

Full Original Paper

A Possible Mechanism of Action of the Chemopreventive Effects of Sarcotriol on Skin Tumor Development in CD-1 Mice

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Received: 28 July 2006 / Accepted: 18 August 2006 / Published: 21 August 2006

Abstract: Sarcophine derivatives have been suggested to be chemopreventive in nature. One of its derivatives, Sarcotriol (ST), was investigated to study the skin cancer chemopreventive effects in female CD-1 mice. Three groups (control, promotion, initiation) of 30 female CD-1 mice each were taken. Carcinogenesis was initiated with 7, 12-dimethylbenz (a) anthracene (DMBA) and promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA). One hour before treating with DMBA (200 nmol/100 µl acetone), control and promotion groups were treated with acetone (100 µl) and initiation group with ST (30 µg/100 µl of acetone). Beginning one week after initiation with DMBA, control and initiation groups were treated with acetone and promotion group with ST (30 µg/100 µl of acetone), one hour before treating with TPA (5 nmol/100 µl acetone). This was carried out twice a week for the next 20 weeks. The effects of ST on ³H-thymidine incorporation in epidermal DNA, the possible role of apoptotic proteins and COX-2 involved in the prevention of skin tumor development of CD-1 mice were investigated. Tumor incidence and multiplicity was found to be 100%, 73%, 100% and 8.2, 4.8, 9.7 in control, promotion and initiation groups respectively. ST treatment resulted in a significant ($P < 0.05$) inhibition in the incorporation of ³H-thymidine in epidermal DNA. The promotion group showed higher levels of caspase-3, -8 and -9 compared to the control. COX-2 expression was significantly lower ($P < 0.05$) in the promotion group as compared to the control. No significant difference in caspase-3, -8, -9 and COX-2 levels were observed in the initiation group compared to control. Together, this study confirms the chemopreventive effects of ST, and for the first time identifies the stage of carcinogenesis at which ST exerts its chemopreventive effect, and elucidates the mechanism possibly by inducing apoptosis and

decreasing the COX-2 levels, contributing to its overall cancer chemopreventive effects in the mouse skin cancer model.

Keywords: Cancer chemoprevention, Skin cancer, Sarcoptriol, Apoptosis, COX-2.

1. Introduction

Skin cancer is the most common type of cancer, accounting to nearly half of all cancers in United States [1]. One third of all the new cancer cases diagnosed annually in the world originates in the skin [2,3]. Therefore, developing novel strategies for management of skin cancer should be pursued [2]. Chemoprevention, a newer dimension in the management of neoplasia, could offer a hope in this direction [4] and, therefore, major focus of research has been the chemoprevention of cancer [5,6]. The National Cancer Institute has 25 chemopreventive agents in clinical trials and has a need of continuous research on chemoprevention [7].

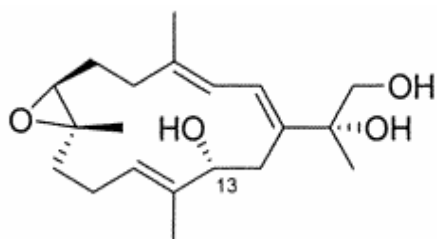
Chemoprevention is a means of cancer control by the use of natural or synthetic agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth [4].

Chemopreventive agents can be broadly classified as blocking and suppressing agents [8]. The blocking agents prevent carcinogenic agents from reaching or reacting with critical target sites whereas suppressing agents prevent the evolution of neoplastic processes in the cells [8].

Chemical and UVB radiation-induced carcinogenesis in murine skin and possibly human skin is a stepwise process of at least three distinct stages: initiation, promotion, and progression [9]. Tumors can be initiated by a single dose of carcinogen such as 7, 12-dimethylbenz (a) anthracene (DMBA) followed by a repetitive application of a tumor promoter such as TPA. Promoters like 12-O-tetradecanoylphorbol-13-acetate (TPA) induce epidermal inflammation, hyperplasia, and an increase in DNA biosynthesis [10].

Research studies have shown that thousands of novel compounds and their metabolites obtained from marine organisms have a diverse range of biological activities ranging from antiviral to anticancer [11]. One of them is sarcophytol A, an oxygenated cembrene-type diterpenoid isolated from the Okinawan soft coral *Sarcophyton glaucum*, which has been shown to have significant inhibitory activity against various classes of tumor promoters and hydrogen peroxide formation by TPA activated human polymorphonuclear leukocytes [12]. Sarcophytol A is available only in minute quantities which is a major problem in carrying out its clinical studies, which prompted to study further on another naturally occurring cembranolide sarcophine, obtained from red soft coral *Sarcophyton glaucum* [13]. Sarcophine is a fish toxin that has inhibitory activity against various vital enzymes including cholinesterase and phosphofructokinase, and is abundantly available [13]. In our previous work [14-18], we have reported a new series of sarcophine derivatives that displayed a potent chemopreventive activity against skin cancer, one of which is sarcotriol (ST).

The purpose of the present investigation is to determine what stage of carcinogenesis ST inhibits during the study of its chemopreventive effects on DMBA-initiated and TPA-promoted skin papillomas in CD-1 mice. To elucidate the possible mechanism of action of ST, ³H-thymidine incorporation in epidermal DNA, Caspase-3, -8, -9 and COX-2 expression were also determined.



2. Results and Discussion

In the present study, we have examined the effect of ST on DMBA-initiated and TPA-promoted tumorigenesis in a two-stage skin carcinogenesis model. The results from this investigation indicated that ST inhibited skin papilloma development as reflected in tumor incidence, to a lesser extent, and tumor multiplicity only in the promotion group which indicates that ST was effective only during the promotion phase of DMBA and TPA protocol. ST treatment did not have any significant effects during the initiation phase. Thus ST may not affect the binding of DMBA to DNA during the initiation phase.

There was no significant difference in weight gain among all experimental groups throughout the duration of the experiment (data not shown). Thus, ST treatment did not influence the normal growth and development of the animals during the experimental period.

The effects of ST treatment on the incidence of skin tumors in CD-1 mice are shown in Fig. 1. Skin tumors appeared in the fifth week of promotion after the initial DMBA application in the control and initiation groups whereas papillomas were seen only after the seventh week in the promotion group. Skin papilloma incidence was 100, 73, and 100% in control, promotion and initiation groups, respectively, after 20 weeks of promotion. Tumor incidence in the promotion group was significantly lower ($P < 0.05$, Chi Square test) than the control and initiation groups. There was no significant difference in the incidence of papillomas between control and initiation group throughout the duration of the experiment.

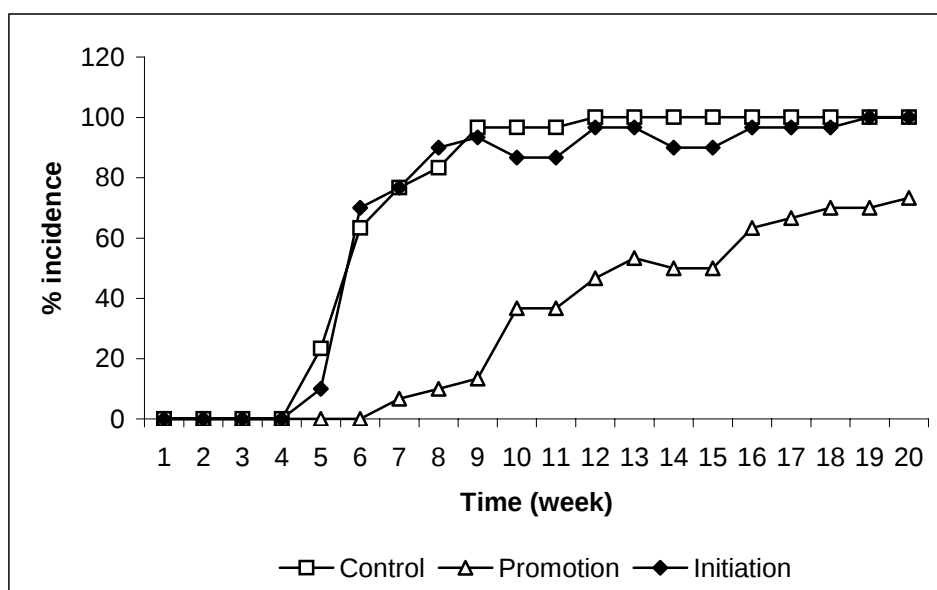


Figure 1. The effects of ST treatment on tumor incidence in CD-1 mice.

The effects of ST treatment on papilloma multiplicity is given in Fig. 2. The promotion group had an average of 4.8 tumors per mouse; whereas, the control and initiation groups had 8.2 and 9.7 tumors per mouse respectively. The mean number of tumors per mouse was significantly lower ($P < 0.001$, ANOVA) in the promotion group compared to control and initiation groups.

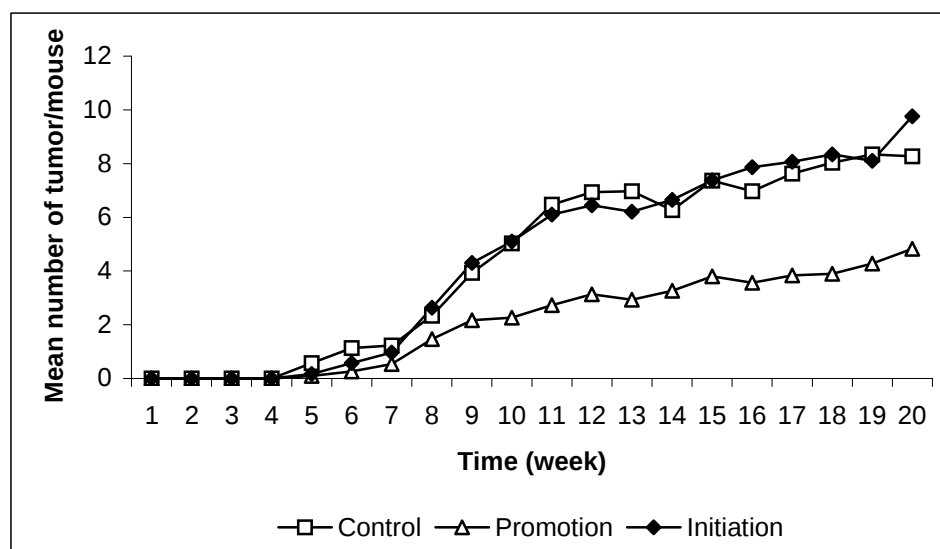


Figure 2. The effects of ST treatment on tumor multiplicity in CD-1 mice.

One of the prominent biochemical response associated with TPA tumor promotion is the increase in epidermal DNA synthesis [10]. As expected, ST treatment significantly ($P < 0.001$) decreased incorporation of ^3H -thymidine in DNA in the skin of mice. Because ST treatment did not influence DMBA-induced initiation, most likely the chemopreventive effects on TPA-induced promotion are caused by systemic absorption rather than simply blocking the penetration of TPA. Topical application of ST (30 μg /100 μl of acetone) significantly ($P < 0.001$) reduced the TPA-induced ^3H -thymidine incorporation in epidermal DNA (68% inhibition). Data is shown in Fig. 3.

Another biological event responsible for tumorigenesis is the loss of apoptotic death of transformed cells [19]. In most cancers, the apoptotic pathway of tumor cells is compromised with a survival advantage over the normal cells [20,21]. Apoptosis, a programmed cell death, which is carried out by caspases, plays an important role in regulating cell proliferation. [22]. Detection of active caspases serves as biomarkers for chemoprevention [23]. Induction of apoptosis is a key event, which controls the outcome of chemopreventive efficacy of an agent under investigation [20,24]. Strategies, therefore, are also needed that lead to induction of apoptotic cell death in both transformed and tumor cells. So based on these results, here we assessed whether ST induces cell death by apoptosis. Consistent with these notions, the results obtained suggest that ST was able to induce apoptosis in tumor cells.

A fundamental biochemical event marking the start of apoptosis is the activation of caspases [25]. Apoptosis occurs through two pathways; extrinsic and intrinsic [22]. The extrinsic pathway is activated by the ligation of death receptors, which ultimately leads to the activation of caspase-8 [22]. In the intrinsic pathway of apoptosis, mitochondrial dysfunction takes place causing the release of cytochrome *c* into the cytosol, which finally leads to the activation of caspase-9 [26]. Finally the

upstream caspases-9 and -8 converge to caspase-3 leading to apoptosis [22]. Accordingly, to elucidate the mechanisms underlying the induction of apoptosis by ST, first we focused our efforts on caspase activation. Employing SDS-PAGE and Western blots, we observed that ST causes caspase-3 activation. In order to assess the mechanism of caspase-3 activation a further study was carried out to find out whether the upstream caspases were activated. As expected ST treatment was able to significantly ($P < 0.001$) elevate the levels of caspase-9 in the promotion group. These findings suggest that caspase-9 activation could be one mechanism of caspase-3 activation and apoptosis induction. However, there was concomitant and almost similar induction of caspase-8 activity in the promotion group too suggesting an additional mechanism(s) of caspase-3 activation by ST. These results suggest that ST induced apoptotic cell death might be mediated by both extrinsic and intrinsic pathways of apoptosis.

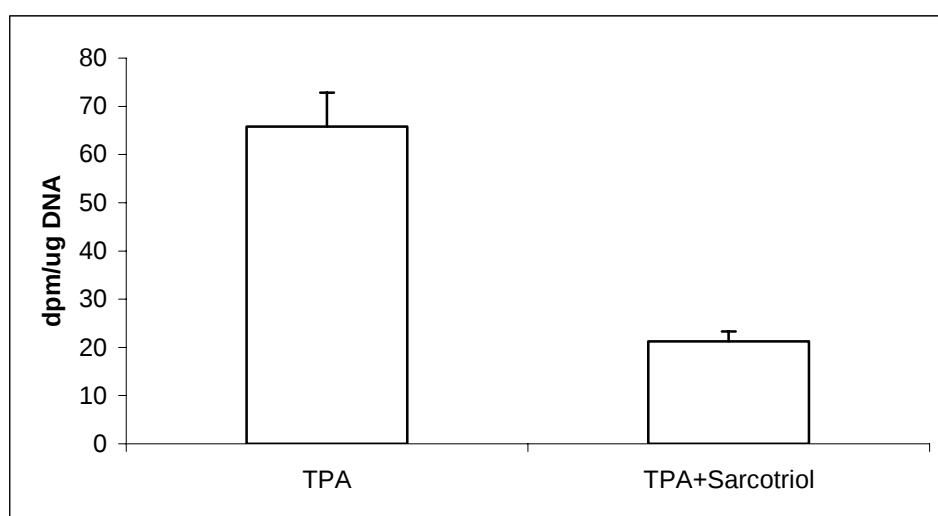


Figure 3: The effects of ST treatment on the incorporation of ^3H -thymidine in epidermal DNA in CD-1 mice.

Recent clinical studies have provided clear evidence for the role of COX-2 in colorectal neoplasia. Epidemiological studies have suggested the capacity of NSAIDS to decrease the incidence of colorectal carcinoma [27,28]. Similarly, elevated prostaglandin levels have been correlated with higher metastatic potential in human breast cancer [29,30,31]. Studies have indicated an increased expression of COX-2 in skin tumorigenesis [32,33]. There is a growing body of compelling evidence that inhibition of COX-2 activity is valuable for not only alleviating inflammation, but also preventing cancer. Consistent with this, ST treatment suppressed the expression of COX-2 in the promotion group. Since chronic inflammation predisposes to malignancy [34], the inhibition of COX-2 by ST is likely to contribute to both anti-inflammatory and chemopreventive effects this compound exerts.

There was no significant increase in the caspase-3, -8, -9 and COX-2 levels in the initiation group. This might be that ST was not able to block the DMBA binding to the DNA and hence it might not be able to act as blocking agent but rather act as a suppressing agent by preventing the evolution of neoplastic processes in the cell probably by activating the caspases and decreasing the COX-2 levels.

In order to determine whether apoptosis is being carried out, caspase-3 levels were detected using Western blots. Levels of caspase-3 and cleaved caspase-3 were significantly ($P < 0.001$, ANOVA) higher in the promotion group than in the control and initiation groups. The values of caspase-3 and

cleaved caspase-3 in promotion and initiation groups were $600.62 \pm 39.61\%$, $329.47 \pm 35.90\%$ and $177.32 \pm 62.68\%$, $125.80 \pm 44.04\%$ respectively with respect to control. Data is given in Fig.4.

In order to understand the pathway behind caspase-3 activation and to assess if upstream effector caspases are being activated, levels of caspase-8 and caspase-9 were detected. Both caspase-8 and caspase-9 levels were significantly ($P < 0.001$, ANOVA) upregulated in the promotion group compared to control and initiation groups. The values of caspase-8 and cleaved caspase-8 in the promotion and initiation groups were $514.39 \pm 45.39\%$, $656.38 \pm 37.52\%$ and $141.93 \pm 57.09\%$, $138.86 \pm 62.01\%$ respectively compared to control. The levels of caspase-9 were $269.87 \pm 32.13\%$ and $157.66 \pm 21.87\%$ in promotion and initiation groups respectively compared to control. Data is presented in Fig. 4.

COX-2 levels are presented in Fig. 4. COX-2 levels were found to be significantly ($P < 0.001$, ANOVA) lowered in the promotion group when compared to control and initiation groups. The values of COX-2 in promotion and initiation groups with respect to control were $31.18 \pm 5.91\%$ and $76.58 \pm 7.18\%$ respectively. There was no significant difference in caspase-3, -8, -9 and COX-2 levels in the initiation group compared to control.

3. Conclusion

In conclusion, ST prevents the skin tumor development in CD-1 mice probably by activating caspase-3, -8, -9 and decreasing DNA synthesis and COX-2 levels. The effectiveness of ST as chemopreventive agent appears to be very promising in skin cancer control. Additional studies on the chemopreventive effects of ST on UV-B induced skin tumor development protocol are in progress. It is worth mentioning that some of the well-known chemopreventive compounds currently under clinical investigation such as Curcumin and Epigallocatechin gallate EGCG produced comparable effects at milligram concentrations, while ST produced its effects at a microgram range

4. Experimental

4.1. General

Sarcophine was isolated from the soft coral *Sarcophyton glaucum* collected from several locations of the Red Sea in Egypt. DMBA, TPA, calf thymus DNA, EDTA disodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). ^3H -thymidine was from American Radiolabeled Chemicals (St. Louis, MO). Primary antibodies against caspase-3, -8, -9 and COX-2 were purchased from Cayman Chemical Company (Ann Arbor, MI). Other reagents were obtained in their highest purity grade available commercially.

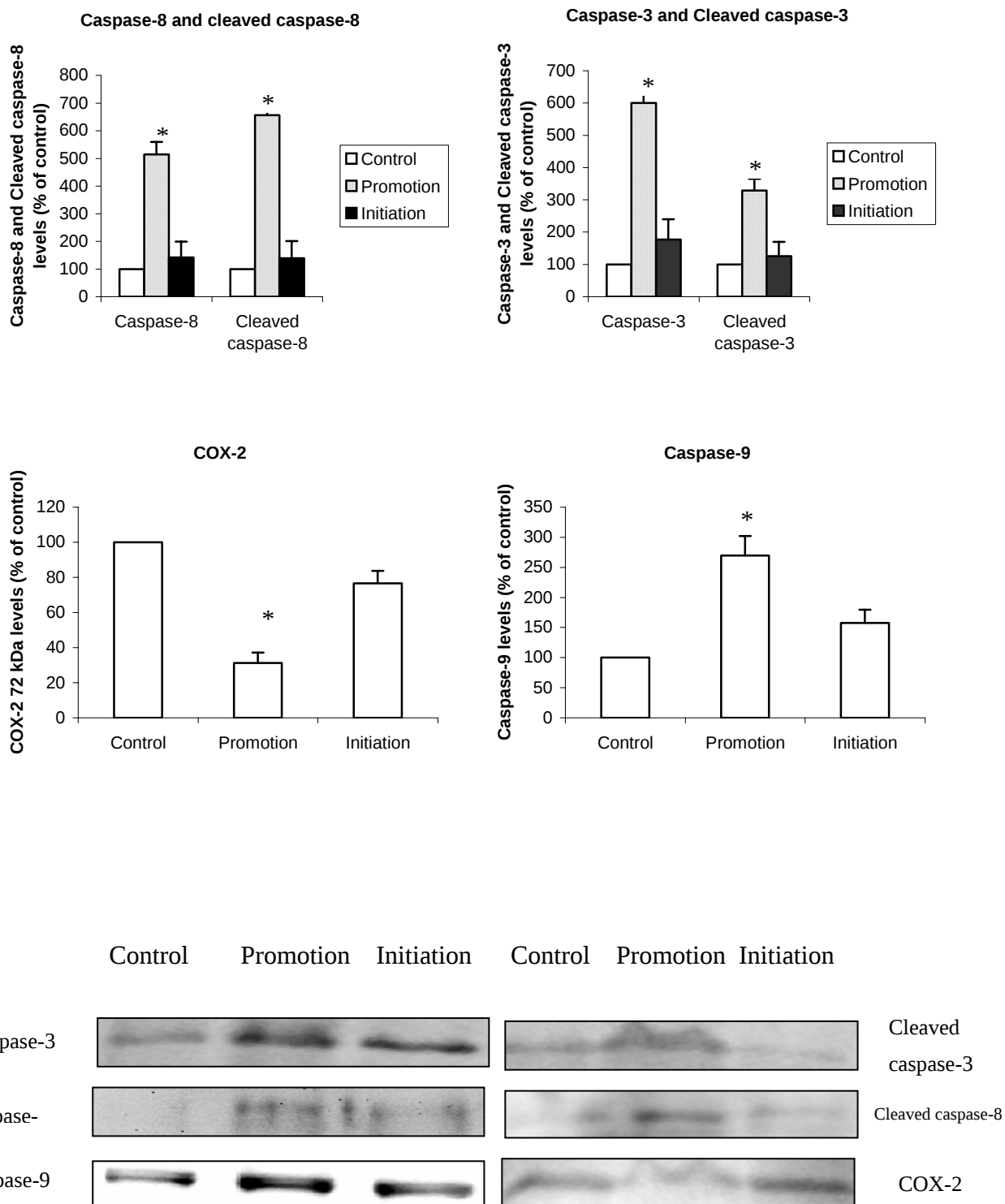


Figure 4. Caspase-3, -8, -9 and COX-2 levels were detected by Western blots. Values (mean $\pm$ SD; n=3) are percentages of control values. * Significantly different than control and initiation group ($P < 0.001$).

4.1.1. Animals

Female CD-1 mice (5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed ten per cage at $22 \pm 1^{\circ}\text{C}$ and $50 \pm 10\%$ relative humidity and subjected to a 12h light/ 12h dark cycle. They were acclimatized for 1 week before use and provided food and water *ad libitum*. The use of mice in this project was approved and in compliance with regulations stated by the Institutional Animal Care And Use Committee (IACUC), South Dakota State University.

4.1.2. Materials

Sarcophine was isolated from the soft coral by extraction several times with petroleum ether at room temperature following the reported procedure [13] in the laboratories of the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt. The dried extract was evaporated under reduced pressure and chromatographed on silica gel column using hexane: ethyl acetate (1:2) as eluent. Pure sarcophine was obtained by crystallization from ethanol.

4.2. Synthesis of ST

Sarcophine was isolated from the soft coral *Sarcophyton glaucum* by using column chromatography on silica gel eluting with EtOAc-hexane (2:1), and crystallized from ethyl acetate [13]. This obtained sarcophine was reduced to its lactone ring-opened analogue (50mg, 0.16 mmol) to which selenium dioxide (35.5 mg, 0.32 mmol) was added in dry 1,4-dioxane (15 ml), and the reaction mixture was stirred at room temperature for 4h. Water was then added, and the product was extracted with CH_2Cl_2 . Saturated NaHCO_3 solution was used to wash the CH_2Cl_2 layer and dried over anhydrous Na_2SO_4 . Solvent was evaporated and the residue was chromatographed on silica gel using hexane - acetone (1:1) as an eluent to obtain ST (12 mg, 23%) [14].

4.3. Two-stage mouse skin carcinogenesis model induced by DMBA/TPA

Tumorigenesis protocol as described by Dwivedi *et al* [35] was used. The backs of the mice were carefully shaved with an electric clipper 2 days prior to the start of the experiment. The mice were randomly divided into three groups of 30 mice each. Group assignments were as follows:

Group I, Control; 100 μl of acetone before DMBA and each TPA application.

Group II, Promotion; 100 μl of acetone before DMBA and 100 μl of ST (30 μg /100 μl of acetone) topical application 1h before each TPA treatment throughout the duration of the experiment.

Group III, Initiation; 100 μl of ST (30 μg /100 μl of acetone) 1 h before DMBA and 100 μl of acetone 1h before each TPA topical application.

Tumorigenesis in mice was initiated with a single dose of DMBA (200 nmol/100 μl acetone) topical application. Beginning 1 week after initiation, mice in all the groups were treated topically with TPA (5 nmol/100 μl acetone) twice a week (Monday and Thursday) for 20 weeks. Group weights were taken on a weekly basis and papillomas appearing on the skin were recorded every week during the experimental period.

4.4. Determination of DNA synthesis

Determination of DNA synthesis was based on the procedure of Burton [36], Huang *et al* [37] and Smart *et al* [38] as reported by Dwivedi *et al* [35].

4.5. Lysate preparation

The cell pellet obtained as a result of ultra centrifugation is taken in an appendorf tube and lysed in 5% SDS containing protease inhibitors leupeptin, pepstatin and PMSF. The resultant suspension was allowed to pass through 25G needle and centrifuged at 13,000 rpm for 20min.

4.6. Western Blot Analysis of Caspase 3, 8, 9 and COX-2

Protein concentration was measured in each cell lysate by the protein assay (Pierce, Illinois) with albumin as a standard. Equal amounts of protein lysates (60 µg) were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 5% skim milk in TBS (10mM Tris, 100mM NaCl), and then probed with primary antibodies against caspase-3, 8, 9 and COX-2. The secondary antibodies conjugated to horseradish peroxidase were used for development with the enhanced chemiluminescence detection kit. The western blots were quantified using a UVP Biochem Gel Documentation system (UVP, Inc., Upland, California).

4.7. Statistical Analysis

The software INSTAT (Graph Pad, San Diego, CA) was used to analyze the data. Chi Square was used for analyzing the data on tumor incidence. ANOVA followed by student's t-test were used to compare the tumor multiplicity, weight gain, DNA synthesis, caspase-3, 8, 9 and COX-2 levels in the three groups.

Acknowledgements

This work was supported by South Dakota State University and College of Pharmacy research Support Fund.

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