into cytosol was measured by immunoblotting.

Western blot analysis

For Western blot analysis cell lysates were prepared after treatment as described previously (16). Protein extracts were resolved on 10–12% SDS-PAGE and analyzed by Western blotting using specific antibodies against either caspase-2, Bid, cytochrome *c*, PARP, c-FLIP, Bcl-xL, cIAP-1, cIAP-2, XIAP, Hsp90, Hsp70, IKK-α, IKK-β, Beclin-1 and LC3. Densitometry of individual bands was determined using Scion Image software (Scion Corporation).

Electrophoretic mobility shift assay

NF-κB DNA binding activity was determined by electrophoretic mobility shift assay (EMSA) as described previously (19).

Statistical analysis

Statistical significance of the differences was determined by the paired two-tailed Student *t* test using Microsoft Excel software. P values < 0.05 was considered as statistically significant.

Results

Curcumin generates reactive oxygen species (ROS) in HuT-78 and MyLa cells

It has been shown that many chemotherapeutic agents induce ROS mediated killing of cancer cells (21). The role of ROS in curcumin mediated apoptosis of HuT-78 cells are yet to be explored. To determine whether curcumin increases ROS production in HuT-78 cells, first we measured the intracellular ROS generation at different time points in curcumin treated cells using anion superoxide-sensitive probe DHE. Our FACS analysis revealed that intracellular superoxide anion free radical ($O_2^{-\cdot}$) level was increased in HuT-78 cells following treatment with 25 μM curcumin compared to untreated control cells. Superoxide anion free radical ($O_2^{-\cdot}$) was detected as early as 0.5 h after treatment with 25 μM curcumin, peaked at 6 h (Fig. 1B). We also tested the effect of curcumin on $O_2^{-\cdot}$ generation in another CTCL cell line, MyLa. Similar results were obtained (Fig. 1B).We also measured extracellular H_2O_2 in the culture supernatants using Amplex ® Red H_2O_2 assay at similar time points. We found that curcumin treatment resulted in enhanced accumulation of H_2O_2 in culture supernatant, peaked at 2 h (Fig. 1C). Prior treatment of cells with antioxidant, N-acetyl-L-cysteine (NAC) (10 mM) markedly blocked curcumin mediated ROS generation (data not shown).

Curcumin shows cyototoxic activity against HuT-78 and MyLa cells in a ROS dependent manner

Recently, it has been reported that curcumin is cytotoxic for CTCL cells (15). So, we were interested to see whether ROS is involved in curcumin mediated killing of HuT-78 and MyLa cells. For this purpose, cells were treated with different concentrations of curcumin (0-25 μM) for 24 h and cell viability was assessed by live PI dye exclusion method. Concentration dependent cytotoxic effects were observed in curcumin treated both the cell lines (Fig. 1D).

Curcumin mediated cell death was found to be ROS dependent because addition of NAC completely suppressed the cytotoxic effect of curcumin. TUNEL assay of curcumin treated cells further confirmed that curcumin mediated killing of HuT-78 cells is ROS dependent. As shown in Fig. 1E, curcumin treatment showed dose dependent increase in the percentage of apoptotic cells from 3% to 78% and pretreatment with NAC markedly reduced the degree of apoptosis. Under similar conditions, NAC (10 mM) alone did not show any effect on cell viability (data not shown).

Curcumin mediated ROS generation induces caspase activation, alters mitochondrial membrane potential (MMP) followed by cytochrome *c* release

To determine the involvement of caspases in curcumin mediated apoptosis of HuT-78 cells, we examined the activation of different caspases. First, we measured the activation of caspase-8, the initiator caspase in curcumin (0-25 μM) treated HuT-78 cells by colorimetric assay. As shown in Fig. 2A, a dose dependent increase in caspase-8 activity was obtained when measured after 24 h of curcumin treatment. Caspase-8 activation was blocked when NAC or broad range caspase inhibitor, z-VAD-fmk was present in the system. When the activation of another initiator caspase, caspase-2 was measured by immunoblotting, we obtained dose dependent activation of caspase-2 in HuT-78 cells after 24 h treated with curcumin. Prior treatment of cells either with NAC or different caspase inhibitors (z-VAD-fmk, z-IETD-fmk and Ac-DEVD-CHO) blocked this activation (Fig. 2B). The cleavage of Bcl-2 family member Bid to t-Bid, a substrate for caspase-8, is involved in linking extrinsic pathway to mitochondrial pathway (6). Therefore, we investigated the cleavage of Bid to t-Bid in HuT-78 cells treated with curcumin. Enhanced cleavage of Bid was observed in the cytosolic extracts of curcumin (25 μM) treated cells as

compared to control cells after 24 h. The cleavage of Bid was suppressed in the presence of NAC or z-VAD-fmk (Fig. 2C).

Next, we wanted to see if mitochondrial pathway is involved in curcumin mediated apoptosis of HuT-78 cells. Cells were treated with different concentrations of curcumin (0-25 μM) for 24 h and then $DiOC_6$ staining was performed. As shown in Fig. 2D, a dose dependent increase in MMP was seen which indicated mitochondrial hyperpolarization. When 25 μM curcumin treated cells were analyzed after staining, depolarization peak was clearly visible and this peak disappeared when cells were pretreated with NAC. Alteration of MMP is known to cause release of cytochrome *c* into the cytosol. Cytochrome *c* release from mitochondria into the cytosol of curcumin treated HuT-78 cells was detected by immunoblotting. Pretreatment with NAC blocked the cytochrome *c* release but z-VAD-fmk pretreatment could not significantly suppress the release of cytochrome *c* (Fig. 2E).

Next, we measured the activation of caspase-9 and then the activation of caspase-3 in curcumin treated HuT-78 cells after 24 h. Concentration dependent activation of both caspase-9 and caspase-3 were observed (Fig. 3A and B). Activation of caspase-3 was markedly reduced in the presence NAC or broad range caspase inhibitor, z-VAD-fmk (Fig. 3B). PARP cleavage was detected by immunoblotting using anti-PARP antibody. PARP cleavage could be detected in cells treated with 12.5 and 25 μM of curcumin but at lower concentration no cleavage was observed. Remarkably, this curcumin mediated cleavage of PARP was completely reverted back in the presence of NAC (Fig. 3C).

We found that curcumin activated initiator (caspase-8, -2 and -9) and executioner caspase, caspase-3, so the next obvious question we asked was that whether caspase mediated cell death pathway is solely responsible for killing of HuT-78 cells induced by curcumin. For this purpose,

the effect of different caspase inhibitors on curcumin mediated death of HuT-78 cells were studied. Cells were first treated with different caspase inhibitors viz. z-VAD-fmk, z-IETD-fmk and Ac-DEVD-CHO for 1 h and then treated with 25 μM curcumin. After 24 h, annexin V staining was performed to confirm cell death. As shown in Fig. 3D, only partial inhibition (12-16%) in cell death were observed with different caspase inhibitors whereas prior treatment with NAC significantly blocked cell death induced by curcumin.

Curcumin downregulates c-FLIP, Bcl-xL, cleaves XIAP and Hsp90 in HuT-78 cells

Western blot analysis revealed that curcumin effectively down regulated the expression of both c-FLIP$_L$ and c-FLIP$_S$ as compared to control (Fig. 4A). Curcumin also inhibited Bcl-xL expression in a dose dependent manner (Fig. 4B). When immunoblotting was performed to detect the expression of c-IAP family proteins, cIAP-1 and cIAP-2, we found that 25 μM curcumin significantly inhibited the expression of cIAP-2 as compared to cIAP-1(Fig. 4C). The expression of c-FLIP, Bcl-xL and cIAP-2 appeared to be ROS dependent since addition of NAC suppressed the inhibitory effect of curcumin on these proteins. We also checked the expression of XIAP in cell lysates prepared from curcumin treated HuT-78 cells after 24 h. Immunoblotting revealed that curcumin at 25 μM concentration cleaved XIAP. The cleaved fragment of XIAP was not visible in NAC pretreated cells (Fig. 4D). Further, to determine whether curcumin induced activation of caspases has any effect on XIAP, we treated HuT-78 cells with 25 μM curcumin in the presence or absence of different inhibitors of caspases. XIAP cleavage was completely blocked in the presence of z-VAD-fmk, a broad range caspase inhibitor while caspase-8- specific inhibitor, z-IETD-fmk slightly inhibited the XIAP cleavage. In contrast,

caspase-3-specific inhibitor, Ac-DEVD-CHO, was not able to block the XIAP cleavage (Fig. 4E).

It is demonstrated that Hsp90 promotes survival of cancer cells providing stability to pro and anti apoptotic proteins (22). We, therefore, studied the effect of curcumin on Hsp90 expression. For this, HuT-78 cells were treated with different concentrations (0-25 μM) of curcumin for 24 h, whole cell lyasates were prepared and used for Western blotting. Significant cleavage of Hsp90 was observed when HuT-78 cells were treated with 25 μM curcumin. This curcumin mediated cleavage of Hsp90 was blocked by antioxidant, NAC (Fig. 4F). In order to see whether curcumin generated ROS specifically cleaves Hsp90, we also checked the expression of Hsp70 in curcumin treated HuT-78 cells. As shown in Fig. 4F, it is clear that curcumin has no effect on Hsp70 expression. Time kinetics experiment showed that Hsp90 cleavage started at 24 h time point with 25 μM curcumin (Fig. 4G). Hsp90 cleavage was inhibited by the addition of NAC or broad range caspase inhibitor, z-VAD-fmk with curcumin (Fig. 4H).

Targeted disruption of Hsp90 by curcumin induced oxidative stress, inhibited IKK α / β, NF-κB

It has been reported that disruption of Hsp90 leads to proteolytic cleavage of its client proteins (22). Thus, we checked the stability of Hsp90 client proteins IKK α and β in curcumin treated HuT-78 cells. Immunoblot analysis clearly showed that curcumin downregulated the expression of IKK-α and IKK-β in ROS dependent manner since pretreatment of cells with NAC abolished the effect of curcumin (Fig. 5A). Interestingly, dose dependent effect of curcumin was seen on IKK-α expression, whereas IKK-β expression was inhibited only at higher concentration of curcumin (25μM).

It is reported that curcumin inhibits constitutive NF-κB in CTCL cells (15). We speculated that inhibition of NF-κB activity may be due to IKK degradation in curcumin treated cells. To see the status of NF-κB, nuclear extracts were prepared from various concentrations of curcumin treated HuT-78 cells and subjected to EMSA. As shown in Fig. 5B, curcumin dose dependently inhibited NF-κB activity and significant inhibition was observed when 25 μM of curcumin was added. Curcumin mediated inhibition of constitutive NF-κB was found to be under the control of ROS as NAC pretreatment suppressed the inhibitory effect of curcumin. The specificity of the binding was examined by competition with unlabeled oligonucleotide (data not shown).

Next, we were interested to see whether Hsp90 is directly involved in NF-κB downregulation. For this, HuT-78 cells were treated with different concentrations of Hsp90 inhibitor, 17-AAG and nuclear extracts were prepared and analysed for DNA binding activity of NF-κB by EMSA. Interestingly, at 2.5 μM concentration, 17-AAG inhibits NF-κB DNA binding activity (Fig. 5C).

Hsp90 cleavage in curcumin treated HuT-78 cells disrupt the stability of Beclin-1

Recently, it has been shown that Hsp90 forms a complex with Beclin-1, a key protein involved in autophagy and thus maintain the stability of Beclin-1 (11). Since curcumin selectively cleaves Hsp90, therefore, we were interested to see the stability of Beclin-1 in curcumin treated HuT-78 cells. For this purpose, HuT-78 cells were treated with 25 μM curcumin for 24 h either in the presence or absence of NAC and thereafter, whole cell lysates were prepared and immunoblotting was done using anti Beclin-1 antibody. We observed that curcumin treatment resulted in the degradation of Beclin-1, which was inhibited by NAC (Fig. 6A). Time kinetics experiments clearly indicated that degradation of Beclin-1 occurred not before 24 h of curcumin

treatment (Fig. 6B). Similar result was obtained when similar experiment was performed in MyLa cells (Fig. 6C).

Beclin-1 plays pivotal role in autophagy formation (23). Down regulation of Beclin-1 in curcumin treated cells indicated that the autophagy formation may be inhibited in this cell. Conversion of autophagy specific marker LC3-I to LC3-II is an indicator for autophagy formation. We, therefore, monitored LC3-I to LC3-II conversion in curcumin (0-25 μM) treated HuT-78 and in MyLa cells. Accumulation of LC3-I was observed when higher concentration of curcumin (12.5 and 25 μM) was present which was inhibited by NAC (Fig. 6D). Rapamycin, a known inducer of autophagy was taken as positive control to induce autophagy and to check the conversion of LC3-I to LC3-II in HuT-78 cells. As expected, rapamycin treatment induced conversion of LC3-I to LC3-II and this conversion was inhibited in the presence of autophagy inhibitors, 3-MA and wortmannin, Wm (Fig. 6E).

Next, we studied the expression of autophagy related proteins Atg5 and Atg7. Curcumin treatment inhibited the expression of Atg7 but not Atg5. Curcumin failed to show its effect in the presence of NAC (Fig. 6F).

Discussion

Recent years have seen a growing trend in developing therapeutic agents from natural sources against various diseases. Curcumin, a yellow pigment from turmeric which shows a wide spectrum of biological function, has proved to be a promising candidate as an anticancer agent. Curcumin induces cell death in different forms of human cancer cells. Accumulating experimental data indicate that curcumin exerts its cytotoxic effect either by acting as an antioxidant or as a pro-oxidant (12). In this study, we have investigated whether curcumin treatment generates oxidative stress in HuT-78 cells and if so then which are the important cell survival mechanisms affected by curcumin generated oxidative stress.

We report here that curcumin through ROS dependent mechanism perturbs multiple cell signaling molecules and eventually induces apoptosis in HuT-78 cells. This conclusion is based on several crucial observations. First, time dependent accumulation of ROS was observed in curcumin treated cells and secondly, ROS scavenger, NAC extensively attenuated all curcumin induced effects such as activation of caspases, down regulation of antiapoptotic gene expressions, cleavage of Hsp90, inhibition DNA binding activity of NF-κB, including apoptosis in HuT-78 cells.

We have found that HuT-78 cells are much more vulnerable to oxidative stress than normal PBMC (data not shown). Induction of substantial apoptosis by curcumin in CTCL cells but not in PBMC from healthy donors, as reported recently by Zhang et al. seems to be due to the generation of excessive ROS in those cancer cells (15). Apoptosis is mediated by the activation of different effector caspases. According to their function and mode of activation caspases are classified as initiator caspases which include caspase-2, caspase-8, caspase-9 and executioner

caspases such as caspase-3, caspase-6 and caspase-7. Active caspases are generated in two distinct pathways the intrinsic or mitochondrial death pathway and the extrinsic or receptor mediated pathway (6, 24). We have found that curcumin through ROS generation activated caspase-8, caspase-2, caspase-9 as well as caspase-3.

Caspase-8 activation is a crucial step for the initiation of the extrinsic pathway on the other hand caspase-9 activation is essential for the execution of intrinsic pathway. The link between these two pathways is mediated by the truncated proapoptotic protein Bid (6). Caspase-2 like caspase-8 is also known to cleave Bid to t-Bid which alters mitochondrial membrane potential (25). We observed truncation of Bid, mitochondrial hyperpolarization, cytochrome *c* release, caspase-9 activation followed by caspase-3 activation and ultimately cleavage of PARP in curcumin treated HuT-78 cells. All these mechanisms are controlled by ROS since prior treatment with NAC completely blocked this cascade of events. Caspases mediated pathways could not account for the 80-90% apoptosis induced by curcumin in HuT-78 since, addition of broad range caspase inhibitor as well as caspase specific inhibitors (caspase-8 and caspase-3) only partially suppressed cell death induced by curcumin.

Many researches have demonstrated that curcumin influences multiple signaling pathways to exert its antiapoptotic effect on various cell types (12). Since, we found that the caspase cascade is minimally involved in curcumin mediated killing of HuT-78 cells, therefore, next we investigated the effect of curcumin on other cell survival molecules to find an explanation for curcumin mediated killing of HuT-78 cells. It is known that HuT-78 cells express high amount of antiapoptotic proteins like c-FLIP, Bcl-xL, cIAP, and XIAP. Bcl-xL, a member of Bcl2 family protein is known to promote cell survival and regulates MMP (26). We found that curcumin at high concentration reduces the expression of Bcl-xL which probably account for the alteration of

mitochondrial membrane polarization seen in curcumin treated cells. XIAP is known to regulate intracellular ROS by up-regulating the expression of antioxidative genes (27). Many cancer cells have been shown to produce high amount of XIAP (28). We observed the cleavage of XIAP in curcumin treated HuT-78 cells which probably account for the high level of ROS generation by curcumin in these cells.

Researches have shown that Hsp90 function is required for the activation of constitutive IKK which then activates constitutive NF-κB (10). We found that curcumin treatment results in degradation of IKK as a consequence down regulation of NF-κB takes place in HuT-78 cells in a ROS dependent manner. Earlier Zhang et al. reported that curcumin inhibits constitutive NF-κB in CTCL cells (15), but for the first time, we demonstrated the involvement of curcumin generated ROS in down regulation of constitutive NF-κB in HuT-78 cells.

Curcumin has been shown to induce autophagy in different cell types (29, 30). It is also known that the IKK complex contributes to the induction of autophagy (31). Moreover, like IKK, Beclin-1, the key autophagy promoting protein has been identified recently as a client protein of Hsp90 (11). We found the degradation of Beclin-1 when HuT-78 cells were treated with 25 μM curcumin. Time kinetics show that noteworthy down regulation of Beclin-1 occurs at 24 h time point, at the same time when Hsp90 function is inhibited by curcumin in a ROS dependent mechanism. Next, we investigated autophagy formation in curcumin treated HuT-78 cells by following the conversion of autophagy specific marker LC3-I to LC3-II. Accumulation of LC3-I rather than its conversion to LC3-II was visible at 25 μM concentration of curcumin. Down regulation of autophagy specific protein Atg7 occurs in curcumin treated HuT-78 cells. Taken

together, our results indicated that curcumin by disrupting Hsp90 also disrupts important cellular pathway, autophagosome formation in a ROS dependent manner.

Cell proliferation and cell survival regulated by a complex, interactive network of cell signaling pathways. Therefore, it may not be sufficient to control malignant cell growth simply by disrupting one or two cellular targets. Research have established that one common biochemical change in malignant cells is the increased production of ROS due to high metabolism (21). This fact is utilized to develop therapeutics against malignant cells. Curcumin successfully employs this strategy, induces oxidative stress in HuT-78 cells, perturbs important cell survival mechanisms, and thus achieves high degree of killing.

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Figure Legends

Figure 1. Curcumin generates reactive oxygen species (ROS) in HuT-78 and MyLa cells and induces cell death**.** A, chemical structure of curcumin. B, HuT-78 or MyLa cells were treated with 25 μM concentration of curcumin for indicated times and the level of $O_2^{-\cdot}$ was assayed by DHE staining using flow cytometer. Data represent the results from one of the three similar experiments. C, at similar time points as in B, H_2O_2 accumulation in supernatants was measured using Amplex Red hydrogen peroxide assay kit. Data represent mean values ± S.D. of three similar experiments. (*P < 0.05 vs. control). D, HuT-78 or MyLa cells were treated with curcumin (0-25 μM) in the presence and absence of NAC (10 mM) for 24 h and examined for cell viability by live PI exclusion method, where M1 represent % dead cells. Bar graph represents killing of HuT-78 and MyLa cells. Values are expressed as mean ± S.D. of three similar experiments. (*P < 0.05 vs. control). E, HuT-78 cells were treated similarly for 24 h and apoptosis was detected by TUNEL assay through flow cytometry at FL-1 channel. The M1 and M2 gates demarcate apoptotic and non-apoptotic populations respectively.

Figure 2. Curcumin induces caspase activation, alters mitochondrial membrane potential in a ROS dependent manner. A, HuT-78 cells were treated with curcumin (0-25 μM) for 24 h and caspase-8 activation was determined by colorimetric assay. B, caspase-2 activation was analysed after curcumin (0-25 μM) treatment for 24 h in the presence or absence of NAC (10 mM) or caspase specific inhibitors (20 μM each) by immunoblotting. C, cells were treated with curcumin for 24 h, Bid cleavage was examined by immunoblotting. D, cells were treated with curcumin (0-25 μM) for 24 h. The mitochondrial membrane potential changes were determined by $DiOC_6$

staining using flow cytometry at FL-1 channel. E, cytosolic fraction was used for analysis of cytochrome *c* by immunoblotting.

Figure 3. Curcumin causes caspase-9, 3 activation and PARP cleavage. A, HuT-78 cells were treated with different concentrations of curcumin (0-25 μM) for 24 h and caspase-9 activity was determined by cell based luminescence assay. Data represent mean values ± S.D. of three similar experiments. (**P* < 0.05 vs. control). B, Cells were treated with curcumin (0-25 μM) in the presence or absence of NAC (10 mM) or z-VAD-fmk (20 μM) for 24 h and caspase-3 activation was determined by colorimetric assay. C, PARP cleavage was detected by Western blotting. D, Cells were treated with 25 μM curcumin for 24 h in the presence or absence of NAC (10 mM) or different caspase inhibitors (20 μM each). Apoptosis was determined by annexin V staining through flow cytometry. The M1 and M2 gates demarcate non-apoptotic and apoptotic populations respectively. Data show mean values ± S.D. of three similar experiments. (**P* < 0.05 vs. control).

Figure 4. Curcumin mediated ROS generation down regulates antiapoptotic proteins. A-D, whole cell lysates prepared from HuT-78 after 24 h treatment with curcumin (0-25 μM) and immunoblotting was performed. A. c-FLIP$_{L/S}$, B. Bcl-xL, C. cIAP-1, 2 and D. XIAP. E, cells were treated with 25 μM curcumin in the presence or absence of different caspase inhibitors (20 μM each) for 24 h and XIAP expression was checked by immunoblotting. The arrow indicates full length and cleaved fragment of XIAP. F, similar experiment as in D, was performed to see the effect of curcumin on Hsp90 and Hsp70 expression by Western blotting. G, cells were treated with curcumin (25 μM) for different time points and Hsp90 expression was examined. H, cells were treated with 25 μM curcumin in the presence or absence of NAC (10 mM) or different

caspase inhibitors (20 μM each) and analyzed for Hsp90 expression by Western blotting. Arrow indicates full length and cleaved Hsp90.

Figure 5. Curcumin induced oxidative stress inhibits IKK α/β and NF-κB. A, HuT-78 cells were treated with curcumin (0-25 μM) for 24 h and cell lysates were used to analyse for the expression of IKK-α/β. B, cells were treated with different concentrations of curcumin (0-25 μM) for 12 h and nuclear extracts were prepared and assayed for NF-κB by EMSA. C, HuT-78 cells were treated with varying concentrations of Hsp90 inhibitor, 17-AAG for 12 h and NF-κB activation was seen in nuclear extracts by EMSA. FP represents free probe alone (no nuclear extracts).

Figure 6. Curcumin mediated oxidative stress degrades Beclin-1, accumulate LC3-I and inhibits Atg7. A, HuT-78 cells were treated with 25 μM curcumin in the presence or absence of NAC (10 mM) for 24 h and cell lysates were used to analyze Beclin-1 expression by immunoblotting. B and C, HuT-78 or MyLa cells were exposed with curcumin (25 μM) for different time points and Beclin-1 expression was checked by Western blotting. D, HuT-78 or MyLa cells were treated with curcumin (0-25 μM) in the presence or absence of NAC (10 mM) for 24 h and cell lysates were used to detect LC3 by Western blotting. E, HuT-78 cells were treated with different concentrations of rapamycin (25 and 40 ng/μL) and then cell lysates were prepared. Immnoblot analysis with antibodies against LC3 or actin was performed. 3-MA (10 mM) or Wm (200 ng/μL) was added 1 h before addition of rapamycin (40 ng/μL). The ratios of intensities of LC3-II and actin are indicated below each lane. F, cells were treated with curcumin (0-25 μM) for 24 h and cell lysates were prepared and analyzed Atg5 and Atg7 by immunoblot analysis.

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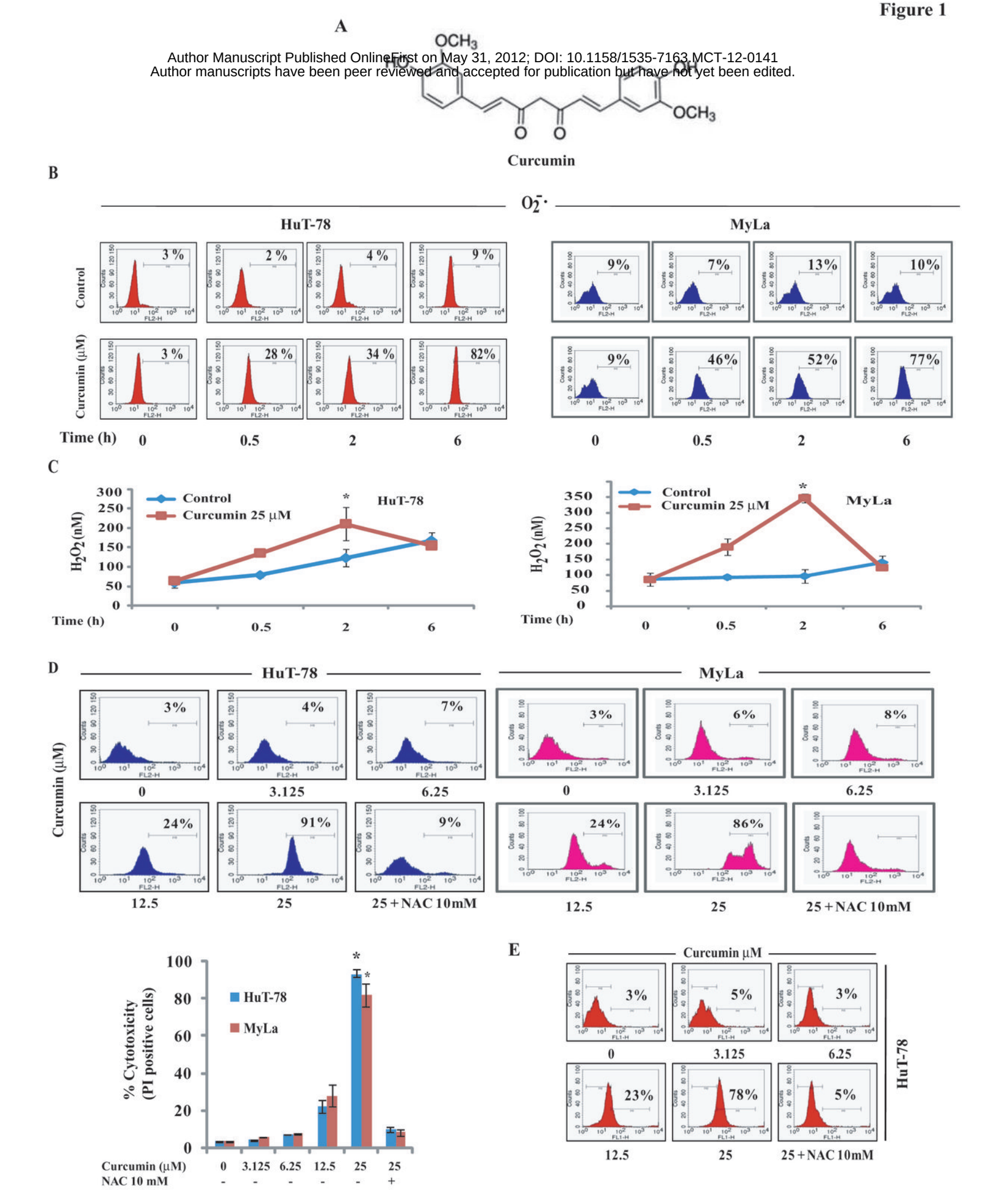
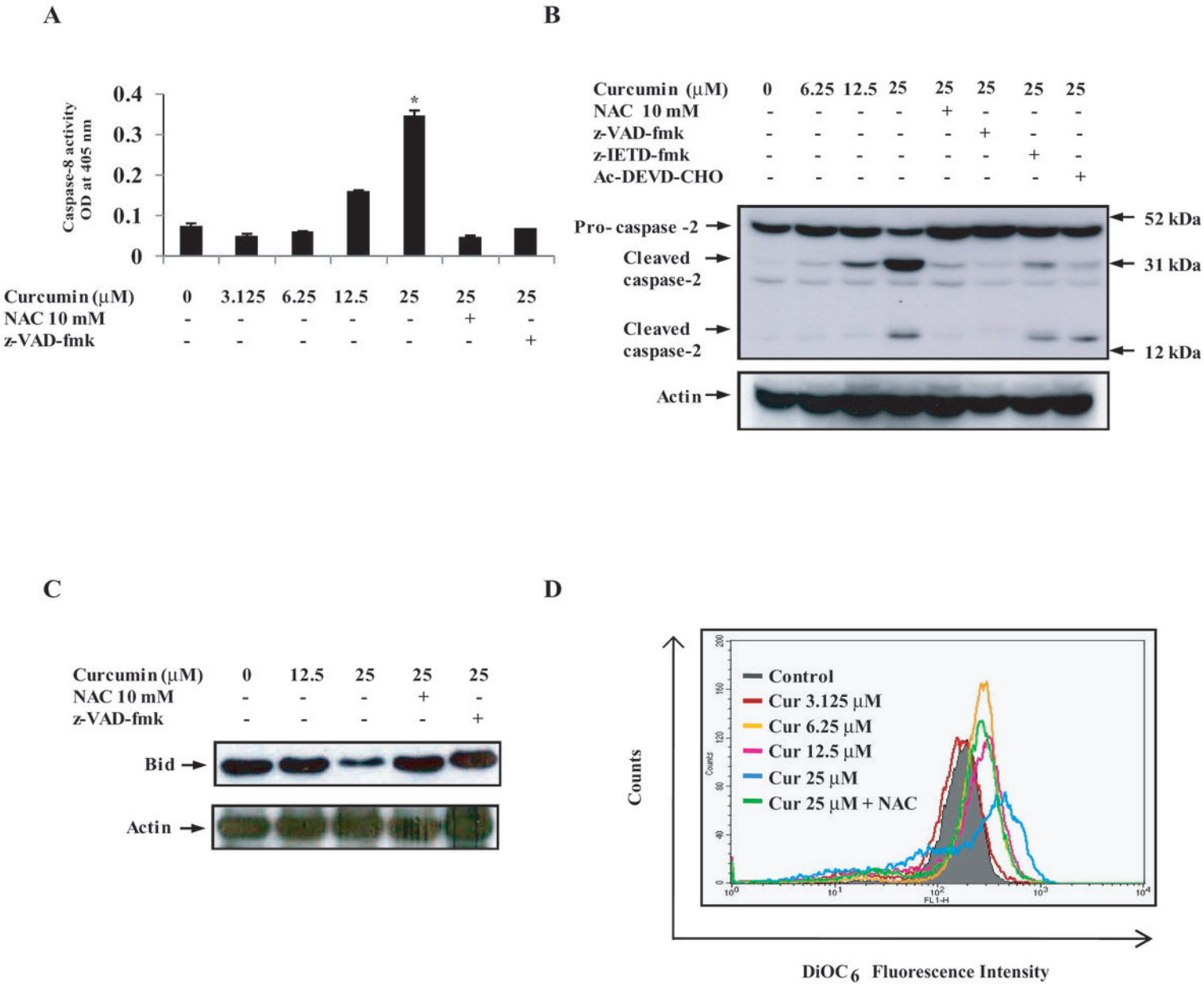


Figure 2



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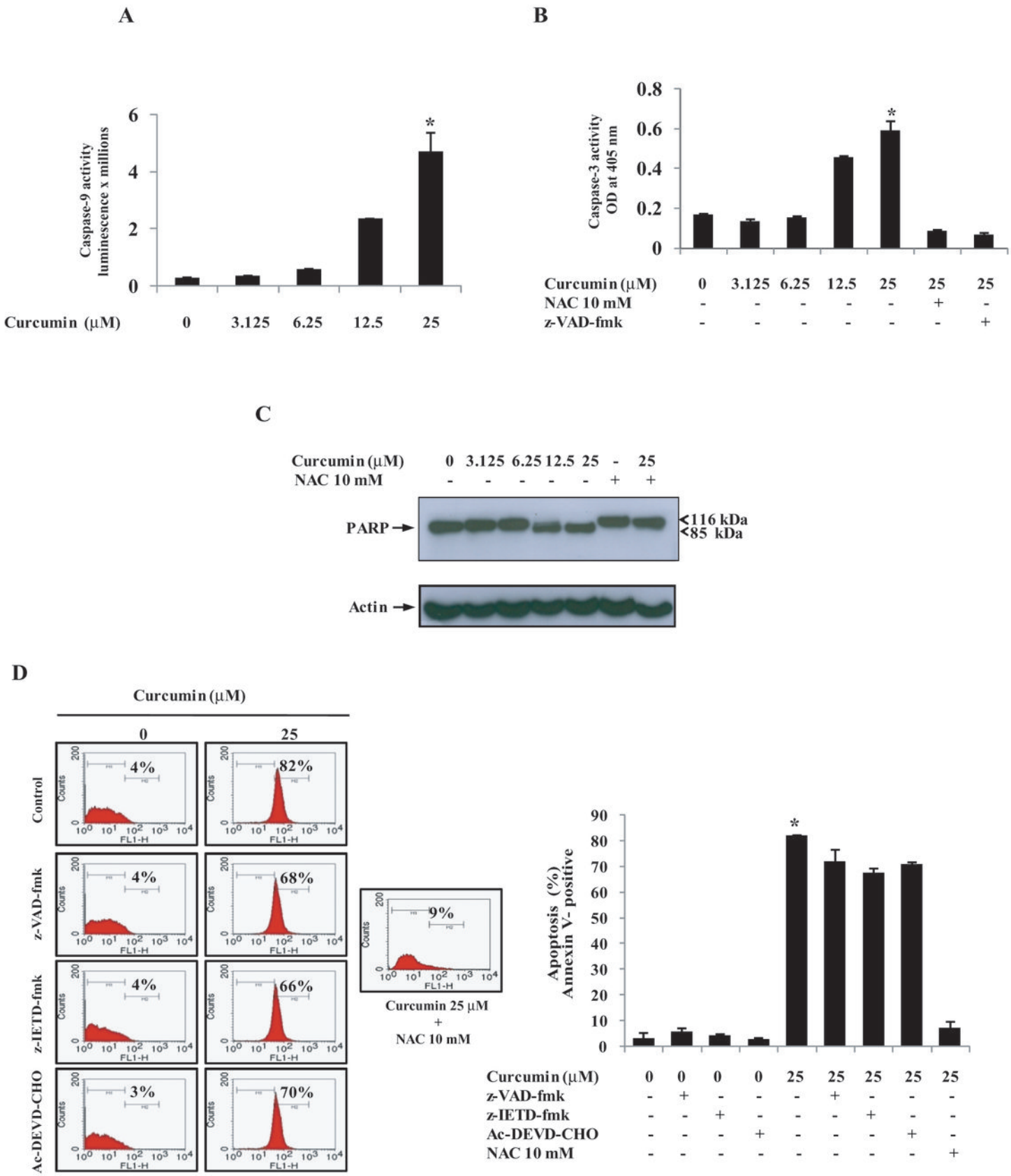

Figure 4

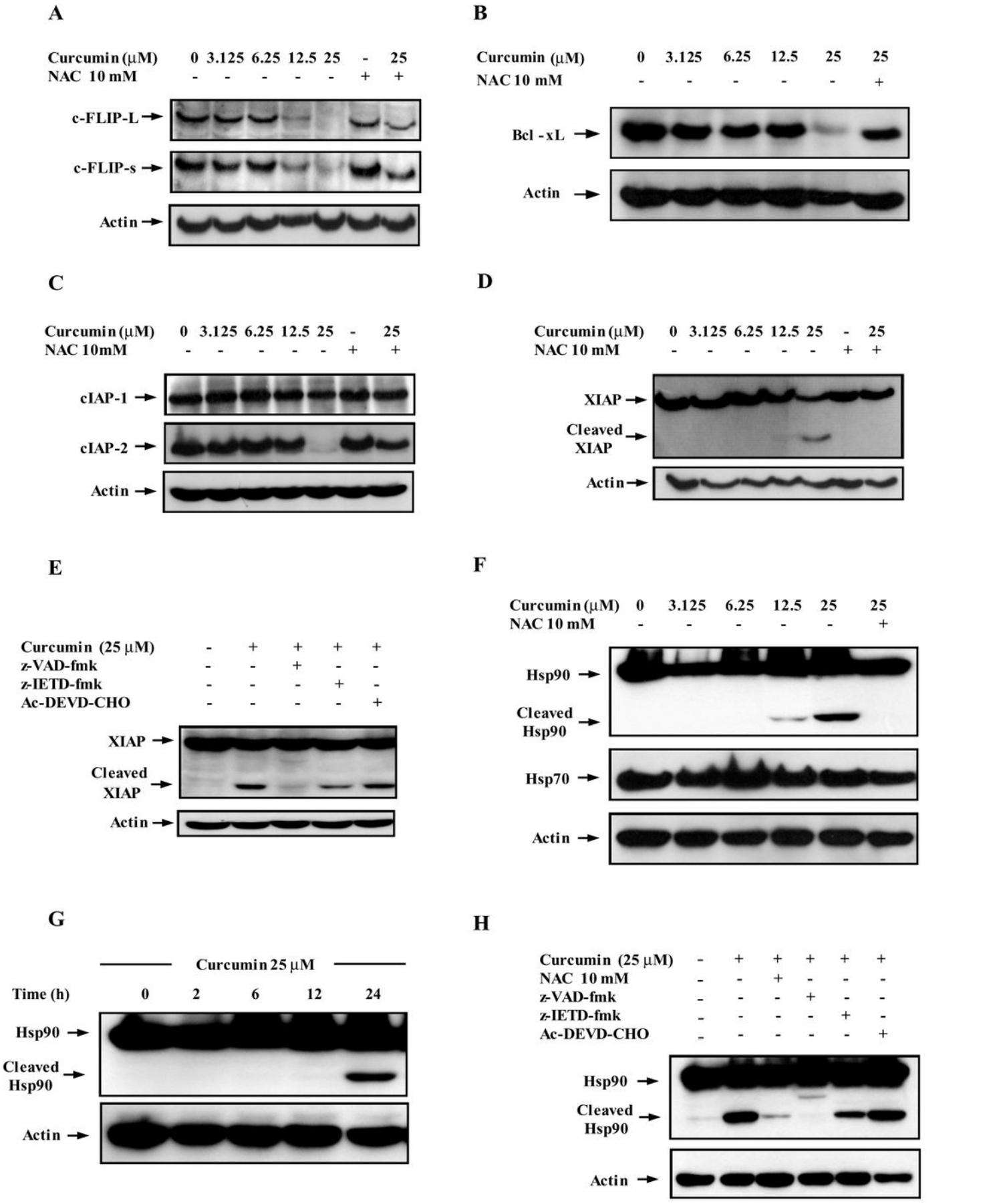

Figure 5

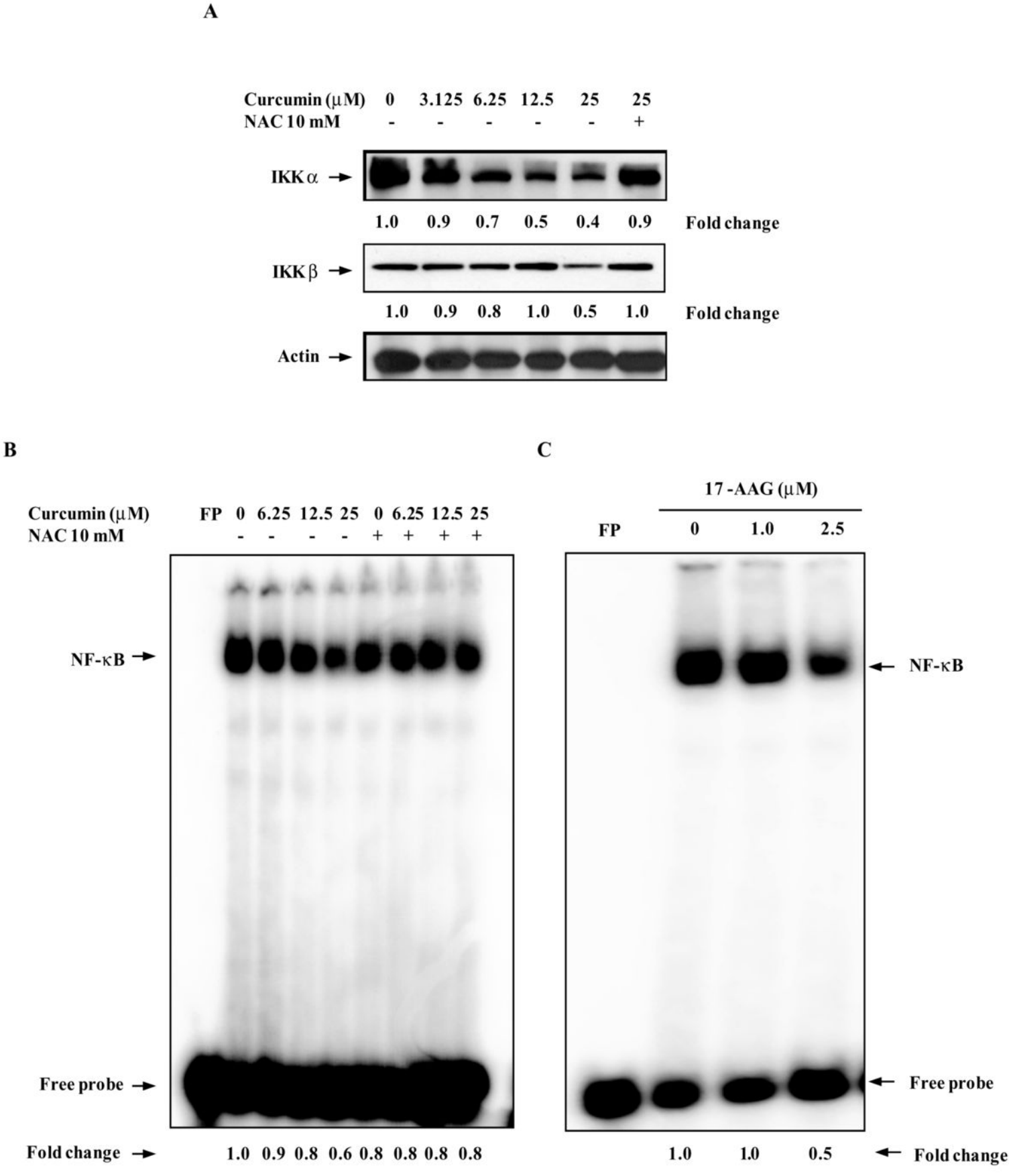

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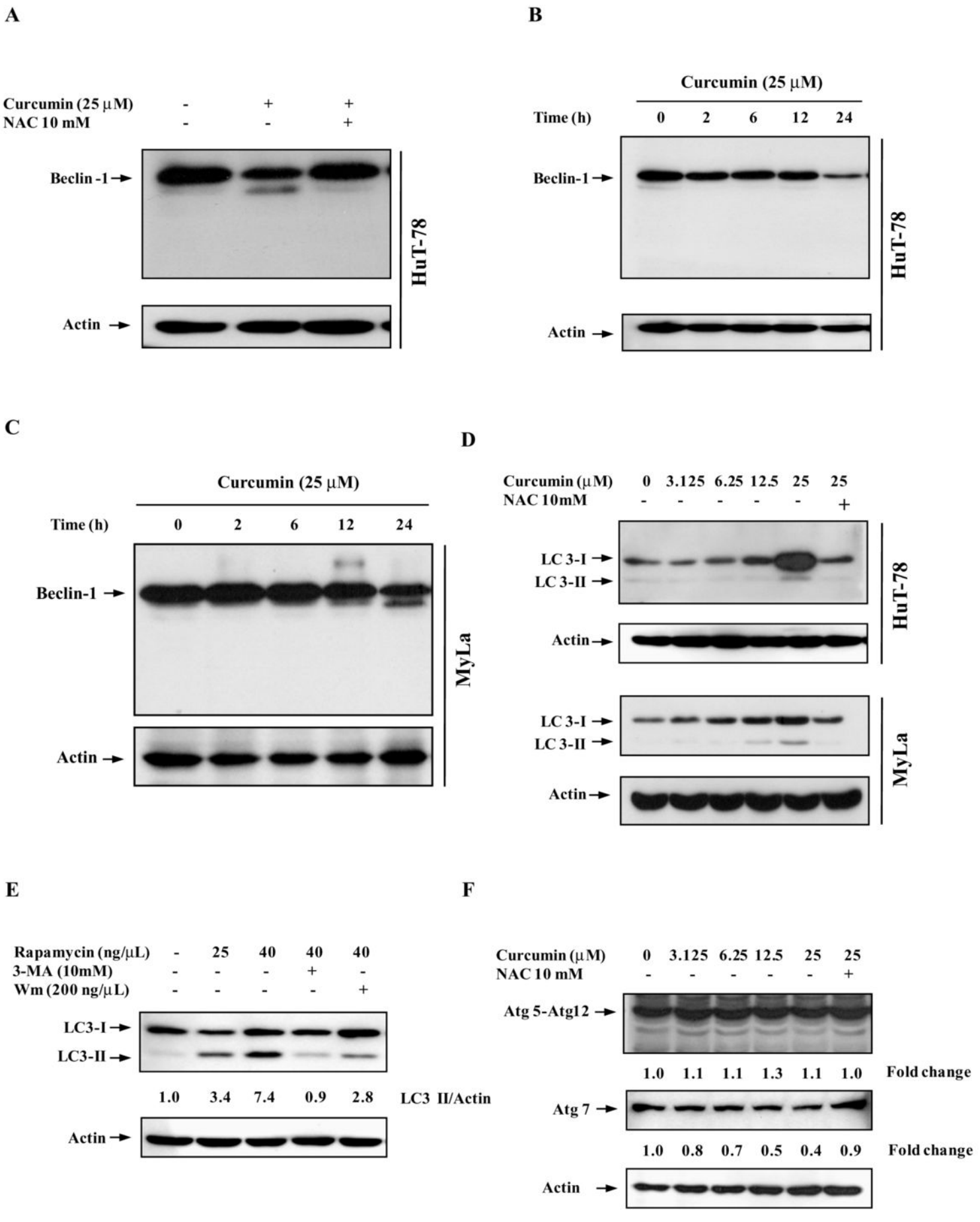
Figure 6



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Molecular Cancer Therapeutics

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