Cristacarpin promotes ER stress-mediated ROS generation leading to premature senescence by activation of p21^{waf-1}

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Abstract Stress-induced premature senescence (SIPS) is quite similar to replicative senescence that is committed by cells exposed to various stress conditions viz. ultraviolet radiation (DNA damage), hydrogen peroxide (oxidative stress), chemotherapeutic agents (cytotoxic threat), etc. Here, we report that cristacarpin, a natural product obtained from the stem bark of *Erythrina suberosa*, promotes endoplasmic reticulum (ER) stress, leading to sub-lethal reactive oxygen species (ROS) generation and which eventually terminates by triggering senescence in pancreatic and breast cancer cells

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through blocking the cell cycle in the G1 phase. The majority of cristacarpin-treated cells responded to conventional SA- β -gal stains; showed characteristic p21^{waf1} upregulation along with enlarged and flattened morphology; and increased volume, granularity, and formation of heterochromatin foci—all of these features are the hallmarks of senescence. Inhibition of ROS generation by *N*-acetyl-L-cysteine (NAC) significantly reduced the expression of p21^{waf1}, confirming that the modulation in p21^{waf1} by anti-proliferative cristacarpin was ROS dependent. Further, the elevation in p21^{waf1} expression in PANC-1 and MCF-7 cells was consistent with the decrease in the expression of Cdk-2 and cyclinD1. Here, we provide evidence that cristacarpin promotes senescence in a p53-independent manner. Moreover, cristacarpin treatment induced p38MAPK, indicating the ROS-dependent activation of the MAP kinase pathway, and thus abrogates the tumor growth in mouse allograft tumor model.

Keywords Cristacarpin · p21^{waf1} · ROS · Senescence · GRP-78 · p38MAPK

Introduction

Cancer cells technically avoid replicative senescence by over-expressing telomerase, but cellular senescence is evident in cancer cells by other mechanisms (Dimri 2005), which are independent of telomere length. In normal cells, progressive shortening of telomere length eventually pushes the cells toward senescence, but

premature senescence, which is a prevalent property in cancer cells, is independent of number of cell divisions or telomere length (Grimes and Chandra 2009). Extensive proliferation of cancer cells due to upregulation of oncogenes or DNA damage triggers cellular senescence. On the other hand, intracellular reactive oxygen species (ROS) generation, caused by metabolic pressure and loss of tumor suppressor genes (*PTEN* or *p53*), leads to another kind of senescence, termed as stress-induced premature senescence (SIPS) (Kuilman et al. 2010; Munoz-Espin and Serrano 2014; Roninson 2003). ROS acts as a cellular sensor for various stresses like hypoxia, DNA damage response, and endoplasmic reticulum-mediated stress due to unfolded protein responses, etc. Interestingly, the level of ROS dictates several processes within the cell by controlling key signaling pathways related to apoptosis and cell cycle. Convincing evidence (Macip et al. 2002) shows that sub-lethal concentration of ROS could escalate cellular senescence by inducing $p21^{waf1}$ leading to G_1 arrest. Although *p53* is the major transcription factor for $p21^{waf1}$ -induced senescence activation, there are several reports underscoring the induction of cellular senescence in *p53*-mutated condition (Colavitti and Finkel 2005). The generation of ROS can induce $p21^{waf1}$, but via a feedback loop existing between *p21* and ROS, cellular senescence is maintained (Passos et al. 2010a). $p21^{waf1}$ is a potential cyclin-dependent kinase inhibitor that promotes the dephosphorylation of Rb protein and subsequent inhibition of E2F transcription factor, a major transcription factor controlling genes related to cell cycle progression and DNA replication. Thus, such hindrance in cell cycle facilitates cellular senescence, where *p21* plays a leading role to trigger pivotal genes responsible for aging and senescence (Chang et al. 2000; Passos et al. 2010b; Qiu et al. 1996), the two sides of a coin.

Intriguingly, ER stress-mediated ROS generation triggers drastic physiological changes within the cell. ROS is a by-product of the endoplasmic reticulum during protein folding through the di-sulfide bond formation. But, as a result of the prevalent cytotoxic threat, advanced UPR responses steadily build up mild to moderate ROS generation. The initial ROS level in the cells puts forward a protective mechanism by triggering G_1 cell cycle arrest, which is a precursor for SIPS (Raghuram and Mishra 2014), but excessive ROS generation within the mitochondria ultimately reverts the membrane potential by releasing cytochrome *c* to

trigger apoptosis (Malhotra and Kaufman 2007). However, ROS generation augments the expression of superoxide dismutase, which detoxifies superoxide ions in mitochondria to maintain the balance of ROS production and ultimately provides a cytoprotective role from oxidative stress (Robbins and Zhao 2012).

The role of ER stress in the induction of premature senescence is still not completely understood. In this report, we have demonstrated a novel mechanism by which ER stress-regulated ROS generation via natural compound cristacarpin, isolated from the stem bark of *Erythrina suberosa*, triggers the cells toward cell cycle arrest, leading to premature senescence by *p53*-independent upregulation of $p21^{waf1}$.

Materials and methods

Isolation of cristacarpin

Air-dried and finely powdered *E. suberosa* stem bark (450 g) was extracted thrice with DCM: MeOH (1:1, 1 L) at room temperature for 12 h; then, the extract was concentrated to dryness in vacuum to yield a dark brown semi-solid (47.2 g). The residue was suspended in 2 % H_2SO_4 (400 ml) and partitioned thrice with $CHCl_3$ (300 ml); the lower layer afforded non-alkaloid crude extract (15 g). The obtained solid was chromatographed by silica gel column chromatography, eluted with hexane/ethyl acetate solvent system. The gradient in polarity (5–20 %) of ethyl acetate afforded four enriched sub-fractions (A–E) 1–8, B 9–13, C 14–19, and D 20–27). Further purification of sub-fraction B (fractions 9–13) over Sephadex LH-20 using MeOH as eluent yielded [(7-hydroxy-2-(4-methoxy-3-(3-methylbut-2-en-1-yl) phenyl) chroman-4-one)] (22 mg). Separation from sub-fraction C (fractions 14–19) via RP-HPLC (conditions: solvent ACN/ H_2O 80:20, flow rate 0.8 ml/min, UV detection at 254 nm) furnished cristacarpin (37 mg, *t*_R 8.0 min). Thus, the cristacarpin obtained was characterized by NMR and mass spectra as previously reported.

Cell culture and reagents

PANC-1, MCF-7, PC3, and human umbilical vein endothelial cell (HUVEC) cells were purchased from European Collection of Cell Culture (ECACC). BPH was a kind gift from Dr. V. M. Rangnekar (University of

Lexington, Kentucky). MCF-7, PC3, and BPH cells were cultured in RPMI 1640, and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). Both RPMI and DMEM were supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin G (100 U/ml)/streptomycin (100 mg/ml). HUVEC cells were cultured in F-12K medium and supplemented with 0.1 mg/ml heparin and 0.03–0.05 mg/ml endothelial cell growth supplement (ECGS). All the cells were maintained at 37 °C in a humidified 5 % CO₂ incubator under standard conditions, respectively. Antibodies for GRP-78, GRP-94, PERK, p21^{waf1}, p16, Cdk-2, cyclinD1, p38MAPK, MnSOD, and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and phospho-p38MAPK from Cell Signaling Technology (Cell Signaling Technologies, MA, USA). *N*-acetyl-L-cysteine (NAC) was procured from Selleck Chemicals (Houston, USA), and salubrinal, Bradford reagent, MTT HEPES, NP-40, DTT, sodium orthovanadate, PMSF, 2',7' -dichlorofluorescein diacetate (DCFDA), X-Gal, and protease inhibitor cocktail were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Cell viability assay

The cell viability was determined by standard MTT dye uptake method. Briefly, PANC-1, MCF-7, PC3 BPH, and HUVEC cells (2500 cells/well) were plated in a 96-well tissue culture plate and were treated with different concentrations of cristacarpin in triplicate so that the final concentration of DMSO solvent was 0.2 %. After 44-h incubation, MTT solution was added, and cells were cultured for another 4 h at 37 °C in 5.0 % CO₂ incubator. The colorimetric readout was measured by TECAN microplate reader (Infinite M200 PRO) at 570 nm. The percent cell viability was determined according to the protocol described earlier (Rah et al. 2012), and the characteristic sigmoid curve was obtained by using GraphPad Prism software.

Immunoblotting

Immunoblotting experiments were performed according to the standard procedure (Rah et al. 2015). Briefly, following treatments with indicated agents, cells were lysed in lysis buffer. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was determined by the standard Bradford method. An equal amount (20–30 μ g) of protein was subjected

to SDS-PAGE, and proteins were transferred to PVDF membranes (PAL), blocked with 5.0 % (w/v) non-fat milk in PBS containing 0.1 % Tween-20. Then, the membranes were probed with indicated antibodies for 3 h at room temperature or overnight at 4 °C. Subsequently, blots were washed and probed with species-specific secondary antibodies coupled to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and exposed to Biomax light film (Eastman Kodak Co., Rochester, NY, USA).

Detection of ROS

The ROS activity was measured by membrane-permeable 2'-7'-dichlorofluorescein diacetate (H2DCFDA), which is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by ROS following cleavage of its acetate groups by intracellular esterases. Briefly, PANC-1 and MCF-7 cells were seeded in a six-well plate and treated with cristacarpin/vehicle DMSO for 24 h. Two hours before the termination of the incubation period, H₂O₂ (positive control) was added to the medium. DCFDA dye solution (10 μ M) was added after the completion of 24 h, and the cells were further incubated for 30 min. Then, the cells were thoroughly washed with PBS, and random field images were captured by FLoid Cell Imaging Station (Invitrogen) using $\times 20$ objective. The fluorescence was measured by Tecan M-200 PRO plate reader. Similarly, for fluorescence-activated cell sorting (FACS) analysis of DCFDA-stained PANC-1/MCF-7 cells, we employed the same treatment as described above. After the completion of the treatment period, the cells were trypsinized and resuspended in PBS following analysis by flow cytometry (BD FACS Aria II).

Flow cytometric cell cycle analysis

Cell cycle analysis was performed as per standard protocol (Rah et al.). Briefly, PANC-1 and MCF-7 cells were seeded in six-well plates and treated appropriately at set time points. Cells were collected by trypsinization, washed with PBS, and incubated in the hypotonic solution (0.1 % sodium citrate, 25 μ g/ml propidium iodide, 0.03 % Triton X-100, and 40 μ g RNase A) for 25 min at room temperature in the dark. The samples were subsequently analyzed by FACS using BD Diva software.

β -Galactosidase assay in vitro

Senescent cells predominantly express β -galactosidase, which are detectable at pH 6, and senescence-associated β -Gal (SA- β -Gal) staining procedure was adopted according to the published protocol (Dimri et al. 1995). Briefly, PANC-1/MCF-7 cells (50×10^3) were seeded per well in a 12-well plate treated with cristacarpin for 6 days. Cells were washed with PBS and fixed with 4 % paraformaldehyde for 5 min at room temperature and then washed thoroughly two times with sufficient PBS. Accordingly, the cells were incubated at 37 °C (without CO₂) in the presence of fresh senescence-associated β -Gal (SA- β -Gal) staining solution containing 1 mg of 5-bromo-4-chloro-3-indolyl P-D-galactoside (X-Gal) per milliliter in 40 mM of citric acid, sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂ for 24–48 h. Post-staining, the cells were fixed in methanol and air-dried before microscopic analysis. Finally, senescent cells were observed under brightfield microscope and were quantified using ImageJ software.

Detection of SAHF by DAPI staining

The senescence-associated heterochromatin foci detection method was carried out as per the previously described method (Aird and Zhang 2013). On the previous day, about 20,000 PANC-1/MCF-7 cells were seeded in eight-well chamber slide following treatment (next day) with cristacarpin (3 μ M) or DMSO as a vehicle. After 10 days, the cells were washed with PBS and fixed with 4 % paraformaldehyde (w/v) at room temperature for 10 min. After discarding the paraformaldehyde, the cells were washed with PBS, stained, and mounted with UltraCruz mounting media containing DAPI (Invitrogen). Fluorescence images were captured in FLoid Cell Imaging Station (Invitrogen) using $\times 20$ objective.

In vivo 4T1 assay

All the in vivo experimental protocols were approved by the Institutional Animal Ethics Committee, CPCSEA, of Indian Institute of Integrative Medicine, Jammu. The animals were maintained at 22 °C with a 12-h light-dark cycle and free access to feed and water inside the institutional animal house. Proper care was taken to maintain

them in a healthy condition and to avoid any risk of possible pathogenic contamination. Healthy, female Balb/c mice (5–7 weeks old) were taken for the study. For subcutaneous implantations, the mice were randomized into three groups of ten mice per group, and 1.5 million 4T1 cells (in serum free RPMI media) were implanted into the mammary pad of each mouse. A week after implantation, mice were injected intraperitoneally with either vehicle (saline) or cristacarpin (50 mg/kg) and positive control 5-FU (22 mg/kg) in every alternate day for 2 weeks. Tumor sizes were recorded on each alternate day, and body weights were recorded once per week. After the completion of the treatment period, the mice were sacrificed, and the subcutaneous tumors were dissected out. The weight of the tumors was measured, and percent tumor growth inhibition was calculated. For analyzing the obtained data, one-way ANOVA (with SAS 9.2) test was carried out to compare the mean tumor weights between the three experimental groups.

SA- β -gal staining of tumor tissue

4T1 tumors were generated in mouse mammary pad by the method as described above, and after the completion of the treatment period with cristacarpin (50 mg/kg for 2 weeks on every alternate day), the tumors were dissected from mouse mammary pad and washed with PBS to remove any blood. The tumors were immediately placed in OCT compound (Fisher Healthcare™) and flash frozen with liquid nitrogen containing 2-methylbutane. The mold was then sectioned using ultramicrotome to obtain about 4- μ m-thick sections and adhered to slide pre-coated with L-lysine. Further, the sections were fixed with 1 % formaldehyde for 1 min at RT, washed with PBS, and immersed in SA- β -gal stain for 12–24 h. Following β -gal staining, the slides were washed with PBS and counterstained with eosin. The stained slides were mounted using DPX and were observed by Nikon D3100 inverted microscope camera at $\times 40$ magnification (Itahana et al. 2013).

Results

Cristacarpin is a potential anti-proliferative agent

Prenylated pterocarpan is a group of phytochemical, known for its anti-cancer activity against diverse tumors (Watjen et al. 2007). Cristacarpin (Fig. 1a), a prenylated

pterocarpan, was isolated from the stem bark of *E. suberosa*, and its purity was checked (>95 %) by HPLC. For further characterization, ^1H NMR, ^{13}C NMR, and mass spectrometry of cristacarpin were accomplished for the confirmation of the compound and to compare with the previously reported data (Ingham and Markham 1980) (Supplementary Fig. 1a, b). Cristacarpin (Fig. 1a) showed its significant anti-proliferative effect in MTT assay on three different, moderately aggressive cell lines, viz. pancreatic cancer, PANC-1, breast cancer, MCF-7 and prostate cancer, and PC3 cells with IC_{50} values 6.3, 6.6, and 10.23 μM , respectively (Fig. 1b, c). In contrast, cristacarpin did not show any significant cytotoxicity against two other normal cell lines viz. HUVEC and BPH-1 (Fig. 1b). This result indicates that cristacarpin is a prospective cytotoxic and anti-proliferative agent in cancer but not in normal cells.

Cristacarpin promotes ER stress in breast and pancreatic cancer cells

The majority of the anti-cancer natural products induce ER stresses in vitro experimental set-up (Lee 2007). Therefore, we assumed whether cristacarpin promoted ER stress in PANC-1/MCF-7 cells. To do this, we first examined the effects of cristacarpin on ER stress sensors. Accordingly, the PANC-1 and MCF-7 cells were treated with cristacarpin (1, 5, and 10 μM) for 12 h, and the levels of potential ER stress markers viz. GRP-78, GRP-94, and PERK were analyzed by western blotting. Although GRP-78 showed a biphasic type of expression, there was a considerable persistence of ER stress at higher concentrations of cristacarpin (5 and 10 μM), as confirmed by elevated expressions of GRP-94 and PERK (Fig. 2a, b). Moreover, cristacarpin could not induce ER stress in the cells pre-treated with ER stress inhibitor

Fig. 1 Cristacarpin is a cytotoxic anti-proliferative agent. **a** Chemical structure of cristacarpin. **b, c** Effects of cristacarpin on proliferation of PANC-1 (pancreatic cancer), PC-3 (prostate cancer), MCF-7 (breast cancer), BPH-1 (normal prostate), and HUVEC

(normal umbilical vein) cell lines were determined by MTT assay; graphical and tabular representations show the comparative IC_{50} of cristacarpin in the mentioned cell lines. The results are the representative of three independent experiments. $*P < 0.05$

Fig. 2 Cristacarpin induces ER stress. **a, b** PANC-1 and MCF-7 cell lysates after exposure to the indicated concentrations of cristacarpin for set time points were analyzed, through western blotting, for GRP-78, GRP-94, and PERK expression along with the loading control β -actin. **c** Cristacarpin- and/or salubrinal-treated

cells, as shown, were analyzed by immunoblotting for the expression of GRP-78 and GRP-94. **d** Time-dependent effect of cristacarpin on GRP-78 and GRP-94 expression in PANC-1 cells was analyzed by western blot

salubrinal (Fig. 2c). Similarly, in MCF-7 cells, GRP-78 and GRP-94 expressions were steadily amplified in the presence of 1 μ M of cristacarpin in a time-dependent manner (Fig. 2d).

Cristacarpin induces ER stress-mediated sub-lethal concentration of intracellular ROS

The exposure of cells to sub-lethal stresses (for example, oxidative, cytotoxic, or DNA damage) could trigger SIPS (Frippiat et al. 2000). To investigate whether ER stress could modulate ROS generation and, thus, senescence, we examined the effects of ER stress on ROS generation. Our results showed that cristacarpin augmented intracellular ROS as analyzed by FACS and confocal microscopy. The FACS data clearly revealed that there was a mild increment (30–40 %, compared to positive control H_2O_2 which yields as high as 69 % ROS generation) in the level of ROS up to 5- μ M concentration of cristacarpin, correlating an oxidative stress condition which was extremely favorable in promoting premature

senescence (Toussaint et al. 2000; Macip et al. 2002) (Fig. 3a; third quadrant, both upper and lower panels). Further, to examine if cristacarpin treatment generated ROS production, PANC-1 cells were incubated with increasing concentrations of cristacarpin and successively stained with dichloro-dihydro-fluorescein diacetate (DCFH-DA) dye and analyzed by microscopy as well as fluorimetric studies. DCFH-DA is a cell-permeable dye, cleaved by cellular esterases forming a non-fluorescent compound, which later on reacts mainly with H_2O_2 and other peroxides to yield green fluorescent dichlorofluorescein (DCF). Our results unveiled a moderate green fluorescence in the presence of cristacarpin, indicating the signatures of ROS generation (Fig. 3b, c). Further, to investigate the correlation between ER stress and ROS generation (in presence of cristacarpin), the addition of ER stress inhibitor salubrinal exhibited a sharp disappearance of green fluorescence in the presence of ER stress inhibitor (Fig. 3b). Intriguingly, addition of salubrinal to cristacarpin-treated cells contributed a significant shifting of peak compared to cristacarpin-alone

Fig. 3 Cristacarpin induces oxidative ER stress. **a** DCFDA staining of PANC-1 and MCF-7 cells illustrating sub-lethal generation of ROS when treated with cristacarpin or H₂O₂. **b** Fluorescence microscopic analysis of ROS generation in PANC-1 cells when treated with cristacarpin and/or thapsigargin and salubrinal. **c** Bar

graph representing the amount of DCFDA fluorescence in terms of relative fluorescence units. **d** Autoradiograph showing the expression of MnSOD in PANC-1 and MCF-7 cells after treatment with the increasing concentration of cristacarpin. The results are the representative of three independent experiments. * $P < 0.05$

treatment (Fig. 3a, fourth quadrant, both upper and lower panels).

Interestingly, the natural product cristacarpin amplified the expression of superoxide dismutase-1 (SOD-1), a potent anti-oxidant enzyme that persists predominantly in the cytosol and also localizes in the mitochondrial intermembrane space, nucleus, and peroxisomes, which detoxified or scavenged the reactive oxygen species as H₂O₂ and molecular oxygen (Robbins and Zhao 2012) (Fig. 3d). Further, with the help of the enzyme catalase, H₂O₂ is detoxified into water (Robbins and Zhao 2012).

Cristacarpin augments ROS-dependent p21^{waf1} expression leading to G₁ phase cell cycle arrest

Senescent cells are believed to arrest in G₁ or G₀ stage of the cell cycle (Fripiat et al. 2000). Therefore, to examine the effects of cristacarpin on cell cycle progression, we found that the expression levels of p21^{waf1} were

significantly amplified in a dose-dependent manner in cristacarpin-treated cells (Fig. 4a). Additionally, the elevation in p21^{waf1} expression in PANC-1 and MCF-7 cells was consistent with the decrease in the expression of Cdk-2 and cyclinD1 (Munoz-Espin and Serrano 2014) (Fig. 4a). To check whether the change in the expression of p21^{waf1} was ROS dependent, PANC-1 cells were treated with cristacarpin and/or ROS inhibitor *N*-acetyl-L-cysteine (NAC). Truly, inhibition of ROS generation by NAC significantly reduced the expression of p21^{waf1}, confirming that the change in p21^{waf1} protein levels by cristacarpin was ROS dependent (Fig. 4b). Cristacarpin treatment (for 24 h) arrested 54 and 53.5 % of cells in G₁ phase compared to control (43 and 40.8 %) in PANC-1 and MCF-7 cells, respectively (Fig. 4c and Supplementary Fig. 1c). The above results were also validated by FACS analysis revealing G₁ phase cell cycle arrest in cristacarpin-treated PANC-1 cells, and this G₁ arrest was abrogated by salubrinal (ER stress inhibitor) treatment as illustrated in Fig. 4c.

Fig. 4 Cristacarpin arrests cells in G₁ phase. **a** PANC-1 and MCF-7 cells were exposed to increasing concentrations of cristacarpin as indicated; p21, Cdk-2, and cyclinD1 expressions were determined by western blotting. **b** Immunoblots show the expression of p21 in PANC-1 cells when treated with cristacarpin and/or NAC. **c**

PANC-1 cells were treated with cristacarpin along with vehicle DMSO and salubrinal- or thapsigargin-treated cells were analyzed by FACS (as indicated); graph and the table show the percentage of cells in G₁, S, and G₂-M phases

Cristacarpin activates p38MAPK and induces ROS-dependent cellular senescence

Generation of ROS is correlated with activation of p38MAPK leading to the stimulation of p21^{waf1}. Although, the ROS-mediated p21^{waf1} activation depends on p53, still, there are concerned reports eliciting p21^{waf1} upregulation independent of p53 (Sun et al. 2007). Here, we have shown that cristacarpin promoted senescence in a p53-independent manner. Intriguingly, Fig. 5a illustrates that phospho-p38MAPK augmented in a dose-dependent manner, but a mild change in total p38MAPK was also observed, indicating the ROS-dependent activation of the MAPK kinase pathway

(Fig. 5a). Additionally, activation of p38MAPK orchestrated the amplification of p16^{INK4a} in PANC-1 cells (Fig. 5a). p38MAPK is a major cell cycle regulator and its activation blocks the cell cycle to induce senescence (Debacq-Chainiaux et al. 2010).

Although, G₁ cell cycle arrest augments the expression of p21^{waf1} or downregulation of Cdk-2/cyclinD1 indirectly refers to cellular senescence, more specific β -galactosidase (β -gal) staining and DAPI-based senescence-associated heterochromatin foci (SAHF) are true signatures of senescent cells. To explore the effects of ER stress in premature senescence, PANC-1 and MCF-7 cells were treated with the indicated concentrations of cristacarpin. Interestingly, cells incubated without cristacarpin showed no detectable SA- β -Gal activity,

Fig. 5 Cristacarpin-induced premature senescence. **a** Total P38 MAPK and p16 expressions in cristacarpin-treated PANC-1 cells were determined by western blotting; the graph represents the densitometry analysis of western blots. **b–d** PANC-1 and MCF-7 cells treated with cristacarpin along with or without doxorubicin and salubrinal for the set time points; the cells were fixed and stained for β -galactosidase. β -Galactosidase-positive cells

were imaged ($\times 20$) under brightfield microscope. **e** PANC-1/MCF-7 cells were treated with cristacarpin (3 μ M), and after the proper time point, as indicated, the cells were visualized for senescence-associated heterochromatin foci (SAHF) under fluorescent microscope. The results are the representative of three independent experiments. $**P < 0.01$

whereas cells treated with 1, 3, and 5 μ M cristacarpin revealed a marked X-Gal staining in a dose-dependent manner as depicted in Fig. 5b, c. Consistently, the SA- β -Gal-positive cells showed an enlarged and flattened morphology with increased volume and granularity, with persistent senescence signatures (Fig. 5b, c). SA- β -Gal-positive cells were counted using ImageJ software, and percentage of SA- β -gal positive cells was plotted in the graph (Fig. 5d). To counterproof the induction of senescence by cristacarpin, we also checked the presence of senescence-associated heterochromatin foci (SAHF) by DAPI staining of the nucleus.

The presence of SAHF is a newly characterize morphological marker of senescence (Aird and Zhang 2013; Raghuram and Mishra 2014). To do this, both PANC-1 and MCF-7 cells were treated with 3- μ M concentrations of cristacarpin for 10 days and following nuclear DAPI staining; beaded nucleus was sharply visible depicting the typical hetero-chromatinized regions within the nucleus, whereas the cells treated with the vehicle DMSO were devoid of such appearances (Fig. 5e). In conjunction with these findings, ER stress inhibitor salubrinal abrogated cells from entering into senescence in the presence of 5 μ M cristacarpin (Fig. 5b, c), whereas

thapsigargin (positive control for ER stress) considerably augmented the number of SA- β -Gal-positive cells, suggesting the role of cristacarpin-induced ER stress in senescence induction (Supplementary Fig. 2a, b).

Cristacarpin shows a reduction of in vivo allograft mouse tumor

The anti-tumor activity of cristacarpin was evaluated in a 4T1 allograft mouse mammary carcinoma model. The mice were treated with 50 mg/kg/body weight (b.w.) of cristacarpin intraperitoneally on each alternative day for

2 weeks, and the animals were sacrificed 15 days after the treatment termination. Tumors were dissected out carefully and weighed separately. The average tumor weight for the vehicle-treated group was 20 ± 0.43 mg and for the cristacarpin-treated group was 6.0 ± 0.32 mg as shown in Fig. 6a, whereas the tumor weight for the positive control 5-FU (22 mg/kg/b.w.)-treated group was 4.9 ± 0.21 . Notably, our results showed ~70 % reduction in tumor growth in the group of animals treated with cristacarpin compared to the 75.5 % reduction in the 5-FU-treated group (Table 1). Moreover, the animals that received the 50 mg/kg dose remained safe

Fig. 6 Cristacarpin inhibits tumor formation in vivo. **a** The reduction in average tumor growth in cristacarpin-treated mice as compared to the positive control 5-FU and normal saline. The graph is the representative of tumor sizes, as shown. SD $\pm$, $**P < 0.01$. **b** The dissected tumors were further processed for SA- β -gal staining as described in “Materials and methods” section. The figures show the distinct SA- β -gal-stained cells

in cristacarpin-treated samples as compared to the normal saline. **c** Schematic diagram showing the molecular route of cristacarpin-induced senescence. The figure depicts that the induction of cristacarpin-mediated ER stress is inducing ROS, which is subsequently followed by G1 arrest, due to p21 and p16 upregulation, leading to senescence

Table 1 In vivo anti-cancer activity of cristacarpin

Treatment groups	Avg. body weights (g) 14th day of animals on days			Avg. body weights (g)	Avg. tumor weights (mg)	% Tumor growth inhibition	Mortality
	First	Fourth	Seventh				
Cristacarpin (50 mg/kg, i.p.)	19.12	21.22	22.14	20.82	6 ± 0.32*	70.0	0/7
5-FU (22 mg/kg i.p.)	21.63	22.17	23.24	22.34	4.9 ± 0.21**	75.5	0/7
Normal saline (0.2 ml, i.p.)	22.21	21.15	22.18	21.84	20 ± 0.43	—	0/10

Values are mean ± SE ($n = 7, 10$ for control). Comparisons were made between the control and treated groups using Student's t test

* $P < 0.05$, ** $P < 0.01$ versus normal saline control

throughout the experimental period without any adverse effects. These results demonstrate that cristacarpin is an effective inhibitor of tumor growth in a safe and tolerable dose. Additionally, as a proof of concept, SA- β -Gal staining of the tumor samples (from treated/untreated group) revealed a characteristic senescence-related blue staining in cristacarpin-treated tumors, not in the untreated ones (Fig. 6b). Therefore, the above results advocate cristacarpin as a potent pharmacophore, which can be exploited to curb tumor growth and development by inducing senescence.

Discussion

This report provides evidence that natural compound cristacarpin isolated from the stem bark of *E. suberosa* (Watjen et al. 2007) is a potent inducer of ER stress in cancer cells (pancreatic and breast) leading to the moderate intracellular ROS generation which eventually promoted SIPS. Reactive oxygen species, a cumulative word for different kinds of superoxide ions, attributes as signaling molecule controlling various events within the cell. Interestingly, in cancer cells, excessive ROS generation activates signaling events related to apoptosis, but sub-lethal production of ROS is known to trigger senescence in normal fibroblast (Gong et al. 2012; Kaur et al. 2011). A handful of evidence suggests that several anti-cancer polyphenolic compounds from fruits and vegetables induce tumor growth arrest largely through the generation of ROS (Yar Khan et al. 2012). Sin et al. demonstrate that chronic treatment with 20(S)-ginsenoside Rg3, a compound isolated from ginseng, at a sub-toxic concentration triggers senescence-like

growth arrest and escalates ROS production in human glioma cells (Sin et al. 2012). Moreover, bisdemethoxycurcumin, a natural derivative of curcumin, abrogates human breast cancer cell proliferation by inducing oxidative stress-mediated senescence (Li et al. 2013). Here, we report that sub-lethal doses of cristacarpin elevated p21^{waf1} and p16^{INK4a} with simultaneous inhibition of the expression of Cdk-2 and hence attenuated the cell cycle in the G₁ phase. Hydroxyurea, resveratrol, and pyrrithione induce premature senescence by ROS generation (Luo et al. 2013; Ong et al. 2011). Thus, drug targeting premature senescence in cancer cells could be a superior approach rather activating apoptosis because at a lower concentration of the drug, activated senescence regresses the tumor growth by neutralizing the toxicity of the drugs (Wu et al. 2007). Of note, senescence can be an alternative route to curb tumor growth where cells get resistance to apoptosis. Generally, apoptosis is a programmed cell death, but an apoptotic cell does not communicate with the surrounding cells, whereas, in contrast to apoptosis, a senescent cell can activate a paracrine signaling within the tumor contributing more benefits than apoptosis (Childs et al. 2014; Hoare and Narita 2013).

This report suggests that cristacarpin could be considered as a potential lead for senescence induction in cancer cells. Recently, Matos et al. have described the role of ER stress in senescence inducing by generating CuSO₄/H₂O₂ models and showed the changes in expression levels of ER stress sensors (Matos et al. 2015). Here, for the first time, we have shown that cristacarpin induced ER stress-mediated intracellular ROS generation, which further intricates the p38-MAPK pathway to

augment p21^{waf1} as well as p16^{INK4a} pursuing SIPS within the cells. P38-MAPK has been reported to induce senescence when expressed ectopically, but pharmacological or genetic inactivation of P38-MAPK or its upstream component blocks the activation of senescence by stimulating p53 and p16 (Iwasa et al. 2003; Parrinello et al. 2003; Wang et al. 2002). Sun et al. recently demonstrate that Ras-Raf-Mek-Erk cascade increases the ROS generation and activates the P38-MAPK leading to activation of p21^{waf1} and finally promotes senescence (Sun et al. 2007). We also observed the activation of p38-MAPK and subsequent premature senescence induction in the presence of cristacarpin to abrogate tumor cell growth arrest.

A handful of recent evidence suggests that there are diverse ways that an external agent could induce senescence, but most widely reported roots are through DNA damage, which directly activates the p21^{waf1}, or by inducing ROS, which can activate the DNA damage responses (Di Micco et al. 2006). Aphidicolin, bleomycin, camptothecin, carboplatin, cisplatin, doxorubicin, 5-fluorouracil, and etoposide activate senescence by inducing DNA damage (Ewald et al. 2010). Whether cristacarpin-mediated senescence induction occurs due to DNA damage responses warrants further detailed study.

Interestingly, our results obtained from p53-mutated PANC-1 cells eventually support the view that p21 can be activated in p53-mutated status upon oxidative stress. It has been reported that p53-independent upregulation of p21 is facilitated by Chk-2 or by downregulation of c-myc (Aliouat-Denis et al. 2005; Jeong et al. 2010). Russo et al. describe that diethylmaleate, an oxidizing agent, can activate p21 in p53-mutated conditions and block the cell cycle in G₁ phase (Russo et al. 1995). Strikingly, our results not only describe the p53-independent activation of p21 (in the presence of cristacarpin), but also demonstrate its (cristacarpin) role in promoting senescence by G₁ arrest. Further, the upregulation of p16^{INK4a} by p38MAPK activation is a p53-independent phenomenon, responsible for SIPS in p53-mutated cells, thus providing substantial evidence that whether p53 is functional (MCF-7) or mutated (PANC-1), oxidative responses and its sensors can push the cells toward SIPS.

In a nutshell, here, we show that cristacarpin-induced growth arrest was associated with increased ROS production, suggesting that cristacarpin may induce senescence via an oxidative-mediated damage in breast

and pancreatic cancer cells (Fig. 6c). To confirm this important contribution of ROS, we demonstrate that anti-oxidant NAC attenuated cristacarpin-induced SIPS of PANC-1 and MCF-7 cells. We have also shown that the level of ROS is maintained to its sub-lethal concentration by upregulation of SOD-1. This can be correlated with a feedback response related to increasing in ROS production by cristacarpin, which eventually maintains the sub-lethal concentration of ROS essential for senescence (Li et al. 2010). The detailed mechanism is under investigation of our laboratory.

Additionally, cristacarpin is an effective inhibitor of tumor growth in a safe and tolerable dose. Taken together, this report unveils a possible mechanism of ER SIPS activation in breast/pancreatic cancer by natural product cristacarpin, thus advocating its therapeutic importance.

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Conflict of interest The authors declare that they have no conflict of interest.

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