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Fascaplysin Induces Caspase Mediated Crosstalk Between Apoptosis and Autophagy Through the Inhibition of PI3K/AKT/mTOR Signaling Cascade in Human Leukemia HL-60 Cells

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ABSTRACT

In this study, we for the first time explored the cellular and molecular mechanism of anticancer properties of fascaplysin, a marine sponge-derived alkaloid. Our study demonstrated that fascaplysin induced a cooperative interaction between apoptotic and autophagic pathways to induce cytotoxicity in HL-60 cells. Fascaplysin treatment not only activated pro-apoptotic events like PARP-1 cleavage and caspase activation but also triggered autophagy signaling as shown by the increased expression of LC3-II, ATG7and beclin. Interestingly, it was found that use of pan-caspase inhibitor completely reversed the fascaplysin mediated cell death as analyzed by MTT and cell cycle assays. It was observed that cell death as well as the expression of pro-death proteins was partially reversed, when key autophagy mediators ATG7 was silenced by siRNA in fascaplysin treated cells. Cooperative involvement of autophagy and apoptotic signaling in cytotoxicity was confirmed when combined silencing of pro-apototic (PARP-1) and autophagic (ATG-7) signaling by respective siRNA's lead to substantial rescue of cell death induced by fascaplysin. Although, apoptosis and autophagy are two independent cell death pathways, our findings provide detailed insight by which both the pathways acted cooperatively to elicit fascaplysin induced cell death in HL-60 cells. Our findings provide molecular insight into the anti-cancer potential of fascaplysin by showing that both autophagic and apoptotic signaling can work together in the induction of cell death. J. Cell. Biochem. 116: 985–997, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: Autophagy; Apoptosis; PI3K/AKT pathway; Caspases; Fascaplysin

The oceans cover more than 70% of the earth's surface, which is largely unexplored in the area of drug discovery. Oceans are attractive source of natural products which are chiefly isolated from sponges, molluscs, tunicates, bryozoans, marine bacteria and cyanobacteria [Donia and Hamann, 2003]. Fascaplysin, a brick red colored pigment was first isolated from marine sponge Fascaplysinopsis Bergquist species in 1988 [Roll et al., 1988; Bharate et al., 2012a]. Fascaplysin (FAS) exhibited an ample array of pharmacological activities such as antibacterial, antifungal, antiviral, antimalarial, anticholinesterase, etc [Roll et al., 1988]. It has been reported to inhibit CDK4, arrest cell cycle at G1 phase, induce apoptosis and inhibit angiogenesis [Soni et al., 2000; Lin et al., 2007; Lu et al., 2009]. However, its effect on autophagy and the PI3K/AKT/mTOR signaling pathway has not been studied.

The discovery of novel PI3K/AKT/mTOR inhibitors has developed a great magnetism in anticancer therapeutics. In recent years,

Abbreviations: AO, Acridine orange; ATG, autophagy related gene; LC3, microtubule-associated protein light chain 3; MTT, 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PARP-1-1, Poly(ADP-Ribose) Polymerase-1; PI, propidium iodide; Rh-123, Rhodamine-123; ZVAD (zVAD fmk), benzyloxycarbonyl-Val-Ala-Asp fluoro-methyl-ketone; mTOR, The mammalian target of rapamycin; mpk, mg/kg.

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extensive amount of efforts have been made to discover inhibitors of PI3K/AKT/mTOR pathway for treatment of cancers and several of these inhibitors viz. NVP-BEZ-235, GSK-690693, PKI-587, XL-765, and PI-103 are being evaluated in clinical trials. The PI3K/AKT/mTOR pathway regulates several customary cellular functions that are also critical for tumorigenesis such as cell proliferation, cell metabolism, angiogenesis, cell cycle progression apoptosis and autophagy [Engelman et al., 2006; Hennessy et al., 2007; Courtney et al., 2010]. Apoptosis and autophagy are two major types of cell deaths, designated as type I and type II programmed cell deaths respectively. Apoptosis is characterized by cell shrinkage, DNA fragmentation, chromatin condensation, pyknotic nuclei and production of the apoptotic bodies and activation of caspases, whereas autophagic cell death is differentiated by formation of autophagosome that give a cell characteristic vacuolated appearance [Budihardjo et al., 1999, Baehrecke, 2005]. There are many evidences in which apoptosis and autophagy are interdependent and most often activation of these two death machinery occur simultaneously [Yu et al., 2004; Wu et al., 2008; Sun and Peng, 2009]. Moreover, apoptosis and autophagy are very well linked with PI3K/AKT/mTOR pathway [Hennessy et al., 2005].

In this study, we report for the first time that fascaplysin induce caspase arbitrated in vitro and in vivo autophagy, apoptosis and hamper PI3K/AKT/mTOR pathway. We also report for the first time that fascaplysin induces sub G1 arrest in HL-60 cells and molecular mechanisms behind all above mentioned cell death events was entirely caspase adjudicated.

MATERIALS AND METHODS

CELL CULTURE, GROWTH CONDITIONS, TREATMENT AND ANIMALS

Human promyelocytic leukemia HL-60 cells were obtained from the National Cancer Institute (NCI), Bethesda, USA. The cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), and 0.37% $NaHCO_3$. Cells were grown in a CO_2 incubator (Thermocon Electron Corporation, USA) at 37°C in an atmosphere of 95% air and 5% CO_2 with 98% humidity. Cells treated with fascaplysin solution which were dissolved in DMSO while the untreated cells received the vehicle (DMSO<0.2%). All animal experiments were approved by the animal ethics committee of Indian Institute of Integrative Medicine, CSIR, Jammu, India.

REAGENTS AND CHEMICALS

RPMI-1640, propidium iodide (PI), 3-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rhodamine 123, penicillin, streptomycin, L-glutamine, Hoechst-33258, pyruvic acid, ribonuclease A, croton oil, freund's complete adjuvant, protease inhibitor cocktail, acridine orange, bovine serum albumin, Z-VAD-FMK were purchased from Sigma-Aldrich, India. Fetal bovine serum was obtained from GIBCO Invitrogen Corporation, USA. Tween-20, AnnexinV-FITC apoptosis detection kit, anti mouse monoclonal antibodies, PARP-1 (sc8007), β-actin (sc1616), caspase-3 (sc7272) and anti rabbit monoclonal antibody γH2AX (sc101696) were purchased from Santa Cruz biotechnology, USA. Primary monoclonal antibodies for AKT (4691), p-AKT (S473) (4060), p-AKT (T308) (4056) , p110α (S4249) (4255), p-p70S6K (T389) (2903), p-mTOR (S2448) (5536), p-GSK3β (5558), raptor (2280), rictor (2114), LC3B (2775), BECN 1 (3495), p-PDK1(S241) (3438) and HRP labeled secondary antibodies to rabbit and mouse were acquired from Cell Signaling Technology, USA. The Caspase-3 activity assay kit was purchased from BD Biosciences, USA. All the electrophoresis reagents, protein markers were from Bio-RAD, USA. Hyper film and ECL plus reagents were purchased from Amersham Biosciences, UK. All other bio-chemicals and reagents used in studies were AR grade and purchased from Sigma Aldrich, India.

SYNTHESIS OF FASCAPLYSIN

Fascaplysin was synthesized using a two-step protocol as described earlier [Bharate et al., 2012b]. The reaction of tryptamine with 2-chlorophenyl glyoxal in the presence of Pd/C in acetic acid under reflux produced β-carboline in 85% yield, which on heating at 220 °C for 20 min resulted in formation of fascaplysin in 80% yield.

CELL PROLIFERATION ASSAY

HL-60 cells were seeded in 96 well plates at a density of 15000/200 μl of the medium. Cultures were treated with different concentrations of fascaplysin for 6–48h time intervals. MTT dye (250 μg/ml) was added three hour before termination of the experiment, and incubated at 37°C. The plates were centrifuged at 400 x g for 15 min and MTT formazen crystals were dissolved in 150 μl of DMSO, absorbance was measured at 570 nm with reference wavelength 620 nm [Bhushan et al., 2007].

PHASE CONTRAST MICROSCOPY

Morphological changes in cell were studied by phase contrast microscopy. HL-60 cells were incubated in six well plates and treated with different concentration of fascaplysin (0.5–1.5 μM) for 6 h, after that cells were subjected to photography on an inverted microscope attached to the DP-12 camera (1 × 70, Olympus).

HOECHST 33258 NUCLEAR STAINING

Cells were treated with different concentrations of fascaplysin (0.5–1.5 μM) for 6 h and washed twice with PBS at 400 x g for 5 min. Cells were then stained with one milliliter of staining solution (10 μg/ml, Hoechst 33258, 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween-20) and stained for 30 min in the dark at room temperature. After staining the cells were resuspended in 50 μl of mounting fluid (PBS: glycerol, 1:1) and 10 μl mounting suspension was observed for nuclear morphology under inverted fluorescence microscope using UV excitation (Olympus 1 × 70, magnification 30X) [Bhushan et al., 2006].

FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS AND NECROSIS

HL-60 cells (1×10^6) were treated with 0.5, 1.0 and 1.5 μM concentrations of fascaplysin for 6 h. Cells were double stained with annexin-V/PI by using kit manufacture's protocol (# sc4252, Santa Cruz Biotechnology, USA). The FACS analysis of apoptosis and necrosis was done as described earlier [Bhushan et al., 2006].

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL

HL-60 cells (1×10^6) were seeded in 12 well plates and incubated with different concentrations of fascaplysin (0.5–1.5 μM) for 6 h. Rhodamine-123 dye (200 nM) was added 30 min before termination of the experiment. Cells were collected at 400 x g, washed once with PBS and mitochondrial membrane potential was measured in the FL-1 channel of flow cytometer (FACS Calibur, Becton Dickinson, USA) [Bhushan et al., 2006].

CELL CYCLE ANALYSIS

Cell cycle phase distribution was studied by propidium iodide fluorescence. HL-60 cells (1×10^6) were incubated with different concentrations (0.5–1.5 μM) of fascaplysin for 6 h. The cells were then washed twice with ice-cold PBS, harvested, fixed in 70% ethanol and stored at 4 °C overnight. After fixation, these cells were incubated with RNAse-A (0.1 mg/ml) at 37 °C for 90 min, stained with propidium iodide (100 μg/ml) for 30 min on ice in dark, and then measured for DNA content using BD FACS flow cytometer (Becton Dickinson, USA). Resulting DNA distributions were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of cells in apoptosis, G1, S, and G2/M phases of the cell cycle [Chanda et al., 2012].

CASPASE ASSAY

Cells were treated with indicated concentrations of fascaplysin for 6 h and collected at 400 g, washed once with PBS. The pellet was lysed with cell lysis buffer and flourimetric estimation of caspase was done as described earlier [Bhushan et al., 2006].

ACRIDINE ORANGE STAINING FOR AUTOPHAGY DETECTION

HL-60 cells (0.5×10^6) were incubated in 12 well plates and treated with 0.5–1.5 μM concentrations of fascaplysin for 6 h. Cells were then incubated with acridine orange (1 μg/ml) for 20 min and washed once with ice cold PBS. Autophagy was determined by red fluorescence under a fluorescence microscope (Olympus 1×70) [Kumar et al., 2013].

WESTERN BLOT ANALYSIS

Cells were treated with indicated concentrations of fascaplysin for 6 h, centrifuged at 400 g at 4 °C, washed in PBS and cell pellets were lysed in RIPA buffer for preparation of whole cell lysate as described earlier [Kumar et al., 2013a]. Equal amount of protein (60–80 μg) was loaded into each well for SDS-PAGE. Blots were incubated with different primary antibodies, and chemiluminiscence was captured on hyperfilm after incubating the blots in ECL plus solution.

IMMUNOPRECIPITATION

Immunoprecipitation was performed as described earlier [Kumar et al., 2013b]. Briefly, cells were lysed in denaturing lysis buffer (RIPA buffer). Lysates were exposed to PARP-1 antibody, lysate antibody mixture was kept overnight under constant agitation at 4 °C. Protein agarose A bead was added to the lysate antibody mixture and was incubated overnight under constant agitation at 4 °C. The immunoprecipites were then immunoblotted for different antibodies; western blot of immunoprecipites was done as described above.

SMALL INTERFERING RNA TRANSFECTION

Human PARP-1 and ATG7 specific siRNA were transfected into HL-60 as described earlier [Kumar et al., 2013b] briefly, cells were incubated in transfection media containing transfection reagent and siRNA for 8 h, followed by addition of complete media for 48 h. Knocking down of the expression of the respective proteins was checked by western blotting.

IN VIVO AIR SAC MODEL ASSAY

The murine chronic granulomatous tissue model of chronic inflammation was developed as per the CPCSEA guidelines [Colville-Nash et al., 1995]. Chronic granulomatous air pouches on the back of mice were induced by subcutaneous injection of 5 ml of sterilized air (filtered through 0.22 μm filter) and injection of 0.2 ml freund's complete adjuvant with 0.1% croton oil. The size of air pouch was maintained by injecting extra three ml of sterile air into the same cavity on fifth and eighth day. The dosing regimen was started on day 1 and continued until day 8 with fascaplysin and RAD001 (5–10 mg/kg/i.p) being solubilized in 0.2 ml of diethylene glycol monoethyl ether (Transcutol) (Sigma)/Cremephor El (Sigma)/ normal saline (10:10:80) (normal saline was used for control groups). On 1, 4, and 8 day the animal's body weight was measured and on day 11 animals were anesthetized, tissue was removed, granulomas weight and volume was measured. Granulomatous tissue was homogenized by using dounce homogenizer (Sigma) in RIPA buffer and immunoblotting was performed to evaluate key apoptotic, autophagic and key proteins of PI3K/AKT/mTOR pathway. The same granulomatous tissue was fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned at a thickness of 5–6 μm. Angiogenesis were evaluated by measurement of micro vessels in paraffin embedded tissue sections stained with eosin.

STATISTICAL ANALYSIS

Statistical analysis was done by using bonferroni method and P value<0.05 was considered to be significant with $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

RESULTS

FASCAPLYSIN INHIBIT PROLIFERATION AND INDUCE APOPTOSIS OF HL-60 CELLS

Fascaplysin significantly inhibited HL-60 cell proliferation with IC_{50} values of 0.5 μM, 0.7 μM, 1 μM and 1.3 μM for 6 h, 12 h, 24 h and 48 h, respectively (Fig. 1A). Fascaplysin was able to induce apoptosis in HL-60 cells, which was confirmed by a battery of the apoptotic assays (Fig. 1B-H). External morphological changes were observed through phase contrast microscopy and fascaplysin was found to induce cellular damage in a dose-dependent manner (Fig. 1B). Fascaplysin was also found to damage DNA in a dose-dependent manner as indicated by increased number of apoptotic body formation in hoechst nuclei staining (Fig. 1C). Apoptotic potential of fascaplysin was further confirmed by annexin V/PI staining and cell cycle analysis. Annexin V/PI staining was done to differentiate between apoptotic and necrotic cell death. Significant apoptosis was observed in cells treated with different doses of fascaplysin, whereas

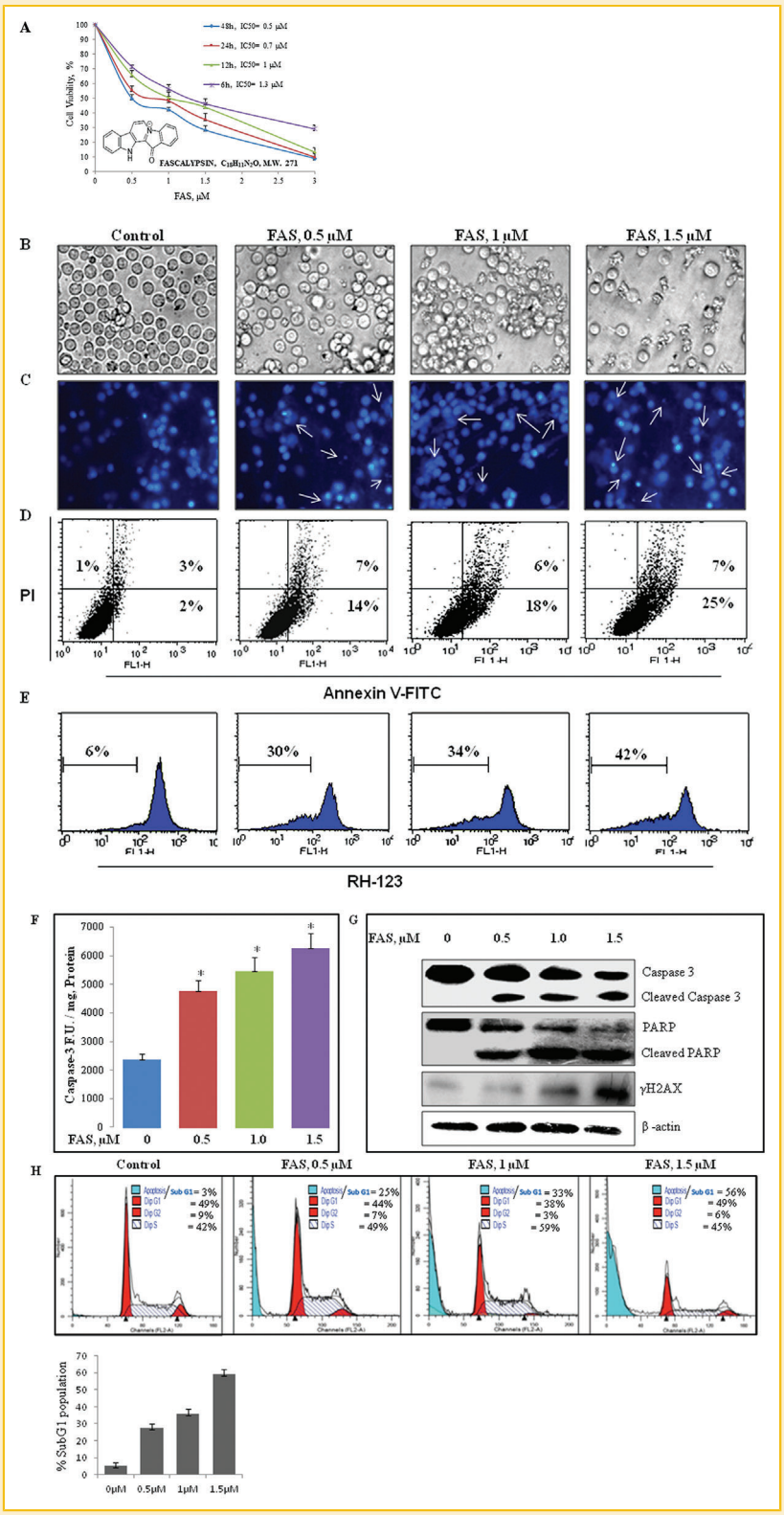

Fig. 1. Continued.

necrotic population was almost negligible. The percentage of apoptotic population was significantly higher in fascaplysin treated HL-60 cells (25%) as compared to untreated control (2%) (Fig. 1D). Furthermore, fascaplysin significantly enhanced mitochondrial membrane potential loss in a dose-dependent manner (Fig. 1E). Apoptosis was further substantiated by activation of caspase-3 and PARP-1 cleavage in HL-60 cells by fascaplysin in a dose-dependent manner (Fig. 1F, G). Several anticancer treatments lead to increased phosphorylation of H2AX due to apoptotic death [Liu et al., 2007]. Fascaplysin also significantly enhanced the expression of γH2AX, a phosphorylated form of histone H2AX, which is associated with DNA fragmentation, or in onset of hurried apoptosis (Fig. 1G).

CELL CYCLE ANALYSIS

Measurement of DNA content makes it possible to identify apoptotic cells and cell cycle phase specificity. Fascaplysin was earlier reported as a potent CDK4 inhibitor, arresting the cell cycle in G1 phase [Soni et al., 2000], but we observed a concentration-dependent rise in hypo-diploid sub-G1 DNA fraction (apoptotic, <2nDNA) in HL-60 cells treated with fascaplysin for 6 h. The sub-G1 fraction was 3% in control cells, which increased to 56% after 1.5 μM of fascaplysin treatment (Fig. 1 H). Sub G1 population has long been associated with apoptosis [Rixe and Fojo, 2007]. Thus, we assumed the role of apoptosis in cell death induced by fascaplysin.

FASCAPLYSIN INDUCED AUTOPHAGY IN HL-60 CELLS

Autophagy is type 2 programmed cell death and is currently one of the most studied fields in cancer biology. Therefore, we decided to check if autophagy has any role in the cell death induced by fascaplysin in HL-60 cells. Interestingly, fascaplysin was able to induce robust autophagy in HL-60 cells. We examined the autophagic potential of fascaplysin through acridine orange staining and found that it significantly increased the number of acridine orange stained cells (red fluorescence) in a dose-dependent manner (Fig. 2A). We also confirmed autophagy induction by immunoblotting of LC3-II expression, which is considered as a hallmark of autophagy [Daido et al., 2004]. Fascaplysin enhanced the expression of LC3-II autophagic protein in a concentration-dependent manner (Fig. 2B). Fascaplysin treated cells exhibited increased expression of beclin (BECN 1) and ATG7 which are considered as key autophagic proteins (Fig. 2B). Autophagy was further corroborated by p62 degradation, also known as sequestosome-1 that targets specific cargoes for autophagy (Fig. 2B). All these data suggested that fascaplysin induced autophagy in HL-60 cells.

FASCAPLYSIN SIMULTANEOUSLY INDUCES THE PROCESS OF AUTOPHAGY AND APOPTOSIS IN HL-60 CELLS

Thus far our results revealed that fascaplysin could substantially induce both autophagy as well as apoptosis. We employed a time dependent experiment to understand the augmentation of autophagy and apoptosis by fascaplysin treatments. Our results showed that fascaplysin simultaneously induced both autophagy as well as apoptosis in HL-60 cells. Mild PARP-1 cleavage was observed in 1 h treatment which increased with increasing time points (Fig. 2C). Similar kind of increase was observed in LC3-II levels (Fig. 2C). These data indicated the role of fascaplysin in simultaneous induction of apoptosis and autophagy.

FASCAPLYSIN INHIBITS THE PI3K/AKT/MTOR SIGNALING PATHWAY

It is well-known that the PI3K/AKT/mTOR pathway plays an important role in cell growth, survival, differentiation and metabolism [Engelman et al., 2006; Hennessy et al., 2007; Courtney et al., 2010]. Inhibition of PI3K/AKT/mTOR signaling pathway causes cell death associated with apoptosis and autophagy [Shrivastava et al., 2011; Wang et al., 2011]. We report here for the first time that fascaplysin assuage PI3K/AKT/mTOR signaling in HL-60 cells. Fascaplysin was found to inhibit all major proteins of this pathway like p110α, pAKT (S473), pAKT (T308), p-mTOR, pP70S6K, raptor and rictor even at 0.5 μM concentrations (Fig. 3A). The degree of inhibition of AKT, and p70S6K expression by fascaplysin was so severe that HL-60 cells completely lost their expression.

FASCAPLYSIN INHIBITS CHRONIC GRANULOMATOUS ANGIOGENESIS AND KEY PROTEINS RELATED TO APOPTOSIS, AUTOPHAGY AND PI3K/AKT/MTOR PATHWAY IN BALB/C MICE GRANULOMATOUS TISSUE

Fascaplysin significantly inhibited all major proteins of PI3K/AKT/mTOR pathway in the murine granuloma tissue lysate. The

Fig. 1. (A) Fascaplysin inhibits HL-60 cell proliferation. HL-60 cells were seeded in 96 well plates, treated with different concentrations of fascaplysin ranging from 0.2–5 μM, for 6 h, 12 h, 24 h and 48 h. MTT was added 3 h before termination of the experiments and the OD was measured at 570 nM. Data are Mean ± SD (n = 8 wells) of three similar experiments. (B,C) Effect of fascaplysin on cellular and nuclear morphology of HL-60 cells. Cells were visualized for cellular and nuclear morphology as described in Materials & Methods. Condensed nuclei and the apoptotic bodies are indicated by white arrows. Data are representative of one of three similar experiments. (D) Flow cytometric analysis of apoptosis and necrosis induced by Fascaplysin. HL-60 cells were stained with Annexin V-FITC and PI to analyze apoptotic and necrotic cell populations. Cells in the lower right quadrant represented apoptosis and in the upper right quadrant indicated post apoptosis (represented by percentage). FACSCan is representative of one of three similar experiments. (E) Fascaplysin induced loss of mitochondrial membrane potential ($\Delta\psi_m$) in HL-60 cells. Cells were incubated with the indicated doses of fascaplysin for 6 h, stained with Rhodamine-123 (200 nM) and analyzed in FL-1 channel of flow cytometer. FACSCan is representative of one of three similar experiments. (F) Fascaplysin induced differential activation of caspase-3 in HL-60 cells. The cells in culture were exposed to 05–1.5 μM of fascaplysin for 6 h. The caspase-3 activities were determined fluorometrically in the cell lysate of HL-60 cells using BD ApoAlert caspase-3 assay kits, as per the instructions provided by the supplier. Data are Mean ± S.D. from three similar experiments. *P* values: -* <0.001 was considered to be significant when compared with untreated control. (G) Influence of fascaplysin on key apoptotic proteins in HL-60 cells. The cells (2×10^6) were treated with 05–1.5 μM of fascaplysin for 6 h. Cell lysates were prepared and equal amount of protein (80 μg) was loaded on SDS-PAGE gel for Western blot analysis as described in Materials and Methods. Data are representative of one of three similar experiments. Specific antibodies were used for detection of caspase-3, PARP-1 and γH2AX. (H) DNA cell cycle analyses in HL-60 cells exposed to fascaplysin. HL-60 cells (1×10^6) were treated with indicated concentrations of fascaplysin for 6 h. After treatment, cells were stained with propidium iodide, PI (10 μg/ml) to determine DNA fluorescence and cell cycle phase distribution as described in Materials and Methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. The fraction of cells from apoptosis, G1, S and G2 phases analyzed from FL2- A vs. cell counts are shown in %. Data are representative of one of three similar experiments.

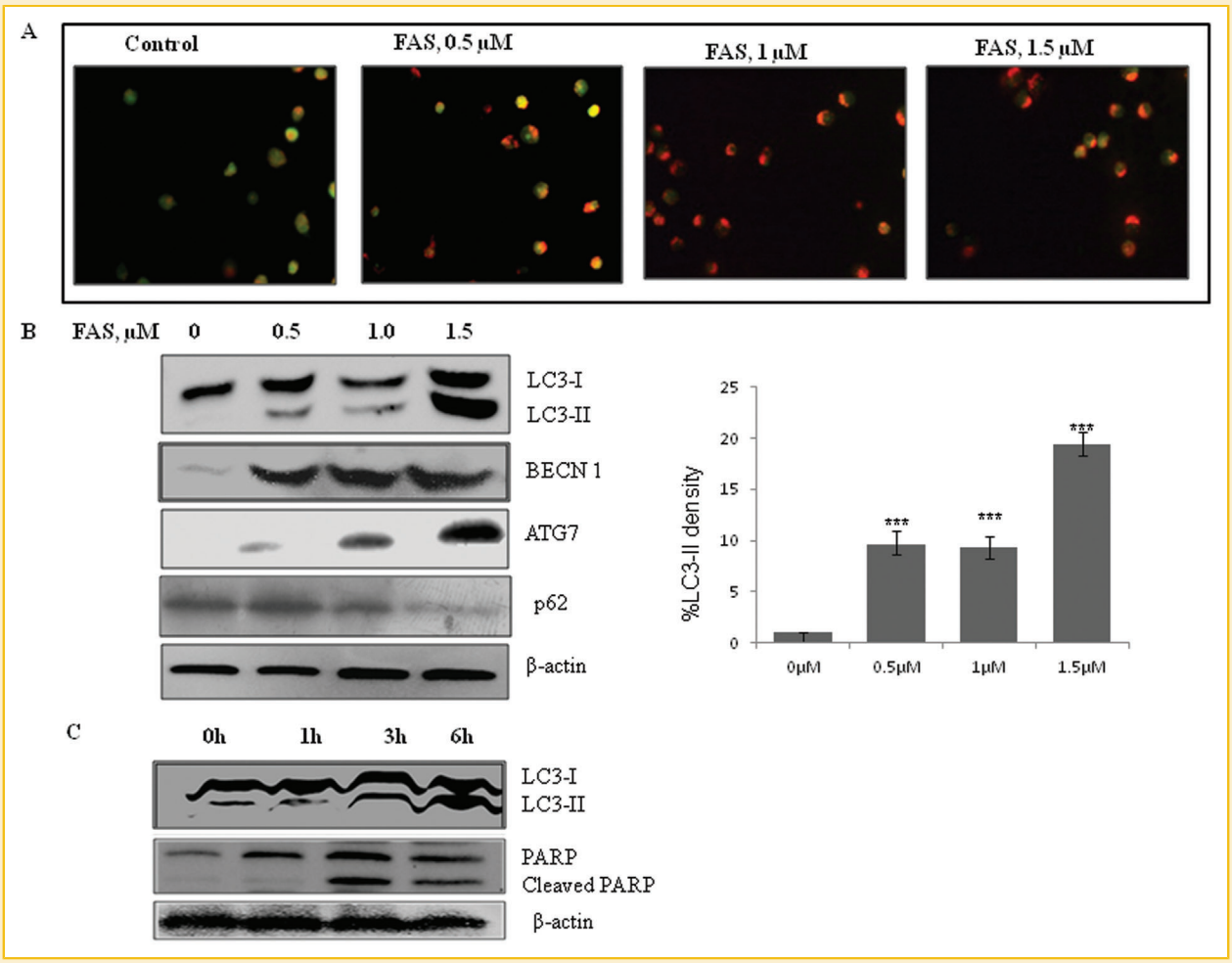

Fig. 2. Fascaplysin induces autophagy in HL-60 cells. (A) HL-60 cells were incubated with different doses of fascaplysin for 6 h. Acridine orange (1 μg/ml) was added 15 min before terminations of the experiment. Cells were collected at 400 g, washed once with PBS and observed under a fluorescence microscope for autophagosome formation. Red fluorescence represents autophagosome formation. (B,C) Cells were treated with 0.5 –1.5 μM of fascaplysin for 6 h and lysed with RIPA buffer and western blotting for the indicated proteins were done as described in Materials and Methods. β-actin was used as an internal control. Densitometry of LC3-II was done using image J software from NIH. Data are representative of one of three similar experiments. Data are mean ± SD of three similar experiments; statistical analysis was done by using bonferroni method and *P* value<0.05 was considered to be significant with ***$P<0.001$, **$P<0.01$, *$P<0.05$.

expression of p110α, p-AKT, p-mTOR and p-p70S6K was completely lost at 10 mpk dose of fascaplysin, which was equivalent to reference compound RAD001 (Fig. 3B). This suggests that fascaplysin is a potent inhibitor of the PI3K/AKT/mTOR pathway by in vitro and in vivo mode of administration. Further, fascaplysin induced in vivo apoptosis via inducing PARP-1cleavage and activating γH2AX in granuloma tissue lysate samples of BALB/c mice (Fig. 3B). Fascaplysin also induced in vivo autophagy as evident by significant increase in LC3-II expression in murine granuloma tissue lysates (Fig. 3B). All these data suggested that fascaplysin induced autophagy and apoptosis by inhibiting PI3K/AKT pathway.

The murine air pouch granulomatous model of chronic inflammation has been used for the profound angiogenic component. The air pouch granulomas were evaluated for weight, histology and vascular index, which were used to assess the rate and extent of angiogenesis. Fascaplysin (5 mpk and 10 mpk, oral) significantly reduced (up to 50%) the granuloma weight, volume and vascular index on day 11 (Fig. 3D, E). Interestingly, we found that fascaplysin treated BALB/c mice did not show any toxic effects as evidenced by no significant loss of body weight and mortality (Fig. 3E). Fascaplysin also caused a dose-dependent reduction in angiogenesis as measured by the degree of vascularization of the granuloma tissue histopathology (Fig. 3F).

FASCAPLYSIN INDUCES PI3K/AKT DEPENDANT AUTOPHAGY AND APOPTOSIS

From our earlier experiments we believed that fascaplysin induced autophagy and apoptosis through the inhibition of the PI3K/AKT signaling pathway as reported earlier [Hennessy et al., 2007]. We activated PI3K/AKT signaling by insulin a known activator of this pathway [Kumar et al., 2013]. Insulin pretreatment dramatically reversed the inhibitory effect of fascaplysin on viability (Fig S1).

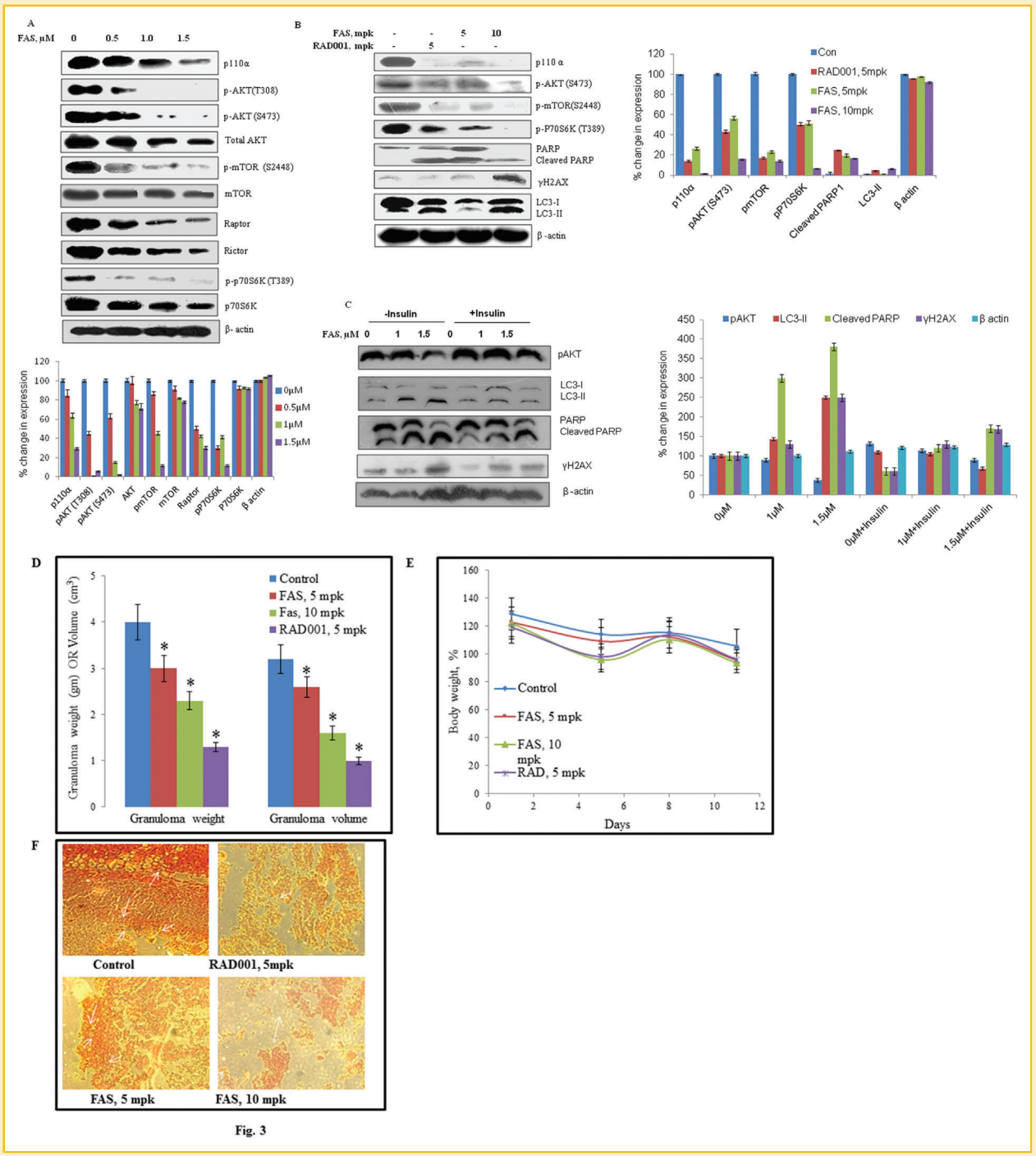

Fig. 3

Fig. 3. (A) Fascaplysin attenuates PI3K/AKT/mTOR signaling in HL-60 cells. HL-60 cells (2×10^6) were incubated with indicated doses of fascaplysin for 6 h, after completion of treatment time cells were collected at 400 g, washed once with PBS and lysates were prepared by using RIPA buffer. Proteins were separated by SDS-PAGE and were transferred to PVDF membranes. Western blots for indicated proteins were done as described in material and method section. Specific antibodies were used for detection of indicated proteins and β-actin was used as an internal control. Data are representative of one of three similar experiments. (B) Fascaplysin inhibits key proteins of apoptosis, autophagy and PI3K/AKT/mTOR pathway in the murine granuloma tissue lysate. Granulomatous tissue was homogenized by using dounce homogenizer (Sigma) in RIPA buffer and immunoblotting of proteins was performed as described in Materials and Methods. Data are representative of one of three similar experiments. (C) Insulin reversed autophagy and apoptosis in HL-60 cells. HL-60 cells (2×10^6) were pretreated with 200 nm of insulin 30 min before treatment with the indicated doses of fascaplysin for 6 h, after completion of treatment time cells were collected at 400 g, washed once with PBS and lysates were prepared by using RIPA buffer. Proteins were separated by SDS-PAGE and were transferred to PVDF membranes. Blots for indicating proteins were done as described in Materials and Method section. Specific antibodies were used for detection of indicated proteins and β-actin was used as an internal control. Data are representative of one of three similar experiments.

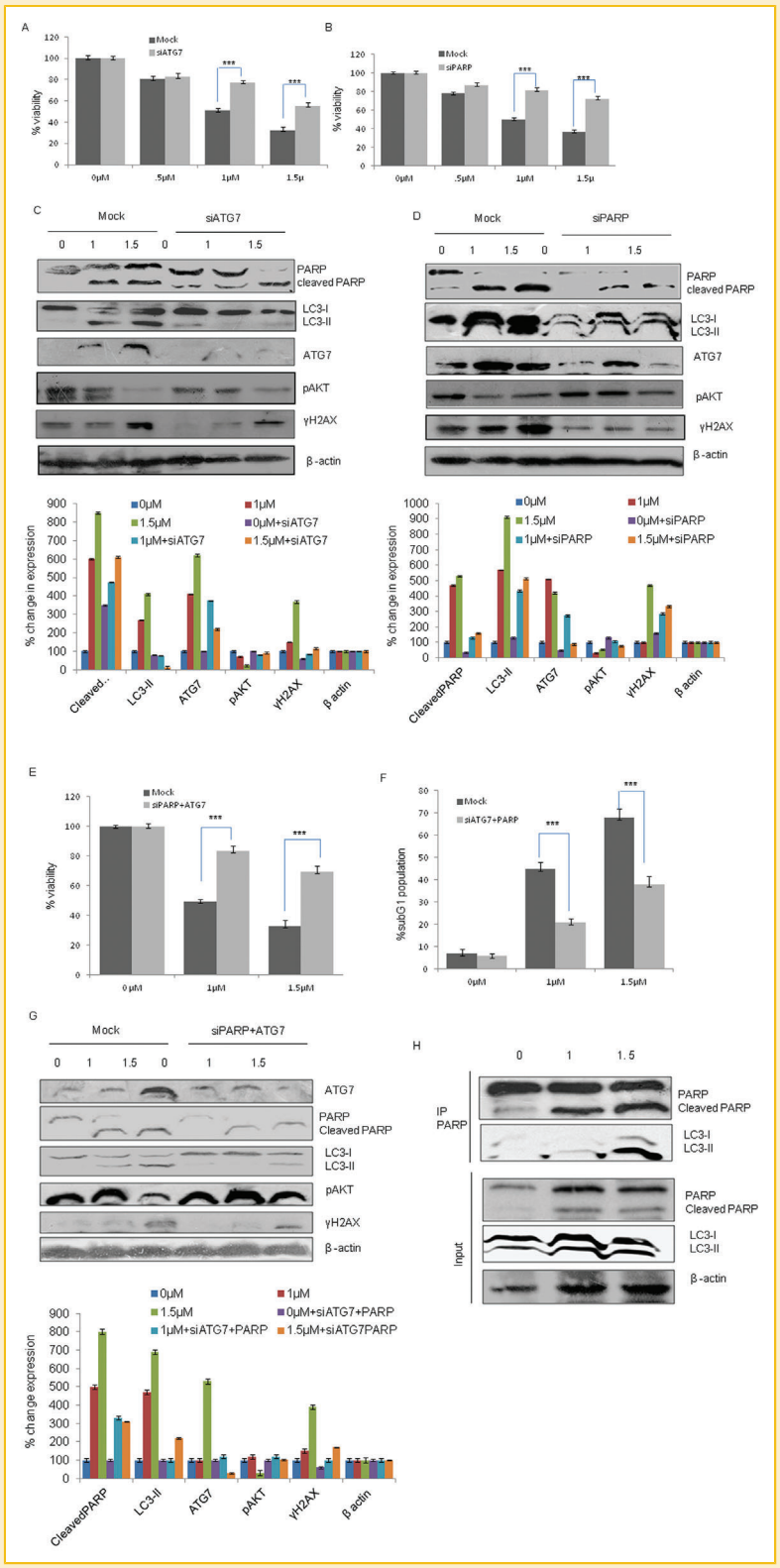

Fig. 4. Continued.

Furthermore, the expression of LC3-II and cleaved PARP-1 was reduced in insulin pretreated samples as compared to samples treated with fascaplysin alone (Fig. 3C). Interestingly the expression of γH2AX was also decreased in insulin pretreated samples as compared with the samples treated with fascaplysin alone (Fig. 3C). This experiment confirmed the partial dependence of autophagy and apoptosis induced by fascaplysin on inhibition of PI3K/AKT pathway.

AUTOPHAGY AND APOPTOSIS ARE INTERDEPENDENT IN CELL DEATH INDUCED BY FASCAPLYSIN

From previous experiments we believed that PARP-1 cleavage and activation of ATG7 might play role in apoptosis and autophagy induced by fascaplysin, as significant PARP-1 cleavage and ATG7 activation was found right from the lowest dose used (Fig 1G and 2B). Therefore, we decided to rule out the possible role of these proteins in autophagy and apoptosis induced by fascaplysin. To find this we silenced these two proteins using siRNAs specific to PARP-1and ATG7. Interestingly, silencing of these proteins improved the viability to significant levels. In ATG7 silenced samples the viability was improved from 49% to 77% and from 32% to 55% percent in 1 μM and 1.5 μM treated samples (Fig. 4A). Similar kind of improvement in viability was observed in samples silenced with PARP-1 siRNA (Fig. 4B). The protective effect of PARP-1and ATG7 silencing was further observed through phase contrast microscopy. A very significant effect was observed on the morphology of the normal cells treated with fascaplysin, while the effect of fascaplysin seemed to be reversed by inhibition ATG7 and PARP-1 silenced samples (Fig S2). Sub G1 population in the cell cycle phase distribution is considered as apoptotic population. We also observed the protective effect of ATG7 and PARP-1 silencing on the sub G1 phase of the cell cycle. The apoptotic population was significantly reversed in ATG7 and PARP-1 silenced samples. The sub G1 population was decreased from 48% to 23% and 37% in PARP-1 and ATG7silenced samples, respectively with 1 μM treatment of fascaplysin and from 66% to 52% and 47% respectively in ATG7 and PARP-1 silenced samples with 1.5 μM treatment (Fig S3). Furthermore, the expression of important apoptotic protein such as PARP-1 cleavage and γH2AX along with autophagic marker LC3-II was reversed in ATG7 silenced cells (Fig. 4C), interestingly, the inhibition of pAKT was reversed in ATG7 silenced samples, suggesting the role of ATG7 in autophagy as well as apoptosis. Similar kind of results was obtained in PARP-1 silenced samples, the expression of LC3-II was reduced significantly along with the expression of γH2AX and ATG7. Also the inhibition of pAKT was reversed in PARP-1 silenced samples (Fig. 4D). Therefore, we concluded that autophagy and apoptosis might be interdependent in cell death induced by fascaplysin.

PARP-1 AND ATG7 ARE REQUIRED FOR AUTOPHAGY AND APOPTOSIS INDUCED BY FASCAPLYSIN IN HL-60 CELLS

From our previous experiment we observed the role of ATG7 and PARP-1 in apoptosis and autophagy induced by fascaplysin. Therefore, we decided to inhibit both of these proteins simultaneously. Interestingly, combined knockdown of these proteins dramatically reversed the inhibitory effect of fascaplysin on viability. The viability was increased from 53% to 84% and from 37% to 70% in samples treated with 1 μM and 1.5 μM of fascaplysin in combined ATG7 and PARP-1 silenced samples (Fig. 4E). The protective effect on cell morphology was observed through microscopy. ATG7 and PARP-1 silenced samples displayed better and intact morphology than the normal cells treated in a similar way with fascaplysin (Fig S4). A further effect of combined knockdown of these proteins was observed in the sub G1 phase of cell cycle, the sub G1 population in ATG7 and PARP-1 silenced samples was decreased from 45% to 21% and from 65% to 38% in 1 μM and 1.5 μM treated samples respectively. Furthermore, western blot analysis also displayed the similar kind of effects; the inhibitory effect of pAKT was significantly reversed in ATG7 and PARP-1 silenced samples, suggesting the role of ATG7 and PARP-1 in the regulation of pAKT (Fig. 4G). Also the expression of autophagic protein LC3-II and apoptotic protein γH2AX was reversed in ATG7 and PARP-1 silenced samples (Fig. 4G). These results suggested that PARP-1 and ATG7 are required for autophagy and apoptosis induced by fascaplysin in HL-60 cells.

Fig. 4. ATG7 and PARP-1 play important role in fascaplysin induced autophagy and apoptosis. (A,B) Inhibition of PARP-1 and ATG7 reversed the inhibitory effect of fascaplysin on viability. HL-60 cells were transfected with ATG7 and PARP-1 siRNA as described in materials and methods section. After transfection, siRNA transfected and non transfected cells were seeded in 96 well plates and viability was determined through MTT assay as described in Materials and Methods sections. Data are mean ± SD of three similar experiments; statistical analysis was done by using bonferroni method and p value <0.05 was considered to be significant with $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. (C,D) Autophagy and apoptosis induced by fascaplysin are interdependent. HL-60 cells were transfected with ATG7 and PARP-1 siRNA as described in Materials and Methods section. siRNA transfected and non transfected cells were seeded in six well plates and treated with the indicated doses of fascaplysin for 6 h. After completion of treatment time cells were collected at 400 g, washed once with PBS and lysates were prepared by using RIPA buffer. Proteins were separated by SDS-PAGE and were transferred to PVDF membranes. Blots for indicating proteins were done as described in material and method section. Specific antibodies were used for detection of indicated proteins and β-actin was used as an internal control. (E) Combined knockdown of PARP-1 and ATG7 reversed the inhibitory effect of fascaplysin on viability. HL-60 cells were transfected with ATg7 and PARP-1 siRNA simultaneously as described in Materials and Methods. Cell viability was determined through MTT assay as described in Materials and Methods. Data are mean ± SD of three similar experiments; statistical analysis was done by using bonferroni method and P value <0.05 was considered to be significant with $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. (F) PARP-1 and ATG7 are responsible for apoptosis induced by fascaplysin in HL-60 cells. HL-60 cells were transfected with ATG7 and PARP-1 siRNA simultaneously as described in materials and methods. Cell cycle analysis was done as described in Materials and Methods. Bar diagram represents the sub G1 population. Data are mean ± SD of three similar experiments; statistical analysis was done by using bonferroni method and P value <0.05 was considered to be significant with $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. (G) siRNA transfected and non transfected cells were treated with indicated concentrations of fascaplysin for 6 h. Western blotting for indicated proteins was done as described in earlier experiments. (H) Fascaplysin induced the interaction between apoptosis and autophagy. Cells were treated with indicated concentrations of fascaplysin for 6 h and immunoprecipitated with PARP-1 antibody as described in Materials and Methods section. Western blots for indicated proteins were done as described above.

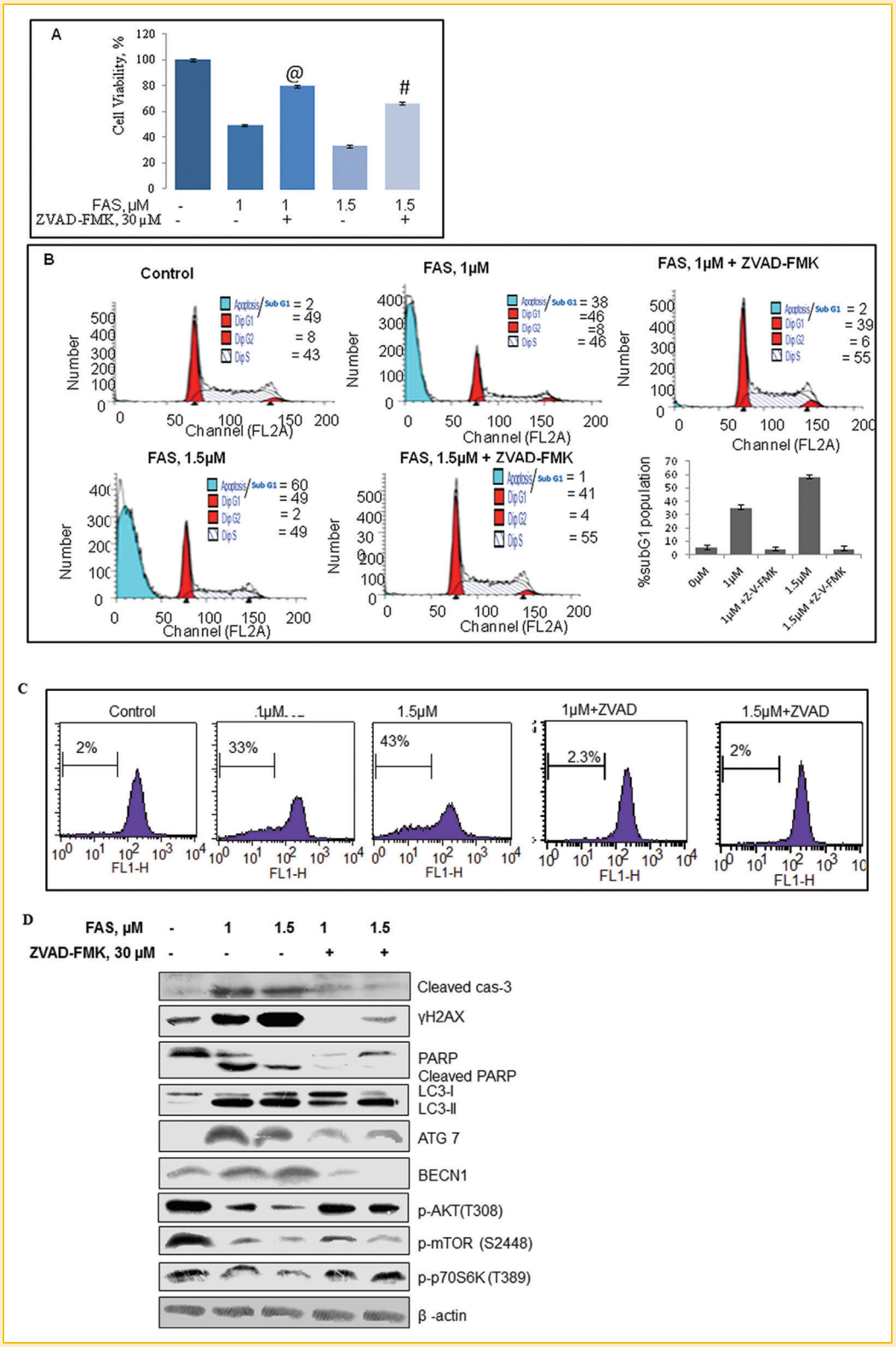

Fig. 5. Continued.

FASCAPLYSIN MEDIATE CO –OPERATIVE INTERACTION BETWEEN AUTOPHAGIC AND APOPTOTIC PATHWAY

In order to understand the mechanism of co-operative signaling between autophagy and apoptosis in fascaplysin mediated cell death, we employed an independent assay involving the Co immunoprecipitation of PARP-1 with LC3 in fascaplysin treated cells. The results showed positive association between PARP-1 cleavage and LC3-II in fascaplysin treated cells (Fig 4 H). This association between PARP-1 and LC3-II depict that apoptosis and autophagy work as partners in cell death induced by fascaplysin in HL-60 cells.

CASPASE-3 IS THE MAIN PLAYER IN AUTOPHAGY AND APOPTOSIS INDUCED BY FASCAPLYSIN

As observed from earlier experiments, caspase-3 is activated by fascaplysin. Moreover, inhibition of PARP-1, which is a downstream effecter of caspase-3, partially protected the cell from fascaplysin induced autophagy and apoptosis. Therefore, we decided to see the role of caspase inhibitor on fascaplysin induced autophagy and apoptosis. Fascinatingly, to our surprise, all the cell death related events induced by fascaplysin in HL-60 were completely reverted back by a universal caspase inhibitor Z-VAD-FMK (Fig. 5A-D). Initially we checked the apoptotic parameters such as sub G1 population in cell cycle and loss of mitochondrial membrane potential and observed the complete reversal of sub G1 phase and MMP loss in samples pretreated with Z-VAD-FMK before treatment with fascaplysin. Interestingly, the expression of γH2AX was also reversed in FMK pretreated samples (Fig. 5B-D). Therefore, we concluded that fascaplysin induced caspase dependent apoptosis. Further, we checked the effect of caspase inhibitor on autophagy and found that Z-VAD-FMK was able to reverse the effect of fascaplysin on the induction of autophagy as the expression of key autophagic proteins LC3-II, ATG7 and BECN1 was significantly reversed in the samples pretreated with caspase inhibitor. Surprisingly, the inhibitory effect of fascaplysin on the expression of all major proteins of AKT pathway like pAKT, p-mTOR, and p70S6K was also reversed by Z-VAD-FMK (Fig. 5D). All these events suggest the role of caspases in the autophagy and apoptosis induced by fascaplysin in HL-60 cells.

DISCUSSION

There are several anticancer agents used clinically and still novel one need to be explored from infinite and hidden resources, like marine. In this study, we have explored the anti-cancer potential of marine indole alkaloid, fascaplysin. Previous studies had shown the apoptotic potential of fascaplysin in human cervical cancer HeLa cells [Lu et al., 2009; Yan et al., 2011], but the molecular mechanism behind the cell death remained unexplored. Here, for the first time, we explored the autophagic potential of fascaplysin along with the molecular mechanism of the cell death induced by fascaplysin in HL-60 cells. We also report for the first time that fascaplysin potentially inhibited PI3K/AKT/mTOR pathway. Fascaplysin inhibited proliferation of human promyelocytic leukemia HL-60 cells through simultaneous induction of autophagy and apoptosis. The early event which was responsible for apoptosis, found to be loss of mitochondrial membrane potential that lead to formation of apoptosome and finally activation of caspase-3 and PARP-1 cleavage. Simultaneous induction of autophagy was seen through acridine orange staining the expression of key autophagic proteins. Fascaplysin was earlier reported for CDK4 inhibition and cell cycle arrest at G1 phase [Soni et al., 2000], but we are first time reporting that it induces sub-G1 arrest in HL-60 cells, which is rather novel and contradictory to earlier reports [Rixe and Fojo, 2007]. However, the CDK4 inhibitory activity of CDK4 has not been reported in HL-60 cells. Therefore, it is evident that the Antiproliferative activities of fascaplysin may vary from one cancer to another.

Importantly, our study is the first to show the effect of fascaplysin on inhibition of PI3K/AKT/mTOR pathway and it was inhibiting all major proteins of this pathway. Expression of p110α was strongly diminished as well as pAKT (T 308), pAKT (S473), mTOR and p70S6K and was abrogated completely. We found that it consistently condenses signaling from mTOR, a serine/threonine kinase stands in a central position on the crossroad of various cell signal pathways including autophagy [Hay and Sonenberg, 2004; Liu et al., 2008]. Inhibition of mTOR is frequently associated with induction of autophagy via ATG regulation [He and Klionsky, 2009]. We also report here for the first time that fascaplysin induces autophagy, a type-II programmed cell death, in HL-60 cells. We showed autophagic induction by acridine orange staining and through induction of important autophagic proteins such as LC3-II, ATG7, BECN 1 and p62 degradation. All these proteins play key role in autophagosome formation [Bursch, 2001; Levine and Klionsky, 2004]. More importantly, fascaplysin inhibited key proteins of PI3K/AKT/mTOR pathway and induced the expression of autophagic protein LC3-II along with PARP-1 cleavage and γH2AX in murine granuloma tissue, which show its potential as both in vitro and in vivo mode of action. There are several reports that confirm the

Fig. 5. Fascaplysin induces caspase dependent apoptosis, autophagy and inhibition of PI3K/AKT/mTOR pathway. (A) Fascaplysin inhibits caspase mediated cell death. HL-60 cells were seeded in 96 well plates and treated with the indicated doses of fascaplysin along with or without caspase inhibitor, Z-V-FMK (20μM), for 48 h. MTT dye was added 3 h prior to termination of the experiment; cell viability was calculated as described in Materials and Methods. Data are Mean ± SD ($n = 8$ wells) of three similar experiments. *P*-values:-$^{@}<0.001$ or $^{\#}<0.001$ considered significant when compared to fascaplysin 1 μM and 1.5 μM treated cells, respectively. (B) Fascaplysin induces caspase dependent sub-G1 arrest. HL-60 cells (1×10^6) were treated with indicated concentrations of Fascaplysin for 6 h with or without caspase inhibitor, Z-V-FMK (20 μM). Cells were stained with propidium iodide, PI (10 μg/ml) to measure the cell cycle phase distribution as described in Materials and Methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) and representative of one of three similar experiments. (C) ZVAD-FMK reversed MMP loss induced by fascaplysin in HL-60 cells. HL-60 cells were pretreated with 30 μM of Z-VAD-FMK before treatment with fascaplysin for 6 h. MMP loss was measured as described in materials and methods. (D) Fascaplysin induces apoptosis, autophagy and inhibition of the PI3K/AKT/mTOR pathway via caspases. HL-60 cells (2×10^6) were incubated with fascaplysin in the presence and absence of caspase inhibitor, for 6 h. Immunoblotting of key proteins related to autophagy, apoptosis and PI3K pathway was performed as described in Materials and Methods. Specific antibodies were used for detection of indicated proteins and β-actin was used as an internal control. Data are representative of one of three similar experiments.

regulation of autophagy and apoptosis by the PI3K/AKT/mTOR pathway [Yu et al., 2004; Hennessy et al., 2005; Wu et al., 2008; Sun and Peng, 2009]. Additionally, AKT activation through known inducer insulin inhibited the fascaplysin induced expression of LC3-II and PARP-1 cleavage suggesting the role of the AKT pathway in autophagy and apoptosis induced by fascaplysin.

Furthermore, to explore the mode of cell death induced by fascaplysin we silenced key autophagic (ATG7) and apoptotic (PARP-1) proteins through siRNA. We found that inhibition of each of these proteins partially blocks fascaplysin induced autophagy as well as apoptosis in HL-60 cells. PARP-1 and ATG7 silenced cells showed significant improvement in the viability when compared with normal HL-60 cells treated with fascaplysin. Furthermore the expression of key apoptotic and autophagic proteins was reversed in both PARP-1 and ATG7 silenced sample. Interestingly, combined knockdown of PARP-1 and ATG7 improved the viability more significantly as compared with individual silencing of each of these proteins. Also, the expression of key autophagic and apoptotic proteins was inhibited in the silenced samples.

Further, we found that fascaplysin induced autophagic and apoptotic signaling converges through the activation of caspase-3. Caspase-3 has been found to have regulatory role in both autophagy and apoptosis, Inhibition of caspase-3 through chemical inhibitor also reverses autophagy [Nan Zhang et al., 2011; Kumar et al., 2013, 2014]. Our results showed augmentation of mitochondrial membrane potential loss along with the DNA damage and autophagy by fascaplysin treatment in HL-60 cells. Caspase -3 has also been demonstrated to have a regulatory role on mitochondrial membrane potential loss along with the autophagy and DNA damage [Kim et al., 2008; Kumar et al., 2013; 2014]. Concomitant with these reports, our data demonstrated that universal caspase inhibitor Z-VAD-FMK reversed the entire key events associated with fascaplysin mediated cell death in HL-60 cells. Caspase inhibitor reversed cell growth inhibition, sub G1 arrest, MMP loss and expression of autophagic, apoptotic and important proteins of the PI3K/AKT/mTOR signaling pathway. These findings put forward the key role of caspases in the induction of cell death via autophagic and apoptotic pathways. There are a number of reports suggesting that PI3K/AKT/mTOR pathway controls the activity of caspase-3 and PARP-1 [Kim et al., 2007; Liu et al., 2007; Jeong et al., 2008] however, there are very few cases where caspase inhibition can activate the expression of this particular pathway [Soares et al., 2009]. In conclusion fascaplysin induces caspase dependent apoptosis and autophagy by targeting the PI3K/AKT/mTOR pathway in both in vitro and in vivo models. Hence, our discovery of this novel mechanism of fascaplysin not only gives further insights into its anti-cancer potential, but also contributes the role of marine natural products in drug discovery and development.

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