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Neem oil limonoids induces p53-independent apoptosis and autophagy

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***Azadirachta indica*, commonly known as neem, has a wide range of medicinal properties. Neem extracts and its purified products have been examined for induction of apoptosis in multiple cancer cell types; however, its underlying mechanisms remain undefined. We show that neem oil (i.e., neem), which contains majority of neem limonoids including azadirachtin, induced apoptotic and autophagic cell death. Gene silencing demonstrated that caspase cascade was initiated by the activation of caspase-9, whereas caspase-8 was also activated late during neem-induced apoptosis. Pretreatment of cancer cells with pan caspase inhibitor, z-VAD inhibited activities of both initiator caspases (e.g., caspase-8 and -9) and executioner caspase-3. Neem induced the release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria, suggesting the involvement of both caspase-dependent and AIF-mediated apoptosis. p21 deficiency caused an increase in caspase activities at lower doses of neem, whereas p53 deficiency did not modulate neem-induced caspase activation. Additionally, neem treatment resulted in the accumulation of LC3-II in cancer cells, suggesting the involvement of autophagy in neem-induced cancer cell death. Low doses of autophagy inhibitors (i.e., 3-methyladenine and LY294002) did not prevent accumulation of neem-induced LC3-II in cancer cells. Silencing of ATG5 or Beclin-1 further enhanced neem-induced cell death. Phosphoinositide 3-kinase (PI3K) or autophagy inhibitors increased neem-induced caspase-3 activation and inhibition of caspases enhanced neem-induced autophagy. Together, for the first time, we demonstrate that neem induces caspase-dependent and AIF-mediated apoptosis, and autophagy in cancer cells.**

Introduction

Plant-derived products are becoming increasingly popular as the source of potential anticancer agents due to the presence of various bioactive phytochemicals. *Azadirachta indica* (neem) is known for its various pharmacological properties in traditional Indian medicine (1). Vast array of biologically active compounds, especially, limonoids such as nimbolide and azadirachtin have been identified from the seeds of neem. Because the process of carcinogenesis is complex, anticancer agents possessing multiple mechanisms of action may be promising candidates for prevention and therapy of multiple types of cancer. Extracts and bioactive compounds isolated from neem show a wide range of activities affecting multiple targets and induce apoptotic cell death in cancer (2–4). Two widely known pathways, extrinsic and intrinsic are critical for apoptotic cell death. The extrinsic pathway is triggered via the formation of the death-inducing signaling complex and activation of caspase-8, which then cleaves executioner caspases. In the intrinsic pathway, the release of mitochondrial cytochrome *c* (Cyt. *c*) induces the activation of caspase-9 (5,6). In addition to apoptotic cell death, whether active components of neem induce other forms of cell death such as autophagy (7,8) has not been investigated.

Available evidences suggest that neem leaf extracts and isolated neem limonoids induce apoptosis through engagement of the mitochondrial pathway (4,9–11). For example, nimbolide and azadirachtin are antiproliferative and induce apoptosis through involvement of Bax/Bak and expression of caspase-3 (4,11,12). Ethanolic neem leaf extract enhanced the expression of proapoptotic genes, such as caspase-8 and -3, and suppressed the expression of Bcl-2 and mutant p53 in the 7,12-dimethylbenz(a)anthracene-induced cancer cells (3,13). Neem leaf extract activates caspase-3, -7, -8 and -9, thus suggesting a caspase-dependent apoptosis (2). These studies suggest that bioactive components of neem induce caspase-dependent apoptosis but the underlying molecular mechanism is still unknown. Additionally, whether caspase-independent apoptotic cell death and autophagy also play a role in neem-induced cell death remains undefined. Although the effects of purified neem limonoids on apoptosis have been studied individually (9), the synergistic effects of neem limonoids has not been fully investigated, which may contribute to efficient anticarcinogenic effects on cancer cells. In this study, we investigated the molecular mechanisms of cancer cell death in response to neem oil, which contains more than 50% of total neem limonoids including azadirachtin. We demonstrate that neem oil (i.e., neem) is potent anticancer agent due to its ability to target mitochondria in inducing caspase-dependent and apoptosis-inducing factor (AIF)-mediated apoptosis, and autophagic cell death.

Abbreviations: 3-MA, 3-methyladenine; AFC, 7-amino-4-trifluoromethyl-coumarin; AIF, apoptosis-inducing factor; Cyt. c, cytochrome *c*; LDH, lactate dehydrogenase; LY, LY294002; neem, neem oil; PI3K, phosphoinositide 3-kinase; TNF, tumor necrosis factor; TNFR-1, tumor necrosis factor receptor 1; z-VAD-fmk, z-VAD-fluoromethyl ketone.

Materials and methods

Cells and reagents

HCT116 cells (colon cancer) and its derivatives were kindly provided by Dr. B. Vogelstein (14,15) and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum. LNCaP and PPC1 cells (prostate cancer), MDA-MB231 breast cancer cells were obtained from the ATCC or from various investigators and were subcultured as described previously (16). The primary antibodies against Cyt. *c* (monoclonal antibody, mAb) and caspase-8 were purchased from BD Pharmingen. Cyt. *c* oxidase subunit II (Mito Sciences); heat shock protein 60 (Hsp60) (Millipore); AIF (Santa Cruz), caspase-3 (Rb pAb; Biomol); caspase-9, LC3, Beclin-1 and ATG5 (Cell Signaling Technology); lactate dehydrogenase (LDH) and actin (mAb; ICN) were obtained from the indicated suppliers. Secondary antibodies and enhanced chemiluminescence reagents were acquired from GE Healthcare. Alexa Fluor 488-conjugated goat anti-mouse was purchased from Molecular Probes. The fluorogenic caspase substrates DEVD-7-amino-4-trifluoromethyl-coumarin (AFC), LEHD-AFC, IETD-AFC and general caspase inhibitor z-VAD-fluoromethyl ketone were obtained from Enzo Life Sciences. Neem oil was kindly provided by Sabinsa Corporation. All other chemicals were purchased from Sigma Chemical Company unless specified otherwise.

Whole cell lysates preparation, subcellular fractionation and western blotting

Preparation of whole cell lysates, mitochondrial and cytosolic fractions and western blotting were performed as mentioned previously (17,18). For the purpose of whole-cell lysate preparation, cells were lysed in NP-40 buffer (50 mM HEPES-KOH, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 mM leupeptin, 1 μg/ml pepstatin A and 1 μg/ml chymostatin). To prepare mitochondrial and cytosolic fractions, cells were harvested, washed in ice-cold phosphate-buffered saline and then resuspended in homogenizing buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium ethylenediaminetetraacetic acid, 1 mM sodium ethyleneglycol-bis(2-aminoethylether)-tetraacetic acid and 1 mM dithiothreitol) containing 250 mM sucrose and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 mM leupeptin, 1 μg/ml pepstatin A and 1 μg/ml chymostatin). After 30-min incubation on ice, cells were homogenized using a glass Pyrex homogenizer (type B

pestle, 15–25 strokes) and centrifuged at 1000*g* for 5 min at 4°C to remove cell debris and intact cells. The resulting supernatant was centrifuged at 10 000 g for 20 min to obtain mitochondria-enriched preparation as pellet. Supernatant was further centrifuged at 100 000*g* for 1 h to obtain cytosolic fraction. Micro-BCA kit (Pierce, Rockford, IL) was used to determine protein concentration. Samples were loaded on sodium dodecyl sulfate–polyacrylamide gel for western blotting. After protein transfer, the membrane was probed/reprobed with various primary and corresponding secondary antibodies followed by immunodetection using enhanced chemiluminescence as previously described (17,18).

Quantification of apoptosis and caspase activities measurement

Trypan blue dye exclusion method was used to quantify both live and dead cells. DEVDase, LEHDase and IETDase activities were measured as described previously (17,18). Briefly, proteins were added to the caspase reaction mixture containing 30 µM fluorogenic peptide substrates, DEVD-AFC (for caspase-3) or LEHD-AFC (for caspase-9), IETD-AFC (for caspase-8), 50 mM HEPES, pH 7.4, 10% glycerol, 0.1% CHAPS, 100 mM NaC1, 1 mM ethylenediaminetetraacetic acid and 10 mM dithiothreitol, in a total volume of 100 µl and incubated at 37°C for 90 min. Production of AFC was monitored on spectrofluorimeter using excitation wavelength 400 nm and emission wavelength 505 nm. The results are presented as fold activation as compared with the controls.

Immunofluorescence

Cells grown on coverslips were treated with neem, and 15 min before the end of treatment, cells were incubated live with 4′,6-diamidino-2-phenylindole and Mito Tracker Orange to label both nuclei and mitochondria, respectively (16,17). Cells were then fixed, permeabilized and immunolabeled for Cyt. *c*. In brief, cells were fixed in 4% paraformaldehyde for 10 min followed by permeabilization in 1% Triton X-100 for 10 min. Cells were blocked in 10% goat whole serum for 30 min at 37°C and then incubated with monoclonal anti-Cyt. *c* antibody for 1 h at 37°C. Subsequently, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000) for 1 h at 37°C. After mounting and sealing, images were captured under fluorescence microscope.

Gene silencing using shRNA lentiviral particles

Green fluorescence protein-tagged short-hairpin RNAs (shRNAs) specific to caspase-9, caspase-8, ATG5, Beclin-1 and negative control shRNA were cloned into the pGIPZ (Open Biosystems) lentiviral vector to generate lentiviral particles. The shRNA sequences were: *caspase-8* (5′-GACTTCAGCAGAAATCTTT-3′), *caspase-9* (5′-CCAGGCAGCTG ATCATAGA-3′), *ATG5* (5′-ACCGTGGAATGGAATGAGATTA-3′) and *Beclin-1* (5′-CCGCTATATCAGGATGAGATAA-3′). Lentiviral particles specific for caspase-9, caspase-8, ATG5, Beclin-1 and control shRNAs were obtained from the Roswell Park Cancer Institute shRNA core resource and were directly utilized to infect cells at a multiplicity of infection of 5. After 48 h, puromycin (1 µg/ml) was added to the medium to select caspase-8, caspase-9, ATG5 or Beclin-1 knockdown cells (16,19).

Cell cycle analysis

HCT116 cells were cultured and treated with neem oil for indicated times. Cells were harvested and washed twice with phosphate-buffered saline. Cells were fixed with 70% ice-cold ethanol for 1 h at 4°C, washed with phosphate-buffered saline, then stained with 4′,6-diamidino-2-phenylindole in the presence of 0.02 µg/ml RNase A for 45 min in dark. Samples were analyzed on a BD LSR Fortessa flow cytometer and Modfit analysis software as described previously (20).

Statistical analysis

Data are presented as mean ± standard deviation of at least three independent experiments. Statistical analysis was performed by analysis of variance using GraphPad Prism. Significant changes (P <0.05 or 0.01) are represented by asterisk (*).

Results

Neem exposure causes increased cell death in multiple cancer cell types

Previous studies demonstrated that individual limonoids induces apoptotic cell death (9,11,12), however, combined effects of multiple limonoids on apoptosis or cell death is not clearly defined. We treated multiple cancer cell types with neem, which contains multiple limonoids including azadirachtin, to quantify cell death. For example, HCT116 cells were incubated with neem for 0, 12, 24 and 36 h. Cell viability was assessed using Trypan blue dye. Our results indicated that neem induced cell death in a time-dependent manner (Figure 1A). Similarly, neem induced cell death in LNCaP, PPC1 and MDA-MB231 cells as depicted in Figure 1B.

Neem induces caspase-dependent apoptotic and autophagic cell death

To determine whether neem-induced cell death is caspase-dependent, we measured caspase-3 activity (i.e., DEVDase activity) in HCT116, LNCaP, PPC1 and MDA-MB231 cells upon neem exposure for different times. As shown in Figure 1C (and data not shown), neem treatment to multiple types of cells resulted in time-dependent increase in caspase-3 activity.

To further explore whether neem induces caspase-dependent apoptotic cell death, we pretreated HCT116, LNCaP and MDA-MB231 cells with a pan caspase inhibitor, z-VAD-fmk followed by treatment with neem. To our surprise, we observed that neem-induced cell death was not inhibited by z-VAD-fmk (Figure 2A). In contrast to quantification of cell death, neem-induced caspase-3 activity was inhibited by z-VAD-fmk (Figure 2B–E). These findings suggest that in addition to apoptotic cell death, neem also activates alternative mechanism of cell death such as autophagy.

To determine whether neem induces autophagy, we treated HCT116 and LNCaP cells with neem and observed that neem treatment induced accumulation of LC3-II (Figure 2F, lanes 2 and 9), a marker for autophagy (21). Neem also induced expression of LC3-I (a precursor of LC3-II). It is interesting to note that the inhibition of neem-induced caspase-dependent apoptotic cell death by z-VAD-fmk caused increased accumulation of LC3-II (Figure 2F, lanes 3, 4, 10 and 11), suggesting that inhibition of caspase-dependent apoptosis by z-VAD-fmk enhances autophagy.

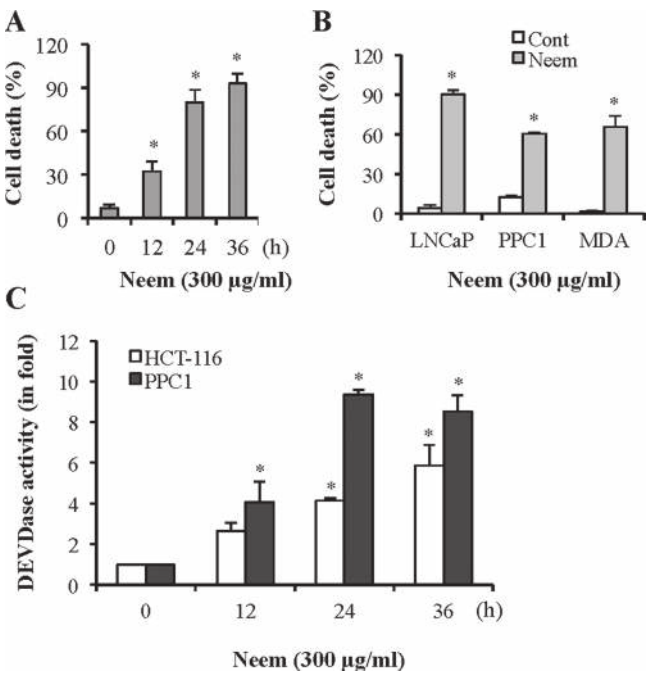


Fig. 1. Neem induces caspase-3 activation and cell death in multiple cancer cell types. (**A**) HCT116 cells were treated with neem for 0, 12, 24 and 36 h, and the percentage of cell death was determined. (**B**) LNCaP, PPC1 and MDA cells were treated with neem for 36 h and the percentage of cell death (mean ± SD) was determined. (**C**) HCT116 and PPC1 cells were treated with neem for 0, 12, 24 and 36 h and equal amounts of protein were subjected to caspase-3 activity measurements (i.e., DEVDase activity). Neem, neem oil; HCT-116, HCT116. Data are mean ± SD, $n = 3$; *$P < 0.01$.

Inhibition of PI3K/AKT pathway enhances autophagy or apoptosis

Because the activation of phosphoinositide 3-kinase (PI3K)/AKT pathway inhibits apoptosis and autophagy (22,23), we determined whether PI3K/AKT plays a role in neem-induced cell death. We pretreated HCT116 and LNCaP cells with LY294002 (PI3K inhibitor) and observed that neem-induced LC3 accumulation (Figure 2F, lanes 2 and 9) was slightly increased upon inhibition of PI3K/AKT signaling (Figure 2F, lanes 5 and 12), suggesting that neem-induced autophagy is not inhibited by LY294002 at the dose that we used.

To determine whether PI3K/AKT inhibition regulates caspase activation in the presence or absence of caspase inhibitor, we measured caspase-3 activity and performed western blotting for caspase-3 processing. As shown in Figure 2, LY294002 alone failed to induce caspase-3 activity (Figure 2B and 2D)/caspase-3 processing (Figure 2C, lanes 7 and 14). Neem-induced caspase-3 processing was slightly increased in HCT116 (Figure 2C, lane 5 versus 2), whereas in LNCaP cells, it was slightly decreased (Figure 2C, lane 12 versus 9). However, substrate cleavage assay did not show difference in HCT116, whereas in LNCaP cells, caspase-3 activity was increased. Additionally, neem-induced cancer cell death was not inhibited upon pretreatment with LY294002 (Figure 2A). These results demonstrate that inhibition of PI3K/AKT pathway increases neem-induced caspase-3 activity; however, the levels of neem-induced cell death do not seem to be modulated.

Neem induces intrinsic apoptotic cell death

To further elucidate the apoptotic cell death mechanism, we treated HCT116 and LNCaP cells with neem. LEHDase (caspase-9 activity) and IETDase (caspase-8 activity) assays as well as western blotting were performed. As shown in Figure 3, neem induced caspase-8 and -9 activities at 36 h. As expected, caspase-inhibitor z-VAD-fmk inhibited neem-induced caspase-8 and -9 activities. Neem-induced processing of caspase-8 and -9 was also inhibited by z-VAD-fmk. Similar to caspase-3 activity as shown in Figure 2, neem-induced caspase-8 and -9 activities were not inhibited by PI3K inhibitor LY294002 (Figure 3A–C).

To determine the underlying mechanism of apoptotic cell death, we first performed time-course analysis of caspase-9 and -8 activation

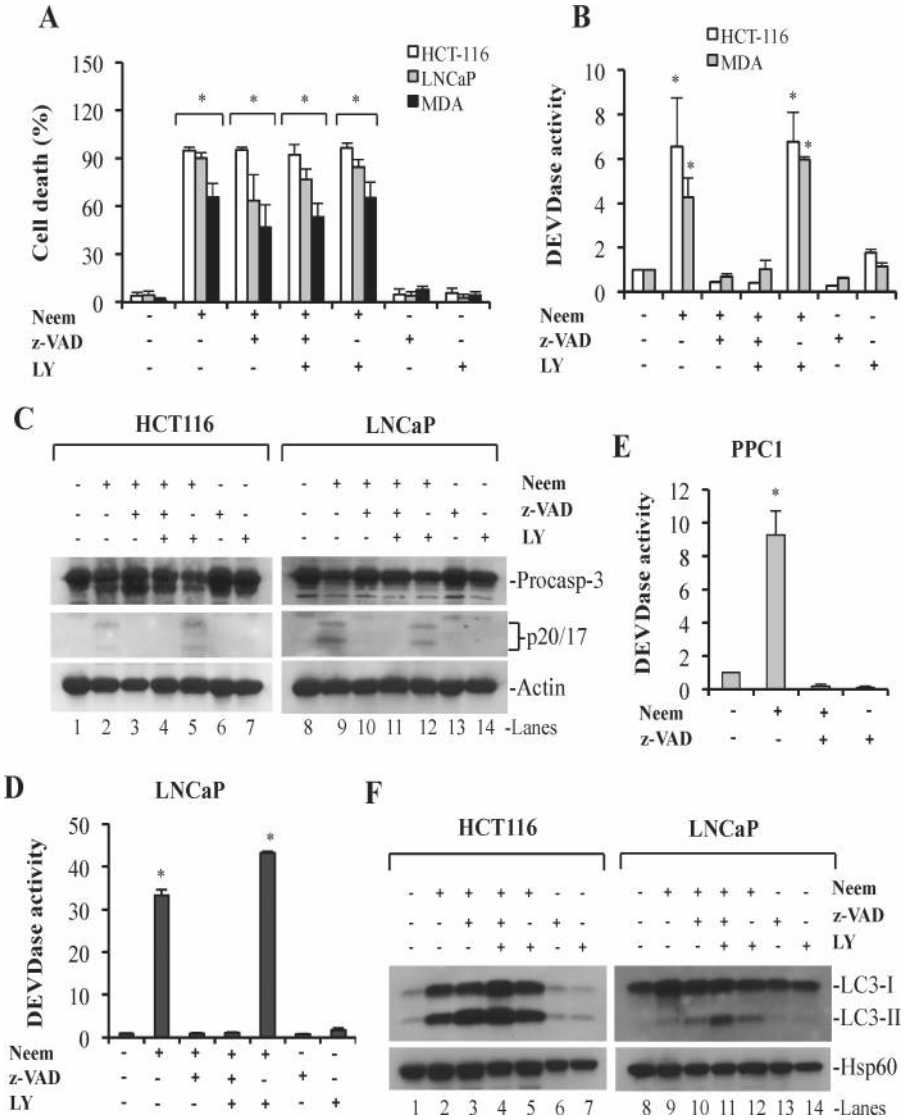


Fig. 2. Neem triggers caspase-dependent and caspase-independent apoptotic, and autophagic cell death. (**A**) HCT116, LNCaP and MDAMB-231 cells, (**B**) HCT116 and MDA-MB231 cells, (**C**) HCT116 and LNCaP cells, (**D**) LNCaP cells, (**E**) PPC1 cells, (**F**) HCT116 and LNCaP cells were pretreated with the pan-caspase inhibitor Z-VAD-fmk (50 μM) or/and LY294002 (10 μM), a potent inhibitor of phosphoinositide 3-kinases (PI3Ks) for 1 h followed by treatment with neem (300 μg/ml) for 36 h. At the end, cells were harvested and subjected to quantification of percentage of cell death, caspase-3 activity (i.e. DEVDase activity) measurements using equal amounts of proteins, and western blotting to detect indicated proteins in C and F. Neem, neem oil; MDA, MDA-MB231 cells; HCT-116, HCT116; LY, LY294002; z-VAD, z-VAD-fmk; Procasp-3, procaspase-3; p20/17, processed fragment of caspase-3. Data are mean ± SD, $n = 3$; $^*P < 0.01$.

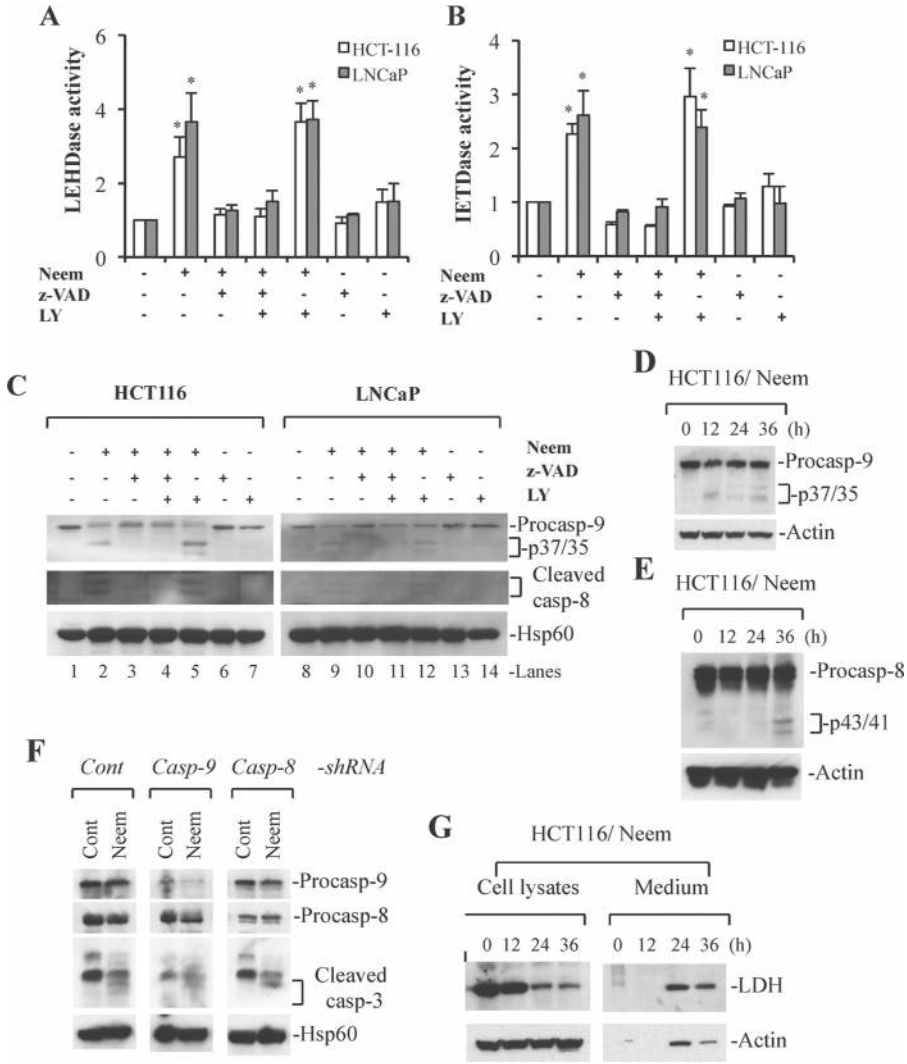


Fig. 3. Neem induces caspase-9-mediated apoptosis, and caspase-8 is also activated late during apoptosis. (**A–C**) HCT116 and LNCaP cells were pretreated with Z-VAD (50 μM) or/and LY294002 (10 μM) for 1 h followed by treatment with neem (300 μg/ml) for 36 h. At the end of treatment, equal amounts of protein were used for caspase-9 activity (i.e. LEHDase activity; A), caspase-8 activity (i.e. IETDase activity; B), and western blotting for indicated proteins (C). (**D** and **E**) HCT116 cells were treated with neem (300 μg/ml) for indicated times. Whole-cell lysates were subjected to western blotting for caspase-9 (D) or caspase-8 (E). (**F**) HCT116 cells were infected with *control* or *caspase-9* or *caspase-8 shRNA* lentiviral particles at multiplicity of infection of 5. Stable cells were treated with neem (300 μg/ml) for 24 h and equal amounts of protein were subjected to western blotting for caspase-3 and other indicated proteins. (**G**) HCT116 cells were treated with neem (300 μg/ml) for indicated times. Equal amounts of protein were subjected to western blotting to detect the release of LDH. HCT-116, HCT116; neem, neem oil. Data are mean ± SD, $n = 3$; $^*P < 0.01$.

and observed that neem induced early activation of caspase-9, whereas caspase-8 activation was observed at later time points (Figure 3D and 3E). These findings suggest that activation of caspase-9 is an initiating event in neem-induced apoptosis. To further confirm the importance of caspase-9 in neem-induced apoptosis, we silenced caspase-9 or -8 in HCT116 cells using shRNA approach (Figure 3F) and treated these cells with neem oil. We observed that silencing of caspase-9 showed reduced levels of caspase-3 activation, whereas silencing of caspase-8 did not alter caspase-3 activation (Figure 3F). Together, our results indicate the involvement of intrinsic pathway of apoptosis during neem-induced cancer cell death; however, caspase-8 could also be activated at later time points to amplify the caspase cascade.

Depending on energy status of cells, apoptotic cell death could also be accompanied by necrosis (24–26). We subjected whole-cell lysates and respective medium for detection of the cellular release of LDH, an indicator of necrosis (27). We observed that LDH was released into the medium late during neem-induced apoptosis (Figure 3G), suggesting that during apoptosis, lack of ATP may contribute to necrotic phenotype in cancer cells.

Neem treatment leads to Cyt. c release from mitochondria

Cyt. *c* release from mitochondria is critical for Apaf-1-dependent caspase activation (28) and the release of Cyt. *c* is also required for amplification of caspase-8 mediated apoptosis in epithelial cancer cells such as breast, prostate and colon cancer cells (29). To understand whether neem induces Cyt. *c* release in order to induce caspase-dependent apoptotic cell death, we purified cytosolic and mitochondrial fractions from unstimulated or neem-treated PPC1 cells followed by western blotting. As shown in Figure 4A, neem treatment induced Cyt. *c* release starting at 12 h in the cytosol of PPC1 cells with concomitant activation of caspase-3 as evidenced by the presence of processed caspase-3 fragments, p20/17. Cyt. *c* oxidase subunit II (a marker for mitochondria) was not detected in the cytosol. Similarly, LDH (a marker for cytosol) was absent in mitochondrial fractions, indicating that cytosolic fractions were not contaminated with mitochondrial proteins and vice versa.

To further demonstrate that neem induces Cyt. c release, we performed immunolabeling to detect Cyt. *c* release after neem treatment. In untreated PPC1 cells, typical mitochondria-like Cyt.

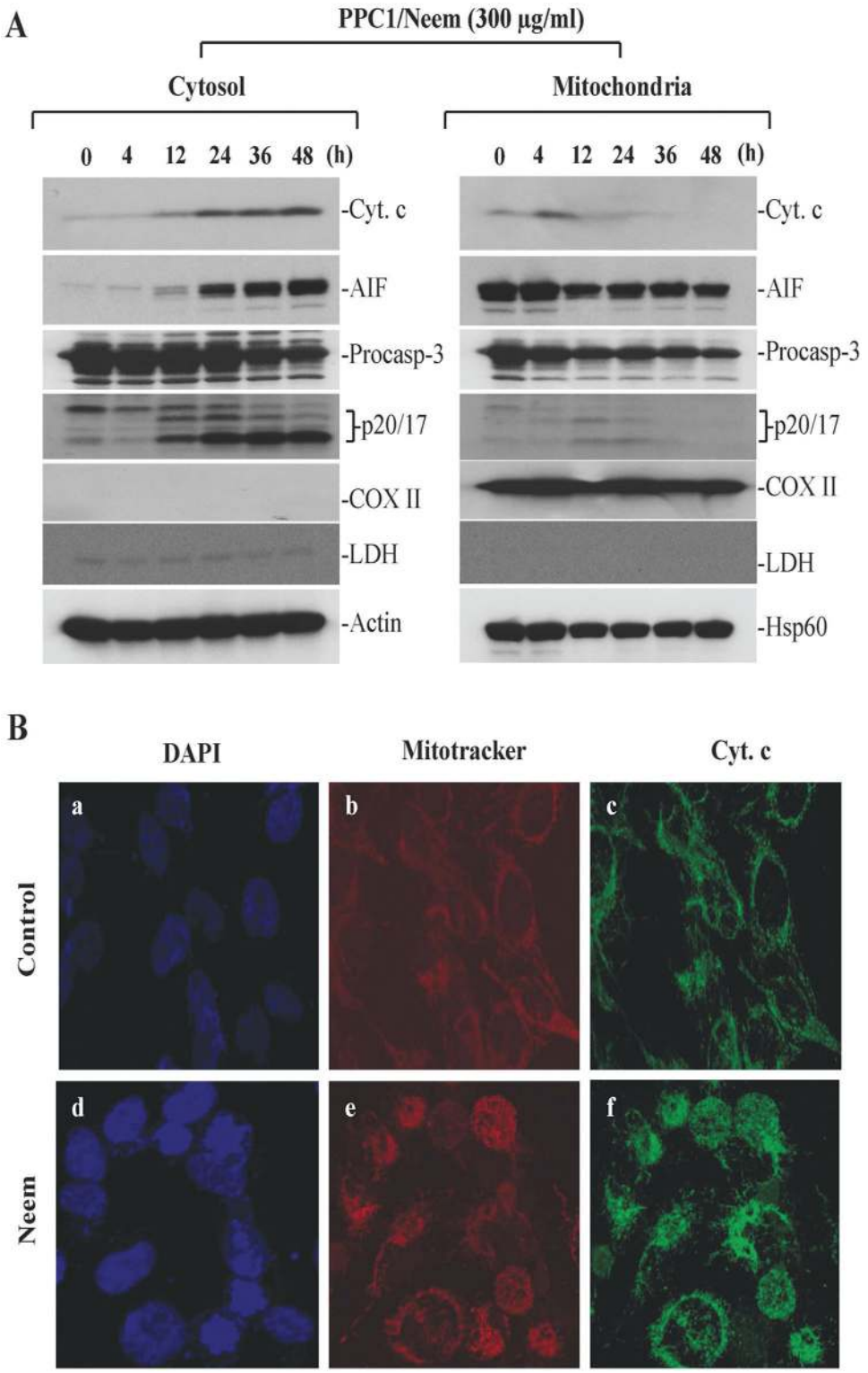


Fig. 4. Concomitant release of cytochrome *c* and AIF is accompanied by caspase activation upon neem exposure to cancer cells. (**A**) PPC1 cells were treated with neem (300 µg/ml) for the indicated times. At the end of treatment, cytosolic and mitochondrial fractions were isolated, and equal amounts of protein were subjected to western blotting for the detection of indicated proteins. *Cyt. c*, cytochrome *c*; *Procasp-3*, procaspase-3; *COX II*, cytochrome *c* oxidase subunit II; *LDH*, lactate dehydrogenase. p20/17, cleaved fragments of caspase-3. Actin or heat shock protein 60 (Hsp60) serve as loading controls. (**B**) PPC1 cells were treated with neem (300 µg/ml) for 24 h. At the end of the treatment, live cells were labeled with DAPI (panels a and d) and Mito Tracker Orange (panels b and e) to detect the nucleus and mitochondria, respectively. Cells were then immunolabeled for cytochrome c (Cyt. c; panels c and f). The diffuse staining for cytochrome c in individual cells reveals that it was released from mitochondria. DAPI, 4′,6-diamidino-2-phenylindole; neem, neem oil.

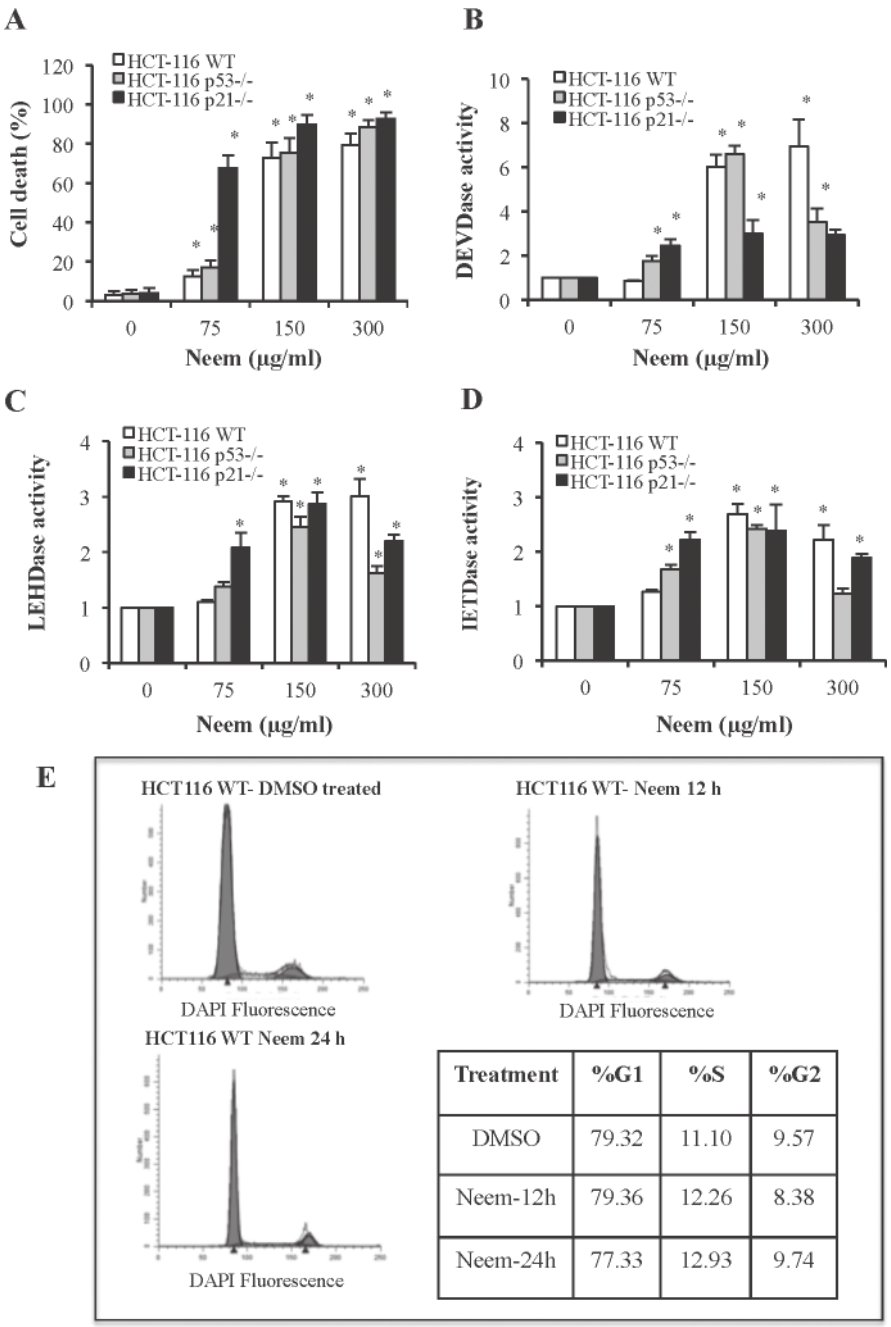
c labeling was observed. Neem treatment led to diffuse Cyt. *c* staining (Figure 4B, panel f), suggesting that Cyt. *c* was released from mitochondria. The released Cyt. c was corroborated with the fragmentation of the nucleus (Figure 4B, panel d). Altogether, these findings demonstrate that neem triggers Cyt. *c* release in cancer cells.

Neem treatment leads to increased accumulation of AIF in the cytosol

AIF is known to be involved in induction of apoptotic cell death via a caspase-independent mechanism (30). Mitochondrial AIF is released in response to death stimuli, and subsequently translocates into the nucleus causing nuclear condensation (31). We determined whether AIF plays a role in neem-induced apoptotic cell death by analyzing changes in the levels of AIF in mitochondrial and cytosolic fractions by western blot. As shown in Figure 4A, neem treatment caused increased accumulation of AIF in the cytosol with concomitant decrease from the mitochondrial compartment. These findings suggest that, similar to Cyt. c release, AIF release is also important for apoptotic cell death, which is evidenced by the lack of inhibition of neem-induced cell death upon z-VAD-fmk treatment (Figure 2).

Neem exerts cytotoxic effect irrespective of p53 status, whereas p21-deficiency promotes apoptosis

p53 promotes Cyt. c release and caspase activation through transcription-dependent and -independent mechanisms, whereas p21 seems to possess both pro-apoptotic and antiapoptotic functions (32–35). Neem treatment induced cell death in p53- or p21-deficient HCT116 cells similar to wild-type (WT) cells (Figure 5A). No significant differences between neem-treated HCT116-WT or -p53$^{-/-}$ cells with respect to cell viability implied that the cytotoxic action of neem was p53-independent. Additionally, we observed that HCT116-p21$^{-/-}$ cells were more sensitive as compared with HCT116-WT at lower doses of neem. We next investigated caspase-3, -9 and -8 activities in HCT116-WT, -p53$^{-/-}$ and -p21$^{-/-}$ cells after 24 h of neem treatment.



Treatment	%G1	%S	%G2
DMSO	79.32	11.10	9.57
Neem-12h	79.36	12.26	8.38
Neem-24h	77.33	12.93	9.74

Fig. 5. Neem induces p53-independent caspase activation and does not require cell cycle arrest. (**A**–**D**) HCT116-WT, -p53$^{-/-}$ and -p21$^{-/-}$ cells were treated with neem for 24 h with indicated concentrations and percentage cell death was determined. Equal amounts of protein were subjected to caspase-3, -8 and -9 activity measurements. (**E**) HCT116 cells were treated with neem (300 µg/ml) for 12 and 24 h. Subsequently, distribution of cells in G1, S and G2 phases was quantified by DAPI staining followed by flow cytometry. DAPI, 4′,6-diamidino-2-phenylindole; HCT-116, HCT116; neem, neem oil. Data are mean ± SD, $n = 3$; *$P < 0.01$.

We observed that neem-induced caspase activities were consistent with increased cell death in HCT116-WT and -p53$^{-/-}$ or -p21$^{-/-}$ cells (Figure 5B–D). Notably, lack of p21 caused increase in caspase activities at lower doses of neem (Figure 5B–D). These findings indicate that p53 does not seem to regulate neem-induced apoptosis, whereas lack of p21 further sensitizes cells to apoptotic cell death.

To demonstrate whether neem induces p21 activation leading to cell cycle arrest, we performed cell cycle analysis in HCT116 cells treated with neem. We observed that neem treatment did not alter cell cycle parameters. For example, the percentage of cells in S-phase was 11.10, 12.26 and 12.93 at 0, 12 and 24 h of neem exposure, respectively (Figure 5E).

Neem induces p53-independent autophagy

To understand whether p53 or p21 regulates neem-induced autophagy, we treated HCT116-WT, -p53$^{-/-}$ and -p21$^{-/-}$ cells with neem and determined the levels of LC3-II. As shown in Figure 6A, neem triggered autophagy in all three cell types as evidenced by accumulation of LC3-II. It is interesting to note that in HCT116-WT or -p53$^{-/-}$ cells both forms of LC3, i.e. LC3-I and -II, were equally detected, whereas in HCT116-p21$^{-/-}$ cells, only LC3-II was accumulated in the cells, suggesting that p21 deficiency further promotes neem-induced autophagy (Figure 6A). These findings suggest that p53 does not seem to play critical role in neem-induced autophagy, whereas p21-deficiency enhances autophagy.

To investigate whether inhibition of autophagy affects neem-induced cell death, we pretreated HCT116-WT cells with 3-methyladenine (3-MA), which is an inhibitor of class III PI3K and blocks autophagy. We observed that neem-induced LC3-II accumulation was slightly increased in the presence of 5 mM of 3-MA (commonly used dose; Figure 6B). However, higher concentration of 3-MA (i.e., 20 mM) inhibited the accumulation of LC3 (data not shown). Neem-induced

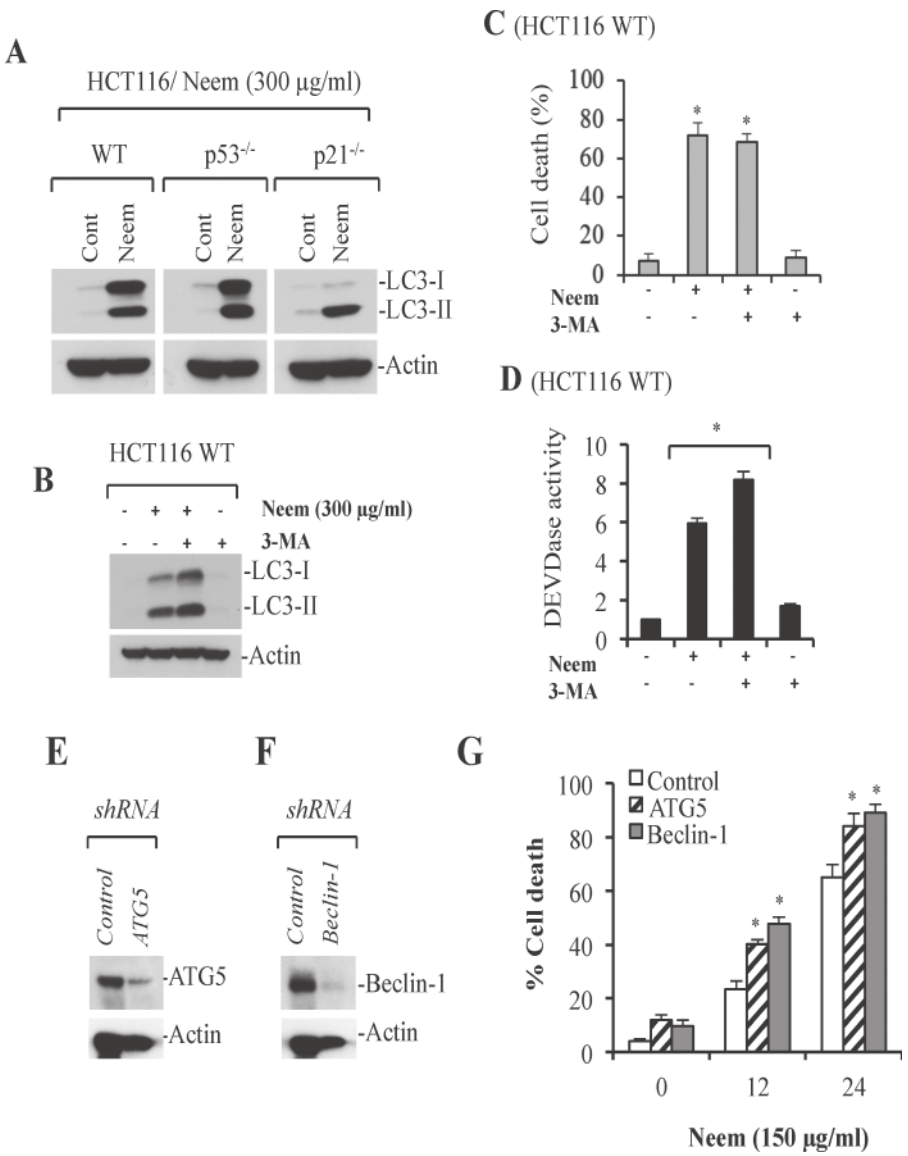


Fig. 6. Neem induces p53-independent autophagy. (**A**) HCT116-WT, -p53$^{-/-}$ and -p21$^{-/-}$ cells were treated with neem (300 µg/ml) for 24 h. Equal amounts of protein were used for western blotting to detect LC3-I and LC3-II (A). Actin serves as loading controls. (**B–D**) HCT116-WT cells were pretreated with autophagy inhibitor, 3-MA (5 mM) followed by neem treatment (300 µg/ml) for 20 h. At the end of treatment, percentage cell death was determined and equal amounts of protein were used for western blotting and caspase-3 activity measurements. (**E** and **F**) HCT116 cells were infected with *control* or *ATG5* or *Beclin-1 shRNA* lentiviral particles at multiplicity of infection of 5. Stable cells were subjected to western blotting to detect indicated proteins. Actin serves as loading controls. (**G**) ATG5 or Beclin-1-silenced or control (non-targeting) cells were treated with neem (150 µg/ml) for 12 and 24 h. At the end of treatment, percentage cell death was determined. Neem, neem oil; 3-MA, 3-methyladenine. Data are mean ± SD, $n = 3$; *$P < 0.05$.

cell death was not affected by treatment with autophagy inhibitor (3-MA) at 5 mM dose, whereas higher doses of 3-MA further enhanced neem-induced cell death (Figure 6C; data not shown). Interestingly, autophagy inhibition enhanced neem-induced caspase-3 activity (Figure 6D), which may compensate cell death activity related to autophagy. To parse the involvement of autophagy-related proteins, we silenced ATG5 and Beclin-1 in HCT116-WT cells (Figure 6E and 6F). These cells were treated with neem followed by quantification of cell death after 12 and 24 h. We observed that ATG5 or Beclin-1 silencing further enhanced neem-induced cell death as compared with control shRNA cells (Figure 6G).

Discussion

Neem limonoids or neem extracts induce cell cycle arrest and apoptosis in many types of cancer cells, thus neem limonoids could be possible anticancer agents (11,12,36–38). However, the underlying molecular mechanism of neem limonoids-induced cell death is not defined. Additionally, whether individual neem limonoids or combination of multiple limonoids could be effective anticancer agents is not known. Neem oil, which contains multiple neem limonoids, prevents mutagenic effects of 7,12-dimethylbenz(a)anthracene (11) indicating that neem plays an important role in the prevention of cancer. Therefore, how combined exposure of neem limonoids induce cell death has significance in prevention and therapy of cancer. This study, for the first time, demonstrates that, neem oil, which contains more that 50% of total neem limonoids, induces efficient cell death by multiple cell-death mechanisms. We observed that mitochondria are the signaling center for apoptotic cell death. Inhibition of caspase-dependent apoptosis enhanced autophagy, and caspase activation was also enhanced by inhibition of autophagy. Concomitant release of AIF and Cyt. c suggests that caspase-independent apoptosis is parallel to caspase-dependent apoptosis. An early activation of caspase-9 suggests that intrinsic cell death mechanism plays a critical role in neem-induced apoptosis. Inhibition of PI3K/AKT pathway by LY294002 or 3-MA enhanced neem-induced caspase activation. Neem-induced Cyt. c release from mitochondria triggered caspase activated cell death. Deficiency of p53 did not modulate caspase activation, whereas absence of p21 resulted in slightly increased caspase activation and autophagy.

The caspase cascade was initiated by the activation of caspase-9, suggesting that neem activates intrinsic apoptotic cell death. However, caspase-8 was also activated at later time points, indicating that later during apoptosis either caspase-8 could be activated via death-receptor mechanism and/or by activated caspase-3. The mechanism of neem-induced apoptosis is consistent with apoptosis induction in response to neem leaf extract or purified limonoids (2,4,9,36,39). One of the limonoids in neem, azadirachtin, interacts with tumor necrosis factor (TNF) binding domain of TNF receptor 1 (TNFR1), and thus, suppresses NF-κB signaling (10). Because activation of TNFR1 upon ligation with TNF-α triggers death-inducing signaling complex assembly leading to the activation of caspase-8 (40), presence of azadirachtin in neem may inhibit death-receptor signaling. Our findings suggest that the presence of other limonoids in neem might have activated caspase-8 at later time points. Therefore, the combined effect of multiple limonoids may activate multiple caspase signaling leading to efficient apoptotic cell death in cancer cells.

Whether neem limonoids induces caspase-independent cell death is not clearly elaborated. Although azadirachtin induces depolymerization of actin, and subsequently leads to caspase-independent apoptosis in *Drosophila* model system (41), such study has not been reported in mammalian or cancer cells. We observed that caspase-inhibitor z-VAD-fmk inhibited caspase-9, -8 and -3 activities but neem-induced cell death was not inhibited, suggesting that cancer cells may undergo cell death also by caspase-independent mechanism. Indeed, inhibition of autophagy and caspase-dependent cell death did not prevent neem-induced cell death. The release of AIF from mitochondria may trigger caspase-independent cell death. Overall, these findings imply that caspase-independent cell death may also play an important role in neem-induced apoptotic cell death.

Does neem induces autophagy? Inhibition of caspase cascade by z-VAD-fmk failed to inhibit neem-induced cell death, suggesting that in addition to apoptotic cell death, neem induces autophagy. Indeed, we observed accumulation of LC3-II, a marker for autophagy, upon neem exposure. Inhibition of PI3K/AKT signaling by LY294002 slightly enhanced accumulation of LC3-II, suggesting that neem-induced autophagy is slightly increased by inhibition of PI3K/AKT pathway. These findings were further supported by slight increased accumulation of LC3-II in the presence of commonly used concentration of another PI3K inhibitor, 3-MA. Apoptosis or autophagy pathways are interlinked and could influence the outcome of neem-induced caspase activation and LC3-II accumulation (42–44). We observed that inhibition of caspase cascade increased accumulation of LC3-II, suggesting that increased autophagy in the absence of caspase activation contributes to cancer cell death. Interestingly, silencing of ATG5 and Beclin-1 enhanced the neem-induced cell death, suggesting that neem-induced autophagy may also have prosurvival role. Thus, inhibition of autophagy by ATG5 or Beclin-1 silencing may lead to increased levels of apoptotic cell death. Although p53 regulates autophagic cell death (45), p53-deficiency did not inhibit or modify LC3-II accumulation in neem-induced cancer cells. In contrast to p53, deficiency of p21 caused accumulation of mostly LC3-II, whereas in HCT116 WT and $p53^{-/-}$ cells, increased expression of both LC3-I and LC3-II was observed upon neem exposure. These findings suggest that, in our system, neem-induced autophagy is not modulated by p53, whereas absence of p21 may enhance autophagy.

Development of multidrug resistance has been associated with the absence of p53 function in many types of cancer (46), which may be associated with mitochondria dysfunction (47). This situation has impelled the search for agents that cause cancer cell death via mechanisms independent of p53. We observed that neem exposure to cancer cells caused equal amounts of caspase-9, -8 and -3 activation irrespective of p53 status. These results are further supported by similar cytotoxicity in HCT116-WT and -$p53^{-/-}$ cells. Increased expression of p53 and p21 was observed upon azadirachtin or nimbolide exposure in the hamster buccal pouch carcinogenesis model (11). Decreased levels of mutant p53 were also observed after administration of ethanolic neem leaf extract to the 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis model (3). Our findings are based on exposure of multiple limonoids, which support p53-independent action of neem. HCT116-$p21^{-/-}$ cells were more sensitive to neem as >60% cell death occurs at 24 h as compared with the HCT116 WT (around 12% cell death) with 75 µg/ml of neem. Earlier reports have also suggested that HCT116-$p21^{-/-}$ cells are more sensitive to anticancer agents or therapy (48,49). This study shows that the presence or absence of p53 does not alter the susceptibility of HCT116 human colon cancer cells to neem-induced apoptosis.

Together, our findings suggest that combined exposure of neem limonoids directly targets mitochondria leading to the release of proapoptotic proteins such as Cyt. c and AIF, which induce caspase-dependent and caspase-independent apoptotic cell death, respectively. Additionally, neem also induces autophagy, suggesting that combined treatment of limonoids may be a new strategy to induce mitochondria damage leading induction of apoptosis and/or autophagy. Because no clinical trials/data are available on neem related to cancer treatment, the doses and duration of neem need to be investigated for anticancer activities *in vivo*. Some studies on animal have used azadirachtin and nimbolide upto 100 µg/kg body weight to test anticancer activity (11), and further studies are needed to establish the importance of neem limonoids in cancer prevention and treatment. Because neem-induced cell death does not depend upon p53 status, multiple cancers irrespective of p53 status could be targeted for efficient cancer therapy.

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