IJBPAS, September, 2023, 12(9): 3892-3901

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANTIPROLIFERATIVE EFFECT OF MOSINONE-A ON EXPRESSION OF APOPTOTIC ASSOCIATED PROTEINS DURING DMBA INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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Received 25th June 2022; Revised 18th Aug. 2022; Accepted 11th Dec. 2022; Available online 1st Sept. 2023

https://doi.org/10.31032/IJBPAS/2023/12.9.6552

ABSTRACT

***Objectives*:** The present study was investigated to evaluate antiproliferative potential of the chemopreventive potential of Mosinone-A on cell proliferation, apoptosis and cell cycle proteins during 7, 12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch (HBP) carcinogenesis.

***Materials and methods*:** A total number of 40 golden Syrian hamsters were randomized into 4 groups of 10 animals in each. Group I animals were served as untreated control. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin three times per week for 14 weeks on the left buccal pouches. Group III animals were orally administered with Mosinone-A (2 mg kg^{-1} b.wt) starting one week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrification of the animals. Groups IV animals were received Mosinone-A alone throughout the experimental period.

***Results*:** Topical application of DMBA for 14 weeks induced buccal pouch carcinomas associated with increased expression of, P^{21}, whereas decreased expression of Bax, Cleaved caspase-9. Oral administration of Mosinone-A significantly inhibited the development of HBP

carcinomas as revealed by decreased expression of P^{21}, and over expression Bax, Cleaved caspase-9,

***Conclusion*:** The result of the present study indicates that Mosinone-A can exerts protective effects against DMBA induced buccal pouch carcinogenesis. These findings suggest that Mosinone-A exerts its anticancer properties by inhibiting cell proliferation and inducing differentiation and apoptosis.

Keywords: Oral cancer; Mosinone-A; DMBA; apoptosis; Westernblot

1. INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents a global public health problem, is the fifth most frequent cancer in men and women worldwide and accounting for approximately 500,000cases annually **[1]**. The incidence rate of oral cancer in India is very high compared with other countries like Sri Lanka, Pakistan, and Bangladesh **[2]**. Various epidemiological studies suggested that tobacco, betal quid chewing and alcohol consumption are major risk factors for the development of oral cancer, more than 90% are squamous cell carcinoma **[3]**.

Establishment of oral cancer cells are associated with increased cell proliferation, prolonged cell survival, dysregulation of cellular differentiation as well as decreased the programmed cell death (apoptosis) **[4]**. Recent studies emphasized the control the incidence of OSCC has focused on developing effective chemoprevention strategies **[5]**. The golden Syrian hamster buccal pouch is an excellent target organ for studying oral carcinogenesis which, under the induction of 7, 12-dimethylbenz[a]anthracene consistently produces squamous cell carcinoma **[6]**.

DMBA, a potent organ and site specific carcinogen is commonly used to induce buccal pouch carcinogenesis in hamsters, Dihydrodiol epoxide, the ultimate carcinogen DMBA, mediate the carcinogenic process by inducing chronic inflammation and through the over production of reactive oxygen species (ROS) **[7-8]**. The present study was designed to determine the antiproliferative potential of Mosinone-A inhamster buccal pouch carcinogenesis. We analysed the expression of biomarkers that are reliable indication of cell proliferation P^{21}, Bax, Cleaved caspase-9 by western blot analysis. Our results indicate that Mosinone-A is a more potent inhibitor of HBP carcinogenesis based on suppression of HBP carcinomas and modulation of cell

proliferation and cell cycle regulation of apoptosis.

P^{21} is a nuclear protein, also known as cyclin dependent kinase inhibitor (CDK1), is frequently expressed in epithelial cells and play an important role in maintaining the cells and p^{53} mediated cell growth arrest and apoptosis considering these functions of p^{21}, it appears to be closely associated with the carcinogenesis **[9-14]** including oral cancer.

Mosinone-A act to inhibit complex I mitochondrial oxidative phosphorylation **[16]** with an activity several times that of rotenone was reported. Mosinone-A is one of the novel mono-tetrahydrofuran ring acetogenin, from the bark of *Annona squamosa*, viewing cytotoxic selectivities for the human pancreatic carcinoma cell line **[15-17]**. However, the mechanism by which Mosinone-A exerts its cytotoxic effect on oral cancer cells are not well understood. Therefore, we undertook this study to investigate the apoptotic and cell cycle regulatory proteins during the DMBA induced hamster buccal pouch carcinogenesis.

2. MATERIAL AND METHODS

2.1. Animals

Eight to ten weeks old male golden Syrian hamsters, weighing 80-120g were purchased from National Institute of Nutrition, Hyderabad, India and were maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided with a standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12h light /dark cycle.

2.2. Chemicals

The carcinogen, 7, 12-dimethylbenz[a]anthracene (DMBA) was obtained from Sigma-Aldrich Chemical Pvt. Ltd. Bangalore, India. All other chemicals used were of analytical grade, marketed by Himedia laboratories, Bangalore and Sisco Research Laboratories Pvt, Ltd, Mumbai, India.

2.3. Isolation of Mosinone-A

Mosinone-A was isolated from *Annona squamosa* bark using the method of Maclaughlin **[18]**. The dried and pulverized bark of *Annona squamosa* was extracted with ethanol. The residues were portioned between chloroform and water, and further portioned between 90% methanol and hexane to get hexane soluble residues. The hexane soluble residue was subjected into column chromatography over silica gel using hexane and chloroform followed by

chloroform and methanol solvent system. The resulting fractions were combined on the basis of HPTLC analysis. Then, the combined fractions were run into column chromatography to get the final product of Mosinone-A, a whity waxy solid substance. The identity of isolated Mosinone-A was done by LC-MS and NMR. Its identity was confirmed by comparison to the reference Mosinone-A, which was purchased from Lock chemicals Ltd China. The yield and purity of the isolated Mosinone-A were found to be 0.21% and >90% respectively. For experimental studies Mosinone-A was first dissolved in 0.5% dimethyl sulfoxide (DMSO).

2.4. Experimental protocol

The local institutional animal ethics committee, Annamalai University, Annamalai Nagar, India, has approved the experimental design. A total number of 40 golden Syrian hamsters were randomized into 4 groups of 10 animals in each. Group I animals were served as untreated control. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin three times per week for 14 weeks on the left buccal pouches (No:4 brush). Group II animals received no other treatment. Group III animals were orally administered with Mosinone-A (2 mg kg^{-1} b.wt) starting one week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrification of the animals. Groups IV animals were received Mosinone-A alone throughout the experimental period. The experiment was terminated at the end of 14^{th} week and all animals were sacrificed by cervical dislocation. Western blotting in protein and quantitative real time PCR in mRNA were expressed in buccal mucosa of control and experimental animals in each group.

2.5. Western Blot Analysis.

Tissues were rinsed with PBS. Cell extracts were prepared using lysis buffer [20 mmol/L Tris-HCl (pH7.5), 0.1% TritonX, 0.5% sodium deoxycholate, 1mmol/L phenylmethylsulfonyl fluoride, 10µg/mL aprotinin, and 10µg/mL leupeptin] and centrifuged at 12,000*g* at 4°C. Total protein concentration was measured using the BCA assay. Cellular extracts containing 50µg total protein were subjected to 10% SDS-PAGE, and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). After blocking with PBS containing 0.2% casein for 1 hr at room temperature, membranes were incubated with primary antibody (1:1000 dilution) in

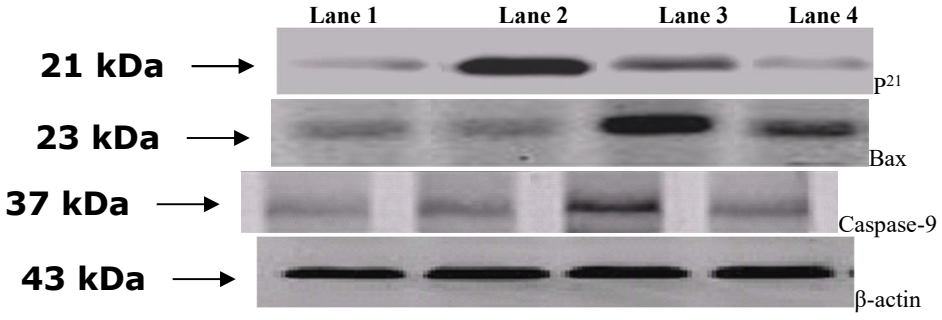
PBS containing 0.2% Tween-20 overnight at 4°C. After 3–5 washes, the membrane was incubated with secondary antibodies (1:8,000 dilutions) for 1 hr. After sequential washes, membranes were scanned on Kodak Image Station 4000MM Digital imaging system (Molecular imaging systems, Carestream Health, Inc, United States). The signals were quantified using ImagePro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to that of β-actin.

2.8. Statistical analysis

Valuesare expressed as mean ± SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The values were considered statistically significant if the p-value were less than 0.0

3. RESULTS

Figure 1, shows western blot analysis of p^{21}, Bax and caspase-9 in the buccal pouch of control and experimental animals in each group. The expression of p^{21}, Bax and caspase-9 were detected as bands of molecular weight 21, 23 and 37 KDa respectively. The expression of p^{21} significantly increased whereas the expression of Bax and caspase-9 significantly decreased in DMBA alone treated animals, Oral administration of Mosinone-A to DMBA treated hamsters' significantly decreased expression of p^{21} and increased the Bax, caspase-9 (Group 3). No significant changes were observed in the protein expression of control and Mosinone-A alone treated hamsters.



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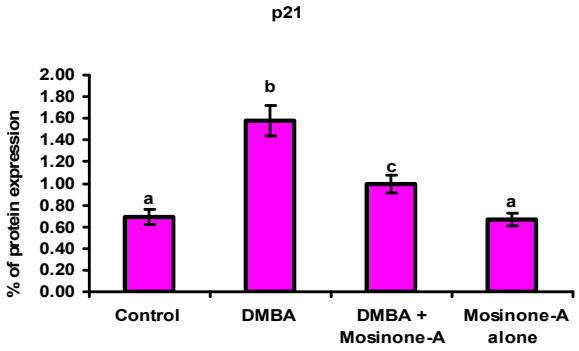

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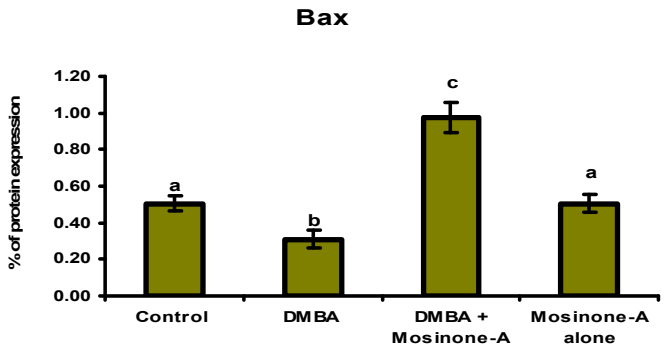

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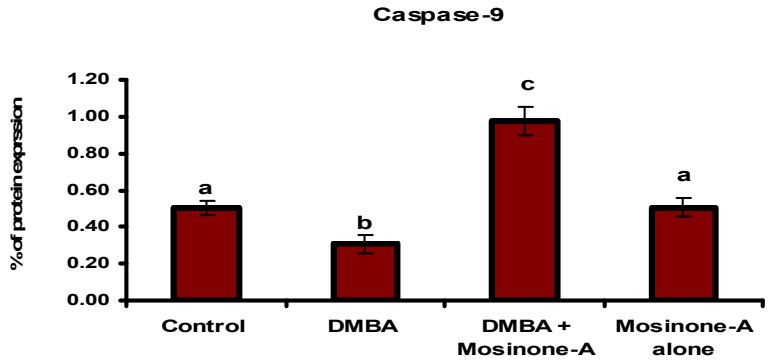

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Figure 1: Western blot analysis of p21, Bax, Caspase-9 (Protein expression) in hamster buccal pouch carcinogenesis. Lane 1- Control, Lane 2 – DMBA, Lane 3 – DMBA+Mosinone-A, Lane 4 –Mosinone-A alone.(A) P21 (B) Cox-2 (C) Bax (D) Caspase-9 in buccal pouch of control and experimental animals in each group. Values are expressed as mean ± SD for six animals in each group. Values not sharing a common superscript letter differ significantly at<0.05(DMRT

4. DISCUSSION

Oral cancer is the most common malignant tumor in the oral and maxillofacial region and has a tremendous impact on health and morbidity **[19]**. A number of chemotherapeutic drugs are resistant to cancer treatment, passably through the modulation of survival cell

components such as proliferative or anti-apoptotic proteins **[20]**.

Chemoprevention is a novel and promising approach to control, inhibit or suppress tumor formation using natural or synthetic entities. A large number of dietary constituents ingest in the human diet exhibit anticarcinogenic and antimutagenic effects **[21]**. The rapid progress in cellular signaling cascades regulating gene expression could provide a rational for developing chemopreventive measures of the naturally occurring compounds for various cancers **[22]**. In the present study, we have examined the molecular evidence to prove chemopreventive efficacy of the Mosinone-A in DMBA induced buccal pouch carcinogenesis.

Profound studies on chemoprevention of human pancreatic and prostate carcinoma cell line offers Mosinone-A as chemopreventive agent due to its diverse pharmacological properties. It has been pointed out Mosinone-A has play a role in the induction of cellular differentiation, apoptosis, and inhibition of cell proliferation and modification of cell cycle progression **[23]**. It has also been suggested that chemopreventive potential of Mosinone-A prevented the development of DMBA induced hamster buccal pouch carcinogenesis. Among the several regulation of cell survival and cell death through apoptosis P^{21}, Bax, caspase-9, are well known primary molecular markers for anti cancer studies. Over expression of p21 and decreased expression of Bax, caspase-9, in buccal pouch carcinogenesis.

P^{21}performlike a signal transduction protein as well as potent cyclin dependent kinase inhibitor **[24]**. The expression of p^{21} is controlled by the tumor suppressor protein p^{53} and it can promote cellular differentiation. Several studies reported that over expression of p^{21} is associated with oral carcinogenesis **[25]**. We observed overexpression p^{21} is associated with oral carcinogenesis in DMBA induced oral carcinogenesis. Oral administration of Mosinone-A to DMBA treated animals significantly reduced the risk of over expression of p^{21} and delayed tumor formation.

Bax and caspase-9 protein expression as representative marker of apoptosis. Bax activates a number of signaling pathways on mitochondria, subsequently release of cytochrome C it binds to adaptor molecules Apaf1 forming an apoptosome which leads to cleavage of procaspase-9 to active caspase-9 **[26]**. We conclude that Bax and caspase-9 exhibits

down regulation of apoptosis by carcinogen (DMBA) might be considered all supplementary indication for the administration of carcinogen (DMBA) treated animal. In the present study, we have confirmed that DMBA inhibits the mitochondrial pathway of apoptosis it leads to downstream of cytochrome C release as well as inhibits caspase-9 activation in cells. Our results are clearly stated that chemotherapeutic effect of Mosinone-A induces Bax and caspase-9 protein expression in tumor cell leading to their apoptosis.

5. CONCLUSION

The results of the present study clearly demonstrated that Mosinone-A acts as a suppressing agents by inhibiting cell proliferation, inducing differentiation and apoptosis and preventive tumourigenesis as exposed by down regulation of p^{21}associated with upregulation of caspase-9, Bax, in protein expression in DMBA induced oral carcinogenesis.

Acknowledgement

Financial assistance from the Department of Science and Technology (DST) New Delhi, India is gratefully acknowledged.

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