



Original Article

In vivo assessment of anti-tumor activity of Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (6) on 7,12 dimethylbenz(a) anthracene-induced mammary tumors in rat

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Abstract

Objective-Breast cancer is one of the most serious problems in oncology. Alkaloids pyrrolizidine are widely found in nature and exhibited versatile biological activities. The aim of the present study was to assess anti-tumor activity of methyl 1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2'-carboxylate (6) on 7,12 dimethylbenz(a) anthracene (DMBA)-induced mammary tumors in rat.

Design- Experimental Study.

Animals- Twenty-one female Wistar rats

Procedures- 7-Week-old female Wistar rats (180 g body weight) were randomized into three groups of seven animals each. DMBA-induced mammary tumors was achieved using DMBA dissolved in 1ml of vehicle (0.5 ml of DMSO plus 0.5 ml of saline) and injected by subcutaneous injection beneath the mammary gland on either side. Tumor yield and size were stabilized after 90 days with the initiation of DMBA. Healthy intact animals (NC) were considered as a normal control feeding on pellets and tap water. In group DMBA, the tumor was induced by DMBA. In group DMBA/6 the animals with tumor received 100 μ L 6 dissolved in DMSO (0.25 μ M) solution interaperitoneally for one week. Synthesis of 6 was accomplished and the structure of new product was assigned by their ¹H/¹³C NMR, IR, CH-COSY and mass spectral data as well as the elemental analysis.

Results-The overall tumor analysis showed that 6 treatment significantly inhibited the breast tumor incidence, tumor multiplicity, and tumor size in the DMBA-initiated rat model. 6 treatment significantly inhibited the total volume of tumors per rat.

Conclusion and Clinical Relevance-The findings of the present study showed that the 6 treatment was significantly effective in reduction of tumors versus the cancer controls.

Key words:Breast carcinogenesis; 7,12-Dimethylbenz(a)anthracene, Indene-2,3'-pyrrolizidine, mammary tumors

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Introduction

One of the major diseases causing death across the world is cancer. Breast cancer is one of the most serious problems in oncology. It is a leading cause of death among women in many countries. The American Cancer Society estimates that in 2015, approximately 2, 11, 240 women are newly diagnosed with this disease and 40,410 die annually.¹

The incidence rates of breast cancer were recorded as 19,240 (29.9%) and mortality rate 5640(18.4%), as per the breast cancer facts and figures.¹

Nitrogen-bridgehead fused heterocycles containing an imidazole ring are a common structural moiety in many pharmacologically important molecules that display a wide range of activities for diverse number of targets. One of the most widely used heterocyclic system from this group is imidazopyridine.² Imidazopyridines show a spectrum of biological activities like inhibitors of aromatase estrogen production suppressors, positive inotropic agents, platelet aggregation inhibitors, thromboxane synthetase inhibitors, antiviral, antibacterial, hypnoselective and anxioselective activities.³⁻⁹ Imidazopyridines exhibit different type of molecular mechanisms in cancer chemotherapy. Recently, Wu and

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coworkers reported 3,7-diarylimidazopyridines as inhibitors of the vascular endothelial growth factor (VEGF)-receptor KDR.¹⁰ VEGF is a regulator of vascular permeability and an inducer of endothelial cell proliferation, migration, and survival. Activation of the VEGF pathway is a fundamental regulation of angiogenesis, the formation of new capillaries from established blood vessels. In molecular mechanisms, the mitogenic signal of VEGF is mediated through the receptor tyrosine kinase KDR (VEGFR-2).¹¹ Substituted 2-(N-trifluoroacetyl amino) imidazopyridines are known to arrest cell cycle at G2/M phase and induce apoptosis in SK-LU-1 human cancer cell line.¹² Oxindoles are important pharmacophores that are known to enhance anticancer activity of some core molecules. Similarly, substituted E-3-(3-Indolylmethylene)-1,3-dihydroindol-2-ones are also reported as anticancer agents and induce apoptosis.¹³⁻¹⁸

Spirocyclooxindoles are valuable synthetic intermediates and constitute the core units of many pharmacological agents and alkaloids.¹⁹ These compounds have attracted much attention from synthetic chemists due to their diverse biological activities including antimycobacterial, antitumor, antimicrobial, antibacterial, antifungal, antiviral, and local anesthetic properties.²⁰⁻²⁶ Hence, a number of synthetic routes have been developed for the preparation of these structural frameworks.²⁷⁻³¹ 1,3-Dipolar cycloaddition provides an efficient approach for the synthesis of five-member heterocycles and spiro-heterocycles, such as poly functionalized pyrrolidines, pyrazolidines and pyrrolizines, which widely occur in natural products and biologically active compounds.³²⁻³⁹ Although there are reports of synthesis of these substituted heterocycles, the development of synthetically important functionalized new spiro heterocyclic is still a challenge and has become a much attempted research endeavor. Spiropyrrolizidineoxindoles are important synthetic targets and several reports of such syntheses exist.^{40,41}

It has been reported that a series of oxindole-derived imidazo[1,5-a]pyrazines derivatives bear anticancer activity with significant cytotoxicity against a panel of 52 human cancer cell lines.²¹

Synthetic compounds of Octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones derivative has been reported to possess a strong antioxidant and radical scavenge properties which inhibit the development of a series of solid tumor, ascites, and leukemic cell lines.²¹

In the present study a novel one-pot protocol for highly diastereo- and regioselective synthesis of some octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones at room temperature or microwave irradiation has been reported.

To the best of our knowledge, however, there are no reports concerning anti-tumor activity of a novel synthetic compound of octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones.

In the present study, anti-tumor activity of **6** was assessed on 7,12 dimethylbenz (a) anthracene-induced mammary tumors in rat. The assessments were based on anti-tumor activity and histopathological findings.

Materials and Methods

Chemicals and reagents

7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents were also obtained from Aldrich (Sigma Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthew Company, Ward Hill, MA, USA) and were used without purification. Reagents were of research grade and stored at 4 °C for further use.

Chemistry

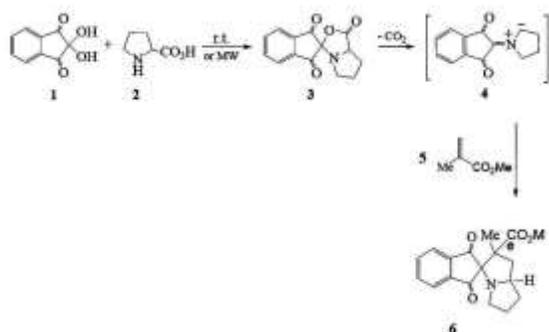
General procedure for the synthesis of Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7','-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (**6**) (0.269 g)

A Typical Procedure for Synthesis of **6** was performed under Microwave Irradiation. In brief, In an erlenmeyer flask (50 mL), a solution of ninhydrin (0.178 g, 1 mmol), proline (0.115 g, 1 mmol) and methyl methacrylate (0.105, 1 mmol) in absolute ethanol (8 mL) was prepared. The flask was irradiated in a microwave oven (800 W) for 2 min. In a meanwhile of irradiation, the reaction was boiled and CO₂ gas was vigorously evolved. As the previous section, the liberation of CO₂ gas should be considered. TLC was monitored the progress of the reaction. After completion of the reaction, the solvent was evaporated under reduced pressure to afford green crystals of (Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7','-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (**6**) (Fig. 1, Table 1).

Table 1. Formation of octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones by the reaction of ninhydrin, proline and acrylates

Entry	Product	R	R'	Room temperature		Microwave irradiation	
				Time (h)	Yield (%) ^a	Time (min)	Yield (%) ^a
1	7d	Me	Me	3	88	2	91

^aYields refer to isolated pure products.

**Figure 1.** Synthesis of octahydrospiro[indene-2,3'-pyrrolizidine]-1,3-diones

Spectral data for product 6 is as follow:

Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7','-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (6): Yellow prism (EtOH), 88–91% yield, m.p. 111–112 °C. ¹H NMR (CDCl₃, 500 MHz) δ 1.67 (3H, s, CH₃), 1.68-1.78 (1H, m, 7'-CH), 1.83-1.93 (1H, m, 7'-CH), 1.94-2.03 (2H, m, 6'-CH₂), 2.06 (1H, dd, J = 6.2, 12.2 Hz, 1'-CH), 2.43-2.48 (1H, m, 1'-CH), 2.71-2.76 (2H, m, 5'-CH₂), 3.29 (3H, s, OCH₃), 3.95-4.04 (1H, m, 'α-H), 7.83-7.92 (3H, m, ArH), 8.01-8.03 (1H, m, ArH). ¹³C NMR (CDCl₃, 125 MHz) δ 14.26 (CH₃), 29.21, 31.21, 33.65, 48.84, 51.91 (OCH₃), 55.17, 68.35 (C_{spiro}), 74.12 (CH-N), 122.12, 123.91, 136.24 (4CH, aromatic), 142.17, 142.22 (2C_{ipso}, aromatic), 171.29, 203.40, 204.71 (3C=O). IR (ν_{max}/cm⁻¹, KBr) 1680, 1584 (2C=O). MS (m/e, %) 313 (M⁺, 75), 254 (M⁺-CO₂Me, 30), 212 (254-C₃H₆, 100), Anal. calcd for C₁₈H₁₉NO₄ (313.348): C, 68.99; H, 6.11; N, 4.47; O, 20.42%. Found: C, 68.98; H, 6.16; N, 4.48; O, 20.42%.

Ethical statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethical Committee of Urmia University of Medical Sciences.

Animals, study design, and tumor induction and inhibition study

7-Week-old female Wistar rats (100 g body weight) were randomized into three groups of seven animals each. They were maintained at 28±1 C, relative humidity 60 °C (12-h light and 12-h dark cycle), and provided with standard food pellets (diet composition, wheat broken—moisture 9.0%, crude protein 11.5%, crude fat 1.9%, crude fibre 4.0%, Ash 0.2%, nitrogen-free extract 73.4%) and tap water *ad libitum*. DMBA-induced mammary tumors was adopted based on a method described by others with slight modifications.^{42,43} DMBA was dissolved in 1 ml of vehicle (0.5 ml of DMSO plus 0.5 ml of saline) and injected by subcutaneous injection beneath the mammary gland on either side. Tumor yield and size were stabilized after 90 days with the initiation of DMBA, and these served as breast cancer control animals without any treatment. The effect of 6 on DMBA-induced tumors was determined after 90 days in the promotional stage of tumor development. Healthy intact animals (NC) were considered as a normal control feeding on pellets and tap water. In group DMBA, the tumor was induced by DMBA. In group DMBA/6 the animals with tumor received 100 μL 6 dissolved in DMSO (0.25 μM) solution interaperitoneally for one week. The experimental rats were regularly monitored for food and water consumption, the apparent signs of toxicity, weight loss, or mortality. The tumor incidence and its multiplicity were also recorded either at the time when carcinoma-bearing rats died, or at the termination of the experiment. After 110 days, all the animals were starved overnight and sacrificed by cervical decapitation. The breast tumor was surgically dissected out, tumor volumes (mm in diameter) of both cancer controls, as well as the experimental groups were measured, and total body weight (g) also recorded based on a method described by others.⁴⁴

Histopathological examination

Macroscopic mammary tumors, fixed in 10% buffered formalin, were embedded in paraffin using a conventional automated system. The blocks were cut to obtain 5 μm

thick sections and stained with hematoxylin–eosin. Serial paraffin sections of each tissue image were captured by light microscopy.

Statistical analysis

Statistical comparisons between control and treatment mean values of two parameters were analyzed using the Student's *t*-test. Multiple comparisons were done using ANOVA. The differences were statistically significant at $P < 0.05$.

Results

Chemistry

The results obviously indicated that using microwaves dramatically increased the inter-collusion of molecules as well as the temperature of reaction to afford higher rate enhancement. All products were assigned by their ^1H and ^{13}C NMR, FTIR, CH-COSY, MS and elemental analysis.

The C-H correlation for adduct **6** again confirmed high regioselectivity of the reaction pattern. (Fig. 2)

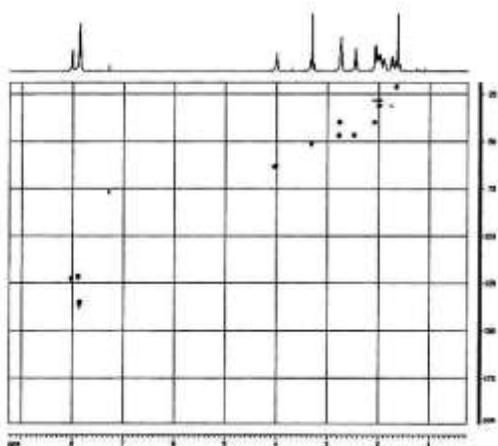


Figure 2: CH–COSY for adduct **6**

Anti-tumor activity

Tumor promotional stage is a reversible stage in the multistage carcinogenesis; therefore, it is the most suitable stage for the anti-carcinogenic agent to prevent, reverse, or slow down the process of carcinogenesis. Animals in the DMBA group attained a promotional stage tumor after 90 days. At the end of the experiment in animal of DMBA group, DMBA-induced breast tumors were increased to the maximum in terms of tumor

incidence (100%), tumor multiplicity per rat, and tumor weight (52%) compared to the normal control rats (NC group) ($P < 0.05$). The animals that administered **6** achieved 52% of tumor reduction after 20 days treatment. Tumor weight analysis also revealed statistically significant reduction of breast tumor weight in **6** treated groups when compared to the cancer controls (DMBA group). The tumor data analyzed in terms of tumor multiplicity recorded, in the DMBA animals, a 39 mm breast tumor size compared to 6 treated rats that showed 33 mm breast tumor size (Fig 3). The overall tumor analysis showed that **6** treatment significantly inhibited the breast tumor incidence, tumor multiplicity, and tumor size in the DMBA-initiated rat model (Table 2).

Table 2: The effect of intraperitoneal administrations of **6** on anti-tumor activity in experimentally DMBA-induced tumor in rats

Groups	Body weight	Tumor Volume (mm)	Reduction of tumor size (%)
NC	173.3± 31.6	0	0
DMBA	92.6± 15.8	45.3± 3.9	0
DMBA/7d	123.7±1.4*	33.2± 2.7*	54.3*

NC: Normal Control group. DMBA: animals with 7,12 dimethylbenz(a) anthracene-induced tumor. DMBA/7d: the animals with tumor received 100 μL **6** dissolved in DMSO (0.25 μM) solution intraperitoneally for one week. Data are represented as mean \pm SD. (n = 7). * $P < 0.01$ vs. DNBA group.

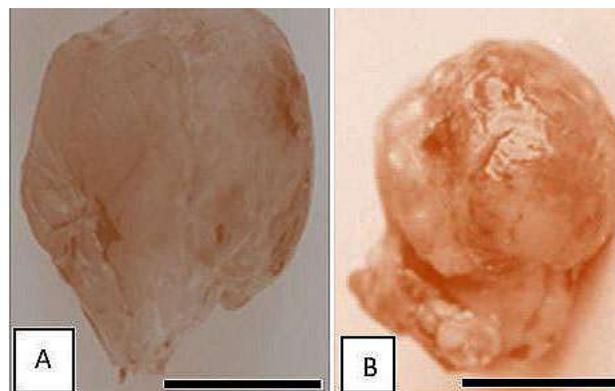


Figure 3. Anti-tumor effect of **6** treatment on DMBA-induced mammary tumors in rats (20 days). (A) DMBA-induced breast tumor development after 90 days served as cancer control rats (DMBA group) Scale bar: 30 mm. (B) Breast tumor bearing rats treated with **6**. Scale bar: 20 mm.

Histopathological examination

The combination **6** treated group showed tumor nodules and formation of intra-tumor vascularization. The vast majority of the lesions that developed in the rat mammary glands were mostly carcinomas. Inflammatory cell infiltration was absent in carcinomas. Most carcinomas exhibited a mixed structural pattern, with invasion of

neighboring tissues, and intense stromal desmoplastic reaction. Histopathology, it was revealed that most carcinomas exhibited an identical nuclear pattern. Most tumors were predominantly epithelial with fibrous tissue surrounding the mammary ducts (Fig 4).

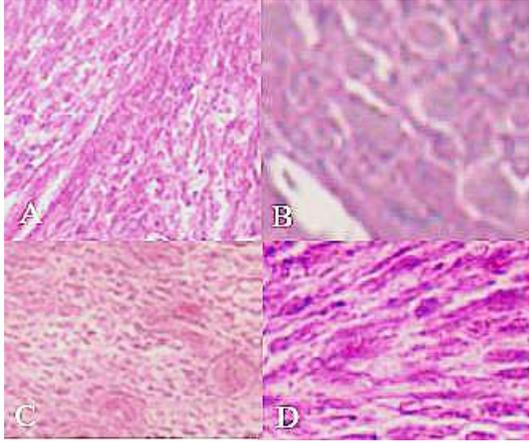


Figure 4. Representative micrographs showing BMBA induced tumors in experimental groups. (A) A typical hyperplastic lobule in DMBA-treated rat after 90 days (B) Carcinoma of the rat mammary gland with extensive solid areas with small cluster. (C) The fibroadenoma of the rat mammary gland, closely apposed alveolar structures are separated by large amount of connective tissue. (D) Necrosis formation along the tumor in rats treated by 6. All sections were stained with hematoxylin and eosin (40× objectives).

Discussion

Animal experimental systems are particularly useful for the study of human mammary carcinogenesis. The mammary gland is one of the few organs that are not totally developed at birth. It undergoes intense evolutive and functional modifications during puberty, pregnancy, and lactation. Others have described the developmental progression of human breast tissue and listed 4 different types of breast lobules.⁴⁵ Type 1 (or virginal) is the most undifferentiated lobule and occurs in the immature female breast before menarche. Type 2 lobule has a more complex morphology, being composed by a higher number of ductular structures per lobule. Type 3 has an average of 80 alveoli (ductules) and is formed under gestational hormonal stimulation. Type 4 is a secreting lobule during lactation. The mammary tumors in rats arise in the epithelium of the terminal end buds, which are comparable structures to the terminal ductal lobular units in the human breast.⁴⁶ The degree of lobular differentiations of importance in the susceptibility to carcinogenesis. Based on studies of the pathogenesis of human mammary cancer, it is possible to say that the type

1 lobule is the site of origin of preneoplastic lesions. Parous women undergo lobular differentiation, whereas nulliparous women seldom reach the type 3 lobule stage. The breasts of parous women free of cancer have the lowest percentage of type1 lobules. Lobules type 1 and 2 are characterized by having a shorter doubling time than type 3, growing faster and having a higher DNA labeling index. The susceptibility of the mammary gland to DMBA carcinogenesis is strongly age-dependent, being maximal when the drug is administered to rats between the ages of 45 and 60days, which is the age of the beginning of sexual maturity.⁴⁶ Active breast organogenesis and high rate of proliferation of type 1 and 2 lobules are characteristics of that period. The chance of chemically inducing breast cancer in rats is greater if DMBA is administered in this phase of the life of the animals. This was the reason why we injected the drug at the age of 47 days. According to the literature, the induced tumors are generally ductal carcinomas or papillary carcinomas, but it is possible that typical fibroadenomas, adenomas, and papilloma as are also formed.⁴⁷ Epithelial and myoepithelial cell proliferation were observed in most of the induced tumors in our experiment. Russo et al. found the same histological aspects. They also carefully presented the correlation between neoplastic and non-neoplastic alterations in the mammary gland of rats and in women.¹¹ Most of the lesions found in rats have corresponding lesions in humans, allowing the translation of basic research in rats into the clinic.⁴⁸

The induced DMBA tumors in this study were multifocal and locally aggressive, but no single case of metastases was identified. This fact is in agreement with studies done by other authors, and metastasis from even the most anaplastic induced tumors are low in frequency.⁴⁹ The absence of metastases in chemically produced mammary neoplasms opens the door for speculation.

The proliferation of epithelial and myoepithelial cells in relatively equal proportions may be a protective factor against metastasis.⁴⁷ In human breast carcinomas, proliferation occurs almost exclusively in epithelial cells. DMBA is highly lipophilic and requires metabolic activation for its carcinogenicity. Several tissues are capable of activating DMBA, and these include the mammary gland. In the breast, DMBA is converted to epoxides, active metabolites with a capacity for damaging the DNA molecule, the main event in carcinogenesis initiation. With the higher cellular proliferative index of

types 1 and 2 lobules, there is higher metabolic activity and more epoxide formation.^{50,51}

Since the rat mammary gland shows a high susceptibility to developing neoplasms which closely mimic human breast cancer, they have been selected in comparison to other animal models.⁵²

In the present investigation, treatment with **6** exhibited potential anticancer activity on DMBA-induced mammary tumors in rats. As a result, the body weight had also slightly increased, the tumor volume decreased, and the percentage of tumor inhibition was statistically significant ($P < 0.05$).

The histopathological examination revealed that most carcinomas exhibited a mixed structural pattern such as nodular well, invasion of neighboring tissues, with intense stromal desmoplastic reaction and necrosis. Upon correlating the histopathological examination, it was evident that most carcinomas exhibited identical nuclear patterns. The tumors were predominantly epithelial, and fibrous tissue surrounded the mammary ducts. **6** treatment significantly inhibited the total volume of tumors per rat. The supply of blood to newly forming tissues and to tumors is a limiting factor that regulates growth.⁵³ The process of neovascularization provides blood to support angiogenesis.⁵³ This observation suggests that vascularization could be helpful in explaining differential effects of cancer preventive agents on angiogenesis in the intra-tumoral region.

The findings of the present study showed that the **6** treatment was significantly effective in reduction of tumors versus the cancer controls. This experimental animal mode closely mimics human breast cancer and can be used as a comparative group in further studies with the purpose of elucidating the role of novel synthetic agents in mammary carcinogenesis. The effects of pretreatment and post treatment of rats with other similar substances that have action on mammary carcinogenesis will be the endpoints of future research in our laboratory using this animal model.

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Conflicts of interests

None

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ارزیابی درون اندامی فعالیت ضد توموری -Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-

octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (6) بر روی تومور پستانی القا شده با

7,12-dimethylbenz(a)anthracene در موش صحرائی

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هدف - سرطان سینه یکی از جدی ترین مشکلات در انکولوژی محسوب میشود. آلكالوئید های پیرولیزیدین به طور گسترده ای در طبیعت یافت شده و داراری فعالیت های بیولوژیکی متنوعی میباشد. هدف از این مطالعه ارزیابی فعالیت ضد توموری -Methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate (6) بر روی تومور پستانی القا شده با 7,12-dimethylbenz(a)anthracene در موش صحرائی میباشد.

طرح - مطالعه تجربی

حیوانات - ۲۱ موش صحرائی ماده نژاد ویستار

روش کار - موش های صحرائی ۷ هفته با وزن تقریبی ۱۸۰ گرم به سه گروه ۷ تایی به طور تصادفی تقسیم شدند. حیوانات با استفاده از تزریق زیر جلدی ۱ میلی لیتر محلول DMBA (نیم میلی لیتر DMSO و نیم میلی لیتر سالین نرمال) در زیر غده پستانی در هر طرف پس از ۹۰ روز مبتلا به تومور پستانی شدند.

حیوانات دست نخورده به عنوان گروه کنترل نرمال در نظر گرفته شده و تحت تغذیه نرمال قرار گرفتند. در گروه DMBA حیوانات با تزریق زیر جلدی DMBA به تومور پستانی مبتلا شدند. در گروه حیوانات مبتلا به تومور ۱۰۰ میکرو لیتر از ماده سنتز شده 6 با غلظت بسیست و پنج صدم میکرو مولار به مدت یک هفته به روش داخل صفاقی تحت درمان قرار گرفتند. سنتز 6 با موفقیت انجام گرفت و ساختار ماده تشکیل شده با استفاده از ¹H/¹³C NMR, IR, CH-COSY و آنالیز عنصری مورد تایید قرار گرفت. نتایج - آنالیز کلی تومورها نشان داد که درمان با 7d به طور معنی داری شیوع، مولتی پلیسیتی و اندازه تومور را مهار کرده است. 6 به طور معنی داری حجم کلی تومورها در هر موش صحرائی را مهار کرد.

نتیجه گیری و کاربرد بالینی - یافته های مطالعه حاضر نشان داد که درمان با 6 به طور معنی داری در کاهش تومورها در مقایسه با گروه کنترل موثر بوده است.

کلمات کلیدی - کارسینوژن پستان، 7,12-Dimethylbenz(a)anthracene, Indene-2,3'-pyrrolizidine, تومور پستانی