



Phenoxodiol, a novel isoflavone derivative, inhibits dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague–Dawley rats

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Abstract

The present study was undertaken to evaluate the potential cancer chemopreventive effects of novel synthetic derivatives of isoflavones. Initially these agents were tested in a mouse mammary organ culture (MMOC) model. Phenoxodiol (2H-1-benzopyran-7-O1,3-(4-hydroxyphenyl)), the most effective in this assay, was selected for further testing in female Sprague–Dawley rats. The agent was tested at 0 (basal diet), 50 and 75 mg/kg diet. Mammary carcinomas in these three groups were induced by dimethylbenz[a]anthracene (DMBA) injected 1 week after the animals started eating the experimental diets. Phenoxodiol significantly reduced tumour incidence rate at both doses ($P \leq 0.05$). Tumour latency was increased from 70.4 days in the control group to 92.9 ($P = 0.04$) days and 97.8 ($P = 0.03$) days in the groups that were fed 50 and 75 mg/kg phenoxodiol, respectively. Compared with the control that was fed basal diet, tumour multiplicity was reduced by 42% ($P = 0.04$) in the group that was fed 50 mg/kg phenoxodiol and by 49% ($P = 0.01$) in the group that was fed 75 mg/kg phenoxodiol. Two additional groups that were not exposed to DMBA, one fed the basal diet and the other a diet containing 75 mg/kg phenoxodiol, were free of tumours. These data suggest that phenoxodiol is an effective chemopreventive agent against DMBA-induced mammary carcinogenesis.

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1. Introduction

A prospective phase III clinical trial was completed in 1998 in which more than 13 000 women at high risk for developing breast cancer participated. In this 5-year study, breast cancer incidence was decreased by 49% in the tamoxifen-treatment group compared with the placebo group [1]. This outcome provided ‘proof of principle’ demonstrating that prevention of breast cancer in high-risk individuals by a single agent is possible. Furthermore, the tamoxifen phase III study suggested that chemically-induced mammary tumours in rodents are suitable models for the identification of agents that can prevent breast cancer in human populations. However,

the field of cancer chemoprevention experienced a major setback after the results of the Carotene and Retinol Efficacy Trial (CARET) became known. In this study, β -carotene was found to increase instead of lower the risk of lung cancer in smokers [2]. These clinical studies underscore the need for vigorous preclinical testing of promising chemopreventive agents in suitable animal models.

Potential chemopreventive agents are initially evaluated in high throughput *in vitro* tests, or organ culture systems, prior to being evaluated in animal models [3]. We have established a mouse mammary gland organ culture (MMOC) model for the purpose of evaluating agents that can be promising in preventing mammary tumours in rats and breast cancer in human beings [4,5]. In this model, mammary glands from normal young female Balb/C mice are exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) for a short

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period. With appropriate hormonal changes during a 24-day experimental period, the mammary epithelial cells attain a transformed phenotype, which, if transplanted in syngeneic mice, can form adenocarcinoma. Incubation of the glands with efficacious chemopreventive agents during the growth phase decreases the incidence and multiplicity of lesions. This can be used as a measure to determine the efficacy of the test agent. There is a very good correlation between the efficacy observed *in vitro* in MMOC and the efficacy in experimental mammary carcinogenesis models *in vivo* [3]. Using this model, we evaluated four potential chemopreventive agents that are natural or synthetic derivatives of the isoflavone daidzein that is found in considerable quantities in soybeans and red clover.

Daidzein and especially genistein attracted much interest in cancer chemoprevention, mainly due to epidemiological studies that suggested that the low incidence of breast cancer in Asian countries might be due to the frequent consumption of soy products [4]. In rodent models of carcinogenesis, soy diets produced a reduction in the incidence and multiplicity of tumours [5–7]. Genistein was effective in protecting female rats against future mammary tumour development only when given during the neonatal or prepubertal period of development [8]. Genistein, when given to adult rodents (i.e. 42 days of age), was either ineffective [7,9] or it increased mammary gland proliferation and tumour growth [10,11].

Phenoxodiol is a synthetic analogue of equol that is currently undergoing clinical testing as an antitumour drug.

2. Materials and methods

2.1. Chemicals and agents

All chemicals, unless otherwise specified, were purchased from the Sigma Chemical Co. (St Louis, MO, USA). 2H-1-Benzopyran-7-0,1,3-(4-hydroxyphenyl) (phenoxodiol), 4-methoxy-7-hydroxyisoflavone (formononetin), 4'-7-dihydroxyisoflavone (dihydrodaidzein), and 6-hydroxy-*O*-desmethylangolensin were provided by Novogen Ltd. (North Ryde, NSW, Australia).

2.2. Mouse mammary organ culture (MMOC)

The procedure of evaluating the chemopreventive agents using the MMOC was previously described in Ref. [12]. Briefly, 4-week-old BALB/c female mice (Charles River, Wilmington, MA, USA) were pretreated for 9 days with 1 µg of oestradiol and 1 mg of progesterone. On the tenth day, the mice were sacrificed, and the second thoracic mammary glands were dissected on silk and transferred to 60-mm culture dishes containing

5 ml of Waymouth's 752/1 MB medium (Gibco, Grand Island, NY, USA) supplemented with 100 units penicillin, 100 µg streptomycin and 35 µg L-glutamine per ml medium. Fifteen glands were used per group. The glands were incubated for 10 days (37 °C, 95% O₂ + 5% CO₂) in the presence of growth-promoting hormones (5 µg of insulin, 5 µg of prolactin, 1 µg of aldosterone and 1 µg of hydrocortisone per ml of medium). Glands were exposed to 2 µg/ml DMBA between 72 and 96 h. After exposure, the glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones, except insulin, for 14 additional days. Test compounds were present in the medium during days 1–10 of culture at five concentrations ranging from 1 ng/ml to 10 µg/ml. Control glands did not receive any chemopreventive agents, only the solvent. At the end of the experiments, the glands were fixed in formalin and stained with alum carmine. The glands were scored for lesions, and the percentage of glands with lesions was calculated for every group. Results were subjected to χ^2 analysis to determine statistical significance.

2.3. Toxicity and dose-selection study

Virgin female Sprague–Dawley rats (Hsd:SD/BR, Indianapolis, IN, USA), 35 days old, were randomised by weight into seven groups of 20 rats each. One week later, dietary treatment was initiated. Phenoxodiol was administered in Teklad global 16% protein (w/w) rodent diet (Harlan/Teklad, Madison, WI, USA) rodent chow at the following doses: 0, 50, 75, 100, 125, 150 and 175 mg/kg diet. Animals were observed twice daily and weighed weekly in order to assess possible toxicity. After 8 weeks on the supplemented diet, all animals except those on the selected dose were sacrificed, and necropsies were performed in order to detect possible gross toxicity of the agent. Mammary glands and liver were removed and processed frozen for biochemical and histopathological analyses.

2.4. Tumour induction and chemoprevention study

Based on the results of the dose selection study, suitable phenoxodiol doses were selected for the chemoprevention study. Virgin female Sprague–Dawley rats at 35 days of age were randomised by weight and received Teklad global 16% protein (w/w) rodent diet as a basal diet. One week later, all, but the control groups, received basal diet supplemented with phenoxodiol. One week later, at age 50 days, the animals received a single intragastric (i.g.) dose of DMBA (15 mg) in sesame oil (or vehicle only).

During the experimental period, the animals were weighed weekly. Palpation of mammary tumours began

4 weeks after animals received DMBA and continued until termination of the study (117 days). The date of appearance and location of every palpable tumour were recorded. Animals were observed daily to assess their general health. Animals were sacrificed by CO₂ asphyxiation. Mammary tumours were coded by location, removed from the animal, and weighed. Each tumour was divided in two portions. One portion was snap-frozen in liquid nitrogen and stored at -70°C for biochemical analysis. The other half was fixed in 10% buffered formalin (v/v) for histopathological examination.

The statistical significance of differences between mean tumour multiplicities was assessed using one-way ANOVA Armitage's test for trend in proportions. Tumour incidence curves were generated by the life table method and compared by Peto and colleagues [13]. A significant inhibition of tumour induction as achieved by administration of an inhibitor was defined as a statistically significant decrease in tumour incidence, multiplicity or increased latency period.

3. Results

We have used the mouse mammary gland organ culture model to identify the most promising chemopreventive agent from a group of four isoflavone derivatives. In this model system, mammary glands from normal young female Balb/C mice are exposed to DMBA following treatment with a combination of hormones. The mammary epithelial cells attain a transformed phenotype [14] and develop mammary alveolar lesions (MAL). Incubation of the glands with an efficacious chemopreventive agent during the first 10 days of culture decreases the incidence of the MAL. A correlation has been established between the efficacy of chemopreventive agents in the MMOC model and animal models of mammary tumour development, demonstrating that the model is of high predictive value [3].

In the group that was treated with DMBA (control), 9/15 glands developed MAL, yielding an incidence of 60% (Table 1). In the group that was treated with phenoxodiol, 3/13 glands developed MAL, representing

62% inhibition over the control levels. The other isoflavone derivatives inhibited MAL development by 22–33%, being clearly less effective. Phenoxodiol was further evaluated in the MMOC model at 0.001, 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$ doses to determine the IC₅₀ and any toxicity. We found that the IC₅₀ was 0.1 $\mu\text{g/ml}$, and it lacked toxicity even at the highest concentration (data not shown). Toxic agents induce dilation of ducts leading to amorphous material with complete disintegration of the glands. Toxicity is determined by the degree of dilation of the ducts. Based on these findings, phenoxodiol was selected for further studies in the rat model.

In the dose selection study, supplementation with phenoxodiol doses of 100–175 mg/kg produced statistically significant reduced rates of weight gain in comparison to the control diet (data not shown). Necropsies after 8 weeks did not show any signs of gross toxicity, indicating that the reduced weight gain is probably due to reduced food intake. Based on these results, we selected phenoxodiol doses of 50 and 75 mg/kg for the long-term chemoprevention study.

The effects of these two doses on mammary tumour development were evaluated in female Sprague–Dawley rats by determining the tumour incidence (percentage of tumour-bearing rats), the tumour multiplicity (Fig. 1), and the median tumour latency (Table 2). The diet containing 50 mg/kg phenoxodiol (low dose) reduced final tumour incidence to 65% ($P=0.03$) compared with 94.7% in the control group. The diet containing 75 mg/kg phenoxodiol (high dose) reduced the tumour incidence to 70% ($P=0.05$). The above statistical comparisons for the final tumour incidence were determined with Fisher's Exact test. Comparison of the incidence rates [13] showed statistical significance between each of the treatment groups and the control group ($P\leq 0.05$). Mean tumour multiplicity was reduced by both experimental doses (Fig. 1b). The diet that was supplemented with 50 mg/kg phenoxodiol reduced tumour multiplicity by 43% ($P=0.04$, unpaired t -test) while the 75 mg/kg diet reduced multiplicity by 50% ($P=0.01$, unpaired t -test). Median tumour latency was increased by 32 and 39% by the low and high doses, respectively, of the experimental diets, as compared with the control diet (Table 2). Both of these increases were statistically

Table 1
Effect of isoflavone derivatives on the development of mammary alveolar lesions (MAL) in mouse mammary organ culture (MMOC)

Agent	Glands with lesions/total glands	Incidence (%)	Inhibition (%)
None	9/15	60	0
Formononetin	6/15	40	33
Dihydrodaidzein	6/15	40	33
Phenoxodiol	3/13	23	62
6-Hydroxy- <i>O</i> -desmethylangolensin	7/15	47	22

Glands were incubated with the different isoflavone derivatives at 10 $\mu\text{g/ml}$. Percent inhibition was calculated from determining glands with MAL in each group consisting of 10–15 glands.

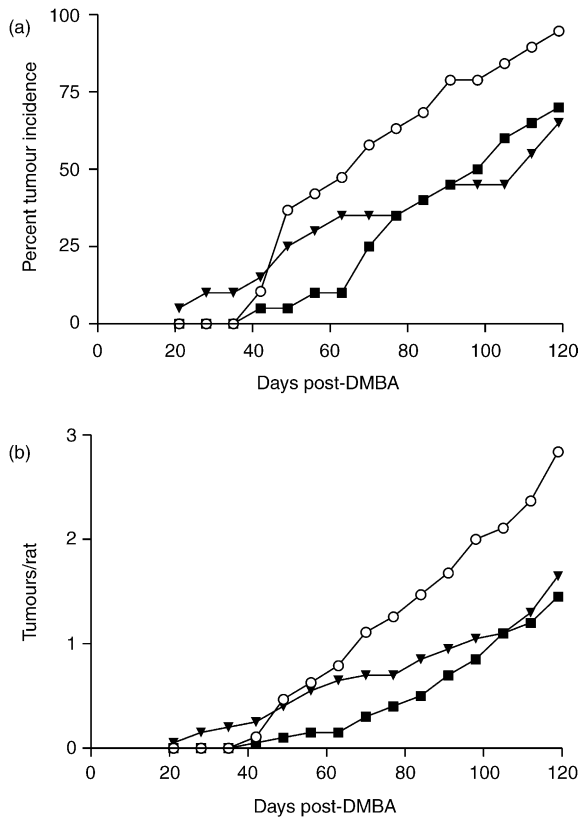


Fig. 1. The effect of phenoxodiol on tumour incidence (a) and mean tumour number (b), in female Sprague-Dawley rats exposed to DMBA. Rats ($n=20$) were started on basal diet (○) or diets containing 50 mg/kg phenoxodiol (▼), or 75 mg/kg phenoxodiol (■) 1 week prior to the administration of DMBA. Rats were maintained on the diet throughout the experiment. Tumours were detected by palpation during the study and verified after removal.

significant ($P \leq 0.05$ and $P \leq 0.01$, respectively) when compared with the control group.

Mean body weight and survival were also determined (Fig. 2). Phenoxodiol reduced weight gain (Fig. 2b). The reduction in weight by the high and low phenoxodiol doses was 4.8 and 5.2%, respectively, compared with the mean body weight of the control. However, the mean body weight of the phenoxodiol-treated groups was not statistically different from that of the control DMBA-treated group. The survival of the groups that were fed the phenoxodiol-supplemented diets seemed to be higher than the control group (Fig. 2a). However,

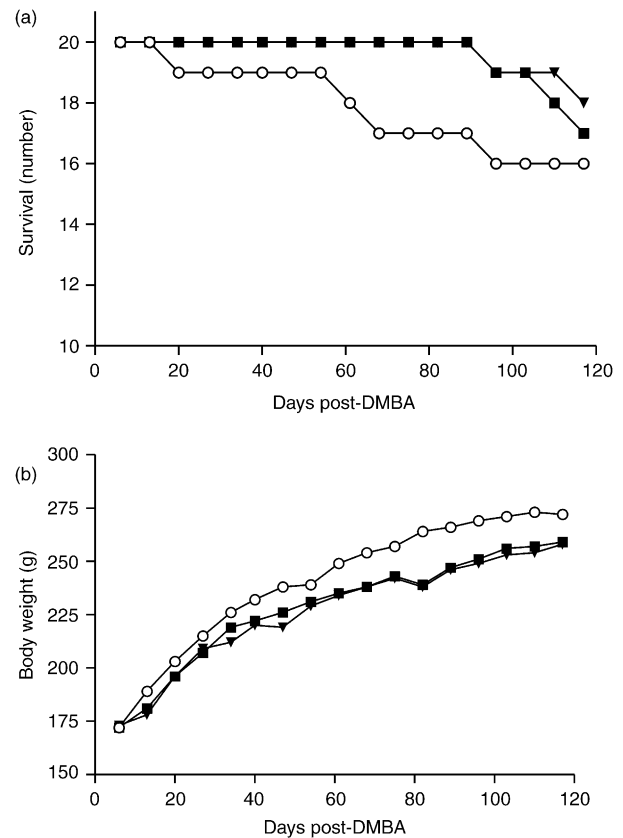


Fig. 2. The effect of phenoxodiol on survival (a), and body weight (b), in female Sprague-Dawley rats exposed to DMBA. Rats ($n=20$) were fed a basal diet (○) or diets containing 50 mg/kg phenoxodiol (▼), or 75 mg/kg phenoxodiol (■) 1 week prior to the administration of DMBA. Rats were maintained on the diet throughout the experiment.

this observation may be due to an unusually large number of animals that died in the control group for reasons unrelated to tumour burden.

4. Discussion

Flavonoids are a class of compounds ubiquitous in the plant kingdom characterised by di-phenolic or benzopyran ring structure. Many of these compounds display a range of novel and potentially important anticancer effects after their ingestion as part of the

Table 2
Effect of phenoxodiol on final tumour incidence, multiplicity and latency

Agent	Final tumour incidence (%)	Mean tumour multiplicity \pm SEM	Median tumour latency \pm SEM (days)
Control	94.7	2.8 ± 0.4	70.4 ± 5.9
Phenoxodiol (50 mg/kg)	65.0*	$1.6 \pm 0.4^*$	$92.9 \pm 8.9^*$
Phenoxodiol (75 mg/kg)	70.0*	$1.4 \pm 0.4^*$	$97.8 \pm 6.3^{**}$

Statistical comparisons were made for experimental groups versus control as follows. Final tumour incidence by Fisher's Exact test. Mean tumour multiplicity (determined as the number of tumours per number of tumour-bearing animals) and median tumour latency by unpaired t -test. Statistical significance is as follows: * $P \leq 0.05$ versus control; ** $P \leq 0.01$ versus control; $n=20$. SEM, standard error of the mean.

human diet. The naturally occurring flavonoids quercetin, found in vegetables such as onions and apples, and genistein, an isoflavone which is considered to be the main bioactive chemopreventive component of soybeans, have been evaluated in this regard, but have not produced sufficient anticancer effects *in vivo* to warrant their use as cancer treatment agents. The synthetic flavonoid, flavopiridol, is currently undergoing clinical studies, but while early studies have revealed promising anticancer activity, it has been found to have a dose-limiting toxicity, the main side-effects being nausea, diarrhoea, fatigue and a pro-inflammatory syndrome [15].

Phenoxodiol (2H-1-benzopyran-7-O1,3-(4-hydroxyphenyl)) is a synthetically derived flavonoid compound structurally related to the isoflavone daidzein. In this paper, we show how an organ culture model (MMOC) enabled us to identify, from a panel of related isoflavone derivatives, phenoxodiol as the most promising chemopreventive agent. From the analysis of data of previous studies representing over 300 test agents, we have determined that when an agent inhibits the formation of mammary lesions by at least 60% compared with the control group the effect is statistically significant ($P \leq 0.05$, [16]). Phenoxodiol was the only agent among the four tested in the present study that inhibited MAL by over 60%, and it consequently was considered effective and was therefore further evaluated in rats. Testing in animal models constitutes the next step in the process of chemopreventive agent evaluation as outlined by Kelloff and colleagues [17].

In the DMBA rat model, phenoxodiol was very effective in reducing mammary carcinogenesis as determined by three different criteria. It significantly reduced the mean tumour incidence and multiplicity, and it increased significantly the median latency period. These effects are comparable to those of other chemopreventive agents such as tamoxifen and leuprolide that have been evaluated initially in rat models and found to be effective in clinical settings [18–23]. There is extensive variation in the carcinogen-induced mammary tumour chemoprevention protocols including rat strain, DMBA dose, route of carcinogen and chemopreventive agent administration. We compared the effects of phenoxodiol

with four other chemopreventive agents that were evaluated with experimental protocols that were similar to the present, and the results are shown in Table 3. As can be seen from this comparison, the effects of phenoxodiol are similar to those of tamoxifen; although the effects of phenoxodiol on tumour incidence and multiplicity are a little less pronounced in comparison to tamoxifen, phenoxodiol's effect on tumour latency is more pronounced. Phenoxodiol is found to be effective (with statistical significance) in all three evaluation criteria of chemoprevention, compared with two criteria for tamoxifen, one for leuprolide, one for soy protein isolate, and none for genistein (Table 3).

Although genistein is found to be effective in inhibiting mammary carcinogenesis in neonatal rats, it seems to be ineffective in inhibiting mammary carcinogenesis in older rats. In previous studies, we found genistein to be only marginally effective in inhibiting parameters of carcinogenesis in the MNU-rat model [24] and ineffective in the DMBA-rat model [7]. In contrast, soy protein isolate and daidzein were found to be effective [7]. It is possible that phenoxodiol, which is closely related structurally to daidzein, may be produced naturally as part of daidzein's normal metabolism, which could have accounted for the chemopreventive effects observed with soy.

The effect of phenoxodiol at the two doses was initially dose-dependent, with the high dose (75 mg/kg diet) being more effective than the low dose (50 mg/kg diet) in reducing both tumour incidence and multiplicity at up to approximately 80 days postcarcinogen administration. Later on the two doses showed diminished differences with respect to multiplicity (Fig. 1b), and their effects on tumour incidence were reversed, with the low dose showing a slightly stronger effect than the high dose during the period of 100–120 days postcarcinogen administration (Fig. 1a). The differences between the two doses are non-statistically significant. However, these results suggest that the low dose might be sufficient to produce the desired effect.

In a previous study, phenoxodiol was demonstrated to be a potent inhibitor of topoisomerase II [25], and we have shown previously that these inhibitors are likely to be chemopreventive agents [26]. Topo II is required for

Table 3

Comparison of the effects of phenoxodiol to that of other chemopreventive agents on tumour parameters in DMBA-treated Sprague–Dawley rats

Agent	Percent decrease		Percent increase tumour latency	Reference
	Tumour incidence	Tumour multiplicity		
Phenoxodiol	31*	50*	39*	Current study
Tamoxifen	56*	57*	9	[23]
Leuprolide	48*	43	9	[23]
Genistein	10	23	17	[7]
Soy protein isolate	10	50*	37	[7]

*Reached statistical significance ($P \leq 0.05$) when compared with the controls as determined in the corresponding references.

DNA replication as it allows chromosomal segregation [27]. Topo II catalytic inhibitors can prevent tumour cell division by inhibiting these critical steps of cell proliferation. Alternatively, reduced topo II levels might trigger cell differentiation [28,29]. The previously reported cell cycle arrest in G1 phase of PC3 prostate cancer cell line by phenoxodiol could be derived from topo II inhibition and can represent cell differentiation [30].

In conclusion, we determined that phenoxodiol has properties that are consistent with those of cancer chemopreventive agents. Although the *in vivo* mode of action is unknown at present, this novel agent had demonstrated *in vitro* activities that suggested that it might act as an inhibitor of cell division and/or an inducer of cell differentiation. Future studies will focus on identifying the *in vivo* mode of action of this new and promising chemopreventive agent and evaluating its chemopreventive efficacy in clinical studies.

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