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Andrographolide Inhibits Oral Squamous Cell Carcinogenesis through NF-κB Inactivation

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APPENDIX

MATERIALS & METHODS

Chemicals

DMBA and Andrographolide (Andro) were purchased from Sigma-Aldrich (St. Louis, MO, USA). We prepared the stock solutions of Andro at 15 mM for animal treatment or 100 mM for cell treatment by dissolving Andro in DMSO (Sigma-Aldrich). 4H-Andrographolide (4H-Andro) was prepared *via* palladium on activated carbon-catalyzed hydrogenation, as previously described (Xia *et al.,* 2004). All other reagents used were of analytical grade.

Animals

Syrian golden hamsters (aged ~ 6 wks and weighing ~ 60 g) were purchased from the Center of Experimental Animals at Sun Yat-sen University, Guangzhou, China. They were housed individually in polypropylene cages with sawdust bedding and were provided with feeding sticks and sterile water *ad libitum*. Clean bedding was provided in the cages twice *per* wk, with constant temperature (22°C) and humidity (60%) and an alternating light/dark cycle every 12 hrs. All procedures for appropriate animal care and handling were conducted in accordance with The Regulations of Chinese National Guidance for the Care and Use of Laboratory Animals.

Buccal Pouch Model of Squamous Cell Carcinogenesis

The DMBA-induced squamous cell carcinogenesis model involving the use of Syrian golden hamsters (~ 6 wks old and ~ 60 g in weight) was carried out exactly as previously described (Wang *et al*., 2008). For collections of tissue samples at different stages of DMBA-induced squamous cell carcinogenesis, 20 male Syrian golden hamsters were randomly divided into an experimental group (n = 16) and a control group (n = 4). For experimental group animals, the right pouch was painted twice with a 0.5% DMBA/acetone solution by means of a No. 4 sable-hair brush, and the left pouch was painted twice with acetone alone as the solvent control. Approximately 0.1 mL of the respective solution was applied topically to the medial wall of each pouch twice. Hamsters were treated 3 times *per* wk (Monday, Wednesday, and Friday) for 10 wks. The control group was untreated throughout the entire experiment. The experimental group animals were simultaneously killed in groups of 4 by inhalation of a lethal dose of diethyl ether after treatment for 6 wks (the hyperplasia stage), 8 wks (the dysplasia stage), 10 wks (the carcinoma *in situ* stage), and 12 wks (the carcinoma stage), whereas animals in the control group were killed after treatment for 12 wks.

For therapeutic treatment, animals were observed and weighed, and the size of the tumor was measured twice *per* wk with a micro-caliper. The tumor volume was calculated according to the formula $V = 0.52 \times L \times W^2$ (V, volume; L, length; W, width) (Wang *et al*., 2008). Fifty-two hamsters with similar tumor masses were selected from among 80 DMBA-treated male hamsters. They were randomly divided into 3 groups: Andro treatment (n = 22), 4H-Andro treatment (n = 16), and saline treatment (n = 14). An intraperitoneal injection of saline, 4H-Andro, or Andro (6.2 μg/g body weight) was given at the beginning of the 14th wk. Hamsters (approximately 100 g body weight at the 14th week) were treated twice *per* wk for 5 continuous wks. Buccal pouches of hamsters were dissected, excised, and fixed in 10% formalin solution for 24 hrs, followed by paraffin-embedding (Wang *et al*., 2008).

Histologic and Immunohistologic Analysis

Hematoxylin & eosin (H&E) and immunohistochemical staining for total p65, phosphorylated p65 (Ser536), and IκBα (Ser32/36), c-myc, cyclin D1 (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA), bromodeoxyuridine (BrdU), and terminal deoxynucleotidyl transferase 2-deoxyuridine, 5-triphosphate nick-end labeling (Tunel; both from Roche Diagnostics Inc., Basel, Switzerland), or von Willebrand factor

Appendix Table. Pathological Changes in DMBA-treated Hamsters

Time of Treatment	No. of Hamsters	Hyperplasia	Dysplasia	Cancer *in situ*	Carcinoma
6 wks	4	3	1	0	0
8 wks	4	0	3	1	0
10 wks	4	0	0	4	0
12 wks	4	0	0	0	4
Total	16	3	4	5	4

(vWF; Peninsula Diagnostics Inc., San Carlos, CA, USA) were performed and analyzed as previously described (Wang *et al*., 2008). Infiltrating leukocytes in the tumors were identified and counted under an oil immersion lens. Semi-quantitation of immunohistochemical staining was performed with the ImagePro Plus 6.0 analysis system.

Measurements of Cell Proliferation

The high metastatic human tongue carcinoma Tb cells (Tb cells; Yao *et al*., 2006) were maintained in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in the presence of 5% CO_2. Cells in the log phase of growth were used for all experiments. For measurement of cell proliferation, Tb cells were plated into 96-well tissue culture plates (~ 5000 cells/well), and the indicated amounts of Andro were added 5 hrs later. They were then cultured for 48 hrs or 72 hrs. Tetrazolium salt was added (20 μL/well) and incubated at 37ºC for 4 hrs. The insoluble blue formazan product was solubilized by the addition of 100 μL/well of 10% SDS/5% isobutanol. The plates were read on a microtiter plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Flow Cytometric Analysis

Apoptosis was measured by means of the Annexin V-FITC apoptosis detection kit from EMD Calbiochem (San Diego, CA, USA). After exposure to the indicated doses of Andro for 24 hrs, both adherent and floating Tb cells were collected and re-suspended for flow cytometric analysis, by means of an EPICS XL cytofluorimeter (Beckman Coulter, Fullerton, CA, USA).

Immunoblotting

Following indicated treatment, Tb cells underwent lysis with the lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mg/mL leupeptin, 10 mg/mL aprotinin, and 10 mM Na_3VO_4) and were subjected to SDS-PAGE, followed by immunoblotting with respective primary Abs and their corresponding horseradish-peroxidase-conjugated secondary Abs (Wang *et al.*, 2008).

Statistical Analysis

Statistical analysis of the experimental data was carried out by Student's *t* test and ANOVA. $p < 0.05$ or 0.01 was considered statistically significant or very significant, respectively.

APPENDIX REFERENCES

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