Research Article

Cancer Prevention Research

5MeCDDO Blocks Metabolic Activation but not Progression of Breast, Intestine, and Tongue Cancers. Is Antioxidant Response Element a Prevention Target?

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Abstract

The preventive efficacy of the triterpenoid 5MeCDDO was tested in two models of mammary cancer, the Min model of intestinal cancer, and a chemically induced model of head and neck cancer. In one model of mammary cancer, female Sprague-Dawley rats were administered MNU at 50 days of age, and 5MeCDDO (27 ppm) was administered in the diet beginning 5 days later for the duration of the study; 5MeCDDO was ineffective. In contrast, in a model examining initiation of mammary cancers by the procarcinogen dimethyl-benzanthracene, 5, 6-benzoflavone (500 ppm, an Ah receptor agonist) or 5MeCDDO (27 or 2.7 ppm) decreased tumor multiplicity by 90%, 80%, and 50%, respectively. This anti-initiating effect which is presumably mediated by altered metabolic activation parallels our observation that 5MeCDDO induced proteins of various antioxidant response element (ARE)-related phase II drug-metabolizing enzymes [e.g., GST Pi, AKR 7A3 (aflatoxicol), epoxide hydrolase, and quinone reductase] in the liver. 5MeCDDO tested in the 4-nitroquinoline-l-oxide (4-NQO) head and neck cancer model failed to decrease tumor incidence or invasiveness. In the Min mouse model of intestinal cancer, a high dose of 5MeCDDO (80 ppm) was weakly effective in reducing adenoma multiplicity [~30% ($P < 0.05$)]; however, a lower dose was totally ineffective. These findings question whether measuring increased levels of certain ARE-related genes (e.g., quinone reductase, GST Pi), indicating decreased carcinogen activation are sufficient to imply general chemopreventive efficacy of a given agent or mixture. *Cancer Prev Res; 9(7); 616–23. ©2016 AACR.*

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Introduction

The field of breast cancer prevention has produced a number of effective agents (SERMs, aromatase inhibitors) that appear unique to breast cancer; and, in fact, specifically to ER^+ breast cancers, by directly or indirectly targeting ERα (1, 2). In contrast, both clinically and in animal studies, the COX-1/2 inhibitors appear to be effective against cancers in a variety of organs [e.g., colon, skin, esophagus, and urinary bladder (3)].

One additional target in the field of chemoprevention that has generated a great deal of interest is agents that activate the antioxidant response element (ARE). These structurally diverse agents alter expression of a variety of genes including many non-CYP 450 drug-metabolizing enzymes (conjugating enzymes). Pickett and colleagues (4) identified specific DNA sequences in the promoter region of genes coding for certain phase II enzymes (e.g., quinone reductase, GST Yc2, GST Pi) which appear to control the transcriptional response to many agents; such as tert-butylhydroxyanisole. As many of the agents that activated these genes were defined as antioxidants, the resulting DNA sequences were initially called AREs. Subsequently, it has been shown that transcription at these AREs is mediated by a complex that includes the transcription factor Nrf-2 (5). This complex involves the binding of Nrf-2 to a cytoskeleton protein KEAP which modulates the degradation of Nrf-2 (5, 6). This binding between Nrf-2 and KEAP is altered by multiple sulfhydryl groups on the KEAP protein whose oxidation/reduction status can be changed by antioxidants (5, 6). Therefore, there was a systematic effort to identify agents that might preferentially or solely activate the ARE and associated phase II drug-metabolizing enzymes (7, 8). This examination was initially performed by screening for agents that would induce one or more of the phase II enzymes; particularly quinone oxidoreductase (9).

On the basis of this screening assay, a wide variety of structurally varied xenobiotics and naturally occurring agents that stimulate the ARE were identified (refs. 9–11; Supplementary Fig. S1). One class of agents that stimulate the ARE (and has recently generated substantial interest in the field of cancer prevention) are the triterpenoids that have been systematically investigated by Sporn and colleagues (12). This class of agents (specifically the CDDO-imidazole derivative) has proven highly effective in prevention of ER^- mammary cancers in animal models (12). In addition, both CDDO-imidazole and the 5MeCDDO ester

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proved to be effective preventive agents in mouse models of lung cancer (13, 14). Further interest in the field has been spurred by mechanistic considerations. Thus, this class of agents has proven to be highly effective agonists for genes modulated by the ARE, and has been proposed to inhibit inflammation via the NF-κb pathway (15). There has been great interest in the potential role of antioxidants and oxidants in development of cancer, and specifically in agents which induce the ARE response. We have employed one specific agent 5MeCDDO in a variety of standard preclinical prevention models. These studies include two carcinogen-induced mammary cancer models in rats. The MNU model has been used by our laboratory and others to determine agents that can prevent progression of ER^+ mammary cancers, whereas the dimethyl-benzanthracene (DMBA) model has been used to look for agents that block cancer initiation routinely by blocking metabolic activation of DMBA (16). In addition, we examined the efficacy of 5MeCDDO in models of intestinal cancer in mice and head and neck cancer in rats (17). Finally, the ability of this agent to modulate expression of proteins associated with the ARE response was determined.

Materials and Methods

Agents

5MeCDDO was obtained from the NCI Chemoprevention Chemical Repository. 5, 6 Benzoflavone, and 7, 12 dimthylbenzanthracene were obtained from Sigma Chemical Co. MNU and 4-NQO were obtained from the NCI Chemical Carcinogen Repository and Aldrich Chemical Company, respectively. 5MeCCDO was mixed directly into the diet (Teklad 4%, Harlan Teklad Corporation) using a Liquid–Solid Blender with Intensifier Bar (Patterson-Kelley). The diets in the animal cages were changed 3 times per week.

Rat mammary cancer models

Two different carcinogen-induced rat mammary cancer models were used in these studies (16).

MNU model

Female Sprague-Dawley rats were administered the carcinogen (50 mg/kg BW) by intravenous injection (jugular vein) at 50 days of age. Five days after MNU, the animals received diet supplemented with 5MeCDDO (81 or 27 ppm). The dose of 81 ppm was found to be toxic. The animals receiving the lower dose (27 ppm) were weighed once a week, palpated for mammary tumors two times per week, and observed daily for signs of toxicity. Mammary tumors were excised, weighed, and processed for histologic classification at termination of the study (126 days after MNU). Differences in cancer multiplicity were determined by the Armitage test.

DMBA model

Starting at 43 days of age, female Sprague-Dawley rats were administered 5MeCDDO (27 or 2.7 ppm) or 5, 6-benzoflavone (500 ppm) in the diet for a period of 14 days, and then returned to standard Teklad diet. At 50 days of age, the carcinogen DMBA dissolved in corn oil (12 mg by gavage) was administered. The animals were weighed once a week, palpated for mammary tumors twice a week, and observed daily for signs of toxicity. Mammary tumors were excised, weighed, and processed for histologic classification at termination of the study (126 days after DMBA). Differences in cancer multiplicity were determined by the Armitage test.

Intestinal tumors in Min mice

We have recently described the use of this model in detail (17). In brief, male $Apc^{+/Min\text{-}FCCC}$ mice (8 weeks old) were obtained from the Laboratory Animal Facility at the Fox Chase Cancer Center. At 9 weeks of age, mice were randomized to receive control diet or diets supplemented with 5MeCDDO (40 or 80 ppm), or (as a positive control) mice were administered the EGFR inhibitor gefitinib at 132 or 400 ppm. Body weights were recorded weekly to monitor toxicity, and mice were maintained on the experimental diets *ad libitum* for 45 days. Mice were sacrificed at the end of the experiment. The entire small intestine was excised and evaluated pathologically in a blinded manner. Adenomas were defined as circumscribed neoplasms composed of tubular and/or villous structures and lined with dysplastic epithelium. Defined cancers had to meet three criteria: (i) invasion into at least the submucosa, (ii) eliciting a desmoplastic reaction, and (iii) exhibiting cytologic features of neoplasia. Body weights and tumor multiplicities were compared among treatment groups using the Wilcoxon rank-sum test.

Squamous cancers of the head and neck

Rats were treated as described in our recent studies (17, 18). Male Fischer-344 rats were exposed to drinking water containing 20 ppm 4-NQO for a period of 10 weeks. Treatment with 5MeCDDO was initiated one day after the final administration of 4-NQO. Animals were monitored daily and weighed twice a week. Rats were sacrificed if they lost weight for two successive weeks, or 18 weeks following the last administration of 4-NQO. All rats underwent a complete necropsy that focused on the tongue, oral cavity, and gastrointestinal tract.

Protein extraction and digestion

Livers from control or 5MeCDDO-treated rats were frozen in liquid nitrogen and cryopulverized using a Biopulverizer. Pulverized tissue was solubilized in a Barocycler (NEP 3229, Pressure Biosciences) at 35,000 psi for 30 seconds, followed by ambient pressure for 20 seconds, 10 cycles) in 2 % RapiGest, 8 mol/L urea in 100 mmol/L Tris-HCl, pH 8.5. Solubilized tissue was transferred to an Eppendorf tube and spun at 16,000 rcf for 20 minutes to pellet any remaining solids at the bottom. The supernatant was transferred to another Eppendorf tube, and the protein concentration was determined. The sample was then treated with endoproteases after precipitation of 20 μg of total protein using the 2D Clean-up Kit (GE Healthcare). The precipitate was solubilized in 8 mol/L urea in 100 mmol/L Tris-HCl, pH 8.5. Peptides were prepared for nano-LC/MS using a previously described method (19). Briefly, the proteins were reduced/alkylated, sequentially digested with endoprotease Lys-C and trypsin, and the peptides extracted using a porous graphite carbon wedge tip (Glygen). The peptides were dissolved in aqueous acetonitrile (1%) containing 1% formic acid for analysis using nano-LC/MS.

Mass spectrometry and data processing

The complex peptide mixtures were analyzed using high-resolution nano-LC/MS on a hybrid mass spectrometer consisting of a linear quadrupole ion-trap and an Orbitrap (LTQ-Orbitrap XL,

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Table 1. Effects of 5MeCDDO and 5, 6 benzoflavone in the prevention of dimethylbenzanthracene (DMBA)-induced mammary cancers in female Sprague-Dawley rats

				Mammary adenocarcinomas[c]		
Group	**No. of rats**	**Carcinogen[a]**	**Treatment[b]**	**Percent incidence**	**Number/rat[d,e]**	**Avg. weight of cancers (mg)[e,f]**
1	15	DMBA	5MeCDDO, 27 mg/kg diet	33	0.60 (79%↓)[g]	139 (85%↓)[g]
2	15	DMBA	5MeCDDO, 2.7 mg/kg diet	80	1.40 (50%↓)[h]	551 (39%↓)
3	15	DMBA	5,6-Benzoflavone, 500 mg/kg diet	13	0.13 (95%↓)[g]	67 (93%↓)[g]
4	15	DMBA	None	73	2.80	900

[a]Female Sprague-Dawley rats received DMBA at 50 days of age.
[b]Agents were administered one week prior to and one week after DMBA.
[c]Data on mammary cancers were obtained at necropsy of the rats (134 days after DMBA).
[d]Final tumor multiplicity different from Group 4.
[e]Numbers in parenthesis are percent differences from control group (Group 4).
[f]Tumor weight different from Group 4.
[g]$P < 0.01$.
[h]$P < 0.05$.

Thermo Fisher Scientific). Chromatographic separations were performed using a NanoLC-1D Plus (Eksigent) for gradient delivery and a cHiPLC-nanoflex (Eksigent) containing a 15 cm × 75 μm C18 column (ChromXP C18-CL, 3 μm, 120 Å, Eksigent). The system and gradient conditions have been described previously (19). The peptide fragmentation spectra (MS2) were processed using MASCOT Distiller (Matrix Science, version 2.3.2.0) with the settings described previously (20). The resulting MS2 centroided files were used for database searching with MASCOT, version 2.3.02, against two protein databases, UniProt-Rat (downloaded 5/2/2011, 31,645 entries) and mouse (downloaded on 5/2/2011, 72,503 entries), using the following parameters: enzyme, trypsin; MS tolerance = 50 ppm, MS-MS tolerance = 0.8 Da with a fixed carbamidomethylation of Cys residues and oxidation of Met residues as a variable modification. For relative protein quantification, LC/MS unprocessed files were imported into Rosetta Elucidator (Rosetta Biosoftware, ver 3.3) for *m/z* and retention time alignment of the peptide ion currents from the five control liver samples and the five samples from animals treated with 5MeCDDO. The aligned, normalized peptide ion currents were annotated within the alignment software by generating database search files (*.dta) and were annotated at the feature level using the MASCOT parameters, as described above. The ion current signals from all charge states for each peptide were concatenated unique using a visual script within the software. The table of peptides and peptide intensities was exported in Excel *.csv format. The protein database searches were qualified using Scaffold software (ver. 3_00_07) and the Protein Prophet algorithm (21) with protein and peptide acceptance thresholds of 95% and 50%, respectively.

The peptides were grouped as products from individual genes (data not shown). The gene-grouped and peptide intensity data were imported into DAnTE-R for statistical analysis (22, 23). Only proteins represented by two or more annotated peptides were considered for subsequent data analyses. For quantification of each protein (gene product), a mean value was calculated from all contributing annotated peptides. The ANOVA analysis to determine relative protein abundances between the 5MeCDDO-treated and control datasets was performed in DANTE-R with a Benjamini–Hochberg correction.

Results

MNU-induced ER$^+$ mammary cancers

In the MNU-induced rat mammary cancer model, we initially treated rats with 81 or 27 ppm of 5MeCDDO beginning 5 days after treatment with MNU. Within 2 weeks, the higher dose caused toxicity: decreasing body weights and inducing moribund conditions and therefore these rats were sacrificed. No toxicity was observed at the 27 ppm dose level. This dose, however, failed to decrease either tumor latency or tumor multiplicity. In fact, this dose caused a significant increase in the average weight of the tumors that did develop (Table 1; Fig. 1).

DMBA-induced ER$^+$ mammary cancers

The DMBA-induced rat mammary cancer model is typically employed to determine whether a specific chemopreventive agent can inhibit cancer initiation routinely reflecting altered metabolic activation of DMBA. 5MeCDDO was given at two doses (2.7 and 27 ppm), and 5, 6-benzoflavone (500 ppm) was administered as a positive control. The agents are administered for 2 weeks when animals were 43 to 57 days of age, whereas DMBA is administered as a single dose when rats were 50 days of age. 5, 6-Benzoflavone profoundly increased tumor latency and decreased tumor multiplicity >90% ($P < 0.005$). The higher dose of 5MeCDDO similarly increased latency and decreased tumor multiplicity by 80% ($P < 0.01$), whereas the lower dose minimally decreased tumor multiplicity (50%, $P < 0.08$; Fig. 2; Table 2).

Table 2. Effects of 5MeCDDO and 5, 6 benzoflavone in the prevention of methylnitrosourea (MNU)-induced mammary cancers in female Sprague-Dawley rats

				Mammary adenocarcinomas[c]		
Group	**No. of rats**	**Carcinogen[a]**	**Treatment[b]**	**Percent incidence**	**Number/rat[d,e]**	**Avg. weight of cancers (g)[e,f]**
1	15	MNU	5MeCDDO	100	8.0 (86%↑)[g]	19.2 (126%↑)[g]
2	15	MNU	None	93	4.3	8.5

[a]Female Sprague-Dawley rats received MMNU at 50 days of age.
[b]5MeCDDO was administered one week after MNU.
[c]Data on mammary cancers were obtained at necropsy of the rats (126 days after MNU).
[d]Numbers in parenthesis are percent differences from control group (Group 2).
[e]Tumor multiplicity different from Group 2.
[f]Tumor weight different from Group 2.
[g]$P < 0.05$.

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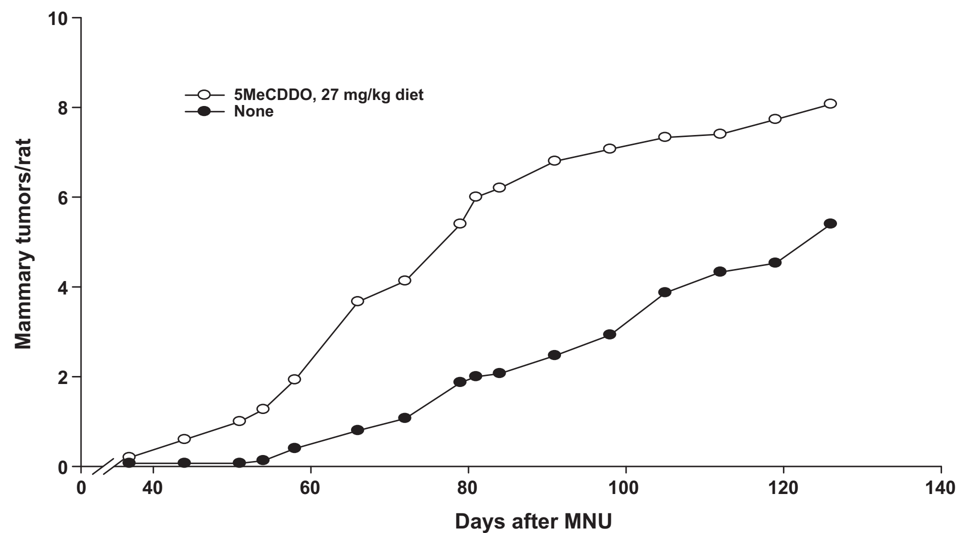

Figure 1.
Effects of 5MeCDDO on time of appearance of MNU-induced mammary cancers. Sprague-Dawley rats were administered MNU (75 mg/kg BW, i.v.) at 50 days of age. Beginning at 55 days of age, rats were administered 5MeCDDO (27 ppm in diet) for the duration of the study. Rats were palpated twice a week. Differences in cancer multiplicity were determined by the Armitage test. There was a significant ($P < 0.05$) increase in cancer multiplicity in 5MeCDDO-treated rats.

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Induction of ARE-related genes in the livers of treated rats using proteomics

Female Sprague-Dawley rats (50 days of age) were treated with 27 ppm of 5MeCDDO or vehicle for a period of 7 days. At that time, rats were sacrificed and proteomics performed on the resulting livers. Approximately 250 of 800 proteins were identified as being significantly increased or decreased (relative increase or decrease of at least 25% and $P < 0.01$; Townsend, Lubet; unpublished data). In Fig. 3, the results are presented for four proteins associated with drug metabolism which are known to be transcriptionally activated via the ARE. The specific peptides which we employed for quantitation are presented in figure legend 3. However, we required that we identify at least two peptides from any given protein to perform quantitation. These proteins are: (i) GST Pi; increased 65×, $P < 10^{-23}$, (ii) AKR 7A3 (aflatoxicol reductase); increased 24×, $P < 10^{-12}$, (iii) microsomal epoxide hydrolase; increased 6×, $P < 10^{-8}$; and (iv) quinone reductase; increased 4×, $P < 10^{-7}$ (Fig. 3).

Development of intestinal tumors in Min mice

These mice have a germline mutation in the APC gene and develop multiple intestinal neoplasias and a limited number of colon neoplasias (17). Mice were treated with doses of 80 or 40 ppm of 5MeCDDO beginning at 6 weeks of age for a period of 8 weeks. The lower dose failed to decrease intestinal tumor multiplicity although the higher dose caused a limited, albeit statistically significant, effect on adenoma multiplicity of 30% ($P < 0.05$). The EGFR1 inhibitor gefitinib at a dose of 132 ppm was tested simultaneously and decreased intestinal tumor multiplicity 55% ($P < 0.01$; data not shown; Fig. 4).

Development of squamous cell cancers of the tongue in 4-NQO–treated rats

F344 rats were treated with 4-NQO in drinking water for 8 weeks. Rats were treated with a dose of 15 ppm of 5MeCDDO beginning 1 week after the final dose of 4-NQO at 14 weeks of age for a period of 16 weeks. This dose failed to decrease either the incidence or the invasiveness of the squamous cell tongue tumors which developed (Table 3).

Discussion

The ARE has been a major focus in the field of chemoprevention. Progress and interest in the ARE response are due in part to the striking efforts of Dr. P. Talalay and Dr. T. Kensler (7, 8) in identifying the wide range of agents that elicited this response. They developed an assay for quinone reductase which allowed one to rapidly screen agents or mixtures for ARE agonist activity has strongly stimulated the field. Transcriptional analysis (4, 5, 6) demonstrated that the ARE activates a wide variety of genes (e.g., phase II drug metabolizing genes and genes related to glutathione metabolism) that would block cancer initiation. Another appeal of ARE agonists is that they affect multiple pathways that contribute to the process of carcinogenesis: (i) oxidation, particularly of nucleic acids, (ii) obesity, which is likely to increase the levels of oxidation, and (iii) ARE response that induces proteins that inhibit the activation of various procarcinogens.

There are structurally diverse agents that activate the ARE (Supplementary Fig. S1). Reflecting the ability of these structurally varied compounds to alter the oxidation state of sulfhydryl groups in the KEAP protein (5, 6) and, thereby, cause activation of NRF2 and transcription at ARE sites. Given the structural diversity of ARE agonists, one might expect huge differences in the effects of these agents on various physiologic effects other than those mediated by the ARE. However, all should increase levels of the ARE-mediated genes. Two particular classes of synthetic agents which exhibited strong ARE agonist activity are the dithiolthiones (24) and the triterpenoids (25). The dithiolthiones are synthetic while the triterpeonids such as 5MeCDDO are synthesized analogues of naturally occurring triterpenoids developed in major part by Sporn and colleagues (12). The imidazole derivative has proven to be highly effective in the prevention of lung adenocarcinomas in mice (14). A further analogue was the methyl ester 5MeCDDO. The advantage of 5MeCDDO is that it was easier to synthesize than the imidazole derivative, had demonstrated efficacy in a mouse lung cancer rodent model, and activated the ARE. In these studies, we examined the ability of 5MeCDDO to prevent cancers in various animal models, primarily in the progression setting as well as determining expression of proteins known to be associated with the ARE. 5MeCDDO was initially evaluated in the MNU-induced rat model that develops ER^{+} mammary cancers. The

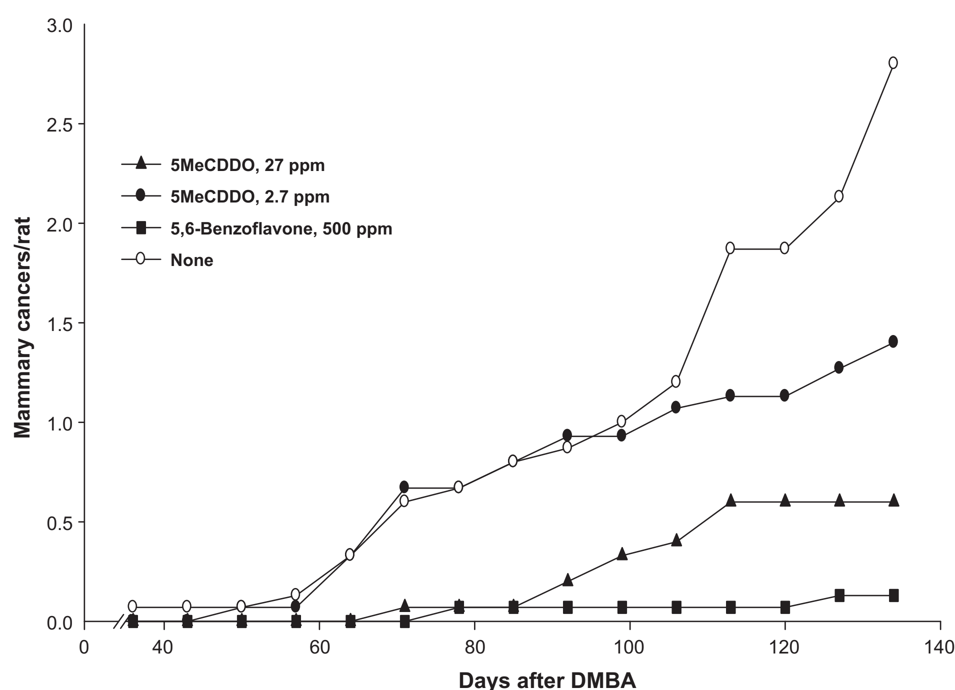

Figure 2.
Effects of 5MeCDDO on time of appearance of DMBA-induced mammary cancers. Female Sprague-Dawley rats were administered DMBA (12 mg by gavage) at 50 days of age. Beginning at 43 days of age, rats were administered 5MeCDDO (27 or 2.7 ppm in diet) or 5, 6 benzoflavone (500 ppm in diet) for a period of 14 days (43–57 days of age). Rats were palpated twice a week. Differences in cancer multiplicity were determined by the Armitage test. There was a significant decrease in mammary cancers in all three treatment groups. The decreases were significant at $P < 0.01$ for the higher dose of 5MeCDDO, and 5, 6 benzoflavone, whereas for the lower dose of 5MeCDDO, it was $P < 0.05$.

doses employed (81 and 27 ppm) were based on published results in mice (13). The higher dose was toxic causing significant weight loss and other toxic signs within 2 weeks of treatment with the agent. Thus, the results in Fig. 1 are with the lower dose (27 ppm) which showed no toxicity. Unexpectedly, rats in this group showed a significant decrease in cancer latency and increase in cancer multiplicity and weights.

The striking induction of the phase II drug metabolizing enzymes (Fig. 3) gives an immediate expectation that the agent should be able to alter carcinogen metabolism, and, thereby,

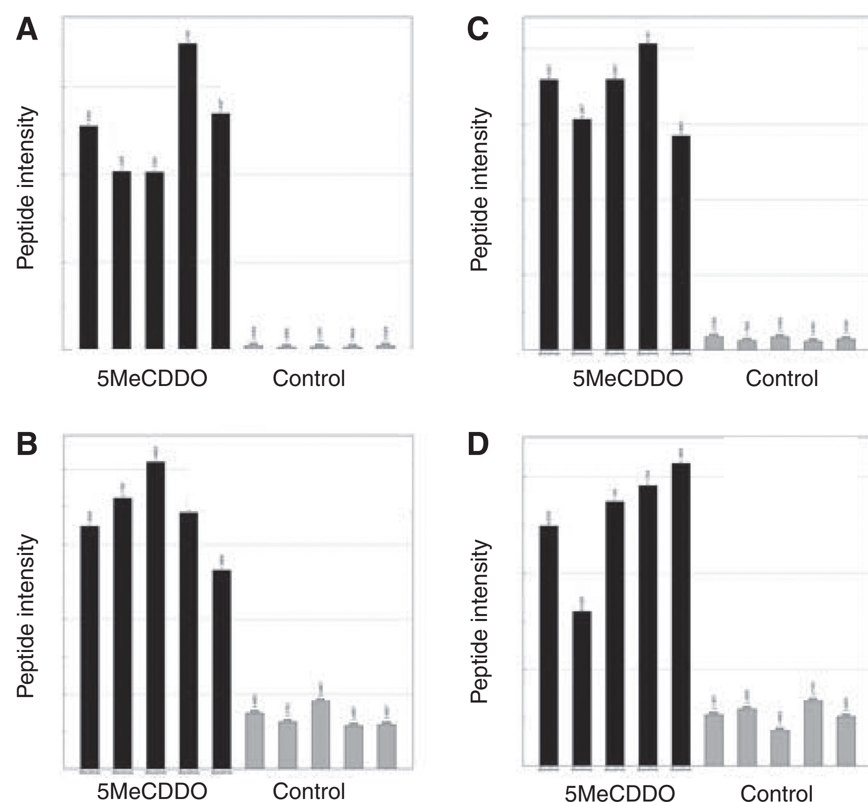

Figure 3.
A–D, determination of expression of various ARE-mediated proteins in livers of 5MeCDDO-treated rats. Female Sprague-Dawley rats were treated 7 days with 5MeCDDO, and livers removed (see Materials and Methods). Increased levels of these proteins: GST Pi; 65×, $P < 10^{-23}$ (A), microsomal epoxide hydrolase; 6×, $P < 10^{-8}$ (B); AKR 7A3 (aflatoxicol reductase); 24×, $P < 10^{-12}$ (C), quinone reductase; 4×, $P < 10^{-7}$ (D). The specific peptides employed in quantitating protein levels are presented GSTP1 FEDGLTYQSNAILR (A); Ephx 1 (microsomal epoxide hydrolase) VFVPTGSAFPSELLHAPEK (B); Akr7a3 FFGNPFSQLYMDR(C); Nqo1 (quinone reductase) FGSLVGHHLGK (D).

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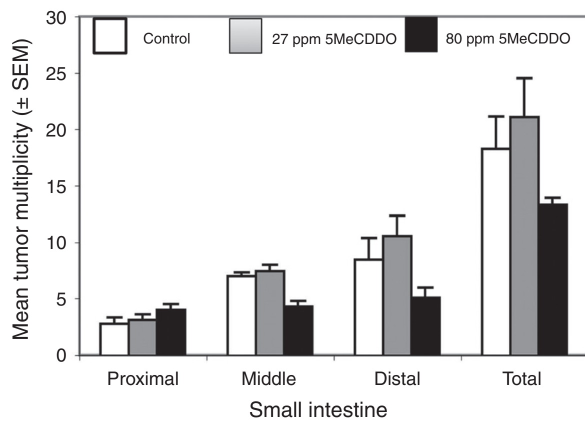

Figure 4.
Effects of 5MeCDDO on small Intestinal Lesions in FCCC Apc^{Min} mice. ApcMin mice were treated with 5MeCDDO beginning at 6 weeks of age for a period of 8 weeks. At that time, animals were sacrificed and lesions were determined as described in Materials and Methods. While the lower dose of 5MeCDDO did not significantly affect adenoma multiplicity, the higher dose reduced multiplicity roughly 30% ($P < .05$).

decrease carcinogen-induced tumorigenesis. We employed the DMBA-induced mammary cancer model in rats to determine anti-initiating activity presumably driven by inhibition of metabolic activation. This model has been used for decades by a wide variety of investigators to identify agents that block the activation of a procarcinogen to reactive intermediates. Various agents with cancer anti-initiating activity decrease both DMBA binding to mammary epithelial DNA and mammary tumorigenesis (26). In Fig. 2, we show that 5, 6-benzoflavone (500 ppm) decreased cancer multiplicity approximately 95%, whereas 5MeCDDO at doses of 27 and 2.7 ppm reduced cancer multiplicity by 80% and 50%, respectively. 5MeCDDO also is profoundly effective in blocking aflatoxin-induced liver carcinogenesis in rats (15). 5, 6-benzoflavone, which induces both phase I and phase II drug-metabolizing enzymes, was used as a positive control (16, 26). While 5MeCDDO (27 ppm) was highly effective at roughly one-half of its toxic dose (27 ppm), it was not as effective as 500 ppm of 5, 6-benzoflavone, roughly one-tenth of its toxic dose. This confirms our prior findings that strong induction of the ARE and phase II enzymes do not appear to be as effective in blocking carcinogenesis as agents which induce both phase I and phase II enzymes (e.g., indole-3-carbinol and 5, 6-benzoflavone; refs. 16, 26). Nevertheless, 5MeCDDO resulted in strong ARE inductions and chemopreventive efficacy in a carcinogen-induced model of cancer initiation.

Table 3. Influence of 5MeCDDO on incidence of neoplastic and hyperplastic oral lesions in rats treated with 4-NQO

	Group	
	4-NQO/basal diet	**4-NQO/CDDO-ME**
Route	—	Diet
Dietary level (mg/kg diet)	0 (control)	15
Microscopic tongue lesion		
Squamous cell carcinoma	21/28 (75%)	24/29 (83%)
Carcinoma invasion score		
+1	1/28 (4%)	1/29 (3%)
+2	2/28 (7%)	1/29 (3%)
+3	2/28 (7%)	3/29 (10%)
+4	16/28 (57%)	19/29 (66%)
Squamous epithelial hyperplasia	4/28 (14%)	5/29 (17%)
Normal	3/28 (11%)	0

We additionally examined the efficacy of 5MeCDDO in the Min intestinal cancer model and the 4-NQO–induced squamous cell head and neck cancer model in rats. Limited activity in the Min mouse model was observed. While the higher dose of 80 ppm reduced adenoma multiplicity 30% ($P > 0.05$), the lower dose of 40 ppm was totally ineffective. In contrast, gefitinib, (an EGFR1 inhibitor) which was examined concurrently at a dose of 132 ppm (roughly the human equivalent dose); reduced tumor multiplicity by 55% ($P < 0.01$; data not shown). Furthermore, NSAIDs and DFMO are profoundly effective in this model reducing adenoma numbers by >65%. No efficacy was observed in the 4-NQO model of head and neck cancer. In this model, both COX-1/2 inhibitors (18) and EGFR1 inhibitors (McCormick and Lubet; unpublished data) have proven to be effective. Finally, in data not presented (Wang, You, Lubet; unpublished data), we confirmed the results of Sporn and colleagues (13, 14) showing that 5MeCDDO could inhibit lung adenoma and adenocarcinoma formation in A/J mice with wild-type or mutated P53 following treatment with vinyl carbamate.

The expression of various proteins coding for ARE-associated phase II drug-metabolizing enzymes was determined by GS-MS/MS (Fig. 3). Approximately 250 of 800 identified liver proteins exhibited increased or decreased levels of >25% with a $P < 0.01$ (data not shown; Townsend, Lubet). Data with four well-known phase II enzymes (GST Pi, AKR7A3, microsomal epoxide hydrolase, and quinone oxidoreductase) are presented. We, and others, had previously shown that the genes coding for these enzymes were highly induced in rat liver following treatment with strong ARE agonists, including dithiolthiones and CDDO-imidazole (11). Thus, 5MeCDDO strongly induces ARE, and transcriptional analysis parallels results at the protein level. Nevertheless, we failed to observe efficacy of this agent in a variety of cancer models. The lack of efficacy in numerous organs agrees with certain of our prior data that 1, 2 dithiol-3-thione (another strong ARE agonist) failed to decrease either MNU-induced ER^+ tumors (27) or colon cancer in rats by AOM (Reddy, Lubet, Steele; unpublished data) although similarly to 5MeCDDO it did decrease initiation of DMBA-induced tumors (26). Furthermore, diethyl maleate, a strong ARE agonist in cell culture, failed to prevent tumorigenesis in multiple models (Lubet, Steele; unpublished data); although this may be based on the effects of PK for this agent. Interestingly, Sporn and colleagues have recently shown that the structurally related dimethylfumarate is effective in inducing the ARE response *in vivo*, but is ineffective in inhibiting lung cancer unlike 5MeCDDO and the CDDO-imidazole (14). The current data, while agreeing with the specific data of Sporn and colleagues that 5MeCDDO inhibits lung tumorigenesis, is in apparent disagreement with the findings of Sporn that 5MeCDDO was effective in multiple models of carcinogenesis, including pancreatic cancer and ER^- breast cancer induced by polyoma middle T MMTV-Neu or BRCA-1 deletion (12). With regards to the efficacy of the CDDO analogues (5Me-CDD) and (CDDO-imidazole) in lung the negative data with dimethylfumarate implies that the efficacy of these CDDO analogues is not related to their ability to induce the ARE response, but rather to other characteristics including its ability to alter the NF-κB pathway (12). One important aspect of this finding is that many investigators have proposed that merely determining that an agent or mixture is an agonist of the ARE

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response (routinely determined by measuring induction of quinone oxidoreductase) implies that it is likely to have significant preventive activity in a variety of cancer models. Such an approach does appear relevant for models where tumorigenesis is induced by a procarcinogen, for example, DMBA or AFB1. However, it appears less relevant for the progression phase of a variety of animal cancer models. In fact, there has been an argument in pancreatic cancer that induction of the ARE response may actually increase tumorigenesis in a Ki Ras–mediated model (28). The rationale is that Ki Ras itself may increase oxidant levels which are potentially toxic to the tumor cells with the Ki Ras mutation. Presumably, the induction of the ARE reduces toxicity, and, thereby, increases tumorigenesis. Finally, in both lung adenocarcinomas and squamous cell cancer of the lung, roughly 15% of cancers have mutations in the KEAP protein which should result in the constitutive activation of the ARE response (29, 30).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.L. Clapper, M.M. Juliana, D.L. McCormick
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Other (involved in the above areas in the development of this manuscript): R.A. Lubet

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References

1. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the national surgical adjuvant breast and bowel project P-1 study. J Natl Cancer Inst 1998;90:371–88.
2. Goss PE, Ingle JN, Ales-Martinez JE, Cheung AM, Chlebowski RT, Wactawski-wende J, et al. Exemustane for breast-cancer prevention in postmenopausal women. N Engl J Med 2011;364:2381–91.
3. Fischer SM, Hawk ET, Lubet RA. Coxibs and other nonsteroidal anti-inflammatory drugs in animal models of cancer chemoprevention. Cancer Prev Res 2011;4:1728–35.
4. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233–60.
5. Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB. Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. J Biol Chem 2005;280:32485–92.
6. Jeong WS, Jun M, Kong AN. Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. Antioxid Redox Signal 2006;8:99–106.
7. Talalay P, Dinkova-Kostova AT, Holtzclaw WD. Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv Enzyme Regul 2003;43:121–34.
8. De Long MJ, Prochaska HJ, Talalay P. Induction of NAD(P)H: quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes and other chemoprotectors: a model system for the study of anticarcinogens. Proc Natl Acad Sci USA 1986;83:787–91.
9. Fahey JW, Dinkova-Kostova AT, Stephenson KK, Talalay P. The "Prochaska" microtiter plate bioassay for inducers of NQO1. Methods Enzymol 2004;382:243–58.
10. Dinkova-Kostova AT, Fahey JW, Talalay P. Chemical structures of inducers of nicotinamide quinone oxidoreductase 1(NQO1). Methods Enzymol 2004;382:423–48.
11. Lubet RA, Yao R, Grubbs CJ, You M, Wang Y. Induced expression of drug metabolizing enzymes by preventive agents: role of the antioxidant response element. Chem Biol Interact 2009;182:22–8.
12. Liby KT, Sporn MB. Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease. Pharmacological Rev 2012;64:972–1003.
13. Liby K, Rinsingsong R, Royce DB, Williams CR, Ma T, Yore MM, et al. Triterpenoids CDDO-methyl ester or CDDO-ethyl amide and rexinoids LG 100268 or NRX194204 for prevention and treatment of lung cancer in mice. Cancer Prev Res 2009;2:1050–8.
14. To C, Ringelberg CS, Royce DB, Williams CR, Risingsong R, Sporn MB, et al. Dimethyl fumarate and the oleanane triterpenoids, CDDO-Imidazolide and CDDO-Methyl Ester, both activate the Nrf2 pathway but have opposite effects in the A/J model of lung carcinogenesis. Carcinogenesis 2015;36:769–81.
15. Yates MS, Kwak MK, Egner PA, Groopman JD, Bodreddigari S, Sutter TR, et al. Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulation pathyways by the triterpenoid 1-{2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole. Cancer Res 2006;66:2488–94.
16. Lubet RA, Heckman BA, De flora SL, Steele VE, Crowell JA, Juliana MM, et al. Effects of 5,6-benzoflavone, indole-3-carbinol(I3C) and diindolylmethane (DIM) on chemically-induced mammary carcinogenesis: is DIM a substitute for 13C? Oncol Rep 2011;26:731–6.
17. Lubet RA, Clapper ML, McCormick DL, Pereira MA, Chang WC, Steele VE, et al. Chemopreventive efficacy of Targretin in rodent models of urinary bladder, colon/intestine, head and neck and mammary cancers. Oncol Rep 2012;27:1400–6.
18. McCormick DL, Phillips JM, Horn TL, Johnson WD, Steele VE, Lubet RA. Over expression of cyclooxygenase 2 in rat oral cancers and prevention of oral carcinogenesis in rats by selective and nonselective COX inhibitors. Cancer Prev Res 2010;3:73–81.
19. Chen ZW, Fuchs K, Sieghart W, Townsend RR, Evers AS. Deep amino acid sequencing of native brain GABAA receptors using high-resolution mass spectrometry. Mol Cell Proteomics 2012;11:M111.011445.
20. Nittis T, Guittat L, LeDuc RD, Dao B, Duxin JP, Rohrs H. Revealing novel telomere proteins using *in vivo* cross-linking, tandem affinity purification, and label-free quantitative LC-FTICR-MS. Mol Cell Proteomics 2010;9:1144–56.
21. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 2002;74:5383–92.
22. Polpitiya AD, Qian WJ, Jaitly N, Petyuk VA, Adkins JN, Camp DG 2nd, et al. DAnTE: a statistical tool for quantitative analysis of -omics data. Bioinformatics 2008;24:1556–8.
23. Karpievitch Y, Stanley J, Taverner T, Huang J, Adkins JN, Ansong C, et al. A statistical framework for protein quantitation in bottom-up MS based proteomics. Bioinformatics 2009;25:2028–34.

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24. Roebuck BD, Curphey TJ, Li Y, Baumgartner KJ, Bodreddigari S, Yan J, et al. Evaluation of cancer chemopreventive potency of dithiolethione analogs of oltipraz. Carcinogenesis 2003;24:1919–28.
25. Sporn MB, Liby KT. NRF2 and Cancer: the good, the bad and the importance of context. Nat Rev Cancer 2012;12:564–71.
26. Izzotti A, Camoirano A, Cartiglia C, Grubbs CJ, Lubet RA, Kelloff, et al. Patterns of DNA adduct formation in liver and mammary epithelial cells of rats treated with 7,12- dimethylbenz(a)anthracene, and selective effects of chemopreventive agents. Cancer Res 1999:59: 4285–90.
27. Cohen LA, Zhao Z, Pittman B, Aliaga C, Lubet R. Feeding thiol-containing compounds, derived from vegetables, fails to inhibit N-methylnitrosourea-induced mammary tumorigenesis. Cancer Detect Prev 2001;25:254–61.
28. DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature 2011;475:107–10.
29. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519–25.
30. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinomas. Nature 2014:511:543–50.

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