Cervical cancer cell proliferation inhibition by vanillin oxime through HIF-1α expression inhibition, ERK1/2 and Akt protein down-regulation

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Cervical cancer is a frequently reported cancer of reproductive tract in females and is worldwide 4th most common malignant tumor. The present study investigated the effect of vanillin oxime on proliferation of cervical cancer cells. Vanillin oxime treatment led to suppression of Caski cell proliferation but could not affect proliferation of (HCvEpC) cells at the tested (2 to 10 μM) concentrations. In vanillin oxime treated Caski cells ROS level showed an increase with enhancement in concentration from 2 to 10 μM. Vanillin oxime treatment significantly (P<0.0487) lowered the count of colonies and inhibited invasive abilities of Caski cells. Treatment with vanillin oxime caused a significant (P<0.0487) suppression in HIF-1α expression in Caski cells. Caski cell apoptotic count reached to 8.76% and 48.65%, on incubation with 2 and 10 μM concentrations of vanillin oxime respectively. After treatment with vanillin oxime a prominent reduction in MMP-2 and -9 levels was observed in Caski cells. A prominent reduction in p-ERK1/2 and p-Akt levels was observed in Caski cells after treatment with vanillin oxime. Vanillin oxime inhibits cervical cancer proliferation, invasive abilities, induces apoptotic signalling, and elevates ROS production. Therefore, vanillin oxime may be developed as an effective therapeutic agent for treatment of cervical cancer.

Keywords: cervical cancer, apoptosis, vanillin oxime, ROS, HIF-1α expression

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Abbreviations: HCvEpC, human cervical epithelial cells; HIF-1α, Hypoxia-inducible factor-1α

INTRODUCTION

Cervical cancer is a frequently reported cancer of reproductive tract in females and is worldwide 4th most common malignant tumor (Chen et al., 2020). Annually cervical cancer is detected in ~500 000 patients globally and reports have shown increasing incidence among younger women over the past few years (Fitzmaurice et al., 2015; Wei et al., 2012). Surgery along with radiotherapy have shown significant improvement in the cervical cancer management in patients with early-stage of cancer (Sousa et al., 2018; McClung et al., 2019). However, the treatments available hardly effect tumor growth in patients having late-stage cancer and tumor recurrence. Patients with late-stage cervical cancer have very poor prognosis and it is reported that 1-year survival rate of such patients is below 20% (Sousa et al., 2018; McClung et al., 2019). The comprehensive strategies, including surgery used for cervical cancer have failed to produce any breakthrough in the treatment of advanced and recurrent tumors (Su et al., 2019; Yang et al., 2014). Thus, identification of molecular targets and discovery of potent anti-tumor agents is required to improve the health and survival of patients and inhibit tumor development.

There are several reactive oxygen species (ROS) in human bodies which play important role in various metabolic regulatory processes like glucose metabolism (Jambunathan et al., 2010; Wang et al., 2018). ROS act as main signalling factors and regulate several processes including proliferation of cells, migration abilities, and cell differentiation by influencing various proteins and lipids (Lu et al., 2018; Diwani et al., 2018; Sun et al., 2019). In addition, ROS also influence the functioning and structure of the blood vessels (Lu et al., 2018; Diwani et al., 2018; Sun et al., 2019). Higher content of ROS induces various disorders in the body by damaging various proteins and membranes. Hypoxia-inducible factor-1α (HIF-1α) has been found to play a prominent role in invasion of cancer cells, their metastatic ability, and proliferation (Zhang et al., 2017; Lai et al., 2018). Reports have demonstrated that HIF-1α expression inhibition by therapeutic agents exhibits anti-tumor effect in many cancers such as breast and pulmonary cancer cells (Sun et al., 2011; Yang et al., 2014).

Vanillin, a phytoconstituent isolated from *Vanilla planifolia* plant is commonly known as 4-hydroxy-3-methoxy-benzaldehyde. Initial screening revealed anti-microbial, anti-inflammatory activities of vanillin in rodent as well as human models (Walton et al., 2003; King et al., 2008; Srinivasan et al., 2008; Imanishi et al., 1990). Mechanistic study revealed that vanillin inhibits invasion and migration abilities of liver cancer cells by down-regulation of nuclear factor-κB expression and suppression of matrix metalloproteinases (Liang et al., 2009). Moreover, vanillin treatment led to the inhibition of PI3K phosphorylation to suppress angiogenesis of lung cancer cells and formation of lamellipodia (Lirdprapamongkol et al., 2010; Lirdprapamongkol et al., 2005; Lirdprapamongkol et al., 2009). Vanillin treatment of cervical and breast cancer cells is associated with activation of apoptosis pathway and thereby inhibition of tumor growth (Lirdprapamongkol et al., 2010; Lirdprapamongkol et al., 2005; Lirdprapamongkol et al., 2009). Compound obtained by modification of many natural products has shown strong anticancer property exhibiting...
81–82% cytotoxicity. Oxime analogs of curcumin have been found to possess strong antiproliferative property against cancer cells compared to the cyclohexanone and tetralone analogs (Qin et al., 2016). Taking into account string anticancer activity of oxime analogs of the natural products the present study investigated vanillin oxime as anti-proliferative agent against cervical cancer cells and elucidated the associated mechanism.

EXPERIMENTAL

Reagents and chemicals

Vanillin oxime (Cas number S539570; purity 99%) and dimethyl sulfoxide were supplied by the Merck Chemicals. Dimethyl sulfoxide (DMSO) and many other chemicals were provided by the Sigma-Aldrich (St Louis, MO, USA). Stock solution of vanillin oxime was prepared in physiological saline.

Cell lines and culture

Caski cervical cancer cell line and human cervical epithelial cells (HCvEpC) cells were supplied by the Shanghai Bioengineering Co., Ltd., China. The cells were cultured overnight in RPMI-1640 medium mixed with FBS (10%) in an incubator containing CO₂ at 37°C. At the time of entering logarithmic growth phase the cells were digested for 3 min on treatment with 0.25% pancreatin at 37°C.

Proliferation assay

Caski and (HCvEpC) cells were inoculated in 96-well plates at a density of 2×10⁴ cells/ well and incubated for 24 h with vanillin oxime at 2, 4, 6, 8 and 10 μM concentrations. Culture solution from the plates was removed at 24 h of incubation and then 10 μl of MTT (5 mg/ml) solution was added to each well. After 4 h of incubation with MTT, 100 μl DMSO was added to solubilise the formazan crystals that formed in each well. Measurement of absorbance was made three times for each plate at 450 nm using a microplate reader (Thermo, BD, USA, VIOLA0D2) for calculation of cell proliferation.

Determination of ROS level

Fluorescent dye namely, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen) was used for ROS content detection in Caski cells. The cells were incubated at 1×10⁶ cells/ well distribution in 6-well plates for 24 h with 2 to 10 μM concentrations of vanillin oxime at 37°C. Afterwards cells were PBS washed twice before incubation for 40 min at 37°C with 20 μM H₂DCFDA as per the instructions of the supplier. Excitation and emission wavelengths used were 489 and 528 nm, for detection of fluorescence using enzyme labeling instrument (Tecan Group, Ltd.) and for the determination of ROS content, respectively.

Colony formation assay

Caski cells growing in logarithmic phase were distributed at 1000 cells/ well density in 60-mm culture dishes and subjected to culture at 37°C. Incubation with vanillin oxime at 2 and 10 μM concentrations was followed by removal of the medium and subsequently medium was replaced after every 3 days. Washing of cells in PBS, fixing for 10 min in methanol (anhydrous) was followed by staining with crystal violet solution (0.1%). Staining was performed for 20 min at room temperature prior to calculation of colonies formed under light microscope (magnification ×200; Carl Zeiss AG).

Analysis of cell invasion

Matrigel was adjusted to a concentration of 1 mg/ml using precooled medium devoid of the serum at 4°C. Upper chambers having filters with 8 μm pore-size were coated for 4 h at 37°C using Matrigel and set for solidification. The cells in groups of 4×10⁵ were suspended in 350 μl serum-free medium and then transwell chambers were put into the 24-well culture plate. Mixture of the cells was put into the upper chamber and the medium mixed with 20% FBS was added into the lower chamber. Removal of the chamber and aspiration of the cell culture was followed by PBS washing of the cells and then fixing for 10 min in methanol (anhydrous). The cells were dyed for 10 min with crystal violet (0.1%), washed in water, dried, and then mounted on the coverslips using neutral gum. Cell invasion was detected randomly in five fields using a light microscope (magnification ×200; Carl Zeiss AG).

Western blot analysis

Caski cells treated with vanillin oxime were lysed using the RIPA lysis buffer mixed with PMSF protease inhibitor (1 μM) on ice over 40 min. Supernatant was isolated after lyase centrifugation at 4°C for 25 min at 13 000×g and protein content was estimated using the BCA method. Protein samples (30 μg protein/lane) were separated on 10% SDS-PAGE and transferred to PVDF membranes which were blocked by incubation with 5% skimmed milk powder for 2 h. Membrane incubation with primary antibodies against p-ERK1/2 (ab196883; dilution 1:1,000), p-Akt (ab196883; dilution 1:1,000), Bel-2 (ab218123; dilution 1:1000) and HIF-1α (ab205833; dilution 1:1,000; all from Abcam) was performed for overnight at 4°C. Washing with PBS-0.1%Tween-20 (PBST) three times was followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000; cat. no. ab6789; Abcam). Protein expression was analysed using Laser scanning imaging (LI-COR Biosciences) and ImageJ 6.0 system.

Reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from vanillin oxime treated Caski cells using TRIzol® reagent (Invitrogen) and for determination of RNA purity and concentration the ultraviolet spectrophotometer (Tecan Group, Ltd.) was employed. The RNA samples were reverse transcribed to cDNA using the EcoDry™ Premix (Takara Biotechnology Co., Ltd.). Conditions used for reverse transcription were 50 min at 42°C, for inactivation of the reverse transcriptase 5 min at 98°C and reverse transcription product saving at 4°C. The BeyoFast™ SYBR-Green quantitative PCR premix was used for detection of HIF-1α expression. Conditions used for thermocycling were: denaturation for 8 min at 94°C, 38 cycles for 12 sec at 94°C and for 55 sec at 58°C. The PCR amplification was followed by plotting of the melting curve to determine HIF-1α expression using the 2−ΔΔCq method.

Flow cytometry assay

Caski cells at 2.5×10⁵ cells/ml distribution were collected as single-cell suspension and put into 96-well
plates. After treatment with vanillin oxime cells were harvested, washed twice with PBS, and then dried with Annexin V-FITC and PI for 20 min at 37°C. The cells were subsequently analysed for apoptosis using the flow cytometry (BD FACSCalibur™; BD Biosciences). Apoptosis was analysed using the CellQuest Pro software (version 5.0; BD Biosciences).

**Statistical analysis**

Data expressed are the mean ± S.D. of triplicate experiments carried out independently. Differences were determined statistically using one-way ANOVA, Bonferroni post hoc test and Unpaired t-test to compare two groups. Data were analysed using the SPSS 20 (IBM Corp.) software. Differences were taken as statistically significant at P<0.05.

**RESULTS AND DISCUSSION**

**Inhibition of Caski cell viability and promotion of ROS level by vanillin oxime**

Cervical cancer, a frequently diagnosed gynecological malignancy in females, was responsible for 311 000 deaths in the year 2018 (Arbyn et al., 2020). Worldwide, cervical cancer has an incidence of around 13·1 cases in every 100 000 women and it was diagnosed in ~570 000 women in 2018 (Arbyn et al., 2020). Treatment strategy presently available for cervical cancer consists of surgical resection followed by radiotherapy in combination with chemotherapy (Menderes et al., 2016). Standard treatment that is effective for cervical cancer inhibition is yet to be established and is urgently required to be developed. Chemotherapeutics used against the cervical cancer are mainly confronted with challenges such as tumor recurrence and rapid distant organ metastasis (Geretto et al., 2017). Highly efficient and novel chemotherapeutic agents possessing least/no side effects need to be developed for the treatment of the cervical cancer effectively (Geretto et al., 2017; Dueñas-González and Campbell, 2016).

In the present study viability changes by vanillin oxime in Caski and (HCvEpC) cells were measured following incubation with 2, 4, 6, 8 and 10 μM concentrations by MTT method (Fig. 1A). Vanillin oxime treatment led to suppression of Caski cell proliferation, and the reduction was concentration dependent. Suppression in Caski cell proliferation by vanillin oxime treatment was maximum at 10 μM concentration and remained constant at 10 μM. Vanillin oxime treatment also caused significant (P<0.05) reduction in Caski cell proliferation at 2, 4 and 6 μM concentrations. In (HCvEpC) cells vanillin oxime treatment could not affect proliferation at the tested (2 to 10 μM) concentrations. Vanillin oxime induced ROS level in Caski cells was detected at 2 to 10 μM concentrations following incubation for 24 h (Fig. 1B). In vanillin oxime treated Caski cells ROS level showed an increase with enhancement in concentration from 2 to 10 μM.

**Vanillin oxime inhibits colony formation and cell invasion**

Colony formation inhibition by treatment with vanillin oxime in Caski cells was detected by treatment with concentrations of 2 and 10 μM (Fig. 2A). Vanillin oxime treatment significantly (P<0.0487) lowered the count of colonies in Caski cells compared to the control. In 10 μM vanillin oxime treated cell cultures very few colonies were detected at 24 h compared to the control cultures. Treatment with vanillin oxime also lowered invasive abilities of Caski cells significantly (P<0.05) at 2 and 10 μM concentrations (Fig. 2B). Transwell assay showed that treatment with 10 μM vanillin oxime lowered invasive abilities of Caski cells to greater extent compared to 2 μM concentrations.

**Vanillin oxime induces Caski cell apoptosis**

A main anti-tumor mechanism for chemotherapeutics involving several targets to inhibit cell proliferation via complicated process is the apoptosis (Lunghi et al., 2008). Anti-tumor effect of some chemotherapeutics have been demonstrated to be associated with the damage induced by ROS formation (Sarkar et al., 2014; Qu et al., 2013). At baseline values ROS induces DNA synthe-

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**Figure 1. Effect of vanillin oxime on cell proliferation and ROS level.**

(A) Caski and (HCvEpC) cells were incubated for 24 h with vanillin oxime and then analysed by MTT method for proliferation. (B) ROS level in Caski cells was detected after 24 h of incubation with 2 to 10 μM concentrations of vanillin oxime. *P<0.05, **P<0.01 vs. control cells.

**Figure 2. Effect of vanillin oxime on colony formation and invasive ability.**

(A) Caski cells were incubated for 24 h with vanillin oxime and then analysed for colony-formation. (B) Invasive ability of Caski cells was monitored after 24 h of incubation with 2 and 10 μM concentrations of vanillin oxime.
sis, subsequent cell mitosis and tumor cell proliferation (Hassani et al., 2013). Excessive formation of ROS leads to induction of apoptotic signals and causes necrotic cell death (Pollak et al., 2013). In the present study Caski cells after vanillin oxime treatment at 2 to 10 μM concentrations were analysed for apoptosis by flow cytometry at 24 h (Fig. 3). Treatment with vanillin oxime significantly (P<0.05) raised Caski cell apoptotic count in a dose-dependent way. Caski cell apoptotic count reached to 8.76% and 48.65%, on incubation with 2 and 10 μM concentrations of vanillin oxime, respectively.

Vanillin oxime inhibits MMP expression

Vanillin inhibits tumor migration and suppresses invasive abilities of liver cancer cells via targeting NF-κB expression and MMP activities (Liang et al., 2009). Angiogenesis as well as lamellipodia formation in lung cancer cells is inhibited through interfering with PI3K pathway by vanillin treatment (Lirdprapamongkol et al., 2010; Lirdprapamongkol et al., 2005; Lirdprapamongkol et al., 2009). Growth of cancer cells such as breast and cervical carcinoma is inhibited by vanillin through induction of apoptotic signals (Lirdprapamongkol et al., 2010; Lirdprapamongkol et al., 2005; Lirdprapamongkol et al., 2009). In the present study treatment of Caski cells with vanillin oxime at 2 to 10 μM concentrations for 24 h was followed by MMP expression measurement by western blotting (Fig. 4). After treatment with vanillin oxime a prominent reduction in MMP-2 and -9 levels was observed in Caski cells compared to the control cells. The MMP-2 and -9 levels in Caski cells were reduced to minimum level by vanillin oxime treatment at 10 μM concentration at 24 h.

Vanillin oxime inhibits malignancy protein levels

In Caski cells vanillin oxime treatment at 2 to 10 μM concentrations for 24 h was followed by protein expression determination by western blotting (Fig. 5). A prominent reduction in p-ERK1/2 and p-Akt levels was observed in Caski cells after treatment with vanillin oxime at 2 to 10 μM concentrations. The Bcl-2 level in Caski cells also showed a remarkable reduction after vanillin oxime treatment in a dose-dependent way.

Vanillin oxime inhibits HIF-1α expression

Activation of HIF-1α at a rapid rate plays crucial role in proliferation of tumor cells, their metastatic potential and infiltration abilities (Su et al., 2011). It is also involved in development of resistance in tumor cells against chemotherapies and radiotherapy (Su et al., 2011). In the present study treatment with vanillin oxime caused a significant (P<0.05) suppression in HIF-1α mRNA expression at 2 to 10 μM concentrations in Caski cells (Fig. 6A). After increasing the concentration of vanillin oxime from 2 to 10 μM, the reduction in HIF-1α mRNA showed a dose-dependent reduction. HIF-1α protein level also showed a prominent reduction in Caski cells after treatment with vanillin oxime at 2 to 10 μM concentrations (Fig. 6B, C). Suppression in HIF-1α protein level by vanillin oxime treatment at 24 h was maximum at 10 μM concentration in Caski cells.

CONCLUSION

In summary, vanillin oxime inhibits cervical cancer proliferation, invasive abilities, induces apoptotic signaling, and elevates ROS production. Moreover, HIF-1α expression inhibition, ERK1/2 and Akt protein down-regulation is induced in cervical cancer cells after treatment with vanillin oxime. Therefore, vanillin oxime may
be developed as an effective therapeutic agent for treatment of cervical cancer.

Statement of Ethics

The approval for present study was obtained from the Research Ethics Committee, Chifeng Second Hospital, Neimenggu Chifeng (2004), China. All the experimental procedures were conducted in accordance with the guidelines issued by National Institute of Health (NIH), China.

Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

Guang-Min Zhang conceived and designed the study, Hong-jing Chen, Nai-ling Li and Qiang Gu performed all the experimental work, analysed and compiled the data. Hong-jing Chen and Nai-ling Li made literature survey and wrote the paper. All the authors approved the research article for publication.

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