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Mechanism of neem limonoids-induced cell death in cancer: role of oxidative phosphorylation

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Abstract

We have previously reported that neem limonoids (neem) induce multiple cancer cell death pathways. Here we dissect the underlying mechanisms of neem-induced apoptotic cell death in cancer. We observed that neem-induced caspase activation does not require Bax/Bak channel-mediated mitochondrial outer membrane permeabilization, permeability transition pore, and mitochondrial fragmentation. Neem enhanced mitochondrial DNA and mitochondrial biomass. While oxidative phosphorylation (OXPHOS) Complex-I activity was decreased, the activities of other OXPHOS complexes including Complex-II and -IV were unaltered. Increased reactive oxygen species (ROS) levels were associated with an increase in mitochondrial biomass and apoptosis upon neem exposure. Complex-I deficiency due to the loss of *Ndufa1*-encoded MWFE protein inhibited neem-induced caspase activation and apoptosis, but cell death induction was enhanced. Complex II-deficiency due to the loss of succinate dehydrogenase complex subunit C (SDHC) robustly decreased caspase activation, apoptosis, and cell death. Additionally, the ablation of Complexes-I, -III, -IV, and -V together did not inhibit caspase activation. Together, we

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

NY, SK, and DC study design and paper writing. NY, SK, RK, PS, LS, PR, TM, AS, JI, JO, RL, RG, AC, and DC data acquisition and analysis. NY, material support and data interpretation.

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demonstrate that neem limonoids target OXPHOS system to induce cancer cell death, which does not require upregulation or activation of proapoptotic Bcl-2 family proteins.

Keywords

Neem; apoptosis; oxidative phosphorylation complex; ROS; mitochondrial DNA; necroptosis

INTRODUCTION

Treatment of metastatic cancer with current anticancer agents associates with severe toxicity to patients [1, 2]. Therefore, there is a need for anticancer agents that harbor minimal or reduced toxicity to patients. We have demonstrated that limonoids, the bioactive components derived from *Azadirachta indica* (neem) known for its various pharmacological properties in traditional Indian medicine [3], induce cell death in various types of cancer cells [4]. Neem limonoids target various signaling pathways to induce apoptotic cell death and thus exert anticancer effects in multiple types of cancer cells [5–7]. However, the role of different organelles especially the mitochondrion in neem limonoids-induced apoptotic cell death is not yet defined.

Mitochondria play central role in apoptotic, autophagic, and necroptotic cell death. There is significant crosstalk among these three major types of cell death pathways in cancer [8]. In apoptosis, an extrinsic pathway is activated by death receptor ligation causing caspase-8 initiated signaling. Inhibition of caspase-8 signaling may also activate necroptosis, suggesting the role of caspase-8 in apoptosis and necroptosis. In the mitochondrial intrinsic pathway, the released cytochrome *c* from mitochondria induces the formation of apoptosome, which initiates caspase cascade [9, 10]. Apoptotic stimuli and bioactive compounds including the plant-derived ones induce mitochondrial membrane permeabilization involving proapoptotic members of the Bcl-2 family proteins such as Bax and Bak [11, 12]. Whether permeabilization of mitochondria in a Bax/Bak-dependent mechanism is primarily responsible for the release of proapoptotic proteins in response to bioactive components from neem is still not defined.

Effects of neem and its purified components such as neem limonoids may induce activation of proapoptotic proteins including Bax and Bak for permeabilization of the mitochondrial membrane [7, 13–15]. Neem also suppresses the expression of Bcl-2 and mutant p53 in the 7, 12-dimethylbenz(a)anthracene (DMBA)-induced cancer cells [6, 16]. Although neem components target various signaling pathways to induce apoptosis [7, 13–15, 17], the molecular mechanisms culminating into apoptotic cell death in response to neem components are not defined. Neem oil contains majority of neem limonoids including azadirachtin and nimbolide, which may lead to efficient cancer cell death induction [7, 17–19]. Therefore, we dissected the role of neem limonoids on cell death pathways and their relation with oxidative phosphorylation (OXPHOS) system towards exploiting them for cancer therapy. Here, we demonstrate that neem limonoids target OXPHOS system and mitochondrial DNA (mtDNA) to induce non-classical mitochondria-dependent apoptotic cell death in multiple cancer types.

EXPERIMENTAL PROCEDURES

Cells and reagents

HCT116 cells (colon cancer) and its derivatives were kindly provided by Dr. B. Vogelstein [20, 21] and cultured in McCoy's 5A medium supplemented with 10% FBS. HT29 colon cancer cells, MDA-MB231 breast cancer cell, LNCaP, Du145 and E006AA prostate cancer cells were obtained from ATCC or collaborators and cultured in recommended growth medium. All human cell lines were authenticated using the STR DNA profiling every 6 months. Chinese hamster lung fibroblasts CCL16-B1 (B1), CCL16-B2 (B2), CCL16-B9 (B9), V79-G3 (G3), and V79-G7 (G7) were gift from Dr. I. E. Scheffler, University of California San Diego [22–26]. B2 cells lack Complex I due to null mutations in *Ndufa1*. B9 cells lack succinate dehydrogenase complex subunit C (SDHC) causing Complex II deficiency. G7 cells are impaired in initiation of the mitochondrial protein synthesis, and therefore, lack mtDNA-encoded subunits of Complexes I, III, IV, and V. B1 and G3 cells are wild types for B-series and G-series mutants, respectively. The primary antibodies against cytochrome c, Bid and caspase-8 were purchased from BD Pharmingen (San Jose, CA, USA). Bax N terminus and Bak (Upstate; Billerica, MA, USA); cytochrome c oxidase subunit II and COX II (MitoSciences; Eugene, OR, USA); heat shock protein 60 (Millipore; Billerica, MA, USA); Bim (Calbiochem; Billerica, MA, USA); caspase-3 (Biomol; Farmingdale, NY, USA); caspase-9 (Cell Signaling Technology; Danvers, MA, USA); lactate dehydrogenase (Abcam; Cambridge, MA, USA); and actin (MP Biomedicals; Santa Ana, CA, USA) were obtained from the indicated suppliers. Secondary antibodies and ECL reagents were acquired from GE Healthcare (Pittsburgh, PA, USA). The fluorogenic caspase substrates DEVD-AFC, LEHD-AFC, IETD-AFC and general caspase inhibitor Z-VAD-fluoromethyl ketone were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Neem oil limonoids was obtained from Sabinsa Corporation (East Windsor, NJ, USA). All other chemicals were purchased from Sigma (St Louise, MO, USA) 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 [27, 28]. For whole cell lysates preparation, cells were lysed in NP-40 buffer (50 mM HEPES-KOH, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, 1 mg/ml pepstatin A and 1 mg/ml chymostatin). To prepare mitochondrial and cytosolic fractions, cells were harvested, washed in ice-cold PBS, and then resuspended in homogenizing buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA and 1 mM DTT (dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, 1 mg/ml pepstatin A and 1 mg/ml chymostatin). After 30 min incubation on ice, cells were homogenized using a glass Pyrex homogenizer (type B pestle, 20–25 strokes) and centrifuged at 1100 g for 5 min at 4°C to remove cell debris and unbroken cells. The resulting supernatant was centrifuged at 12,000 g for 12 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 SDS polyacrylamide gel for Western blotting. After protein transfer, the membrane was probed/reprobed with various primary and corresponding secondary antibodies followed by immunodetection using ECL as previously described [27, 28].

Quantification of apoptosis and caspase activity 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 [27, 28]. Briefly, proteins were added to the caspase reaction mixture containing 30 μ M fluorogenic peptide substrates, DEVD-AFC (for caspase-3) or LEHD-AFC (for caspase-9) or IETD-AFC (for caspase-8), 50 mM HEPES, pH 7.4, 10% glycerol, 0.1% CHAPS, 100 mM NaCl, 1 mM EDTA, and 10 mM DTT, in a total volume of 100 μ l and incubated at 37°C for 90 min. Production of 7-amino-4-trifluoromethyl- coumarin (AFC) was monitored on spectrofluorimeter using excitation wavelength 400 nm and emission wavelength 508 nm. The results were presented as fold activation over the control.

Cellular reactive oxygen species (ROS) and mitochondrial ROS (mtROS), mitochondrial membrane potential (mtMP), and mitochondrial biomass (Mito mass) using flowcytometry

Flowcytometry analysis was carried out to illustrate cellular and mtROS, mtMP and mitochondrial mass in control and treated groups as described previously [29]. The cellular ROS, mtROS, mitochondrial mass, and mtMP were estimated in the control and treated groups using DHR123, MitoSox Red, MitoTracker Green, and MitoTracker Orange probes (Thermo Fisher Scientific; Waltham, MA, USA), respectively. In brief, control and treated cells were harvested by centrifugation at 1000 rpm for 5 min. Further, cells were re-suspended in 1 ml of 1X PBS and centrifuged for 5 min at 2500 rpm. The pellets were stained with respective probes and incubated for 30 min in dark at 37°C. Cells were harvested at 1500 rpm for 5 min and pellets were re-suspended in 1X PBS and 10,000 cells were analyzed using flow cytometry (LSR II, BD Biosciences; San Jose, CA, USA). Data were analyzed by WinList 3D 7.1 software and presented as the fold changes of geometric mean in comparison to untreated control.

Annexin/PI staining

Cells were treated with neem or vehicle for various time periods followed by staining with annexin-V-Alexafluor 488/PI kit (Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry (LSR II, BD Biosciences; San Jose, CA, USA) collecting 10,000 events. Graph was plotted using Win List 3D software.

Analysis of mtDNA content by real-time PCR

Total DNA, containing both mtDNA and nuclear DNA, was isolated from unstimulated and neem treated HCT116 WT cells using the ZR Genomic DNA II Kit (Zymo Research; Irvine, CA, USA). After quantification of DNA samples by the NanoDrop8000 (Thermo Fisher Scientific; Waltham, MA, USA), mtDNA content was determined on the Applied Biosystems 7300 real-time PCR system (Grand Island, NY, USA). *β -actin* and cytochrome c

oxidase subunit II (*COX II*) were used for amplification of nuclear and mitochondrial DNA respectively. Primers for *COX II* and β -actin were used as described previously [30]. *COX II* (forward): 5'-CCCCACA TTAGGCTTAAAAACAGAT-3', *COX II* (reverse): 5'-TATACCCCGGTCGTGTAGCG GT-3', β -actin (forward): 5'-TCACCCACACTGTGCCCATCTACGA-3', β -actin (reverse): 5'-CAGCGGAACCGCTCATTGCCAATGG-3'. The real-time PCR reaction was carried out in a total reaction volume of 10 μ l containing 5 μ l of 2X iTaq SYBR Green Supermix with ROX (Bio-Rad; Hercules, CA, USA), 10 ng of template DNA, 200 nM each of forward and reverse primers, and nuclease-free water. A melting curve analysis done at the end of amplification showed the absence of nonspecific amplification or primer dimer formation. The threshold cycle number (Ct) values for each reaction were calculated using the 7300 system SDS software. Standard curves generated from DNA obtained from untreated LNCaP cells using 10 ng to 10 pg provided PCR efficiency based on the equation $E = 10^{(-1/\text{slope})} - 1$ [31]. Average threshold cycle number (Ct) values were obtained by amplification of *COX II* (mtDNA-specific) and β -actin (nDNA-specific). MtDNA content was determined as $2^{Ct - Ct}$. Data presented as mtDNA content normalized to nDNA [32, 33].

Assay of OXPHOS system complexes

Mitochondria were isolated from neem treated or untreated cells. Equal amounts of mitochondria were subjected to three freeze-thaw cycles and 25–50 μ g/ml mitochondrial proteins were used for activity assays as described previously [34, 35]. All reactions were carried out at 30 °C in a reaction volume 100 μ l using a Beckman spectrophotometer.

Complex I (NADH ubiquitin oxidoreductase) activity was determined by measuring the rotenone sensitive NADH oxidation at 340 nm using coenzyme Q10 as an electron acceptor. Complex II (succinate dehydrogenase) activity was determined by measuring reduction rate of 2,6-dichloroindolphenol (DCIP) in the presence and absence of coenzyme Q1. Complex III (decylubiquinol cytochrome c oxidoreductase) activity was determined by monitoring the antimycin A sensitive reduction rate of cytochrome c in the presence of fully reduced CoQ2 as the electron source. Complex IV (cytochrome c oxidase) activity was measured by KCN sensitive oxidation of cytochrome c [29].

Establishment of cancer cells stably expressing caspase-9 or caspase-8 siRNA using shRNA lentiviral vectors

Green fluorescence protein (GFP)-tagged short hairpin RNAs (shRNAs) specific to caspase-9, caspase-8, and negative control shRNA were cloned into the pGIPZ (Open Biosystems; Pittsburgh, PA, USA) lentiviral vector to generate lentiviral particles. The shRNA sequences were: *caspase-8* (5'-GACTTCAGCAGAAATCTTT-3'), and *caspase-9* (5'-CCAGGCAGCTGATCATAGA-3'). Lentiviral particles specific for caspase-9, caspase-8, and control shRNAs were obtained from the Roswell Park Cancer Institute (RPCI) shRNA core resource and were directly utilized to infect cells at a multiplicity of infection (MOI) of 3. After 48 h, puromycin (1 μ g/ml) was added to the medium to select caspase-8 and caspase-9 knockdown stable cells. These cells used for neem treatment to

measure caspase activity and detect indicated proteins by Western blotting as described previously [12].

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) of at least three independent experiments. Statistical analysis was performed by ANOVA using GraphPad Prism. Significant changes ($p < 0.05$ or 0.01) are represented by *.

RESULTS

Caspase silencing inhibits neem-induced caspase activation

We previously demonstrated that neem induced apoptotic cell death in caspase-dependent mechanism [4]. The caspase cascade is initiated by either caspase-8 or caspase-9 signaling via death-receptor or mitochondrial pathway, respectively [36]. To investigate the underlying mechanism of apoptosis, we silenced caspase-8 and caspase-9 in MDA-MB231 (Figure 1A) and measured caspase activation and cell death. We observed that silencing of caspase-8 and -9 blocked caspase activation and attenuated cell death (Figure 1B and C). Similarly, silencing of caspase-8 or caspase-9 in HCT116 inhibited neem-induced caspase-3 activation (Figure 1D and E). Since activation of caspase-9 requires the release of cytochrome c from mitochondria to the cytosol [36, 37], we purified cytosol from HCT116 cells and observed a robust cytochrome c release in the cytosol in response to neem treatment (Figure 1F). Together, we demonstrate that neem-induces cytochrome c release from mitochondria into the cytosol triggering caspase activation and apoptotic cell death.

Neem treatment downregulates expression of proapoptotic Bcl-2 family proteins whereas prosurvival proteins were not modulated

Proapoptotic BH3-only proteins such as Bim and Bid activate Bax and Bak to undergo oligomerization that results in channels formation on the outer mitochondrial membrane [38–40] causing cytochrome c release, which triggers caspase activation. To determine whether proapoptotic Bcl-2 family proteins play a critical role in neem-induced cytochrome c release, HCT116 WT cells were treated with different doses of neem, and the levels of Bim, Bid, Bax, and Bak were determined. We observed that neem treatment lead to depletion of Bim, Bid, Bax, and Bak (Figure 2A and B). In contrast, the levels of prosurvival proteins including Bcl-2, Bcl-xL, and XIAP were not modulated in response to neem treatment (Figure 2C). Since p53 interacts with Bax and Bak to mediate cytochrome c release and deficiency of p21 increases sensitivity to apoptosis [4, 41, 42], we also determined the levels of proapoptotic proteins (Bim, Bid, Bax and Bak) in HCT116-p53^{-/-} and -p21^{-/-} cells. Similar to WT type cells, downregulation of Bid, Bim, Bax and Bak was observed upon neem exposure in HCT116-p53^{-/-} and -p21^{-/-} cells (Figure 2A and B). Although neem treatment depleted the proapoptotic Bcl-2 family proteins, increased caspase activity was observed in neem treated HCT116-WT, -p53^{-/-}, and -p21^{-/-} cells (Figure 2B). These findings suggest that neem-induced cell death does not require the formation of Bax/Bak channels on the mitochondrial membrane and neem renders anticancer effects in p53 and p21-independent mechanisms.

Neem treatment causes decreased levels of proapoptotic Bcl-2 family proteins on mitochondria and induces Bax-independent caspase activation

Since proapoptotic Bcl-2 family proteins translocate to the mitochondrial membrane [43], a decrease/depletion of total levels of these proteins may also affect their levels on mitochondria leading to a lack of Bax channel formation on mitochondria. Therefore, we isolated cytosol and mitochondria from control and neem treated HCT116-WT cells. We observed that neem treatment resulted in reduced accumulation of Bid and Bax on mitochondria (Figure 3A). These findings suggest that neem-induced cytochrome c release does not require pro-apoptotic Bcl-2 family proteins including Bax. To further demonstrate that neem induces Bax-independent caspase activation, HCT116-WT and -Bax^{-/-} cells were treated with different doses of neem followed by determination of caspase-3 activity. We observed no difference in caspase-3 activation between HCT116-WT and Bax^{-/-} cells (Figure 3B). These findings demonstrate that neem induces Bax-independent caspase activation causing apoptotic cell death in cancer cells.

Neem depletes Drp-1 from mitochondria

Apart from Bax, the mitochondrial fission regulates mitochondrial outer membrane permeabilization during apoptosis. It is regulated by dynamin-related protein-1, Drp-1 [44], which seems to be localized with Bax on mitochondria, suggesting a cross-talk between Drp-1 and Bax [45]. To understand whether cytochrome c release is mediated by mitochondrial fission, we measured the levels of Drp-1 in the cytosolic and mitochondrial fractions. Similar to Bax, Drp-1 level was also depleted from mitochondria, whereas cytosolic Drp-1 level was decreased at higher doses (Figure 4). These findings suggest that neem does not promote mitochondria fission during apoptosis induction in cancer cells.

Mitochondrial permeability transition pore formation is not required during neem-induced cell death

Permeability transition pore (PTP) also regulates cytochrome c release [46] and permeabilization of mitochondria is an early event for mitochondria-mediated cell death. The effect of cyclosporin A (CsA), a PTP inhibitor, on early event will allow us to determine whether PTP is critical for neem-induced apoptosis. We pretreated HCT116-WT cells with CsA prior to neem exposure. Caspase activities and cell death at earlier time periods such as 16 h after neem treatment were determined. As shown in Figure 5A–C, neem induced equal levels of caspase activities in the presence or absence of CsA. Equal levels of cell death (Figure 5D) further supported that PTP-mediated channels may not play a significant role in neem-induced caspase activation and cell death. Together, these studies indicate that PTP is not critical for neem-induced caspase activation and apoptotic cell death.

Neem enhances mtDNA content during apoptosis

Mitochondria and OXPHOS system are also primary targets for anticancer agents, including phytochemicals [47, 48]. MtDNA encodes 13 polypeptides, 22tRNAs, and 2 rRNAs that support biogenesis of OXPHOS complexes [49, 50]. Thus changes in mtDNA can affect OXPHOS system, therefore, we determined the level of mtDNA upon treatment with neem.

We observed that neem enhanced mtDNA content during apoptosis, 12 h and onwards, in HCT116 cells (Figure 6A). To further demonstrate that neem-induced mtDNA copy number is not cell type-dependent phenomenon, we treated prostate (Du145 and E006AA) and colon (HT29) colon cancer cell lines. Our finding demonstrated that similar to HCT116 cells, neem induced mtDNA copy number in various types of cancer cells (Figure 6B). Because cyclophilin D can also modulate mitochondria structure and function [47], we determined whether inhibition of cyclophilin D by cyclosporin A (CsA) modulates mtDNA content. Our findings demonstrate that CsA did not modulate neem-induced mtDNA content (Figure 6C).

Increased mtDNA content suggests mitochondrial biogenesis, which may involve upregulation of mitochondrial replication machinery such as polymerase gamma (POLG) and Twinkle, which are encoded by the nuclear genome [51, 52]. Indeed, we observed upregulation of Twinkle protein, however, POLG level was downregulated upon neem treatment (Figure 6D and E). These findings suggest that neem induces mitochondrial biogenesis by modulating mtDNA replication machinery. Together, above data suggest that neem enhances mtDNA content that occurs without the involvement of known mechanisms such as cyclophilin D-mediated PTP and Bax/Bak channels.

Inhibition of OXPHOS Complex I activity associates with increased mitochondria ROS (mtROS), cellular ROS, mitochondrial membrane potential (mtMP), and mitochondrial biomass (Mito mass)

The induction of mtDNA content upon neem treatment may trigger mitochondria-nucleus crosstalk [49]. The induction of mtDNA content by neem suggests onset of mitochondrial biogenesis. To test whether increased mitochondrial biomass translates into higher OXPHOS function, we measured the activity of different complexes. We observed a significant inhibition of OXPHOS Complex I activity. However, the activities of other complexes such as Complex II and IV were not affected in response to neem treatment (Figure 7A). This suggests that neem inhibits Complex I specifically. Inhibition of OXPHOS Complex I activity can result in increased ROS in cells [53, 54]. To determine whether neem treatment causes increased ROS production, we measure cellular and mitochondrial ROS in HCT116 upon treatment with neem. Indeed, we observed significant increase in mtROS and cellular ROS early during apoptosis in response to neem treatment (Figure 7B and C). Increased mtDNA content and ROS are indicative of mitochondrial proliferation. Mitotracker Green probe showed a significant increase in mitochondrial biomass (Figure 7D). This correlated with higher staining with Mitotracker orange, which accumulates in mtMP-dependent manner in cells (Figure 7E). To further validate that neem-induced changes such as mitochondrial ROS are not cell type specific, we also determined mtROS in LNCaP prostate cancer cells in response to neem treatment. Similar to colon cancer cells, neem induced significant mtROS production upon neem exposure to prostate cancer cells (Figure 7F). These findings suggest that neem inhibits Complex I, which induces ROS production leading to mitochondria dysfunction, apoptosis and cell death.

OXPPOS Complex I and Complex II-deficiencies inhibited neem-induced caspase activation

Inhibition of Complex I activity prompted us to evaluate its importance in neem-induced apoptosis. Therefore, we used pre-characterized OXPPOS Complex I-deficient CCL16-B2 cells (B2) to measure caspase activation in response to neem treatment [22, 23]. Compared to wild type B1 cells, the Complex I-deficiency in B2 cells inhibited neem-induced caspase activities and apoptotic cell death. However, the total cell death in Complex I-deficient B2 cells upon neem exposure was higher compared to control B1 cells treated with neem (Figure 8 and 9). The Complex II deficiency in B9 cells [24, 25] also inhibited caspase activation in response to neem treatment. In Complex II deficient B9 cells, the inhibition of caspase activation was associated with significant decrease in both apoptotic and total cell death upon neem exposure (Figure 8 and 9). Together, we conclude that pharmacological and genetic ablation of OXPPOS Complex I may lead to high levels of ROS production may induce other cell death mechanisms such as necrotic or autophagic cell death, whereas OXPPOS Complex II is required for apoptosis induction.

Combined abrogation of Complexes I, III, IV, and V (gross complex-deficiency) did not inhibit caspase activation

We observed increased mtDNA level in response to neem treatment and mtDNA encodes for proteins required for Complex I, III, IV, and V. Therefore, we next determined how gross depletion of mtDNA-encoded subunits of Complexes I, III, IV, and V affects neem-induced apoptosis. We treated control (G3) and gross complex deficient (G7) cells that are impaired in intra-mitochondrial protein synthesis [26]. We observed that impairment of mtDNA-encoded proteins did not inhibit neem-induced caspase activation (Figure 10). These findings suggest that proper functioning of OXPPOS complexes is important for apoptotic cell death induction and Complex II plays an important role during neem-induced apoptosis in cancer cells.

DISCUSSION

This study shows that neem limonoids induce cell death in multiple types of cancer cells [15, 55–58]. Therefore, it could be considered as potent anticancer agents. The detailed analysis of cell death mechanisms in response to neem may lead to design of novel neem-based anticancer agents. Our previous study, demonstrated that neem oil, which contains majority of total limonoids, induces efficient cell death by multiple cell death mechanisms [4]. This manuscript describes the underlying mechanisms of apoptotic cell death induced by neem limonoids in cancer cells. Neem induces apoptosis, which requires caspase activation initiated by initiator caspases such as caspase-9. Although conventional apoptosis signaling involving proapoptotic Bcl-2 family proteins are not involved, neem-induced cell death requires both initiator caspase-8 and caspase-9. This study also describes the role of OXPPOS complexes during neem-induced apoptosis. Interestingly, total cellular levels of Bax and mitochondrial Bax were depleted, suggesting that neem-induced cytochrome c release does not require Bax activation and oligomerization. This was supported by the observation that Bax-deficiency did not inhibit caspase activation. These data together support our conclusion that neem-induced caspase activation, and thus apoptotic cell death

was not regulated by Bax. Similarly, total cellular Bak was decreased upon neem treatment. Our data also support that PTP does not play an essential role in neem-induced cytochrome c release and caspase activation. It is also important to note that both wild type and p53-deficient cells shows Bax/Bak-independent cell death, suggesting that neem-based anticancer agents will be effective in alleviating drug resistance even in p53 mutant tumors.

How does neem induce permeabilization of the outer mitochondria membrane? Caspase-dependent and caspase-independent mechanisms require release of cytochrome c and apoptosis inducing factor (AIF), respectively, into the cytosol from mitochondria. Bax and Bak channels play key role in mitochondrial permeabilization. However, our findings demonstrate that Bax/Bak channels are not formed during neem-induced cell death. Neem treatment decreased total Bim, Bid, Bax, and Bak. More importantly, the reduced levels of Bax and lack of Bax oligomerization were observed upon neem exposure to cancer cells. The expression of proapoptotic proteins such as Bax is regulated by functional p53 [59], however, similar to wild type counterpart, we observed depletion of total cellular levels of proapoptotic Bcl-2 family proteins in p53 or p21-deficient HCT116 cells. Absence or reduced level of Bax on mitochondria further support that permeabilization of mitochondrial membrane does not require Bax channels. Equal levels of caspase activation and cell death in HCT116-WT and -Bax^{-/-} cells further support our conclusion that neem induces Bax-independent caspase activation and apoptosis in cancer cells. Exposure to purified limonoids have been shown to alter expression of Bcl-2 family proteins in HeLa cells [7], whereas our findings are based on combined exposure of multiple limonoids, and suggest Bax-independent permeabilization of the mitochondrial membrane. Since Bax and Bak are normally sequestered by Bcl-2/Bcl-xL [60], our findings suggest that combination of limonoids could directly target mitochondria to induce release of proapoptotic proteins in Bax/Bak-independent manner.

Permeabilization of the mitochondrial membrane is a very complex process and requires multiple mechanisms of action. For example, in addition to Bax/Bak-mediated channels, mitochondrial fission and PTP also regulate release of proapoptotic proteins from mitochondria [46, 47]. We observed depletion of Drp-1, which regulates mitochondrial fission during apoptosis, suggesting the exclusion of mitochondrial fission during neem-induced apoptotic cell death. We also observed that permeabilization of the mitochondrial membrane was not regulated by PTP as neem-induced caspase activation was not inhibited in CsA treated cells. Since PTP is a multiprotein complex, it is possible that neem activates different proteins or complexes that are not inhibited by CsA.

Our data raise the possibility that neem directly affects mitochondrial functions such as inhibition of Complex I. However, we also find that mtDNA content was increased in neem-exposed cells. This may represent a compensatory response to Complex I inhibition. Previous studies indicated that mitochondrial respiration is also a target for caspase-independent cell death [61]. Our previous findings demonstrate that neem limonoids induce cytochrome c release, however, the conventional cytochrome c releasing machinery including mitochondrial outer membrane potential (MOMP), PTP, and mitochondrial fragmentation do not actively participate during neem-induced apoptotic cell death. That raises a question, how increased mtDNA content causes the permeabilization of the

mitochondrial membrane. Although the exact mechanism is still under investigation, it is possible that increased mtDNA content may lead to enhanced synthesis of polypeptides required for Complex I, III, IV and V leading to activation of the respiration complexes [49]. It is also known that Complex-I and III regulate permeabilization of the mitochondrial membrane and Complex-I could also be a substrate for active caspases [62–64]. We observed that even though mitochondrial biomass has been activated but Complex-I activity was significantly inhibited, which leads to higher mtROS generation leading to mitochondria dysfunction and cell death. Interestingly, our data also suggest that different OXPHOS complexes may differently be targeted by neem leading to prodeath or prosurvival function of complexes. Thus, present study identifies a novel mechanism of permeabilization of mitochondria independent of Bax, PTP, or mitochondrial fission.

Together, our findings suggest that combined exposure of limonoids directly targets OXPHOS complexes in mitochondria leading to enhanced caspase activation and cell death. Although cancer cells seem primed for cell death due to increased expression of proapoptotic proteins [65, 66], the presence of various unknown factors do not allow efficient cancer cell death causing resistance to currently available therapies. Our findings in this study identified that OXPHOS complexes could be novel targets for restoration of apoptosis in cancer cells. Current anticancer agents are unable to induce efficient cancer cell death as well as these agents also cause enrichment of cancer-initiating cells, which ultimately results in recurrence of cancer such as colon and ovarian cancers, and metastasis to distant sites [67, 68]. Neem limonoids induce efficient apoptosis via targeting OXPHOS complexes. Since neem-induced cell death does not require p53 and is Bax-independent [4], multiple cancer cell types could be targeted. Thus, cancer cell death without the engagement of upstream mitochondrial events may benefit in targeting drug resistant cancer cells. Thus neem limonoids could be novel agents for the treatment of solid cancers.

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ABBREVIATIONS

3-MA	3-methyladenine
AFC	7-amino-4-trifluoromethyl- coumarin
AIF	apoptosis-inducing factor
COX II	cytochrome c oxidase subunit II
CsA	cyclosporin A
Ct	threshold cycle number
Cyt. c	cytochrome c

mtDNA	mitochondrial DNA
nDNA	nuclear DNA
neem	neem oil limonoids
OXPHOS	oxidative phosphorylation
PTP	permeability transition pore
ROS	reactive oxygen species
z-VAD-fmk	z-VAD-fluoromethyl ketone

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Highlights

- Neem induces caspase activation and apoptosis in Bax/Bak-independent mechanism
- Neem enhances mitochondrial DNA and mitochondrial biogenesis
- Complex I inhibition by neem associates with production of reactive oxygen species
- Complex I-deficiency inhibits caspases but enhances cell death upon neem exposure
- Complex II-deficiency inhibits caspases and apoptosis/cell death upon neem exposure

FIGURE 1. Neem triggers cytochrome c release leading to caspase-dependent apoptotic cell death

A–C, Caspase-8 and caspase-9 silenced MDA-MB-231 cells were treated with various doses of neem for 24 h. At the end, images were taken and cells were harvested and subjected to caspase-3 (i.e., DEVDase), caspase-9 (i.e., LEHDase) and caspase-8 (i.e., IETDase) activities. **D and E**, Caspase-8 or caspase-9 silenced HCT116 cells were treated with neem for 24 h. Cells were harvested and subjected to caspase-3 (i.e., DEVDase) activity measurements. **F**, HCT116 WT cells were treated with neem (150 µg/ml) for 24 h followed by purification of cytosol. Equal amounts of proteins in A, D, E and F were subjected to Western blotting to detect indicated proteins. Actin serves as a loading control. Data are mean $\pm$ SD, n=3. *, $P < 0.05$ compared to respective control shRNA cells. C8, caspase-8; C9, caspase-9; N-150, neem (150 µg/ml); N-300, neem (300 µg/ml).

FIGURE 2. Neem treatment causes decreased expression of proapoptotic proteins while expression of prosurvival proteins were not modulated

A–B, HCT116-WT, p53^{-/-}, and p21^{-/-} cells were treated with indicated doses of neem for 24 h. Equal amounts of protein were subjected to Western blotting for the detection of indicated proteins. Protein bands p20/17 represent cleaved products of caspase-3. Actin or Hsp60 serve as loading controls. **C**, LNCaP and HCT116 cells were treated with indicated concentration of neem for 24 h followed by Western blotting for indicated proteins. Actin serves as a loading control. WT, wild type; Cont, control; Neem, neem; Hsp60, heat shock protein 60; XIAP, X-linked inhibitor of apoptosis.

FIGURE 3. Neem promotes depletion of proapoptotic proteins, Bid, and Bax on mitochondria and induces Bax-independent caspase activation and cell death

A, HCT-116 WT cells were treated with indicated doses of neem for 24 h. Cytosols and mitochondria were purified and equal amounts of protein were subjected to Western blotting for detection of indicated proteins. Actin serves as loading control. **B**, HCT116-WT and -Bax^{-/-} cells were treated with indicated doses of neem for 24 h. At the end, equal amounts of proteins were used for caspase activity measurements. Neem, neem; Cont, control. Data are mean $\pm$ SD, n=3. *, $P < 0.05$ compared to control.

FIGURE 4. Neem-induced apoptosis is not dependent upon mitochondrial fission
HCT116-WT cells were treated with indicated doses of neem for 24 h. Cytosols and mitochondria were purified and equal amounts of protein were subjected to Western blotting for detection of indicated proteins. Actin serves as loading control. Neem, neem.

FIGURE 5. Neem induces CsA-independent caspase activation and cell death

HCT116-WT cells were treated without or with neem in presence or absence of CsA for 16 h. Caspase activities were determined (A–C) in whole cell lysates. Percentage of cell death was also quantified upon neem treatment (D). Neem, neem; CsA, cyclosporin A. Data are mean $\pm$ SD, $n=3$. *, $P < 0.05$ compared to control.

FIGURE 6. Neem enhances mtDNA content, which does not require CsA-dependent permeability transition pore (PTP) but involve mitochondrial replication machinery
A, HCT116-WT cells were treated with different doses of neem for indicated times followed by isolation of total DNA. **B**, HT29 colon cancer cells, Du145 and E006AA prostate cancer cells were treated with neem (300 μg/ml) and total DNA was isolated. **C**, HCT116-WT cells were treated with different doses of neem for 12 h in the presence or absence of CsA and total DNA were isolated. MtDNA content in A, B, and C were determined as described in the experimental procedures. **D and E**, HCT-116 WT cells were treated with indicated doses of neem for 24 h. Equal amounts of protein were subjected to Western blotting for the detection of indicated proteins. Actin served as protein loading control. Neem, neem; CsA, cyclosporin A; N-150, neem (150 μg/ml); N-300, neem (300 μg/ml); POLG, polymerase gamma. Data are mean ± SD, n=3. *, $P < 0.05$ compared to control.

FIGURE 7. Neem inhibits OXPHOS Complex I activity, and associates with induction of ROS production and mitochondrial biomass (Mito mass)

A, HCT116 cells were treated with neem at 150 or 300 µg/ml and at the end of treatment mitochondria were isolated and OXPHOS complexes activities were assessed as described in experimental procedures. **B–E**, HCT116 cells were treated with neem (Neem; 150 or 300 µg/ml for 24 h). Mitochondrial ROS (mtROS), Cellular ROS, mitochondrial biomass (Mito mass), and mitochondrial membrane potential (mtMP) were estimated by flow cytometry analysis of MitoSox Red, dihydrorhodamine 123 (DHR123), MitoTracker green, and MitoTracker orange, respectively. **F**, LNCaP cells were treated with neem for 24 h. MtROS were estimated by flow cytometry analysis. Data are mean $\pm$ SD, $n=3$. *, $P < 0.05$ compared to control. MtROS, mitochondrial ROS; Mito mass, mitochondrial biomass; MtMP, mitochondrial membrane potential.

FIGURE 8. Differential effects of OXPHOS Complexes I and II deficiencies on caspase activation and cell death

Wild type (B1), Complex I-deficient (B2), and Complex-II deficient (B9) Chinese hamster lung fibroblasts cells were treated with neem at various doses for 24 h. Images were taken (A) and equal amount of whole cell lysates were subjected to caspase activity measurement (B–D). Data are mean $\pm$ SD, $n=3$. *, $P < 0.05$ compared to respective treatment WT control cells. Neem-150, neem (150 $\mu\text{g/ml}$); Neem-300, neem (300 $\mu\text{g/ml}$).

FIGURE 9. Differential effects of OXPHOS Complexes I and II deficiencies on neem-induced apoptotic and total cell deaths

A and B, Wild-type (B1), Complex I-deficient (B2), and Complex-II deficient (B9) Chinese hamster lung fibroblasts were treated with neem at various doses for 24 h. At the end of treatment, total cell death and apoptosis were quantified using Annexin/PI staining. 10,000 events were captured using LSRII and data were analyzed by Win List software. Data are mean $\pm$ SD, $n=3$. *, $P < 0.05$ compared to respective treatment WT control cells. Neem-150, neem (150 μ g/ml); Neem-300, neem (300 μ g/ml).

FIGURE 10. Gross OXPHOS deficiency (i.e. Complex I, III, IV, & V deficiencies together in same cells) does not inhibit caspase activation

Wild type (G3) and mutant (G7, with defects in complexes I, III, IV & V) Chinese hamster fibroblasts were treated with neem at various doses for 24 h followed by caspase-3 activity measurements. Data are mean $\pm$ SD, $n=3$. Neem-150, neem (150 $\mu\text{g/ml}$); Neem-300, neem (300 $\mu\text{g/ml}$).