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5-Demethyltangeretin is more potent than tangeretin in inhibiting dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumorigenesis

Nianhan Ma [a,1], *Ching-Shu Lai* [b,1], *Chih-Han Chung* [b], *Jinn-Moon Yang* [c,d,e], *Kai-Cheng Hsu* [d,e], *Chin-Yu Chen* [f], *Tao-Sheng Chung* [g], *Shiming Li* [h,i], *Chi-Tang Ho* [i], *Min-Hsiung Pan* [b,j,*]

[a] *Institute of Systems Biology and Bioinformatics, National Central University, Taoyuan 32001, Taiwan*
[b] *Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan*
[c] *Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu 30050, Taiwan*
[d] *Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30050, Taiwan*
[e] *Center for Bioinformatics Research, National Chiao Tung University, Hsinchu 30050, Taiwan*
[f] *Department of Life Sciences, National Central University, Taoyuan 32001, Taiwan*
[g] *Division of Radiation Oncology, Taiwan Landseed Hospital, Taoyuan 32001, Taiwan*
[h] *Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization, Huanggang Normal University, Hubei 438000, China*
[i] *Department of Food Science, Rutgers University, New Brunswick, NJ 08901, USA*
[j] *Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 40402, Taiwan*

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ABSTRACT

5-Demethyltangeretin (5-DTAN), an autohydrolysis product of tangeretin (TAN) found in citrus peel, exhibited more potent anti-proliferative activity in human cancer cells than TAN itself. In this study, we investigated the anti-tumor promoting effect and underlying molecular mechanism of 5-DTAN on 7,12-dimethyl-benz(a)anthracene (DMBA)-induced and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted skin carcinogenesis. Application of 5-DTAN prior to each TPA-treatment was more effective than that of TAN on reducing the number, incidence and size of papillomas in DMBA-initiated mouse skin. Moreover, 5-DTAN suppressed cyclooxygenase-2 (COX-2) protein expression more strongly than TAN through interfering with phosphatidylinositiol 3-kinase (PI3K)/Akt signaling and further activation of transcription factor NF-κB. Taken together, these results revealed for the first time the *in vivo* chemopreventive efficacy of 5-DTAN on inhibition of skin carcinogenesis through promoting apoptosis and molecular interactions with residues of PI3K, COX-2, and AKT that may potentially serve as a novel functional agent capable of preventing inflammation-associated tumorigenesis.

* *Corresponding author.* Tel.: (886)-2-33664133; fax: (886)-2-33661771
E-mail address: mhpan@ntu.edu.tw (M.-H. Pan)

[1] These authors contributed equally to this work

Abbreviations: 5-DTAN, 5-demethyltangeretin; DMBA, 7,12-dimethyl-benz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; IκB, inhibitor κB; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase

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1. Introduction

Polymethoxyflavones (PMFs) exist almost exclusively in citrus peels and have been demonstrated to have various biological activities including anti-carcinogenic, anti-inflammatory and antitumor activities (Li et al., 2009). Tangeretin (5,6,7,8,4′-pentamethoxyflavone, TAN) (Fig. 1A) is one of the most abundant PMFs in peels of sweet oranges and mandarin oranges and has been suggested to have anti-inflammatory (Chen et al., 2007; Tominari et al., 2012), anti-cancer (Morley et al., 2007; Arafa et al., 2009) and anti-diabetic (Kim et al., 2012; Onda et al., 2013) properties *in vitro* and *in vivo*. We recently demonstrated that the 5-hydroxylated PMFs, produced during storage by autohydrolysis of PMFs, also have excellent chemopreventive activity in inhibition of skin inflammation, tumor promotion (Lai et al., 2007), colonic carcinogenesis (Lai et al., 2011), and obesity (Lai et al., 2013) as well as induction of apoptosis (Pan et al., 2007). In addition, 5-hydroxylated PMFs display more potent anti-proliferative and anti-angiogenesis activities than their PMF counterparts in different human cancer cells (Qiu et al., 2011; Charoensinphon et al., 2013), including 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5-demethyltangeretin, 5-DTAN) (Fig. 1A), but the *in vivo* chemopreventive activity is still unknown. Furthermore, a previous study reported TAN inhibited UVB-induced COX-2 expression in mouse epidermal cells (Yoon et al., 2011).

Inflammation is recognized as critical component in various human diseases including a wide range of malignancies (Libby, 2007). Increasing evidences suggest that chronic inflammation is the seventh hallmark of cancer development and involved in different stages of carcinogenesis such as initiation, promotion, malignant conversion, invasion, and metastasis (Colotta et al., 2009). The pathological mechanism of inflammation implicated in carcinogenesis is complicated. In the setting of inflammation, high levels of free radicals were released from recruited immune cells injury biomolecules and cause cellular damage. Later, immune/inflammatory cells produce excessive proinflammatory cytokines that provide growth and survival advantages for initiated cells to further malignant transformation and proliferation. Once tumor occurred, tumor cells create an inflammatory microenvironment with sufficient growth factors, chemokines and cytokines to potentiate tumor growth and progression (Mantovani, 2010). In these processes, deregulation of inflammatory signalings and over-production of pro-inflammatory mediators are critical events for inflammation-driven cancer development (Colotta et al., 2009; Pan et al., 2009).

Nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) are both considered key factors for linking inflammation and cancer (Lu et al., 2006). COX-2 is a rate-limiting enzyme of prostaglandin biosynthesis from arachidonic acid in responding to inflammatory stimulations and has been found implicated in inflammation and cancer development (Kanaoka et al., 2007). NF-κB is a pivotal transcription factor in regulation of gene expression involved in inflammation and tumorigenesis, including COX-2 (Inoue et al., 2007). Activation of intracellular signaling cascades such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/Akt and inhibitor κB (IκB) kinase (IKK), which in turn phosphorylates IκB, degrades and leads to NF-κB translocation to the nucleus (Inoue et al., 2007). A number of studies suggest that COX-2 is overexpressed in various inflammatory diseases and human cancers and correlated with a poor prognosis (Kargi et al., 2013; Lin et al., 2013). Up-regulated COX-2 increases the production of PGE_2 and contributes to proliferation, tumor growth, angiogenesis and invasion while COX-2 inhibitors are able to counteract the pathological events (Sobolewski et al., 2010), suggesting targeted inhibition of COX-2 serves as a promising strategy for the prevention and treatment of inflammation-driven tumorigenesis.

In the present study, we compared TAN with 5-DTAN on the anti-tumor promoting effect by using classical mouse skin two-stage carcinogenesis model which use initiator 7,12-dimethylbenzanthracene (DMBA) to cause gene mutation in epidermal cells followed by tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) to elicit skin inflammation, and edema and epidermal hyperplasia. We also explored the underlying molecular mechanisms of 5-DTAN on inhibition of skin tumorigenesis and compared with its PMF counterpart, TAN.

2. Materials and methods

2.1. Reagents

TAN and 5-DTAN were isolated and purified from orange peel extract with 40% PMFs (Danisco, Lakeland, FL, USA) as described previously (Li et al., 2006). The purity of both compounds is over 99.5% by high-performance liquid chromatography analysis according to the method of Li et al. (2010). The HPLC was equipped with a reversed phase amide C16 column (Ascentis RP-Amide, 3 μm, 150 × 4.6 mm ID) from Supelco (Bellefonte, PA, USA). Gradient elution was used with a mobile phase that was composed of water (solvent A) and acetonitrile (ACN, solvent B). The optimized conditions were as follows: a 20 min gradient was started with 40% of solvent B, which was linearly increased to 55% over a period of 10 min, then linearly increased to 70% after 15 min, and finally ramped up to 80% after 20 min. The flow rate was 1.0 mL/min, and the column temperature was maintained at 35 °C. The detection wavelength was 326 nm, and the injection volume was 10 μL. TPA and DMBA were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were in the purest form available commercially.

2.2. Animals

Institute of Cancer Research female mice at 5–6 weeks old were obtained from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd., BioLASCO, Taipei, Taiwan). All animals were housed in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) and with a 12 h light–12 h dark cycle. Animals had free access to food and water at all times. After 1 week of acclimation, the dorsal skin of each mouse was shaved with surgical clippers before the application of tested compound. DMBA, TPA, TAN of 5-DTAN and HBA were dissolved in 200 μL of acetone and applied topically to the shaved area of each mouse. All animal experimental protocols used in this study

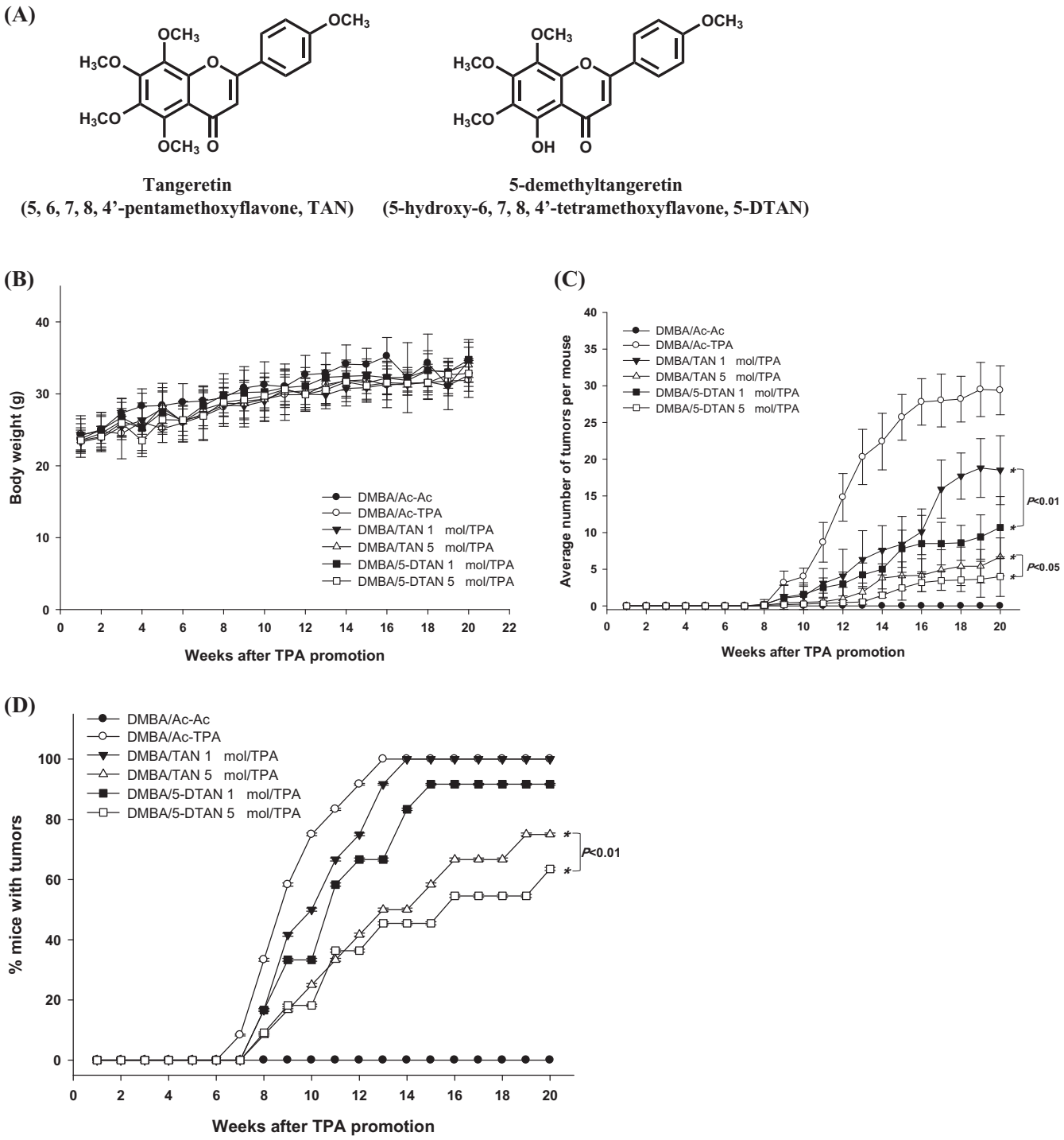

Fig. 1 – Antitumor-promoting effects of TAN and 5-DTAN on DMBA/TPA-induced skin tumorigenesis in ICR mice. Tumor promotion in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 week after initiation. TAN and 5-DTAN (1 and 5 μmol) were dissolved in 0.2 mL acetone and topically applied 30 min prior to each TPA treatment. Tumors of at least 1 mm in diameter were counted and recorded weekly, as described in Materials and methods section. (A) Chemical structures of TAN and 5-DTAN. (B) The body weight of mice during skin tumor promotion. (C) Average number of tumors per mouse. (D) Percentage of tumor-bearing mice (tumor incidence). *P < 0.001 were compared with DMBA/Ac-TPA-treated group. Ac, acetone.

were approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU, #099-AAA9-02, validity dates: 08/01/2009–07/31/2012).

2.3. *Skin two-stage carcinogenesis*

To examine anti-tumor promoting activity of TAN and 5-DTAN, the dorsal skin of each mouse was shaved for DMBA and TPA treatment. For initiation, all animals were treated with DMBA (200 nmol) twice a week and then randomly distributed into six groups of 12 animals each. These animals were given commercial rodent pellets and fresh tap water *ad libitum*, both of which were changed twice a week. After 1 week, DMBA-initiated animals in groups 1 and 2 were pre-treated with acetone for 30 min, and group 2 were then administrated TPA (5 nmol) whereas animals in group 1 were applied with acetone (as vehicle) twice weekly for 20 weeks. DMBA-initiated mice in groups 3 and 4 were pre-treated with TAN for 1 and 5 μmol while groups 5 and 6 were pre-treated with 5-DTAN in the same concentration before each TPA application. Tumors of at least 1 mm of diameter in an electronic digital caliper were counted and recorded once weekly and the diameters of skin tumors were measured at the same time. The results were expressed as the average number of tumors per mouse, percentage of tumor-bearing mice and tumor size distribution per mouse. All mice were sacrificed by CO_2 asphyxiation at the end of 20th week and the tumor tissues were collected for further analysis.

2.4. *TPA-induced COX-2 expression in mouse skin*

Mice (5–6 weeks of age, five per group) were treated topically with 0.2 mL acetone or TAN and 5-DTAN (1 and 5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA and were killed at 4 h after the TPA treatment (Lai et al., 2007). The epidermal proteins were extracted and analyzed for COX-2 by western blotting analysis.

2.5. *Western blot analysis*

For protein analyses, skin tumors in 1–3 mm diameter and epidermis of mice were collected and homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL ice-cold lysis buffer [50 mM Tris–HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM ethylene glycol-bis(aminoethylether)-tetraacetic acid, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% Nonidet P-40 (NP-40) and 10 μg/mL leupeptin] on ice for 30 min, followed by centrifugation at 10,000 *g* for 30 min at 4 °C. The total protein in supernatant was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Equal amount of total protein (50 μg) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide minigels and transferred onto immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was then blocked at room temperature for 1 h with blocking solution (20 mM Tris–HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide) followed by incubation with the primary antibody, overnight, at 4 °C. The membrane was then washed with 0.2% TPBS (0.2% Tween-20/PBS) and subsequently probed with anti-mouse, anti-rabbit, or anti-goat IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY, USA) and visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Buckinghamshire, UK). The primary antibodies used were as follows: COX-2 monoclonal antibody (Transduction Laboratories, BD Biosciences, Lexington, KY, USA); p50, p65, phospho-PI3K (Tyr508) and lamin B polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-Akt (Ser473), Akt, Bcl-2 and Bax polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA); caspase 3 monoclonal antibody (Imgenex, San Diego, CA, USA); β-actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) and GAPDH polyclonal antibody (Genetex, Irvine, CA, USA). The densities of the bands were quantitated with a computer densitometer (AlphaImagerTM 2200 System). All the membranes were stripped and reprobed for β-actin (Sigma Chemical, Co.), lamin B or GAPDH as loading control.

2.6. *Preparation of cytosolic and nuclear extracts from epidermis*

Cytosolic and nuclear protein extractions from skin tumors were prepared as described previously (Lai et al., 2007). Briefly, skin tumors of mice in 1–3 mm diameter were collected and homogenized in 0.5 mL of ice-cold hypotonic buffer A containing 10 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM PMSF with a Polytron for 1 min. The homogenates were incubated on ice with gentle shaking for 15 min and centrifuged at 3000 *g* for 5 min. The supernatant were collected as cytosolic fraction. The pellet was washed by re-suspending in buffer A supplemented with 50 μL of 10% NP-40, vortexed and centrifuged for 2 min at 12,000 *g* for three times. The nuclear pellet was re-suspending in 300 μL of high salt extraction buffer C (50 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol). It was kept on ice for 30 min followed by centrifugation at 10,000 *g* for 30 min. The supernatant were collected as nuclear fraction. Both cytosolic and nuclear fractions were stored at –80 °C for further western blot or EMSA analysis.

2.7. *Electrophoretic mobility shift assay*

The electrophoretic mobility shift assay analysis was performed with a non-radioactive (biotin label) gel shift assay according to the manufacturer's protocol. The NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) consensus oligonucleotide probe was end labeled with biotin (Pierce, Rockford, IL, USA) with terminal deoxynucleotidyl transferase. For the binding reaction, 6 μg of nuclear extract protein was incubated in a total volume of 20 μL with binding buffer containing 50 fmol of biotin end-labeled oligonucleotide. The mixture was incubated at room temperature for 20 min. Following addition of 5 μL of sample buffer, the DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel in a 0.5× Tris–borate–EDTA buffer at 100 V for 2 h and then transferred to nylon membrane. Finally, the biotin-labeled DNA was detected by chemiluminescence using the LightShift Chemiluminescent EMSA Kit (Pierce) and exposed to X-ray film.

2.8. Histological analysis

Skin tumors from different treatment groups were fixed in 10% formalin and embedded in paraffin for histological examinations. Sections (4 μm in thickness) of the skin tumors were cut and mounted on polylysine-coated slides. Each section was deparaffinized in xylene, rehydrated through a series of graded alcohols and subjected to stain with hematoxylin and eosin (H&E). Skin tumors were graded histologically as papilloma and squamous cell carcinoma (SCC) according to Thomas-Ahner et al. (2007).

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To investigate the apoptosis-inducing activity of TAN and 5-DTAN in DMBA/TPA-induced skin tumors, TUNEL assay (Biovision Inc., Mountain View, CA, USA) was performed to measure the apoptotic cells according to the manufacturer's protocol. Skin tumor sections were treated with proteinase K (20 μg/mL) for 20 min at 37 °C and then incubated with the TUNEL reaction mixture for 1 h at 37 °C. Each section was then exposed to an antibody solution for 30 min at room temperature. The TUNEL-stained cells were determined by a Nikon light microscope (Japan) equipped with an ocular micrometer by the magnification (400×) in randomly 5 fields per section. The apoptotic index (%) was expressed as the average number of stained cells per field divided by the total number of cells and multiplied by 100.

2.10. Statistical analyses

All data are presented as means ± standard deviation (SD) of at least three independent experiments. Comparisons were subjected to one-way analysis of Student's t-test and statistical significance was defined as $P < 0.05$.

3. Results

3.1. 5-DTAN was more potent than TAN on suppression of DMBA/TPA-induced mouse skin carcinogenesis

The anti-tumor promoting activity of TAN and 5-DTAN was investigated in DMBA-initiated and TPA-induced tumor promotion in mouse skin. Figure 1B showed that the body weight of animals in each group did not differ throughout the treatment of 20 weeks. We also measured the mean weights of liver, spleen and kidney and showed no significant difference among the groups (data not shown), suggesting long-term treatment of TAN and 5-DTAN did not cause any noticeable side effect to animals. At the end of 20th week, the average tumors per mouse in DMBA-initiated and TPA-promoted group was 29.4 ± 3.3 and no tumor was observed in acetone-treated group (vehicle control) (Fig. 1C). In contrast, the average number of tumors was decreased in pre-treatment with TAN at 1 and 5 μmol 30 min prior to each TPA application for 20 weeks as 18.5 ± 4.7 and 6.7 ± 2.6 ($P < 0.001$ compared with TPA-promoted group), respectively. Pre-treatment with 5-DTAN at 1 and 5 μmol also resulted in reduced average number of tumors as 10.7 ± 4.2 and 4.0 ± 2.7 ($P < 0.001$ compared with TPA-promoted group), respectively. Moreover, 5-DTAN administration displayed stronger inhibition on average number of tumors than TAN at both concentrations ($P < 0.01$ and $P < 0.05$, respectively). The tumor incidence in TPA-promoted and TAN 1 μmol group was 100% but lowered in TAN 5 μmol group as 75% and 5-DTAN groups as 91.7% (1 μmol) and 63.5% (5 μmol) at the end of experiments (Fig. 1D). We also analyzed the size distribution of skin tumors of TAN and 5-DTAN treated groups and compared with TPA-promoted mice. As presented in Table 1, the number of skin tumors in different sizes was dose-dependently decreased in both the TAN and 5-DTAN administrated groups. Application of TAN and 5-DTAN resulted in a significant inhibition of tumors with size of ≥5 mm even at 1 μmol dosage treatment ($P < 0.001$ compared with TPA-promoted group), indicating both TAN and 5-DTAN can lower TPA-induced tumor growth. It was notable of pretreatment with 5-DTAN at 1 and 5 μmol significantly decreased the number of tumors in size ≥5 mm when compared with TAN-treated group.

Table 1 – Effects of TAN and 5-DTAN on tumor diameter (mm) in DMBA/TPA-induced skin carcinogenesis.

Group	Treatment	Tumor diameter (mm)		
		1 to <3	3 to <5	≤5
1	DMBA/Ac-Ac	–	–	–
2	DMBA/Ac-TPA	12.0 ± 2.8	7.2 ± 2.2	10.2 ± 2.3
3	DMBA/TAN 1 mmol-TPA	9.4 ± 2.4	5.1 ± 2.5[a]	4.0 ± 2.4[c]
4	DMBA/TAN 5 mmol-TPA	3.5 ± 2.4[c]	2.3 ± 2.0[c]	0.9 ± 0.6[c]
5	DMBA/5-DTAN 1 mmol-TPA	6.7 ± 2.2[b]	2.2 ± 1.7[c,##]	1.8 ± 1.3[c,#]
6	DMBA/5-DTAN 1 mmol-TPA	2.7 ± 2.3[c]	1.1 ± 1.0[c]	0.4 ± 0.5[c,&]

During the experiment of tumor promotion, the diameters of skin tumors were measured by an electronic digital caliper twice every week. The tumor size was recorded as the average of length × width (mm) per mouse. Statistical analysis was done by Student's t-test.
[a] $P < 0.05$ compared with group 2.
[b] $P < 0.01$ compared with group 2.
[c] $P < 0.001$ compared with group 2.
[#] $P < 0.05$ compared with group 3.
[##] $P < 0.05$ compared with group 3.
[&] $P < 0.05$, compared with group 4.

We also investigated the histopathological features of skin tumors in all treated groups. Results showed that skin tumors in all groups were papillomas characterized by pronounced outgrowth papillary pattern and well-differentiated regard to tumor morphology. There was no papilloma progressed to SCC (data not shown). The epidermal hyperplasia was observed in papillomas in TPA-promoted, 1 μmol of TAN and 5-DTAN-treated groups (Fig. 2). This phenomenon was markedly suppressed in 5 μmol of TAN and 5-DTAN-treated mice that shared a similar epidermal morphology to acetone-treated group.

3.2. 5-DTAN suppressed COX-2 expression in skin tumors and TPA-stimulated mouse epidermis

Because COX-2 is implicated in inflammation-driven carcinogenesis (Lu et al., 2006), we first examined the effect of TAN

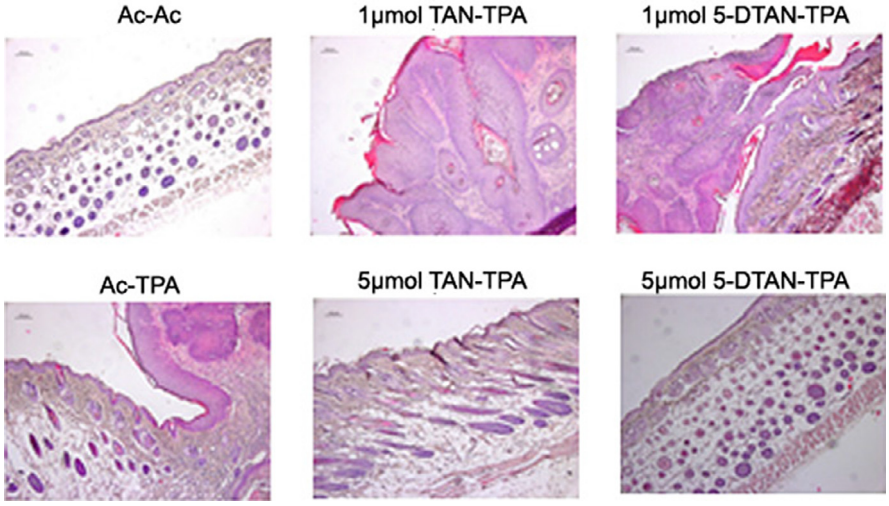

Fig. 2 – Histologic examination of mouse skin and papillomas treated with TAN and 5-DTAN in DMBA/TPA-induced skin carcinogenesis. All mice were initiated by DMBA for 1 week and then treated with TAN, 5-DTAN and TPA as described in Materials and methods section. After promotion for 20 weeks, skin section and tumors were removed and fixed in formalin for H&E stain and tumor grading (100× magnification).

and 5-DTAN on the expression of COX-2 in skin papillomas of mice. Our previous studies revealed that up-regulated protein level of COX-2 occurred in skin papillomas by DMBA-initiated and TPA-promoted mice for 20 weeks (Lai et al., 2007). Similar result was obtained in the present study as shown in Fig. 3A, COX-2 expression was elevated in skin papillomas in DMBA/TPA-treated mice. Pre-treatment with TAN and 5-DTAN dose-dependently reduced the protein levels of COX-2 in papilloma induced by DMBA/TPA. In addition, 5-DTAN displayed stronger inhibitory effect than TAN on COX-2 expression. We also performed a single dose of TPA treated experiment in mouse skin to confirm the inhibitory effect of TAN and 5-DTAN on COX-2 expression. Figure 3B illustrated that topically applied TPA in mouse skin evoked protein expression of COX-2 at 4 h that was consistent with our previous studies (Lai et al., 2007). Topical application of TAN (5 μmol), prior to TPA treatment, resulted in reduction in the level of COX-2 protein in mouse epidermis. In contrast, the protein expression of COX-2 was markedly decreased by 5-DTAN application in a concentration-dependent manner and dramatically suppressed at 5 μmol of 5-DTAN-treated group. These results suggested that 5-DTAN displayed more effective anti-tumor promoting effect than TAN that might contribute to its inhibitory potential on COX-2 expression in mouse skin.

3.3. 5-DTAN suppressed TPA-induced NF-κB activation and IκB degradation in mouse skin tumors

We next examined the effect of 5-DTAN on activation of NF-κB, a critical transcription factor in regulation of COX-2 expression. IκB is an inhibitor of NF-κB by formation of a NF-κB-IκB complex that locates in the cytoplasm. Phosphorylation and proteolytic degradation of IκB by upstream kinases in response to inflammatory stimulation lead to the activation of NF-κB by releasing from inhibitory complex and nuclear translocation, in turn, regulates gene expression (Inoue et al., 2007). Increased serine-phosphorylation of IκBα protein and its degradation were occurred in papillomas by TPA-promoted for 20 weeks (Fig. 3C). Pre-treatment of TAN and 5-DTAN before TPA application for 20 weeks both effectively repressed the phosphorylation and degradation of IκBα in skin papillomas. We further prepared the nuclear and cytosolic extracts of papillomas to measure the translocation of NF-κB by western blot analysis. As presented in Fig. 3D, elevated nuclear translocation of NF-κB subunits, p50 and p65 was found in papillomas of TPA-promoted mice. Pre-treatment of TAN and 5-DTAN inhibited nuclear translocation of p50 and p65. Moreover, application of both TAN and 5-DTAN before TPA treatment strongly suppressed NF-κB DNA-binding activity in papillomas (Fig. 3E). These results suggest that TAN and 5-DTAN reducing COX-2 expression in mouse skin epidermis and papillomas might be through blocking the degradation of IκBα protein and subsequently translocation of NF-κB to the nucleus.

3.4. 5-DTAN suppressed PI3K/Akt signaling in mouse skin tumors

PI3K/Akt signaling is known to activate NF-κB and downstream inflammatory genes such as COX-2 and is repressed by specific inhibitor treatment in TPA-stimulated mouse skin and human keratinocyte (Van Dross et al., 2005; Hwang et al., 2007). Our previous studies have demonstrated that phosphorylation of PI3K and its downstream target Akt was increased in skin papilloma by DMBA-initiation followed by TPA treatment after 20 weeks (Chiou et al., 2013). Here, we investigated the effects of TAN and 5-TAN on the phosphorylation of PI3K and Akt in papillomas by western blotting. As shown in Fig. 4A, phosphorylation of PI3K and Akt was markedly observed in papillomas of DMBA/TPA-treated mice compared to acetone-treated mice. Pre-treatment of TAN and 5-DTAN effectively attenuated phosphorylation of PI3K and Akt in a dose-dependent manner. The inhibitory effect was more effective

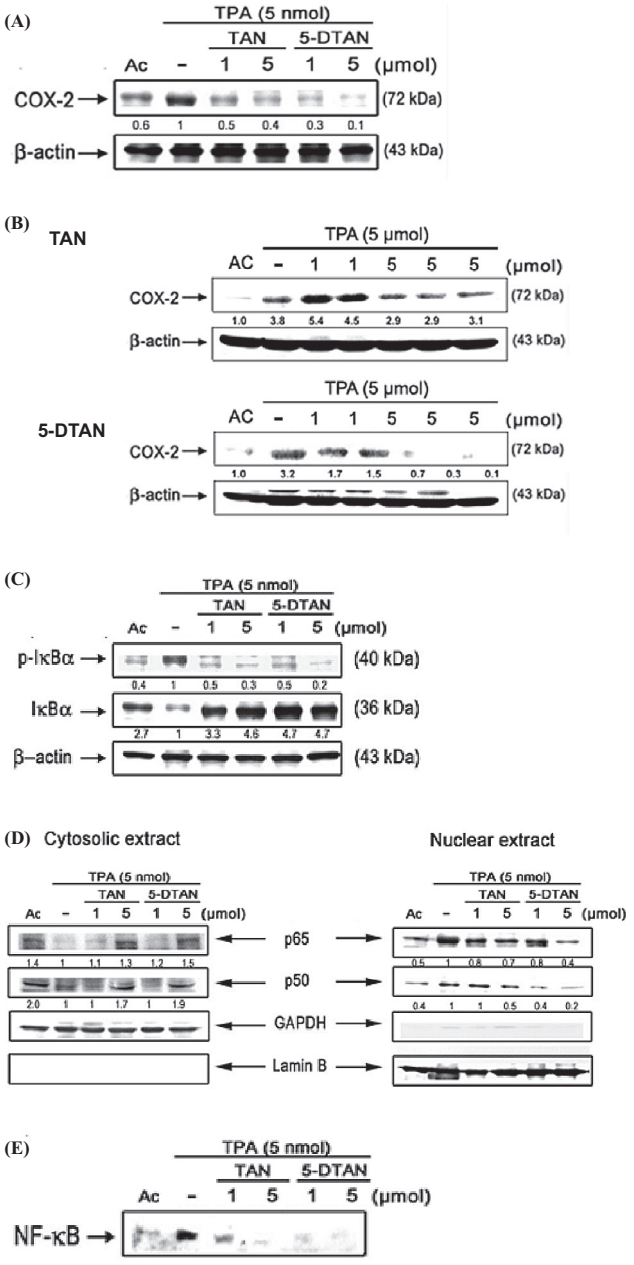

Fig. 3 – Inhibitor effects of TAN and 5-DTAN on COX-2 protein expression and NF-κB activation. Animals were treated as described in Materials and methods section. (A) Skin papillomas (size of 1–3 mm was selected) and (B) TPA-stimulated epidermis were subjected to with western blotting analysis for detection COX-2 by using specific antibody. Ac, acetone. (C) After promotion for 20 weeks, skin papillomas of 1–3 mm size in each group were collected for total protein for phospho-IκB, and (D) nuclear and cytosolic fractions were assayed for p65 and p50. Lamin B and GAPDH were used as internal controls for nuclear and cytosolic fraction, respectively. (E) Electrophoretic mobility shift assay analysis was performed by equal 6 μg of nuclear extracts from papillomas with a biotin-labeled NF-κB probe (non-radioactive). Data are representative of at least three independent experiments, which showed a similar result. Ac, acetone.

in 5-DTAN-treated mice compared with TAN groups. The total protein levels of PI3K and Akt were not affected by TPA, TAN and 5-DTAN treatment. These results indicated that topical pre-treatment of TAN and 5-DTAN suppressing NF-κB activation in TPA-promoted skin papillomas might be through interfering with PI3K/Akt signaling, with further blockage of COX-2 expression.

3.5. *5-DTAN induced apoptosis in mouse skin tumors*

5-Hydroxylated PMFs are found to have more potent anti-proliferative activity in human cancer cells than their respective parent PMFs through induction of apoptosis (Pan et al., 2007; Qiu et al., 2011; Charoensinphon et al., 2013). We thus performed TUNEL assay to examine whether the anti-tumor promoting effect of 5-DTAN was associated with its *in vivo* pro-apoptotic effect in skin papillomas. Results of immunofluorescence assay showed that TUNEL-stained cells were found in papillomas by DMBA/TPA-treated mice that present as apoptotic index of 26.4% ± 2.6 (Fig. 4B and 4C). In contrast, treatment 1 and 5 μmol of 5-DTAN increased apoptotic index to 36.3% ± 2.8 and 39.0% ± 1.8, respectively. Pre-treatment with 5-DTAN also resulted in a cleavage of caspase-3 in papillomas but it was not found in TAN-treated groups (Fig. 4D, upper panel). The protein level of anti-apoptotic Bcl-2 was decreased in both groups of mice with TAN and 5-DTAN treatment while only 5-DTAN caused increased protein expression of pro-apoptotic Bax in papillomas (Fig. 4D, lower panel). These results indicated that the 5-DTAN suppressed tumor promotion possibly through the induction of apoptosis.

4. Discussion

Chemoprevention is defined as the use of natural or synthetic chemicals to reverse, suppress, delay or prevent the development of cancer (Pan and Ho, 2008). Chronic inflammation is implicated in different stages of carcinogenesis and has been recognized as potential target of chemoprevention (Pan et al., 2009). The promotion stage is characterized by transformation and clonal expansion of preneoplastic and premalignant cells. Blockage or interference with promotion stage is considered appropriate approach for cancer chemoprevention due to the fact that it is reversible and requires long-term stimulation (Marks et al., 2007). Many dietary natural compounds are found to be chemopreventive agents through intervention in tumor promotion stage with various anti-inflammatory mechanisms, including suppression of inflammatory cytokines production, interfere with intracellular signaling and diminishing recruitment and activation of inflammatory cells (Pan and Ho, 2008). Our previous studies demonstrated that 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, a 5-hydroxylated PMF, inhibited TPA-induced skin inflammation and tumor promotion through down-regulation of inflammatory iNOS and COX-2 gene expression in mouse skin (Lai et al., 2007). Moreover, we found that 5-hydroxylated PMFs showed potent anti-proliferative and apoptosis-induction activities than their respective parent PMFs in various human cancer cells (Pan et al., 2007; Qiu et al., 2011; Charoensinphon et al., 2013). In the present

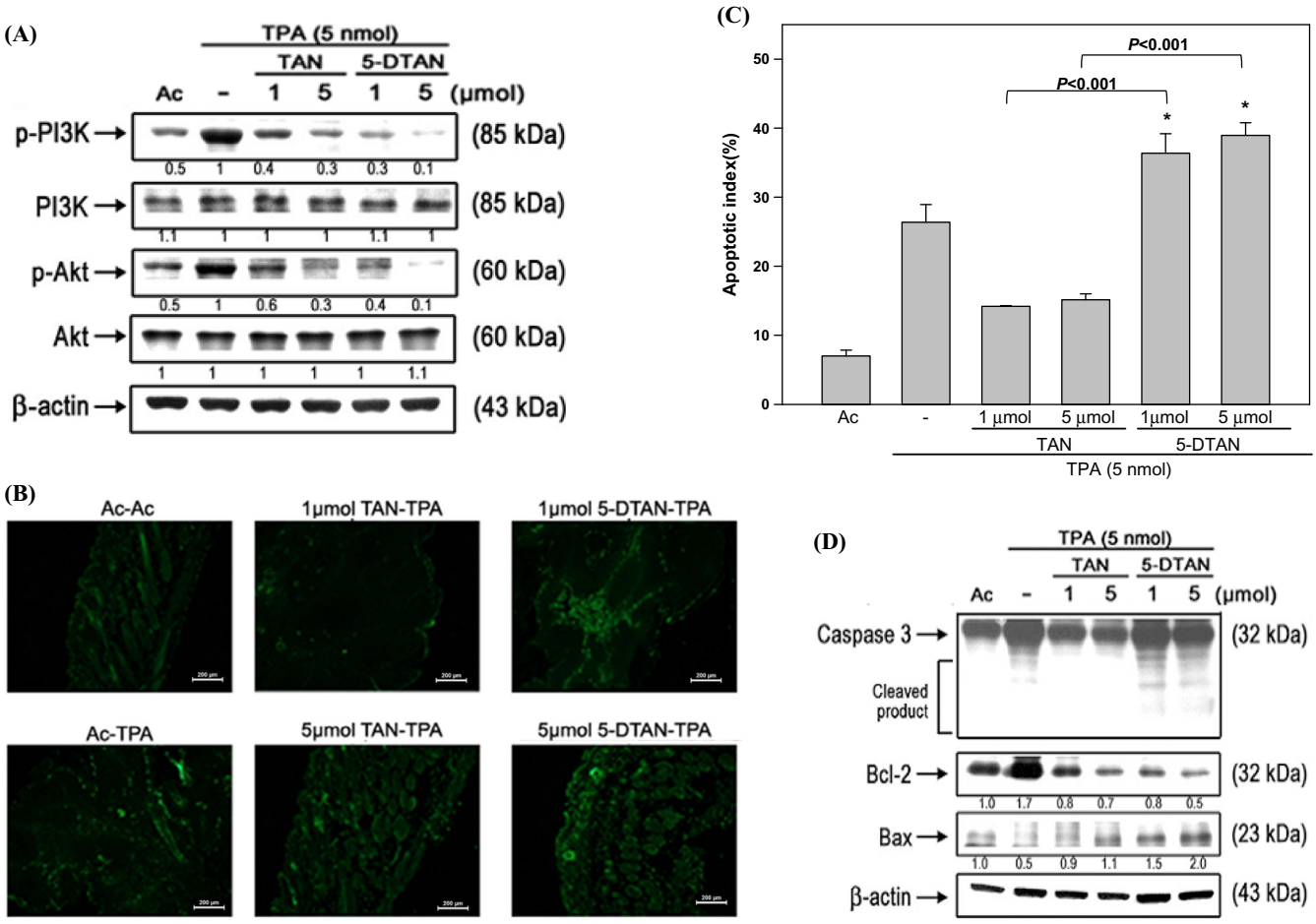

Fig. 4 – Inhibitory effects of TAN and 5-DTAN on PI3K/Akt signaling and apoptosis in mouse skin papillomas. Mice treatment as described in Materials and methods section. (A) Protein lysates were prepared from skin papillomas (size of 1–3 mm was selected) after promotion for 20 weeks. Levels of p-PI3K, total PI3K, p-Akt and total Akt were analyzed by western blot analysis. Ac, acetone. (B) Tumor and skin sections were embedded in paraffin for TUNEL immunostaining as described in Materials and methods section. Tumor tissue sections (size of 1–3 mm was selected) from 5-DTAN treated mice were showed more TUNEL-positive cells (green). Scale bar = 200 μm (C) The apoptotic index was calculated as a ratio of the apoptotic cell numbers to the total cell numbers and multiplied by 100. Data were presented as mean ± SD of five random regions of tumor sections for each group. (D) Western blot analysis of caspase-3, Bcl-2 and Bax in mouse skin papillomas. *P < 0.01 was compared with Ac-TPA-treated group. Ac, acetone.

study, for the first time, we compared the anti-tumor efficacy of TAN and 5-DTAN and showed that topical application of 5-DTAN was more effective on suppression of DMBA/TPA-induced skin carcinogenesis than that of TAN (Fig. 1). The potent anti-tumor promoting effect of 5-DTAN may be attributed to the hydroxyl group at the C5 position and considered an important structural feature for its biological properties (Qiu et al., 2010; Liu et al., 2012).

Overexpression of COX-2 is a common feature in inflammatory conditions and human cancers as well as serves as a potential biomarker of tumor evaluation and prognosis (Kanaoka et al., 2007). Administration of COX-2 inhibitors has been shown to have chemopreventive effect in animals and clinical patients (Kanaoka et al., 2007; Sobolewski et al., 2010). COX-2 and its product PGE_2 play multifarious roles in carcinogenesis including stimulation of proliferation and inhibition of apoptosis, enhancement of angiogenesis and invasion, and impairment of immune system (Kanaoka et al., 2007). Yoon et al. (2011) reported that mouse epidermal cells pre-treated with TAN reduced UVB-induced COX-2 protein expression. Here we found that topical application of 5-DTAN resulted in stronger inhibitory effect than that of TAN on single dose of TPA-stimulated COX-2 protein expression in mouse epidermis (Fig. 3). Similar pattern was also found in DMBA/TPA-induced skin papillomas. Topical administration of 5-DTAN to mice for 20 weeks was more potent than that of TAN on reduction of protein level of COX-2 in papillomas (Fig. 3). This effect was also associated with a decrease of average tumor numbers and tumor size in 5-DTAN treated mice due to the role of COX-2 in proliferation and tumor growth (Fig. 1 and Table 1). These results suggested that anti-inflammatory activity of 5-DTAN might be one of the mechanisms for its anti-tumor promoting efficacy in inhibiting TPA-promoted skin carcinogenesis.

Up-regulation of PI3K/Akt signaling and transcription factor NF-κB is implicated in regulating transcription of inflammatory COX-2, and plays a crucial role in inflammation and carcinogenesis (Tak and Firestein, 2001; Luo et al., 2003). Pre-application of PI3K and NF-κB inhibitors, LY294002 and pyrrolidine dithiocarbamate suppressed epidermal COX-2 expression by TPA stimulation (Hwang et al., 2007; Lai et al., 2008). In the current study, TAN and 5-DTAN treatment was found to attenuated nuclear translocation of p50 and p65 as well as DNA binding activity by reducing phosphorylation and degradation of IκBα protein in TPA-promoted skin papillomas (Fig. 3). In addition, 5-DTAN revealed more effective effect on suppression of nuclear translocation of NF-κB and activation of PI3K and Akt than TAN (Figs. 3 and 4). Akt signaling is known to be involved in the regulation of NF-κB activation by activating IKK (Ozes et al., 1999). Seo et al. (2011) have reported that TAN directly inhibited AKT activity in an *in vitro* kinase assay, indicating 5-DTAN may have similar effect on Akt activity due to their similar structures. However, the detailed mechanism remains to be elucidated. These results suggested that the down-regulation of COX-2 by 5-DTAN in papillomas was through interrupting PI3K/Akt signaling and activation of NF-κB.

Decreased apoptosis is correlated to tumor development and progression while increase of apoptosis is one of attracting mechanisms in cancer chemoprevention (Sun et al., 2004). Another important finding in this study is the observation that 5-DTAN markedly induced apoptosis in skin papillomas but not in TAN-treated groups (Fig. 4C and 4D). Consistent with previous studies by us and Charoensinphon et al., 5-hydroxylated PMFs including 5-DTAN was more effective than parent PMFs on induction of apoptosis in human cancer cells. Increased TUNEL-stained cells were observed in papillomas by 5-DTAN administration following by increased Bax and decreased Bcl-2 protein level (Fig. 4D). This pro-apoptotic activity could be an important mechanism for 5-DTAN against DMBA/TPA-induced skin carcinogenesis.

To dissect the aforementioned molecular mechanisms of 5-DTAN, we further performed the molecular docking regarding the binding conformations of 5-DTAN and TAN. The results shown that 5-DTAN comprises a 5-hydroxy-6,7,8-trimethoxy-4H-chromen-4-one moiety and an anisole moiety. These docking analyses show that 5-DTAN consistently has more hydrogen-bonding interactions with residues of PI3K, COX-2, and AKT compared to TAN, which may account for the better anti-inflammatory effects of 5-DTAN (Supplementary Fig. S1).

In summary, our study showed *in vivo* anti-tumor promoting effects of TAN and 5-DTAN in DMBA/TPA-treated mouse skin. Although topical application of TAN and 5-DTAN reduced skin carcinogenesis, 5-DTAN was more effective than TAN as evidenced by decreased tumor numbers, incidence and tumor size. We suggest that the potential mechanisms for the chemopreventive effect of 5-DTAN were through interfering with PI3K/Akt signaling through molecular interaction with residues of PI3K and Akt, further blocking the degradation and phosphorylation of IκB as well as the activation of NF-κB, thus blocking expression of COX-2. Additionally, 5-DTAN inhibited TPA-promoted skin carcinogenesis possibly not only through lowering COX-2 expression and targeting on COX-2 protein but also by up-regulating apoptosis in papillomas. More importantly, the intracellular accumulation of 5-DTAN was much higher than TAN which increased the biological potential within the cells (Charoensinphon et al., 2013). Briefly, this is the first investigation to evidence that 5-DTAN have more effective potential than TAN as a novel chemopreventive agent to be used in the treatment of inflammation associated with tumorigenesis.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2014.03.032

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