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Chalcones, *myo*-Inositol and Other Novel Inhibitors of Pulmonary Carcinogenesis

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Abstract The objective of the studies reported here has been to find novel chemopreventive agents effective against carcinogenesis of the lung. In particular, identification of suppressing agents, *i.e.*, compounds preventing the evolution of the neoplastic process, has been sought. For this purpose, inhibition of pulmonary neoplasia in female A/J mice given the test agent starting one week after the last administration of three doses of benzo[*a*]pyrene has been employed as the experimental model. Under these conditions, chalcone, 4'-methoxychalcone, *myo*-inositol, dexamethasone, and "terpeneless" orange oil added to the diet suppressed pulmonary adenoma formation. Chalcone and 4'-methoxychalcone are open chain flavonoids, neither of these compounds occurs naturally, and their mechanism of action is not known. *myo*-Inositol is a naturally occurring compound of particular interest because of its exceedingly low toxicity. Dexamethasone is a potent glucocorticoid. Amongst its biological properties is the capacity to induce maturation of Type 2 alveolar cells and to stimulate production of surfactant by these cells. "Terpeneless" orange oil is a fraction of orange oil consisting predominantly of compounds with carbonyl or hydroxyl groups. The constituent or constituents responsible for the inhibitory effects observed is not known. The above studies are in an early phase of development and their ramifications remain to be determined.

Key words: Chalcones, chemoprevention, dexamethasone, inositols, lung carcinogenesis, "terpeneless" orange oil

The optimal way for dealing with virtually all diseases is prevention and this certainly is the case for cancer. Chemoprevention provides one means of obtaining this objective. Chemopreventive agents can be placed into two categories, blocking agents and suppressing agents. Blocking agents prevent cancer-producing compounds from reaching or reacting with critical target sites: they exert a barrier function. Suppressing agents prevent the evolution of the neoplastic process in cells which would otherwise become malignant [1–3]. In experimental systems, the two can generally be separated by the fact that blocking agents are effective when administered prior to or simultaneously with cancer-producing compounds, whereas suppressing agents are active when given after exposure to cancer-producing compounds. Suppressing agents have the positive attribute that they are directed at carcinogenic mechanisms which are generic and, accordingly, have the potential of inhibiting the effects of a variety of carcinogens or combinations of carcinogens that impact a particular tissue. Unlike blocking agents, in which the timing of administration in relationship to carcinogen exposure is critical for their action, this stricture is less pertinent to suppressing agents. Only a

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small number of suppressing agents have been identified. Of those identified, very few are applicable to preventing cancer of the lung [1–3]. The inhibitory capacities of several suppressing agents novel for preventing pulmonary neoplasia will be presented. These are chalcones, *myo*-inositol, dexamethasone and "terpeneless" orange oil.

EXPERIMENTAL PROCEDURES

An *in vivo* animal model was used to determine if test compounds exert chemopreventive activity against pulmonary carcinogenesis. For this purpose, female A/J mice were employed. Benzo[*a*]pyrene (B*a*P) served as the carcinogen. Test compounds were fed in the diet starting one week after the last of three administrations of B*a*P. These conditions were used to determine if the test material was acting as a suppressing agent. The experiments reported were carried out over a period of two years. The mice used in all experiments were obtained from the Jackson Laboratory, Bar Harbor, ME, and fed a semipurified diet consisting of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (USP XIV) and a complete mixture of vitamins (Teklad, Madison, WI). At nine weeks of age, the mice were given the first of three administrations of B*a*P (Aldrich Chemical Co., St. Louis, MO); 2 mg in 0.2 ml cottonseed oil by oral intubation. The second administration was given three days later, and a third dose 7 days after the initial administration. One week following the last dose of B*a*P, the animals were randomized by weight into experimental groups. At that time they were placed on the semipurified diet to which the test agents under investigation had been added. These diets were fed for the duration of the protocol. Control groups were fed the semipurified diet without any additions. The mice were weighed at two-week intervals. The experiment was terminated 21 or 22 weeks after the last dose of B*a*P. Pulmonary adenomas were counted following the general procedure of Shimkin [4]. The adenomas are derived from the peripheral airway cells [5]. Peripheral airway cell marker expression has been reported in approximately 40% of adenocarcinomas of the lung in humans; results obtained with the animal model employed may have special relevance to this group of human tumors [6].

CHALCONES

Chalcones are open chain flavonoids in which the two aromatic rings are joined by a three carbon α,β-unsaturated carbonyl system. Two chalcones, *i.e.*, chalcone and 4′-methoxychalcone, were chosen for study. Neither is a natural product. Chemical attributes that make chalcone and 4′-methoxychalcone of interest are their stilbene configuration with the bracketing of an α,β-unsaturated carbonyl group by two phenyl groups. Stilbene derivatives include a variety of synthetic compounds, such as tamoxifen, which bind to steroid hormone receptors. The α,β-unsaturated carbonyl moiety is a reactive chemical species. Compounds containing this grouping have been shown to bind to receptors that induce increased

TABLE I. Effects of 4'-Methoxychalcone on Benzo[*a*]pyrene-Induced Pulmonary Adenoma Formation in Female A/J Mice

Test Compound	Concentration in the diet (mg/g)	Number of Mice	Pulmonary Tumors		Weight[1] Gain (g)
			Tumors per Mouse	Ratio: Test/Control	
None (control)	—	13	20.5 ± 7.4[2]	—	7
4'-Methoxychalcone	1	14	20.3 ± 5.7	0.99	5
4'-Methoxychalcone	5	14	12.3 ± 7.1[3]	0.60	6

[1] Weight gain from 11–33 weeks of age; [2] mean ± SD; [3] 4'-methoxychalcone versus control, $p < 0.01$.

activities of phase II enzymes responsible for metabolizing xenobiotic compounds [7,8].

The vast majority of naturally occurring chalcones are polyhydroxylated. Bohm [9] lists 58 such compounds. Several polyhydroxylated chalcones have been shown to inhibit lipoxygenase activity and TPA-induced tumor promotion of the mouse epidermis [10,11]. Isoliquiritigenin (4,2',4'-trihydroxychalcone) is particularly potent in this regard [11]. The biological effects produced by chalcones containing other types of substitutions have also been investigated. In one such study, (*E*)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid was found to induce differentiation of HL-60 leukemia cells and teratocarcinoma cells [12,13]. In another, (*E*)-1-(2,5-dimethoxyphenyl)-3-[4-dimethylamino)phenyl]-2-methyl-2-propen-1-one and related compounds were reported to induce antimitotic activity against tumor cells *in vitro* [14]. Lichochalcone A has been shown to inhibit tumor promotion in mice and exhibits antitumor activity against L1210 leukemia and B16 melanoma cells *in vivo* [15]. More recently, 3'-methyl-3-hydroxychalcone has been reported to be a potent inhibitor of proliferation of several lines of malignant human cells *in vitro*, and to suppress TPA-induced tumor promoting activity in mouse skin *in vivo*. An additional attribute of this chalcone is its capacity to inhibit the binding of estradiol to type II estrogen binding sites in HGC-27 cells [16]. Thus, several substituted chalcones have been shown to have effects such as inhibition of cell proliferation and tumor promotion that might endow them with chemopreventive properties.

Chalcone and 4'-methoxychalcone were selected as test compounds based on mechanistic implications derived from their chemical structure and their relationship to other chalcones studied in different test systems [11]. In an initial study, chalcone was found to inhibit B*a*P-induced pulmonary adenoma formation in female A/J mice [17]. In the present study, 4'-methoxychalcone also was found to inhibit the formation of pulmonary tumors in this experimental model (Table I). The potency of the two compounds is similar.

INOSITOLS

Studies of inhibition of pulmonary adenoma formation by *myo*-inositol have their origins in the very interesting work by Shamsuddin *et al* [18–20]. These investigators showed that inositol hexaphosphate (phytate) inhibits carcinogen-induced neoplasia. Their studies focused primarily on inhibition of carcinogenesis in the large bowel of rats and mice. An important finding was that inhibition occurred when phytate was adminis-

TABLE II. Effects of *myo*-Inositol, Dexamethasone and Ambroxol HCl® on Benzo[*a*]pyrene-Induced Pulmonary Adenoma Formation in Female A/J Mice (n=14 per group)

Test Compounds	Concentration in Diet (mg/g)	Pulmonary Tumors		Weight[1] Gain (g)
		Tumors per Mouse	Ratio: Test/Control	
None (control)	—	17.5 ± 6.1[2]	—	3
myo-Inositol	10.0	11.4 ± 3.5[3]	0.65	3
Dexamethasone	0.0005	9.3 ± 4.0[3]	0.53	5
Ambroxol HCL®	0.1	18.5 ± 5.4	1.06	3
myo-Inositol + Ambroxol HCL®	(as above)	12.1 ± 5.3[3]	0.69	3
Dexamethasone + Ambroxol HCL®	(as above)	7.4 ± 3.2[3]	0.42	3

[1] Weight gain from 11–32 weeks of age; [2] mean ± S.D.; [3] test compound versus control, $p < 0.05$.

tered in the post-initiation period. *myo*-Inositol, which is not phosphorylated, inhibits large bowel tumor formation to a similar magnitude under the same conditions as phytate [19]. Rodent small bowel mucosa contains the enzyme, phytase, which removes the phosphate groups from inositol hexaphosphate [21]. Thus, in the studies in which phytate was administered, it is not clear as to what extent the inhibitory effects are due to the parent compound or its dephosphorylated products. Phytate has a broad distribution in foods of plant origin. It occurs in relatively large concentrations in beans and seeds [22,23]. In addition to phytate, lipid-bound and free inositol also occur naturally. Data as to the dietary consumption of each of the three forms of inositol have not been published. However, studies of the aggregate amounts of inositol in foods have been tabulated [24]. The methodology entails hydrolysis of the food under study with 6 N HCl. The data obtained with this procedure are a summation of the various dietary forms of inositol and its phosphorylated derivatives. The data indicate that consumption of the order of one gram per day could be readily achieved [24]. The amounts actually consumed would be dependent on the choice of foods.

The use of phytate as a chemopreventive agent poses problems in that it chelates cations [25]. In contrast, *myo*-inositol, which does not have phosphate groups, is devoid of this potentially harmful attribute. It can be consumed at high dose levels without evidence of toxicity. *myo*-Inositol has been used clinically to minimize diabetic neuritis and cataract formation. The administration schedules employed entailed dose levels of several grams per day over long periods of time [26,27], and in none of these studies was toxicity encountered.

In a previous investigation, *myo*-inositol was shown to inhibit pulmonary adenoma formation in female A/J mice [28]. This observation has been confirmed in the present work as is shown in Table II. The mechanism(s) of inhibition is not known. A biochemical property of *myo*-inositol of some interest is its capacity to inhibit the polyol shunt. This pathway functions when high glucose levels exist in cells. Excess glucose is oxidized by aldoreductase to produce sorbitol which is further metabolized to fructose [29]. Possibly of more relevance is the reported modulation of pulmonary surfactant production by *myo*-inositol and its clinical use for this purpose [30]. Much of the detail of this effect is not clear. Type II alveolar cells are active in production of the constituents of surfactant. It is possible that modification of this metabolic system could play a role in the inhibitory effects produced by *myo*-inositol. Because of this background information, the administration of Ambroxol-HCL® [31], a medicinal compound enhancing surfactant production, was studied for its effects on pulmonary adenoma formation. As shown in Table II, no inhibition was found. In addition, the administration of Ambroxol-HCL® in a diet containing *myo*-inositol did not enhance the inhibitory potency of this latter compound. Although this experiment gave negative results, studies with dexamethasone, which will be described below, further focuses on a possible role of alteration in surfactant metabolism in inhibition of pulmonary adenoma formation.

TABLE III. Effects of "Terpeneless" Orange Oil and Orange Oil on Pulmonary Adenoma Formation in Female A/J Mice

Test Material	Number of Mice	Pulmonary Tumors		Weight[1] Gain (g)
		Tumors per Mouse	Ratio: Test/Control	
None	14	24.7 ± 4.9[2]	—	4
Terpeneless Orange Oil 1%	14	16.3 ± 7.5[3]	0.66	3
Orange Oil 1%	14	16.9 ± 6.9[3]	0.68	4

[1] Weight gain from 11–33 weeks of age; [2] mean ± SD; [3] $p < 0.05$.

DEXAMETHASONE

The selection of dexamethasone for studies of its capacity to inhibit pulmonary adenoma formation had its origins in work showing that it inhibits tumor promotion in mouse skin models [32,33]. Other compounds that inhibit epidermal carcinogenesis under these conditions have been found to prevent the occurrence of neoplasia in the post-initiation period in other tissues in experiments in which no tumor promoter was administered [34–36]. Dexamethasone is a glucocorticoid with a wide range of biological activities. Several of these, singly or in combination, could account for its ability to suppress pulmonary adenoma formation. Early studies showed that dexamethasone inhibits phospholipase A_2 hydrolysis, an early step in arachidonic acid and prostaglandin production. More recently, the activated glucocorticoid receptor has been shown to exert inhibitory effects by direct interaction with nuclear transcription factors regulating cell growth [37]. Further attributes of dexamethasone which appear to be particularly relevant to its chemopreventive effects against pulmonary adenoma formation are its capacity to produce differentiation of type II alveolar cells and to stimulate production of surfactant in these cells [38, 39]. This maturation effect on type II alveolar cells has been shown to occur in the human as well as in rodent species. It is the basis for use of dexamethasone in preventing respiratory distress syndrome in premature infants.

In a previous study, dexamethasone was shown to inhibit B*a*P-induced pulmonary adenoma formation in female A/J mice when administered starting one week after the last dose of carcinogen. In addition, administration of the combination of *myo*-inositol and dexamethasone to A/J mice given B*a*P produced an additive effect of the two compounds on inhibition of pulmonary adenoma formation [28]. As discussed in the section on inositols, *myo*-inositol has been reported to effect surfactant metabolism; thus both *myo*-inositol and dexamethasone have an impact on the surfactant system. However, data are not available as to whether alterations of surfactant production or metabolism are related to inhibition of pulmonary adenoma formation. In Table II, the results of a confirmatory study of the inhibitory effects of dexamethasone on pulmonary adenoma formation are shown. In addition, the effects of the combined administration of dexamethasone with Ambroxol HCl®, an enhancer of surfactant synthesis, was investigated. This combination had a slightly greater inhibitory effect on pulmonary adenoma formation than dexamethasone by itself, but the results are not definitive.

"TERPENELESS" ORANGE OIL

A large number of epidemiology studies have shown that the consumption of diets rich in fruits and vegetables is associated with a decreased risk of cancer of the lung. In accord with these findings has been the identification of plant constituents that have chemopreventive activity. Studies of this nature have resulted in chemopreventive compounds being isolated from citrus fruit oils. Of particular note in this regard, is the monoterpene *d*-limonene [40]. As an extension of these investigations, we have studied "terpeneless" orange oil which is prepared by the fractionation of cold pressed orange oil. "Terpeneless" orange oil is employed as a flavoring for beverages and foods. The term "terpeneless" refers to the fact that unoxidized terpenes such as *d*-limonene have been largely removed (less than 5%) from the original orange oil. It consists mostly (approximately 85%) of oxygenates, *i.e.*, compounds containing either hydroxyl or carbonyl groups. In contrast, *d*-limonene and other unoxidized terpenes comprise over 95% of the content of the original orange oil. The "terpeneless" orange oil comprises only a small fraction of the composition of the original orange oil.

The results of feeding "terpeneless" orange oil and orange oil on B*a*P-induced pulmonary adenoma formation in female A/J mice are shown in Table III. A modest inhibitory effect was obtained with both test materials. The magnitude of the inhibition was very similar. The compound or compounds occurring in the terpeneless" orange oil which exert the inhibitory activity observed are not known. Efforts at identifying the active constituent(s) are in progress.

SUMMARY

The inhibitory effects of several suppressing agents novel for preventing pulmonary neoplasia have been presented. These are chalcones, *myo*-inositol, dexamethasone and "terpeneless" orange

oil. These studies are in an early phase of development and their ramifications remain to be determined.

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