Anti-Proliferative Activities of Vasicinone on Lung Carcinoma Cells Mediated via Activation of Both Mitochondria-Dependent and Independent Pathways

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Abstract
Vasicinone, a quinazoline alkaloid from Adhatoda vasica Nees. is well known for its bronchodilator activity. However its anti-proliferative activities is yet to be elucidated. Here-in we investigated the anti-proliferative effect of vasicinone and its underlying mechanism against A549 lung carcinoma cells. The A549 cells upon treatment with various doses of vasicinone (10, 30, 50, 70 µM) for 72 h showed significant decrease in cell viability. Vasicinone treatment also showed DNA fragmentation, LDH leakage, and disruption of mitochondrial potential, and lower wound healing ability in A549 cells. The Annexin V/PI staining showed disrupted plasma membrane integrity and permeability of PI in treated cells. Moreover vasicinone treatment also lead to down regulation of Bcl-2, Fas death receptor and up regulation of PARP, BAD and cytochrome c, suggesting the anti-proliferative nature of vasicinone which mediated apoptosis through both Fas death receptors as well as Bcl-2 regulated signaling. Furthermore, our preliminary studies with vasicinone treatment also showed to lower the ROS levels in A549 cells and have potential free radical scavenging (DPPH, Hydroxyl) activity and ferric reducing power in cell free systems. Thus combining all, vasicinone may be used to develop a new therapeutic agent against oxidative stress induced lung cancer.

Key Words: Vasicinone, Antioxidant, Anti-proliferative, A549 cells

INTRODUCTION
Lung cancer is considered to be the leading causes of mortality worldwide. In India, lung cancer alone causes 9.3 per cent of all cancer related deaths and the commonest cancer mortality in men (Malik and Raina, 2015). Plants have been used for their potential health benefits across the world especially in Indian Ayurveda, Unani, and Homeopathy, Chinese medicines, and Japanese folklore system of medicines from time immemorial (Shankar et al., 2012; Ningthoujam et al., 2013; Deb et al., 2015; Roy et al., 2015).

Adhatoda vasica Nees (AV), a traditional medicinal plant in Ayurveda is well known to have profound effect on human broncho-alveolar diseases. In vivo and in vitro studies showed that leaf extracts of AV have expectorant (Liu et al., 2015), bronchodilator (Amin and Mehta, 1959; Liu et al., 2015), anti-inflammatory (Singh and Sharma, 2013), antitussive (Liu et al., 2015) and antimicrobial activities (Singh and Sharma, 2013). Vasicine and Vasicinone are the two most biologically active quinazoline alkaloids found in the leaf extracts of AV and it has been reported that vasicinone is the main metabolite of vasicine (Amin and Mehta, 1959; Claeson et al., 2000). Though most of the studies were performed based on the leaf extracts, but the seasonal variation of these alkaloids may limit the efficiency of these studies (Pandita et al., 1983). It has been reported that synthesized vasicinone analogues possesses apoptotic properties in a cell specific manner (Qazi et al., 2014, 2015). In addition, the vasicinone analogues also act as potent inhibitor of the PI3K/Akt/FoxO3a pathway under in vitro and tumor regression in vivo model (Qazi et al., 2014, 2015).
Various studies reported that up regulation of reactive oxygen species (ROS) is a prerequisite for cancer development suggesting it as one of the key factors for cancer progression regulation (Trachootham et al., 2009; Liou and Storz, 2010). Therefore, antioxidant therapy may attenuate the ROS mediated cancer progression. The aim of the present study is to examine the anti-proliferative and antioxidant activities of vasicinone (VAS) in human lung epithelial cells and also to investigate the possible signaling pathways.

MATERIALS AND METHODS

Materials
The human specific antibodies PARP (ab6079), Caspase 3 (ab2666S), Bad (ab11531) and Bcl-2 (ab962) were purchased from Abcam, Inc (Cambridge, MA, USA). Vasicinone (CAS 486-64-6) was purchased from Cayman Chemicals, Michigan, USA. All other chemicals were purchased from Sigma (Saint Louis, USA) unless otherwise mentioned.

Cell culture
The human alveolar epithelial cell line (A549) was obtained from the National Centre for Cell Sciences (Pune, India). The cells were cultured in Ham’s F-12K (Kaighn’s modification of Ham’s F-12 and Coon’s F-12 supplemented with higher concentrations of amino acids and pyruvate, as well as modified salts). The medium was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fetal bovine serum (10%) and 29 mM sodium bicarbonate in a humidified atmosphere containing 5% (v/v) CO₂. The cell cultures were grown to form a monolayer (100% confluence) and then growth arrested for 24 h in the absence of FBS before conducting experiments. Experiments used A549 cells of passage 68-78. The normal skin fibroblast cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 22 mM sodium bicarbonate in a humidified atmosphere containing 5% (v/v) CO₂.

Treatment of cells with VAS
The compound was dissolved in 70% ethanol and further diluted with PBS (1X) and added to the cells at different concentrations. The different concentrations used for VAS treatment were 10, 30, 50 and 70 µM. The time interval fixed for the experiment is 72 h based on the time dependent assay (data not provided). After treatment, cells were lysed in radio immuno precipitation assay (RIPA) buffer (50 mM Tris in pH 8, 150 mM, NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 mg ml⁻¹ leupeptin, 2 mg mL⁻¹, aprotinin, 1 mM EDTA, 10 mM NaN₃, and 1 mM NaVO₃). Lysates were cleared using centrifugation and total protein concentrations were determined using the BCA assay as per the manufacturer’s protocol (Pierce/Thermo Scientific, Rockford, IL, USA).

Cytotoxicity (MTT) assay
The cytotoxicity was evaluated by in vitro MTT assay. Cells (100% confluency) were treated with respective concentrations for 72 h in a humidified incubator at 37°C and 5% CO₂. Cell viability assay was performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] method. The absorbance was read at a wavelength of 570 nm using micro-plate reader (BioTek Instruments, Inc, USA).

Lactate dehydrogenase (LDH) assay
LDH release from cells was determined using commercially available assay kit. The experiments were carried out following the manufacturer’s protocol (Coral Clinical Systems, INDIA).

Wound healing assay
A549 cells were seeded in a 6 well plate under proper conditions as mentioned above. After 24 hours of plating, a wound was gently made by scratching the surface with the help of a 200 µl pipette tip. Then half the wells were left untreated and half were treated with 70 µM of VAS. The cells were photographed by a phase contrast microscope after 0, 24, 48 and 72 hours of VAS exposure.

Assessment of apoptosis by Annexin V/PI/DAPI staining
The A549 cells (100% confluency) were cultured in Milli-cell® EZ slides (Merck Millipore Ltd., Carrigtwohill, Ireland) and incubated respective concentrations of the compound. After 72 h treatment, the cells were washed with serum containing media before incubation with Annexin V-FITC Binding Buffer. After incubation, the cells were suspended in 1 µl of Annexin V-FITC and PI and incubated for 5 minutes in dark (at room temperature). The cells are then washed with PBS and fixed in 2% paraformaldehyde before visualization. Then the slides were observed using a fluorescence microscope (Leica DM-3000LED, Leica Microsystems, Wetzlar, Germany). Image of the same field was captured with appropriate filters and merged with Adobe Photoshop CS5 (Adobe Systems, San Jose, US).

DNA fragmentation assay
The extent of DNA fragmentation was assayed by electrophoresis of genomic DNA samples, isolated from treated and control A549 cells on ethidium bromide stained agarose gels. Briefly, after the 72 h treatment of cells, the DNA extraction was done with the help of DNAeasy blood and tissue kit (Qiagen, Netherlands) following manufacturer’s protocol. The gel was run at 80V for 1 h and visualized under Biorad Chemidoc system (Bio-Rad Laboratories, Hercules, US).

Detection of mitochondrial membrane potential (MMP)
The Mitochondrial membrane potential was measured using the JC1 Mitochondrial Membrane Potential Assay Kit (Mitosciences, Abcam, Cambridge, UK) following manufacturer’s instructions. Briefly, after the treatment, cells were washed once with PBS and then incubated with JC-1 (10 µM). The cells were then incubated at 37°C for 10 min. After washing, the cells were analyzed on a fluorescence spectrofluorometer (Horiba, Japan) at 519 nm excitation and 590 nm emission. All the experiments were done in triplicate and the results were as Mean ± SE.

qPCR array
Customized RT2 profiler qPCR array plates for apoptosis pathway were procured from SA Bioscience, QIAGEN (Fredrick, MD, USA) and gene expression profile of FAS ligand, Fas, caspase-3, PARP-1, BAD, Bcl-2, Cytochrome C was studied with Applied Biosystems Step one plus real-time PCR. Data were analyzed using relative fold change (2⁻ΔΔCT) as
compared with control using the relative housekeeping gene GAPDH with genomic DNA contamination and no template control. Data were analyzed using online SABiosciences RT² profiler PCR array data Analysis software.

**Immunoblotting**

All samples which contained approximately the same amount of protein (40 µg), were run on a 10-12% SDS PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 1 h in blocking buffer containing 1% BSA to prevent nonspecific binding and then incubated with anti-BAD (1:1000), anti-caspase-3 (1:1000), anti-Bcl2 (1:500), and anti-β-actin (1:20000) primary antibodies at 4°C overnight. The membranes were washed in TBS-T (50 mmol L⁻¹ Tris HCl, pH 7.6, 150 mmol L⁻¹ NaCl, and 0.1% Tween 20) for 30 min and incubated with the appropriate HRP conjugated secondary antibody (1:5000) for 2 h at room temperature, then images were developed using the ultrasensitive ECL substrate (Bio-Rad Laboratories, Hercules, US). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5 (Adobe Systems, San Jose, US).

**Quenching of DPPH radical**

The DPPH radical scavenging activity of the VAS was measured following the method reported earlier (Manna et al., 2008; Dutta et al., 2016). Various concentrations of VAS (10, 30, 50, 70, 80 and 100 µM) were added to DPPH solution in methanol (125 µM, 1 mL). The solution was shaken and incubated at 37°C for 30 minutes in dark. The final volume was adjusted to 2 ml by adding water. The decrease in absorbance was measured at 517 nm against methanol blank using microplate reader (BioTek, USA). Percent inhibition was calculated by using the equation,  \[ \text{I} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100, \]  where \( A_0 \) is the absorbance of the blank and \( A_1 \) is the absorbance of test sample. Ascorbic acid was used as a positive control.

**Quenching of hydroxyl radical**

The hydroxyl radical scavenging activity of VAS was examined using the 2-deoxyribose oxidation method (Manna et al., 2010). 2-Deoxyribose is oxidized by the hydroxyl radical generated by Fe²⁺/Ascorbate/EDTA/H₂O₂ system (Fenton reaction) and degraded to malondialdehyde. The extent of deoxyribose degradation was measured by TBA method. The reaction mixture contained 2-deoxy-D-ribose (2.8 mM), FeCl₃ (0.1% w/v, 20 µl) and ferricyanide (1% w/v, 20 µl) were added to DPPH solution in methanol (100 µM) followed by incubation at 50°C for 20 min. After incubation, TCA (10% w/v, 20 µl), distilled water (75 µL) and ferric chloride (0.1% w/v, 20 µl) were added and the reaction mixture was further incubated for 30 min at room temperature in the dark. Absorbance was recorded at 630 nm. Ascorbic acid (0-250 µg/mL) was used to develop a standard curve and the results expressed as ascorbic acid equivalents per µM of sample.

**Detection of intracellular ROS levels in A549 cells**

Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye DCFDA (2′,7′-dichlorofluoresceindiacetate). After treatment, cells were washed once with PBS and then loaded with 5 µM DCFDA in PBS with 4% FBS. The cells were incubated at 37°C for 30 min in the dark and subsequently washed with PBS, and centrifuged at 12,000 g for 10 min at 37°C. After washing, the cells were analyzed on a fluorescence spectrofluorometer (Horiba, Japan) at 488 nm excitation and 519 nm emission as described based on a modified FRAP procedure described elsewhere (Fawole et al., 2010). Different concentrations of VAS (10, 30, 50, 70, 80 and 100 µM) were mixed with 20 µl of 0.2 M potassium phosphate buffer (pH 7.2) and potassium ferri cyanide (1% w/v, 20 µl) followed by incubation at 50°C for 20 min. After incubation, TCA (10% w/v, 20 µl), distilled water (75 µL) and ferric chloride (0.1% w/v, 20 µl) were added and the reaction mixture was further incubated for 30 min at room temperature in the dark. Absorbance was recorded at 630 nm. Ascorbic acid (0-250 µg/mL) was used to develop a standard curve and the results expressed as ascorbic acid equivalents per µM of sample.
All the experiments were done in triplicate and the results were as Mean ± SE.

**Statistical analysis**
Data were analyzed statistically using one way analysis of variance (ANOVA) with Sigma Stat statistical software (Jandel Scientific, San Rafael, CA, USA). When data passed a normality test, all groups were compared using the Student–Newman–Keuls post hoc method. A difference was considered significant at the \( p < 0.05 \) level. The data were represented as mean ± SE.

**RESULTS**

The present study for the first time examined the anti-proliferative activities of VAS against A549 lung epithelial cells and normal skin fibroblast cells. Results are depicted below.

**Effect of VAS on cell viability in lung cancer cells and normal cells**

Fig. 1A and 1B represent the effect of VAS on the cell viability of A549 lung cancer cells and normal skin fibroblast cells. Cells were treated with different concentrations of VAS (10, 30, 50, 70 \( \mu M \)) for the treatment period of 72 h. All four concentrations were shown statistically significant cytotoxic effect against lung epithelial cancer cells. However, treatment with VAS did not cause any cytotoxic effect on normal skin fibroblast cells (Fig. 1B).

**Effect of VAS on lactate dehydrogenase (LDH) activity of lung cancer cells**

LDH is an intracellular enzyme which releases upon plasma membrane leakage into plasma. This enzyme activity is directly proportional to the disruption of plasma membrane. Treatment with VAS at a dose of 50 \( \mu M \) and 70 \( \mu M \) significantly increased the LDH activities compared to those seen in control (Fig. 1C).

**Effect of VAS on cellular motility of lung cancer cells**

Wound healing assay was performed to investigate the effect of VAS on cellular motility of A549 cells. The untreated
cells gradually grew up and filled the wounded region, whereas cells exposed to 70 \( \mu M \) of VAS did not grow much in the wounded region compared to untreated cells. Results suggest that the wound healing ability of A549 cells was decreased after exposure to VAS for 72 h (Fig. 1D).

**Effect of VAS on the mode of cell death by annexin V/PI/DAPI Staining**

Fluorescence microscopy showed the mode of VAS-induced cell death in A549 cells. An increase in annexin-V staining was observed in VAS-treated cells at higher doses (50 and 70 \( \mu M \)) suggesting the apoptotic mode of cell death. DAPI staining also showed an increase in nuclear condensation upon treatment with VAS as compared to untreated cells. Treatment with higher doses of VAS further showed an increase in PI staining, which suggested an increase in DNA damage (Fig. 2A).

**Effect of VAS on DNA fragmentation in lung cancer cells**

DNA fragmentation in VAS-treated A549 cells was examined using agarose-ethidium bromide gel electrophoresis. DNA fragmentation is considered to be a hallmark of apoptotic process in various cell types, whereas the non-apoptotic cells maintain their structure (Kitazumi, Maseki et al., 2010). The agarose gel image as shown in Fig. 2B confirms the DNA fragmentation in VAS (50 and 70 \( \mu M \), 72 h) treated A549 cells.

**Effect of VAS on mitochondrial membrane potential (MMP) level in lung cancer cells**

In this study, we examined the effect of VAS on MMP level in A549 cells (Fig. 3A). Results suggest that treatment with VAS at a dose of 50 and 70 \( \mu M \) caused a decrease in MMP level by 1,802 ± 233.33 and 19,01.84 ± 106.84 respectively compared to control (3,141.33 ± 285).

**Effect of VAS on cell signaling molecules involved in cell death of lung cancer cells**

An increase in Fas/FasL, decrease in mitochondrial transmembrane potential, release of cytochrome C, and subsequent activation of caspase-3 represent a key step in the mitochondrion-dependent apoptotic cell death pathway. Whereas, Poly (ADP-ribose) polymerase (PARP) mediated programmed cell death is said to be caspase independent cell death pathway. In this study we examined both the pathways to evaluate the effect of VAS. The gene expression profile showed a few fold increases of FAS ligand (FasL), Fas, BAD, Cytochrome C, caspase-3, and PARP (Fig. 3B) genes and a decrease in Bcl-2 in VAS-treated lung cancer cells. VAS treatment also up-regulated the protein expression of PARP (Fig. 3C), caspase-3 (Fig. 3D), and BAD (Fig. 3E) and down-regulated Bcl-2 in A549 cells (Fig. 3F).

The antioxidant activities of VAS were examined in both cell free and cellular system. The results are demonstrated below.
Effect of VAS on radical scavenging activities (DPPH and hydroxyl), and ferric reducing antioxidant power in cell free system and intracellular ROS production in lung cancer cells

In this study, we evaluated the antioxidant activities of VAS at different concentrations as shown in Fig. 4A, 4B and 4C. Results suggest that with the increasing concentrations of VAS, the inhibition of DPPH and hydroxyl radical increases and the maximum inhibition were observed at the concentration of 70 µM and stabilized thereafter. The maximum percentage of ferric reducing power was also observed at 70 µM of VAS.

The present study examined the effect of VAS on intracellular ROS production in A549 cells. Results suggest that VAS treatment at a dose of 50 and 70 µM lead to a decrease in ROS production by 1,030.11 ± 104.32 and 1,032.37 ± 81.63 a. u. as compared to control (1,312.55 ± 63.77 a.u.) (Fig. 4D).

DISCUSSION

As a traditional folk medicine, Adhatoda vasica is quite well known in the Indian subcontinent for their bronchodilator activities. In terms of active compounds, the leaves of A. vasica is rich in many phytochemicals, such as alkaloids, tannins, saponins, phenolics, and flavonoids (Claeson et al., 2000). Gibbs et al. (2009) revealed that ambroxol, a natural compound of A. vasica inhibited IgE-dependent basophil mediator release. Among the quinazolinone alkaloids, vasicine and VAS are the most biologically active molecules used for broncho alveolar diseases. Several investigators have synthesized various analogues and derivatives of vasicine and VAS and reported their pharmacological properties, such as anti-mycobacterium, acaricidal to name a few (Gautam and Sharma, 1982; Grange and Snell, 1996; Shevyakov et al., 2006a, 2006b; Shang et al., 2016). The present study evaluated the anti-proliferative and antioxidant properties of VAS using A549 human lung cancer cell culture model.

The study for the first time reports the anti-proliferative properties of VAS in A549 cells. MTT assay demonstrated a cytotoxic effect of VAS against lung cancer cells but not on normal skin fibroblast cells. Apart from the cell viability assay, we also examined the LDH leakage to assess the cytotoxic effect of VAS. Results showed that increasing the concentration of VAS caused an increase in LDH leakage, which suggest the disruption in plasma membrane integrity of lung cancer cells by VAS. The wound healing assay also validated the cytotoxicity of VAS in A549 carcinoma cells.

Cell death pathways are basically of two types, programmed (apoptosis) and non-programmed (necrosis). Necrosis form of cell death is caused by external factors such as trauma or infection whereas apoptosis is known to be a delicately maintained programmed cell death pathway. Among the death receptors in apoptosis, Fas/FasL system is quite well known. The binding of Fas to its ligand on the cell surface leads to the activation of downstream caspases such as caspase 8 and caspase 3, and initiates cell death (Pinkoski et al., 2000). Among other apoptotic cell death pathways, the Bcl-2 family proteins were known to cause cell death via mitochondrial dysfunction (Wong and Puthalakath, 2008; Su et al., 2014). Several investigations suggested that the change in mitochondrial membrane potential may lead the committed cells to apoptotic death (Ly et al., 2003; Galluzzi et al., 2007; Manna et al., 2010; Gruber et al., 2015). The Bcl-2 family proteins are of two types, pro-apoptotic (Bax and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl). An imbalance in Bcl-2 family protein leads to the decrease in mitochondrial membrane potential and subsequent release of cytochrome C into the cytosol (Marsden et al., 2004). In cytosol, cytochrome C along with procaspase-9 interacts to form the apoptosome that triggers the activation of caspase-3.

The present study shows that VAS treatment lowered the mitochondrial membrane potential along with increase in number of annexin-V/PI double stained cells suggesting apoptotic mode of cell death. Results demonstrated VAS treatment lead to up-regulation of mRNA expression of Fas, FasL, BAD, cytochrome C and caspase 3, and down-regulation of Bcl-2. VAS treatment also led to higher protein expression of BAD and caspase 3 of lung cancer cells.

PARP plays a central role in the DNA damage and repair process (Virag et al., 1998). The over-expression of PARP initiates the apoptosis by promoting the release of mitochondrial apoptosis inducing factors and the fracture of double stranded DNA. Our investigation on revealed that VAS treatment lead to an increase in both mRNA and protein expression of PARP and DNA fragmentation in lung cancer cells.

Several investigators have shown that increased ROS production in cancer cells correlates with the aggressiveness of cancer suggesting a pivotal role of ROS in the cancer pathogenesis (Tsao et al., 2007; Kumar et al., 2008). Increased ROS was also shown to be associated with decrease in antioxidants in leukemic and colorectal cancer cell lines (Oberley and Buettner, 1979; Oltra et al., 2001; Skrzydlewsk et al., 2010; Gruber et al., 2015). The Bcl-2 family proteins are of two types, pro-apoptotic (Bax and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl). An imbalance in Bcl-2 family protein leads to the decrease in mitochondrial membrane potential and subsequent release of cytochrome C into the cytosol (Marsden et al., 2004). In cytosol, cytochrome C along with procaspase-9 interacts to form the apoptosome that triggers the activation of caspase-3.

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2005). Thus reducing the intracellular ROS level may be an alternative therapeutic strategy against cancer. In the present study apart from anti-proliferative activity, VAS was also shown to possess a significant free radical scavenging activity (DPPH, hydroxyl) and ferric reducing power (Fig. 4A, 4B, 4C). Moreover, VAS-treatment significantly decreased the intracellular ROS production in A549 cell (Fig. 4D). Thus the study suggests that VAS may mediate cell death in lung cancer cells via Fas death receptors and mitochondrial dependent signaling pathways without increasing the cellular oxidative stress in lung cancer cells.

Based upon the results, a probable role of VAS in inducing apoptosis in lung cancer cells were proposed as shown in Fig. 5. Although detailed studies are needed to examine the molecular mechanism underlying the mitochondrial dysfunction with lowering the intracellular ROS levels, the present study provides scientific evidences of using the compound as a possible lead molecule for in depth research against oxidative stress induced lung cancer for developing therapeutics in near future.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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