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Pristimerin mediated anticancer effects and sensitization of human skin cancer cells through modulation of MAPK signaling pathways

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ABSTRACT

Squamous cell carcinoma is a frequent skin cancer still demanding to understand the underlying mechanisms for better clinical outcomes. Pristimerin, a natural quinonemethide triterpenoid, has shown promising therapeutic outcome due to its anti-cancer activity and multi-targeting potential. We explored the underlying mechanisms of pristimerin-induced programmed cell death of primary (A431) and metastatic (A388) cutaneous squamous cell carcinoma (cSCC) cells. Our results show that pristimerin inhibits growth and proliferation of cSCC through JNK activation. Moreover, pristimerin causes cell cycle arrest and induces cell death via apoptosis and autophagy. Interestingly, use of apoptosis (z-VAD-FMK) and autophagy (3-methyladenine) inhibitors confirmed vital role of programmed cell death in pristimerin-mediated anti-cancer actions. JNK inhibitor, SP600125, also mitigated pristimerin-induced apoptotic and autophagic actions. Moreover, pristimerin-mediated anti-cancer activity acts by generating reactive oxygen species (ROS) thereby inducing JNK signaling. Use of N-acetyl cystine (NAC), a universal ROS scavenger, significantly reversed pristimerin-induced programmed cell death through down-regulation of JNK. Pristimerin sensitized skin cancer cells to conventional anticancer drugs cisplatin, azacytidine and doxorubicin through JNK activation, as confirmed by SP600125. Our results indicate that pristimerin mediates programmed cell death and sensitized skin cancer cells to conventional anti-cancer drugs via ROS-mediated JNK activation.

1. Introduction

Skin cancer is one of the most common human malignancies with increasing morbidity and mortality, posing huge socioeconomic burden to patients and family [1], [2]. Albeit melanoma is the most aggressive type of skin cancer, non-melanoma skin cancers (NMSC) such as basal cell carcinoma and squamous cell carcinoma, are the most frequent among all malign tumors world-wide [3,4].

Accumulating data on the anti-cancer properties of natural products has markedly increased due to their suppressive effects on proliferation, and induction of apoptosis and autophagy [5–8].

Along this line, we aimed to explore the underlying mechanisms of pristimerin-mediated anticancer actions against human cutaneous squamous cell carcinom (cSCC) cells. Pristimerin has potent antioxidant, anti-inflammatory, anti-microbial and anti-cancer potential against various human malignancies [9–12]. Pristimerin also showed promising

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outcomes in sensitizing cancer cells to other therapeutic agents, suppressing cancer stemness features such as recurrence and drug resistance [11,13–15].

It is well known that reactive oxygen species (ROS) are vital in cell survival and biological homeostasis, its levels being tightly controlled by various cellular and biochemical steps in the human body [16,17]. In disease state, the level of ROS increases due to deregulation of their regulatory mechanisms [18–21]. Usually cancer cells harbor elevated ROS levels thereby playing a significant role deregulating intracellular cell signaling pathways including MAP Kinases (MAPK) and a range of other vital regulatory mechanisms converging towards cancer hallmarks [21,22]. Hence, ROS can be of great clinical importance both in the characterization and elimination of cancer cells [23].

A range of natural and conventional therapeutic cancer drugs produce ROS to target cancer cells. Hence, in the current study we explored the crucial role of ROS in skin cancer pathogenesis and drug resistance in two different squamous cell carcinoma cell lines [8,23,24]. For the first time we demonstrate the underlying mechanisms associated with pristimerin-induced anti-cancer activity in cSCC. We have assessed morphological, cellular, and molecular changes to highlight the potential role of ROS in pristimerin-induced anti-cancer actions in skin cancer cells in vitro. Our findings show that pristimerin inhibits growth and stemness features of skin cancer cells through programmed cells death via ROS- mediated activation of the MAPK pathway.

2. Material and methods

2.1. Chemicals and Reagents

Pristimerin, cisplatin, doxorubicin, azacytidine, 3-methyladenine, NAC (N-Acetyl-L-cysteine), and other high grate reagents were purchased from Sigma Aldrich (St. Louis, Missouri, United States). VAD (Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) was purchased from SelleckChem. Antibodies such as p-p38,P38,p-p44/42, p-44, p-JNK, JNK, actin, p21, caspase3, cleaved caspase3, cleaved caspase-8, PARP, LC3A/,B p62, p-H2AX, HSP60, GAPDH, etc., were procured from Cell Signaling Technologies (3 Trask Lane, Danvers, MA, USA) and Cruz Biotechnology, Inc. (Finnell Street Dallas, Texas, USA). Laemmli Sample buffer 1X, resolving Gel Buffer, acrylamide/Bis solution, stacking gel solution, developer kit (Clarity Western ECL) purchased from BIO-RAD (Hercules, California, USA). E-plates for RTCA are purchased from Roche, (San Diego, CA, USA).

2.2. Cell Culture

Human cutaneious squamous cell carcinoma cell lines (cSCC), A431 (primary) and A388 (metastatic) were procured from American Type Culture Collection (ATCC),10801 University Boulevard Manassas, VA 20110 USA. Cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 ^{0}C in a humidified atmosphere containing 5% CO2. Treatments were done in 5% DMEM medium.

2.3. Cell Counting Kit-8 (CCK-8) assay

The anti-proliferative effect of pristimerin, anticancer drugs and inhibitors against cSCC cells was performed by using Cell Counting Kit-8 (CCK-8) assay as described earlier [25]. Briefly, we plated 10^4 cells in each well in a 96-well plate and then incubate at 37 C^{o} / 5% Co2. After 24-hour, cells ware treated with pristimerin alone and in combination with anticancer drugs, and pharmacological inhibitors. After 24 h, CCK-8 solution was added as per the manufacturer's instruction followed by incubation at 37 C^{o} / 5% Co2. Finally, the optical density (OD) was taken at 450 nm. The percent cell viability was calculated as OD of the experiment samples / OD of the control sample x 100. Blank is used to normalize the of treatment conditions including control.

2.4. Real time cell analyzer (RTCA) analysis

To explore the effect of pristimerin on the proliferation of primary and metastatic skin cancer cells in real time, we used real time cell analyzer (RCA). To do so, we first plated specific number of cells using E-Plate VIEW 16 cells in the form of monolayer on top of the electrodes as reported earlier [25]. Next day, cells were treated with different concentrations of pristimerin. The real time cell analyzer and E-Plate VIEW 16 (RTCA; xCELLigence, Roche, San Diego, CA, USA) were used to determine the cell index of pristimerin treated and untreated cells.

2.5. Annexin V staining

A431 and A388 cells were treated with pristimerin alone and in combination with NAC and or anti-cancer drugs followed by 24-hour incubation. Next cells were harvested, washed with PBS, and then stained with flourescein-conjugated Annexin V and Propidium Iodide. Finally, apoptosis was measured by flow cytometry (BD LSRFortessa analyzer, BD Biosciences).

2.6. Measurement of mitochondrial membrane potential

A431 and A388 cells were treated with pristimerin alone and in combination with NAC and or anti-cancer drugs. After the treatment, cells were harvested, washed, and finally stained with JC1 stain kit as per the manufacturer's instruction and then analyzed using flow cytometry (BD LSR Fortessa analyzer, BD Biosciences, USA) as described previously.

2.7. Determination of reactive oxygen species

Skin cancer cells (A431 and A388) were treated with pristimerin alone and in combination with NAC and or anti-cancer drugs. Then cells were washed using HBSS and stained with CellROX ™ Green Reagent (10 μM) for 30 min at 37 ∘C, to quantify level of ROS using flow cytometry (BD LSR Fortessa analyzer, BD Biosciences, USA).

2.8. Live/dead assay

Skin cancer cells (A431 and A388) were treated with pristimerin alone and in combination with NAC and or anti-cancer drugs or inhibitors. Live/dead stain were prepared by adding 20 μL of ethidium homodimer-1 (EthD-1), and 5 μL calcein AM to 10 ml of DPBS and cells were stained as per the manufacturer's instructions. Finally, cells were visualized, and images were captured using EVOS FLc cell imaging system from Invitrogen (Thermo Fisher Scientific).

2.9. Cell Lysis and immunoblotting

To find out the modulatory effect of pristimerin and to explore the underlying mechanisms cells were treated with pristimerin, anticancer drugs, inhibitors, and pharmacological agents. Next, the cells were lysed and immunoblotted with antibodies using as described earlier [25,26]. In brief, cell lysate was separated using SDS-PAGE and proteins were transferred using polyvinylidene difluoride (PVDF) membrane. Next after blocking, membrane blots were incubated with primary antibodies at 4° C for overnight. Next morning blots were washed and incubated with secondary antibodies and finally after washing blots were visualized under a Chemi-Doc System by using ECL substrate (Bio-Rad, Hercules, California, USA).

2.10. Tumorsphere culture

Skin cancer cell spheroids form A431 and A388 cell lines were generated in the lab in the form of spheres based on previous studies [25,26]. At first, NMSC cells were cultured in 10 % DMEM media. After that,

the cells are subjected to an enzymatic treatment to make single cell suspension, using trypsin. Next, NMSC cells were cultured and treated in ultralow attachment plates (Corning, USA) in complete cancer stem cell medium (3D Tumorsphere Medium XF, Promo Cell, Germany, C-28070) in a humidified chamber containing 5 % CO_2 at 37 °C. After the growth for 7 days, spheres were photographed using an EVOS FLc cell imaging system from Invitrogen (Thermo Fisher Scientific) at a magnification of 4 × (scale bar 1000 μm).

2.11. Statistical analysis

Data from various experimental group is presented as mean±S.D. Statistical calculations (Anova, Student's t-test) were performed by using Graph pad prism (GraphPad Software Inc., San Diego, CA, http://www.graphpad.com). Values of * $p < 0.05$ were considered statistically significant.

3. Results

3.1. Pristimerin inhibits cell growth and proliferation, and induces cell cycle arrest in cSCC cells

To investigate the antiproliferative effects of pristimerin on human squamous cell carcinoma, A431 (primary) and metastatic (A388) cells were treated with indicated (see figure legends) concentrations of pristimerin for 24 h, followed by CCK-8 based cell proliferation assay. Pristimerin dose-dependently inhibited cell viability and proliferation in both cancer cell types (Fig. 1A, 1C). Next, we investigated anti-proliferative effect of pristimerin in *real time* using RTCA to further support the strong anti-proliferative action of pristimerin (Figs. 1B, 1D). Morphological cell analysis also supported anti-proliferative action of pristimerin (Figs. 1E, 1F). Moreover, live, and dead cell staining reveals that pristimerin treatment caused several cellular alterations including nucleic acid condensation and membrane damage in A431 (Fig. 1G) and A388 (Fig. 1H) cells. Pristimerin also inhibited colony formation of cSCCs further supporting its effective anti-neoplastic action against primary (Fig. 1I) and metastatic (Fig. 1J) skin cancer cells in vitro. Next, we determined whether pristimerin-mediated inhibition of skin cancer

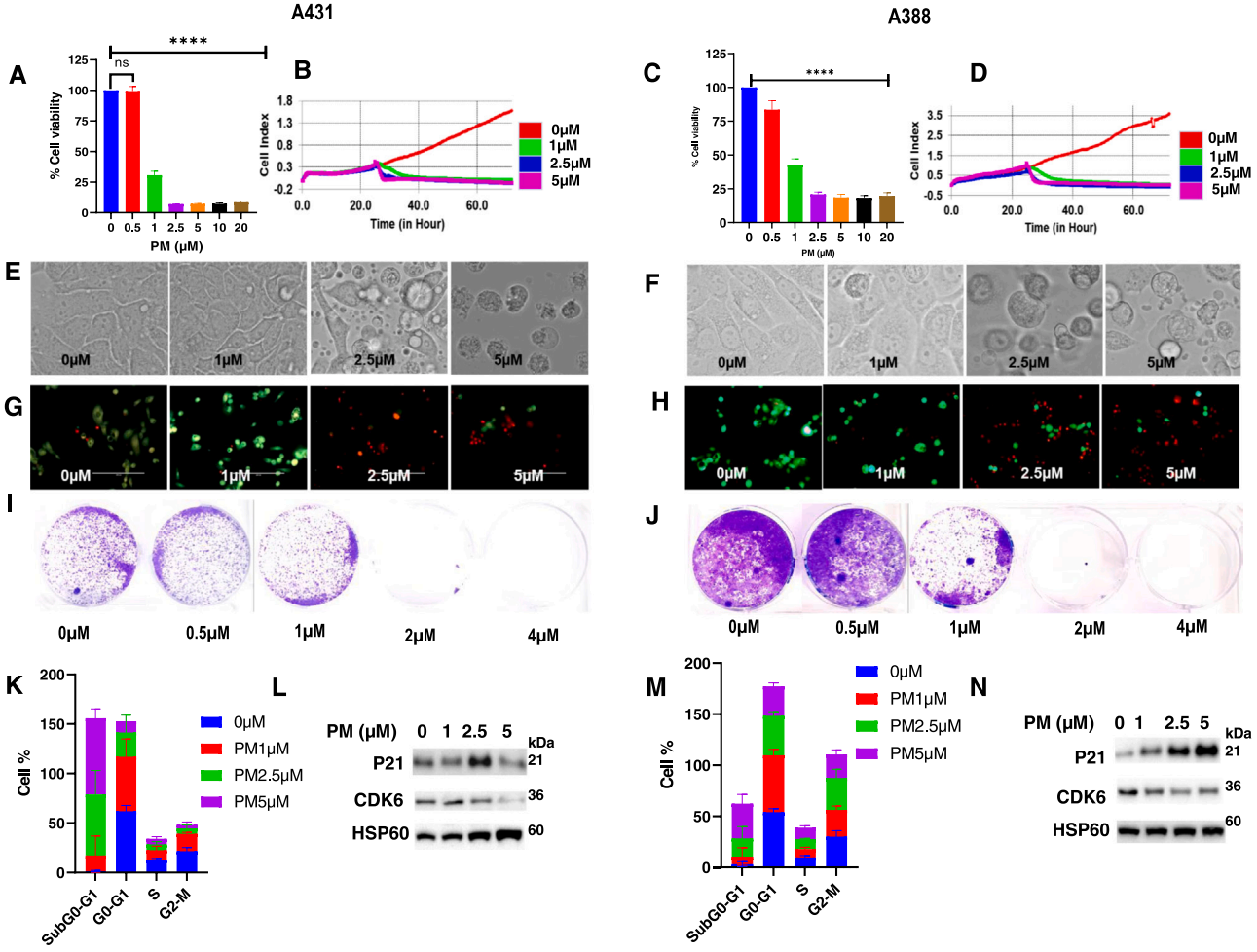

Fig. 1. Pristimerin inhibits cell growth and proliferation and induces cell cycle arrest in cSCC cells. Skin cancer cells, A431 (A) and A388 (C) were treated with different concentrations (0 μM, 0.5 μM, 1 μM, 2.5 μM, 5 μM, 10 μM and 20 μM) of pristimerin for 24 h. (B and D), A431 and A388 cells were treated with indicated concentrations of pristimerin, and cell index was evaluated using RTCA. (E, and F), morphological changes in A431 and A388 cells treated with indicated concentrations of pristimerin for 24 h. (G and H), A431 and A388 cells were stained with live and dead cells staining kit after treatments with 0 μM, 1 μM, 2.5 μM, and 5 μM pristimerin for 24 h. We have used green-fluorescent calcein-AM dye which gives green color that represents the intracellular esterase and red-fluorescent ethidium homodimer-1 gives read color which indicates loss of membrane integrity. (I and J), effect of pristimerin treatments on colony formation in A431 and A388 skin cancer cells. (K and M), A431 and A388 cells were challenged with the indicated concentrations of pristimerin for 24 h and cell cycle distribution percentage was analyzed by flow cytometry. (L and N), A431 and A388 cells were treated with the indicated concentrations of pristimerin for 24 and then the expression of regulatory proteins related to cell cycle was done by western blot. ** **$P < 0.0001$ represents level of significance between treatment groups.

cell proliferation affected the cell distribution pattern by inducing cell cycle arrest. We found that pristimerin treatment caused significant accumulation of cells/cycle arrest at G0-G1 phase in both A431 (Fig. 1K, L) and A388 (Fig. 1M, N) cells (Supplementary Fig. S1).

3.2. Pristimerin mediates antiproliferative actions by apoptosis induction and modulation of apoptotic regulatory proteins

To find out the underlying mechanisms of pristimerin-induced cSSC cell death, we explored the expression of apoptosis-regulating proteins. Indeed, treatment of cSCC cells with pristimerin lead to nuclear condensation, shrinkage, and fragmentation (Fig. 1E, F). Moreover, annexin/PI staining revealed a significant ($P < 0.05$) increase in the number of apoptotic cells due to pristimerin treatment in A431 (Fig. 2A, C) and A388 (Fig. 2B, E) cells, suggesting the major role of cell apoptosis and its regulatory proteins/pathways. To better understand the regulatory mechanisms, we observed a remarkable modulation in the expression of various apoptosis-associated or regulatory proteins. Pristimerin-treated cSCC cells showed changed levels in p-H2AX, caspase-3, cleaved caspase-3, and cleaved caspase-8 (Figs. 2D, 2F).

Further, we explored the role of caspases associated with apoptosis, in cSCC proliferation and cell death due to pristimerin treatment, using z-VAD-fmk, a pan-caspase inhibitor. Our data show that cells treated with z-VAD-fmk showed significant ($p < 0.05$) increase in cell survival as compared to only pristimerin-treated cells (Fig. 2G, 2J). Activation of apoptosis-regulating genes and DNA damage markers was also attenuated due to z-VAD-fmk (Figs. 2H, 2I). Hence, pristimerin induces apoptotic cell death due to activation of apoptotic regulatory proteins in cSCC cells.

3.3. Pristimerin induces autophagy in A431 and A388 cSCC cells

Autophagy, a second type of programmed cell death, plays important role in cellular and biological homeostasis. To explore whether pristimerin-induced skin cancer cell death occurs through autophagy, A431 and A388 cells were challenged with different concentrations of pristimerin. Our results show increased expression/accumulation of autophagy-related proteins, LC3 and p62 (Fig. 3A, 3C).

To further explore the role of autophagy in pristimerin-induced anticancer actions, we pretreated A431 and A388 cells with autophagy inhibitor, 3-methyladenine (3-MA) and observed significant reduction in pristimerin-induced cell viability loss (Figs. 3B, 3D). Next, we explored the possible association between apoptosis and autophagy due to pristimerin treatment. Therefore, skin cancer cells were pretreated with and 3-MA and z-VAD-FMK. Our data show that use of autophagy inhibitor, 3-MA, reversed the expression levels of apoptotic markers (Figs. 3E, 3F) in vitro. Moreover, pretreatment with pan-caspase inhibitor z-VAD-FMK, slightly blocked autophagy marker expression levels (Supplementary Fig. S2, A). This data indicates that pristimerin causes cell death via triggering both apoptosis and autophagy.

3.4. Pristimerin mediates antiproliferative actions by ROS generation and activation of MAPK pathway

Increasing data suggests a crucial role of ROS in antiproliferative cell activity due to various natural products/ anticancer agents and in chemotherapy, at least in part by modulating MAPK cell signaling. Hence, we explored potential activation of MAPK pathways in pristimerin-treated cSCC cells in vitro, as these pathways regulate various underlying mechanisms of cell growth and proliferation.

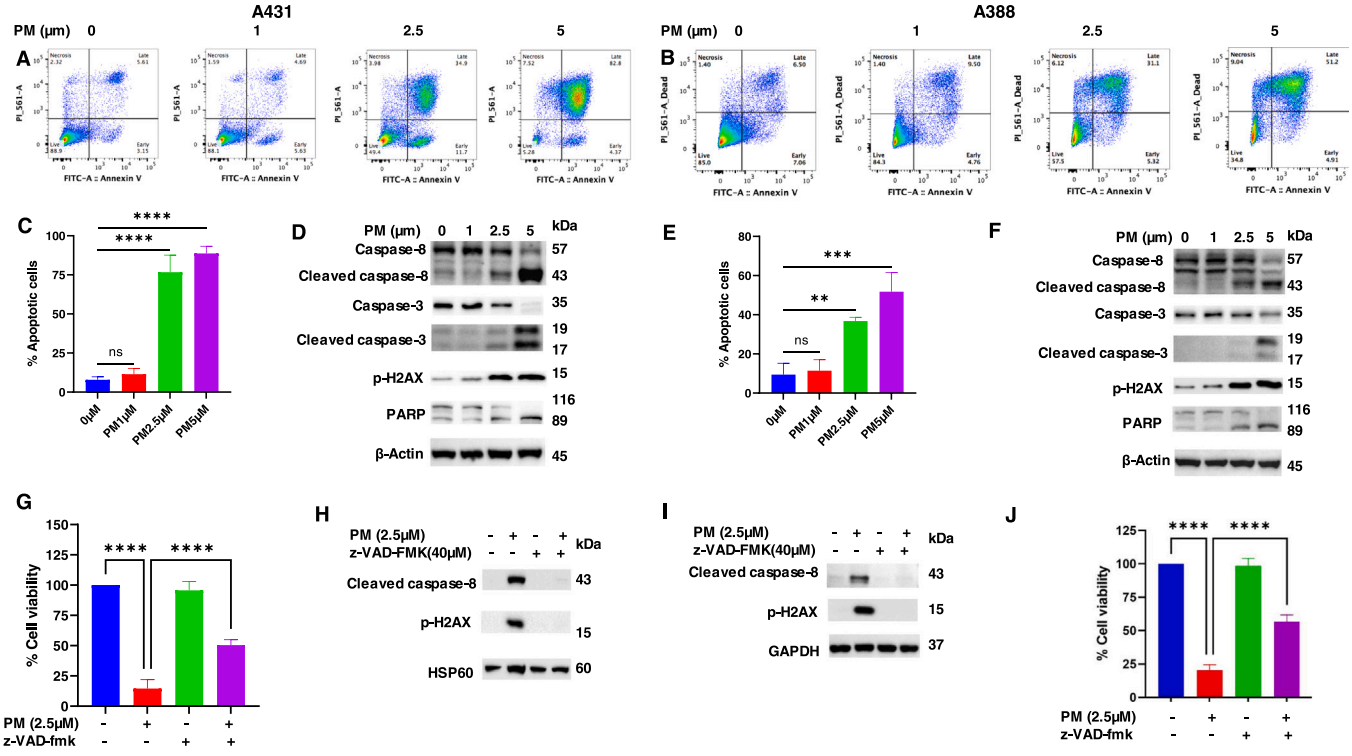

Fig. 2. Pristimerin inhibits cell growth and proliferation through apoptosis. Skin cancer cells, A431 (A, C) and A388 (B,E) were treated with 0 μM, 1 μM, 2.5 μM, and 5 μM of pristimerin for 24 h and percent of cell apoptosis stained by fluorescein-conjugated annexin-V PI was analyzed by flow cytometry. (D and F), A431 and A388 cells were treated with indicated concentrations of pristimerin and cell lysates were prepared. Expression of different proteins associated with apoptosis was done by western blotting. To further confirm the role of apoptosis in pristimerin mediated growth inhibition of skin cancer cells, we pretreated skin cancer cells with pan caspase inhibitor Z-VAD-FMK and then challenged with pristimerin. Cell viability and expression of various apoptotic regulatory proteins in A431 (G, H) and A388 (I, J) was evaluated. Results from three independent experiments are presented. **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ represents level of significance between treatment groups.

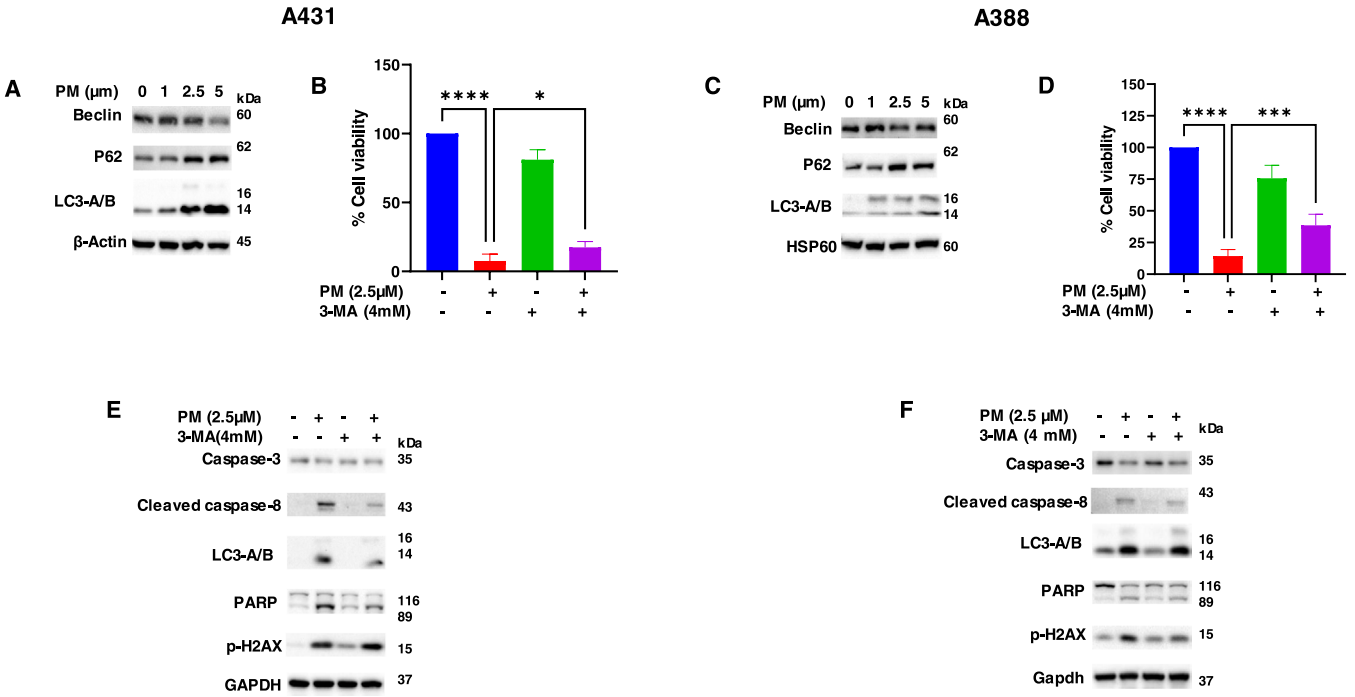

Fig. 3. Pristimerin induces autophagy in skin cancer cells. Primary and metastatic skin cancer cells, A431 (A) and A388 (C) were treated with 0 μM, 1 μM, 2.5 μM, and 5 μM of pristimerin for 24 h then cell lysates were prepared, and western blotting was done for the expression of autophagy associated proteins. (B) A431 and (D) A388 cells were pretreated with 3-methyladenine (4 mM) and then treated with 2.5 μM pristimerin and finally the cell viability was measured using CCK-8 solution. (E, F) A431 and A388 cells were pretreated with 3-methyladenine (4 mM) and then treated with 2.5 μM pristimerin and finally expression of apoptosis and autophagy markers was done by western blotting. *P < 0.05 and ***P < 0.001 and *** *P < 0.0001 represents level of significance between treatment groups.

Pristimerin treatment markedly increased expression (phosphorylation) levels of JNK in both A431 and A388 cells, while the expression of phosphorylated p38 and ERK expression was also changed in both cell types (Fig. 4 A, 4B). In addition, we did not detect remarkable changes in total forms of these proteins due to pristimerin treatment (Fig. 4 A, 4B).

Next, we explored potential ROS generation due to pristimerin treatment in skin cancer cells in vitro. As shown in Fig. 4C-4F, pristimerin markedly induced increased ROS levels in cSCC cells, which was markedly reversed by NAC strongly supporting a crucial role of ROS due to pristimerin treatment. We also sought to investigate the underlying role of pristimerin-induced ROS generation in the modulation of MAPK signaling. Therefore, we pretreated skin cancer cells with NAC, and then challenged them with pristimerin. Indeed, NAC pretreatment reversed the modulation of MAPK proteins confirming a critical involvement of ROS in this process (Fig. 4G, 4H). Use of inhibitor of JNK, further strengthen the idea of an important role of these pathways in pristimerin-treated cancer cells (see also below). Together, our results support a crucial role of ROS in pristimerin-induced antiproliferative activity in cutaneous cSCC through JNK activation.

3.5. Pristimerin-induced cell cycle arrest via apoptotic and autophagic action is mediated through ROS generation

ROS has been the crucial player in various physiological and pathological conditions including cancer. To explore the underlying mechanisms of pristimerin-induced cSCC cell death, we pretreated A431 and A388 cells with NAC. Our results show that ROS is integral in various mechanisms converging towards pristimerin-induced skin cancer cell death. As shown in Fig. 5A-5 F use of NAC reversed the effects of pristimerin on cellular morphology, staining of live and dead assay, and colony formation. Moreover, NAC also reversed the pristimerin-induced cell cycle arrest, annexin staining and mitochondrial membrane potential (Supplementary Figure S 2, B), hence signifying the crucial role of ROS in pristimerin induced cell death. Next, indeed our data clearly shows that NAC treatment reversed pristimerin-induced reduction of cell viability and attenuated apoptosis via modulating the expression of apoptotic markers (Fig. 5G-5 J).

Autophagy, which is an important mechanism of type-II programmed cell death, often deregulated in cancer pathogenesis. Our data reveals important role of autophagy in pristimerin-induced cSCC cell death, as demonstrated by the enhanced expression of the autophagy markers. Moreover, NAC treatment reversed the autophagic action of pristimerin signifying that ROS is integral in autophagic death of cSCC cells (Supplementary Fig. S3; A, B).

3D-Sphroid culture, a model widely used to study the tumor microenvironment and enrichment of cancer stemness features, was used to explored effect of pristimerin on spheroid formation and the underlying role of ROS. The date shown in Supplementary Fig. S3; C, D, clearly shows that pristimerin inhibits the spheroid formation of skin cancer cells. Interestingly, expression of stemness markers was also attenuated due to pristimerin treatment in both primary and metastatic skin cancer cells (Supplementary Fig. S3; E, F). Next, we wanted to explore the role of pristimerin generated ROS on spheroid formation. In this line, the data shown in Supplementary Fig. S3; G, H, clearly suggests that ROS scavenger, NAC attenuated spheroid deformities and confirming that pristimerin-induced ROS is critical in the inhibition of spheroid

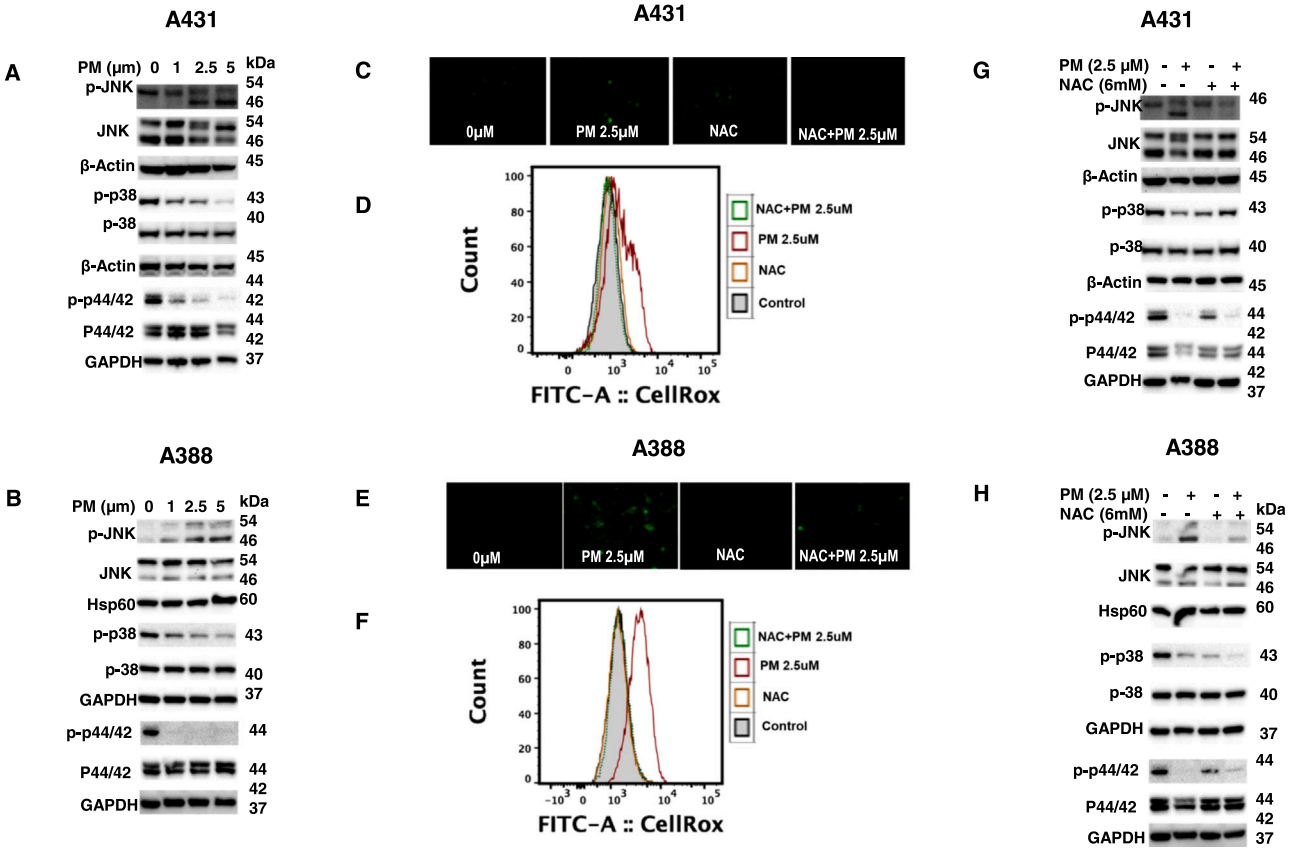

Fig. 4. Pristimerin modulates MAPK signaling though ROS generation in skin cancer cells. (A, B,) A431 and 388 cells were treated with the indicated concentrations of pristimerin for 24 h and expression of MAPK signaling proteins was done through western blotting respectively. (C, D) A431 and (E, F) A388 cells were treated with NAC alone and in combination with pristimerin for 24 and finally the cells were stained with DCFDA and imaging and counting of ROS positive cells was done using EVOS FLc Cell Imaging System from Invitrogen (Thermo Fisher Scientific) and flow cytometry respectively. (G, H), A431 and A388 cells were pretreated with NAC alone and in combination with pristimerin for 24 and then cells then cell lysates were prepared, and western blotting was performed for the expression of MAPK signaling proteins.

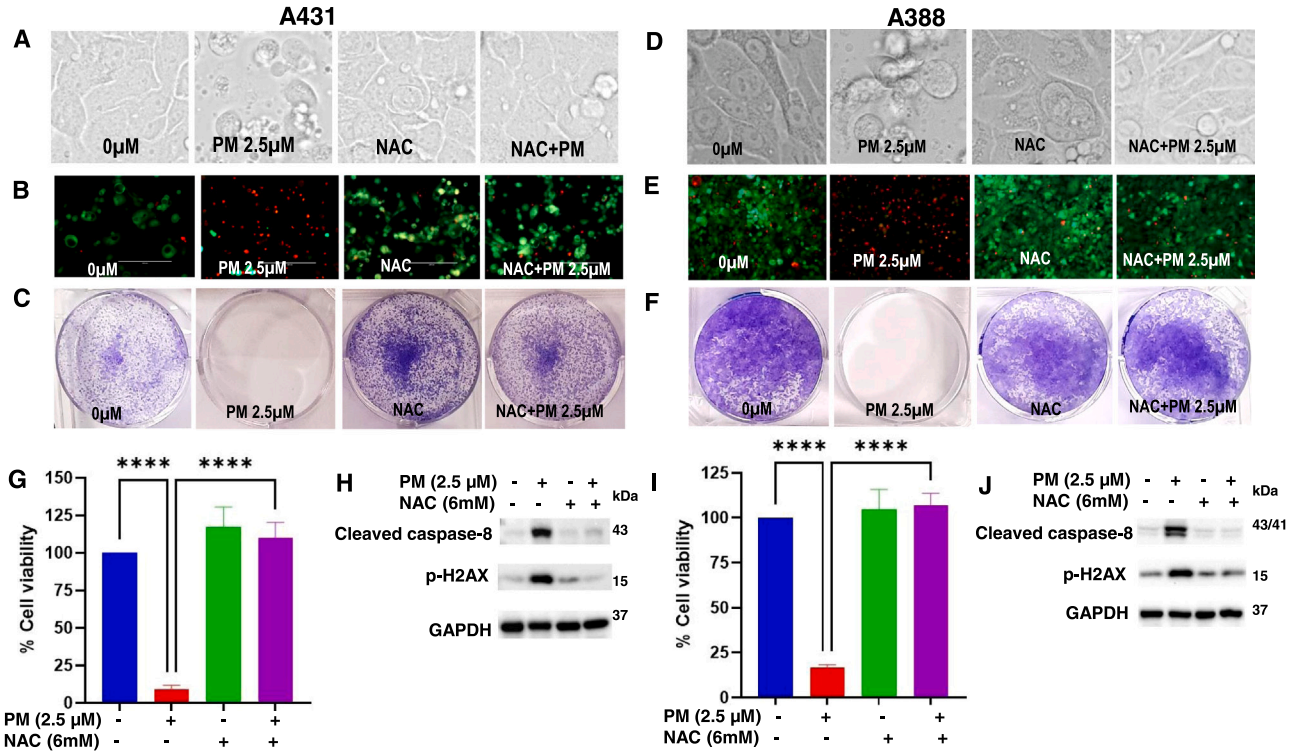

Fig. 5. Pristimerin induced ROS generation is essential for the skin cancer cell death. (A-C, and D-F), represents cell morphology, live and dead cell staining and colony formation of A431 and A388 cells respectively, pretreated with NAC (6 mM) alone and in combination with 2.5 μM pristimerin for 24 h. (G, I, and H, J), shows the percentage cell viability and expression of apoptotic markers of A431 and A388 cells respectively, pretreated with NAC (6 mM) for and then treated with 2.5 μM pristimerin for 24 h. * ** *P < 0.0001 represents level of significance between treatment groups.

formation.

3.6. Pristimerin induced apoptotic and autophagic action through JNK activation

MAPK signaling pathways are critical regulators of different stages of development, growth, proliferation, and biological homeostasis. Our data indicates that pristimerin mediates its anti-cancer action through JNK activation of the MAPK pathway. Along this line, we investigated the effect of JNK inhibition on cell proliferation and programmed cell death. As shown in Fig. 6A-6D, inhibiting JNK with SP600125 significantly reversed the pristimerin-induced morphological alterations and anti-proliferative action in both primary and metastatic cSCC cells. To further explore the mechanisms underlying the role of JNK activation in pristimerin-mediated cell death, we checked expression of markers of apoptosis and autophagy. As depicted in Fig. 6 E, 6F, treatment of cSCC cells with SP600125 significantly suppressed pristimerin- induced activation and cleavage of PARP, p-H2AX, cleaved caspase 8 and LC3. Interestingly, SP600125 also prevented pristimerin-induced spheroid distortion supporting an underlying role of JNK activation in anticancer actions of pristimerin (Fig. 6G, 6H). Overall, our results strongly indicate that pristimerin induces programmed cell death via JNK activation.

3.7. Pristimerin sensitizes cSCC cells to anticancer drugs (cisplatin, doxorubicin and azacytidine) and inhibits spheroid formation

Many of the available anti-cancer therapeutics (e.g., chemotherapy, radiotherapy, surgery, targeted therapy, and immunotherapy etc.) are associated with therapeutic challenges including toxicity, resistance, safety, tolerability, and recurrence. Hence, we explored the combined treatment strategy of pristimerin with available anticancer drugs azacytidine, cisplatin and doxorubicin. As shown in Fig. 7A, pristimerin sensitized A388 cells to anticancer drugs and significantly reduced the cell viability ($p < 0.001$).

Next, increased cellular morphological alterations, live and dead cell staining in the cells treated with both pristimerin and anticancer drugs (azacytidine, doxorubicin and cisplatin) as compared to their alone counterparts further strengthened the sensitizing potential of pristimerin (Figs. 7B,7 C) and Supplementary Fig. S4; A. In this line, Fig. 7B, and 7 C and Supplementary Fig. S4 reveals significantly elevated cellular and morphological alterations, especially suppressed or distorted spheroids, increased dead cells staining in the cells treated with both pristimerin and anticancer drugs as compared to alone treatments. This prompted us to explore the underlying mechanisms associated with the sensitizing potential of pristimerin. As depicted in Supplementary Fig. S5, annexin staining and cell cycle analysis by flow cytometry showed increased arrest of cells in G0-G1 phase and apoptotic cells in combinational group as compared to alone treatments. Moreover, western blots data showed significant increase in expression of apoptotic markers in the cells treated with both pristimerin and anticancer drugs (Figs. 7D, 7E) and Supplementary Fig. S4, B.

Next, we investigated the role of MAPK signaling in cancer cells exposed to combination of pristimerin and anti-cancer drugs. In this line our data showed that JNK activation is critical in the combination anticancer action effects of pristimerin as shown in Fig. 7 F,7 G. Use of SP600125, a JNK inhibitor, markedly suppressed the markers related to the anticancer action of pristimerin with anticancer drugs and thus further signifies critical involvement of JNK.

4. Discussion

Skin malignancies, including nonmelanoma skin cancers (NMSC), are posing increasing therapeutic challenges with pouring socioeconomic burden despite great progress made on exploring its etiopathogenesis [27–30]. Therefore, there is still a need for a better understanding of disease pathogenesis, and development of effective,

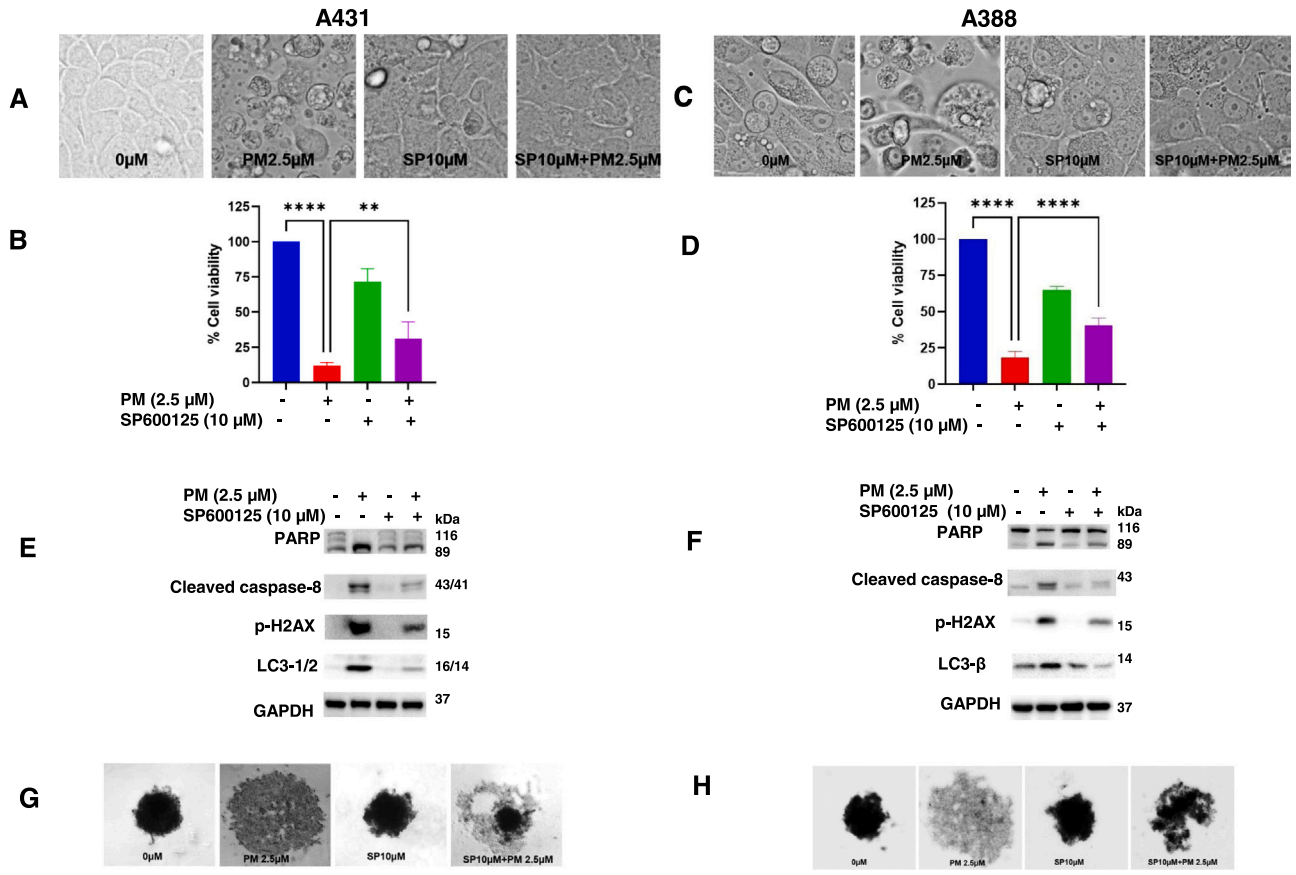

Fig. 6. Pristimerin induces apoptosis and autophagy through JNK activation. (A, B) A431 and (C, D) A388, represents the cellular morphological changes and percentage viability of skin cancer cells pretreated with JNK inhibitor, SP600125 (10 μM) at least two hours and then were challenged with 2.5 μM, pristimerin for 24 h.(E and F), A431 and A388 cells were pretreated with JNK inhibitor, SP600125 (10 μM) and then treated with 2.5 μM pristimerin and then cell lysates were prepared, and western blotting was done for the expression of proteins associated with apoptosis and autophagy. (G and H), A431and A388 cells were cultured in ultralow attachment plates with and without SP600125 (10 μM) and pristimerin (2.5 μM) for at least 6 days and imaging was done at 4x using EVOS FLc Cell Imaging System from Invitrogen (Thermo Fisher Scientific). * *P < 0.01 and ** **P < 0.0001 represents level of significance between treatment groups.

safe, and tolerable treatments. Our study for first time explored the anticancer actions of pristimerin in human cSCC. Earlier studies highlighted the anticancer potential of pristimerin due to its strong antioxidant, anti-inflammatory, anti-microbial and immunomodulatory properties in cancers including lung, colon and breast [10,31–33]. Whether pristimerin mediates anti-cancer activity in cutaneous SCCs, and if yes, how, is not known. We show that pristimerin exerts strong anti-cancer activity in cSCC via ROS-mediated MAPK signaling, and JNK activation. Moreover, pristimerin sensitizes skin cancer cells to conventional anticancer drugs through ROS-induced JNK modulation.

Targeted inhibition of the cancer cell proliferation and growth is one the major therapeutic challenges; there has been increasing demand for such therapeutics for better clinical outcomes. Naturally-derived drugs have shown promising outcome for various cancers [34,35]. Our present study supports evidence for a strong anti-cancer therapeutic potential of pristimerin in cSCC. First, pristimerin inhibited the incessant cell proliferation, a hallmark of cancer cells, in both, primary as well as metastatic cSCC in vitro. Second, pristimerin inhibited growth of cancer cells via induction of programmed cell death i.e., apoptosis and autophagy. Apoptosis is a complex process that play crucial role in biological homeostasis including the development and physiological process [36]. However, deregulated apoptosis or unresponsiveness of cancer cells to therapeutics is one of the major clinical challenges that leads to resistance and disease recurrence [37,38]. Generally, both extrinsic (death receptor-mediated) and intrinsic (mitochondrial) mechanisms induce cell apoptosis through activation and cleavage of caspases such as caspase-8 or caspase-3, which in turn activates PARP cleavage [39]. Our data also show that pristimerin-induced apoptosis through activation and cleavage of caspases, thus supporting its strong anti-tumorigenic potential. Use of pan-caspase inhibitor, z-VAD-FMK confirms crucial role of apoptosis in pristimerin induced antiproliferative actions as supported by the attenuation of morphological, and molecular changes. Moreover, we also investigated role of autophagy in pristimerin-induced anticancer actions in cSCC, as demonstrated by previous investigation in other human malignancies. Autophagy, another major type of programmed cell death that play major role in biological homeostasis through regulation of metabolic adaptation, intracellular quality check, and renovation during development and growth [40,41]. Increasing data also shows that deregulated autophagy is associated with human disease pathogenesis and therapeutic challenges [42,43]. Interestingly, we observed that pristimerin markedly activated autophagy markers suggesting a crucial role of autophagy in its anticancer activity in cSCC.

Accumulating data entails a crucial role of ROS in anticancer activity within conventional anti-cancer therapies including chemotherapy, radiotherapy, and immunotherapy. Hence, we explored a potential role of ROS in pristimerin-mediated anti-cancer activity in non- metastasizing and metastasizing cSCC. Indeed, pristimerin induced ROS generation which may play a role in the induction of programmed cell death i.e., apoptosis and autophagy in cSCC. Use of NAC, a universal scavenger of ROS, revert/suppressed pristimerin-induced antiproliferative actions such as cell viability, cell cycle arrest, annexin staining, cellular ROS staining, activation of caspases and autophagy markers, supporting a curial role of ROS in cSCC in vitro. Overall, these data provide crucial evidence for pristimerin to be a promising anticancer drug for cSCC.

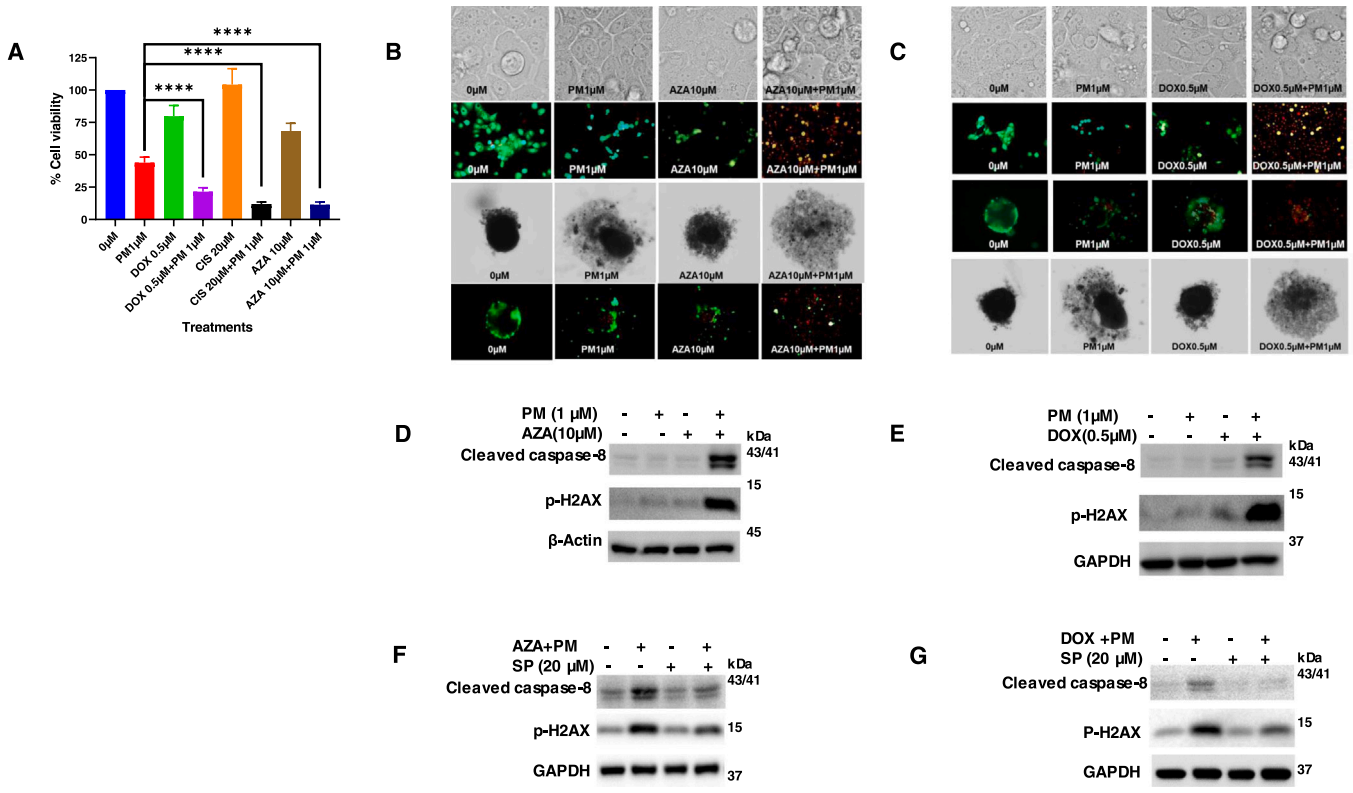

Fig. 7. Pristimerin potentiates anticancer activity of chemotherapeutic drugs and enhanced sensitization of cancer cells towards anticancer agents. (A), pristimerin enhances growth inhibitory action of anticancer drugs. Skin cancer cells, A388 were cultured in the presence and absence of pristimerin (1 μM), azacytidine (10 μM), cisplatin (20 μM) and doxorubicin (0.5 μM) or their combinations for 24 h and cell proliferation assay was performed using CCK-8 as described in materials and Methods. (B, and C), A388 cells were treated with indicated concentration of pristimerin, azacytidine and doxorubicin alone and in combination for 24hors and imaging of morphological changes, live and dead cell staining, and spheroids formation was done using EVOS FLc Cell Imaging System from Invitrogen (Thermo Fisher Scientific). (D-E), A388 cells were treated with, with indicated concentration of pristimerin, azacytidine and doxorubicin alone and in combination for 24hors and western blot analysis of caspase-8 and p-H2AX was done. (F and G), A388 cells were treated with pristimerin+azacytidine and pristimerin+doxorubicin alone and in combination with SP600125 (20 μM) and western blotting of different apoptosis markers was done. * ** *P < 0.0001 represents level of significance between treatment groups.

The present study also explores the role of MAPK in pristimerin-induced anticancer actions in primary and metastatic skin cancer cells. MAPK pathways are integral for normal biological homeostasis including the growth and survival. Indeed, MAPK pathway activation plays a crucial role in anti-cancer therapies and sensitization of cancer cells [44,45]. Moreover, mechanistic data reveals that stress due to anticancer agents induce apoptotic and autophagy cell death via JNK [45–47]. Several studies confirm the crucial role of the MAPK pathway in anti-cancer actions for various therapeutic agents in skin cancer malignancies [48–50]. Our present study demonstrates evidence for pristimerin-mediated anti-cancer actions in cSCC via stress induction, particularly through ROS generation. Accordingly, pristimerin-induced JNK activation confirmed that stress- mediated JNK activation is crucial in pristimerin-induced anti-cancer effects. Additionally, inhibition of JNK by SP600125 reversed pristimerin-induced inhibition of cell proliferation, activation of apoptosis and autophagy markers; thus, clearly signifies involvement of JNK in pristimerin-induced anti-cancer actions including mediating programmed cell death via ROS generation through JNK activation.

Complications such as adverse toxicity, drug resistance and stemness features are the major drawbacks of conventional cancer treatment options including chemotherapy. Combined therapeutic regimen have shown increased clinical benefits in cancer patients for various malignancies, including cSCC. Therefore, we explored whether pristimerin sensitize or enhance the inhibition of cSCC proliferation, formation of spheroids and apoptosis in combination with anti-cancer drugs cisplatin, doxorubicin or azacytidine, potentially important treatment options in the clinical setting.

Overall, our findings suggest a strong anti-cancer potential of pristimerin alone in cSSC, and in combination with conventional anti-cancer drugs against primary and metastatic cSCC cells. Moreover, pristimerin-induced ROS generation may play a major role in the induction of programmed cell death through activation of MAPK pathway JNK. Thus, pristimerin may be a promising novel anti-cancer drug for cSCC and potentially other cancers.

CRediT authorship contribution statement

Maha Al-Tamimi: Data curation, Visualization, Investigation, Methodology, Writing – original draft. **Abdul Q. Khan:** Conceptualization, Data curation, Visualization, Funding acquisition, Investigation, Methodology, Writing – original draft. **Rasheeda Anver**: Data curation, Visualization. **Fareed Ahmad:** Data curation, formal analysis, Visualization. **Jericha M Mateo**: Data curation. **Syed Shadab Raza:** Writing – review & editing, preparation of the graphical abstract. **Majid Alam**: Writing – review & editing. **Joerg Buddenkotte**: Writing – review & editing. **Martin Steinhoff:** Conceptualization, Resources, Supervision, Writing – review & editing. **Shahab Uddin:** Conceptualization, Supervision, Writing – review & editing.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113950.

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