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A novel acetylenic tricyclic *bis*-(cyano enone) potently induces phase 2 cytoprotective pathways and blocks liver carcinogenesis induced by aflatoxin

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Abstract

A novel acetylenic tricyclic *bis*-(cyano enone), TBE-31, is a lead compound in a series of tricyclic compounds with enone functionalities in rings A and C. Nanomolar concentrations of this potent multifunctional molecule suppress the induction of the inflammatory protein, iNOS; activate phase 2 cytoprotective enzymes *in vitro* and *in vivo*; block cell proliferation; and induce differentiation and apoptosis of leukemia cells. Oral administration of TBE-31 also significantly reduces formation of aflatoxin-DNA adducts and decreases size and number of aflatoxin-induced pre-neoplastic hepatic lesions in rats by more than 90%. Because of the two cyano enones in rings A and C, TBE-31 may directly interact with dithiothreitol and protein targets such as Keap1 that contain reactive cysteine residues. The above findings suggest that TBE-31 should also be tested for chemoprevention and chemotherapy in relevant models of cancer and against other chronic, degenerative diseases in which inflammation and oxidative stress contribute to disease pathogenesis.

Keywords

Chemoprevention; aflatoxin; Nrf2; triterpenoid; tricyclic bis-enone; TBE-31

INTRODUCTION

Cancer is the leading cause of death in people under 85 years of age in the United States, and mortality from this disease is not declining at an appreciable rate (1). Therefore, there is an immense need to develop new compounds and strategies to decrease the incidence and mortality of cancer. There are numerous processes which participate in the transformation of a normal cell to a malignant state, as hundreds of different genes have been found to be mutated frequently in breast and colorectal cancers (2;3). Both inflammation and oxidative

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stress are key components contributing to the process of carcinogenesis (4–7). Uncontrolled proliferation, failure to differentiate correctly and the ability to evade apoptotic processes are also highly pertinent to this disease. Therefore, compounds which prevent or reverse these processes should be useful as chemopreventive and chemotherapeutic agents (8).

Over the past decade, we have successively reported that a series of novel pentacyclic oleanane triterpenoids, including 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) and imidazolide (CDDO-Im), potently inhibit the ability of cytokines to induce iNOS in primary mouse macrophages or in RAW264.7 macrophage-like cells. These semisynthetic triterpenoids also induce phase 2 cytoprotective enzymes, inhibit proliferation, and induce differentiation and apoptosis of cancer cells, and are effective for the prevention and treatment of cancer in a variety of preclinical animal models (9). CDDO-Me (10) is currently in early clinical trials for the treatment of cancer.

More recently, we have described the total synthesis and preliminary biological properties of novel tricyclic compounds with the same enone functionalities in rings A and C required for the potent biological activities of CDDO (11;12). Moreover, structure-activity studies indicate that the entire structure of CDDO might not be necessary for potency. In designing tricyclic-*bis*-enone (TBE) compounds (Fig. 1), we wished to avoid some of the limitations of synthetic oleanane triterpenoids, in which only a single functionality at C-17 could be modified and an expensive 11 step synthesis is required to convert oleanolic acid into CDDO. In contrast, the TBEs are entirely synthetic and thus are not hindered by limited functional groups or by steric hindrance, and these new compounds may have more structural diversity, better stability and enhanced pharmacokinetic and pharmacodynamic profiles compared to the oleanane triterpenoids. An early TBE-compound, TBE-9, is nearly equivalent to CDDO in potency, both *in vitro* and *in vivo* (11;13), so we have continued the design, synthesis, and biological evaluation of new TBE analogues. The acetylenic tricyclic *bis*-(cyano enone), TBE-31 [(±)-(4a,8a,10a)-1,2,4a,6,8a,9,10,10a-octahydro-8a-ethynyl-1,1,4a-trimethyl-2,6-dioxophenanthrene-3,7-dicarbonitrile], synthesized from cyclohexanone as a starting material, is much more active than CDDO (12). Here, we report that TBE-31 potently inhibits iNOS, activates the phase 2 response, and induces differentiation and apoptosis of cancer cells. TBE-31 also is orally available and is exceptionally effective at blocking both the formation of aflatoxin-B₁ (AFB₁)-DNA adducts and AFB₁-induced tumorigenesis *in vivo*; moreover, it has better pharmacokinetic and/or pharmacodynamic properties than many pentacyclic oleanane triterpenoids.

MATERIALS AND METHODS

Reagents

The synthesis of the TBEs and CDDO-Im have been described (11–15). Compounds were dissolved in DMSO, and controls containing equal volumes of DMSO (0.1%) were included in all experiments. Sources of reagents and antibodies were as follows: H₂DCFDA, Molecular Probes (Eugene, OR); antibodies against iNOS, HO-1, I B , Id1 and Id2, Santa Cruz Biotechnologies (Santa Cruz, CA); pSTAT3, Cell Signaling Technology (Beverly, MA), PARP, Upstate (Charlottesville, VA), and CD11b-RPE (Dako, Carpinteria, CA). Aflatoxin B₁ (AFB₁) was obtained from Sigma Chemical Co. (St. Louis, MO).

In vitro assays

U937 and RAW264.7 cells, from the American Type Culture Collection (Manassas, VA), were grown in RPMI supplemented with 5% or 10% FBS, respectively. Cells were treated with TBEs or CDDO-Im, and cell extracts were analyzed by Western blotting with iNOS, HO-1, pSTAT3, I B , Id1 and Id2 or tubulin antibodies, by flow cytometry for production

of ROS or induction of CD11b, or for NQO1 enzyme activity. For proliferation assays, cells were treated with compounds for 3 days, pulsed with ^3H -thymidine for 2 h and its incorporation into cells measured by scintillation counting. Apoptosis was analyzed by FACS using the TACS Annexin V-FITC Apoptosis Detection Kit (R & D Systems, Minneapolis, MN) or by Western blotting with PARP antibodies. Additional details are included in the figure or table legends using previously published methods (15–18).

TBE-31 absorption spectra

1 μl of TBE-31 (freshly dissolved in acetonitrile) was added to 999 μl of 20 mM Tris-HCl/0.005% Tween 20 (pH 8.0; final concentration of TBE-31, 0.1 mM). The UV spectrum was recorded by using a double-beam UV/VIS spectrophotometer (Hitachi Instruments Inc., Danbury, CT). Then, 1 μl of DTT (final concentration 1 mM) was added, and the spectrum was recorded again to test for changes in absorbance. DTT was present at the same concentration in the reference cuvette.

Tissue levels

CD-1 male mice ($n = 4$ per group) were gavaged with 1 μmole TBE or CDDO-Im dissolved in DMSO or DMSO alone. Six h later, tissues were harvested and blood was collected into heparinized tubes. Tissues and blood were extracted in acetonitrile, separated by reverse phase liquid chromatography (Waters 2695 HPLC and Waters XTerra MS C18 5 μm particle column with an 8 min 46–94% acetonitrile gradient), and detected using a single quadrupole mass spectrometer with electrospray ionization (Waters Micromass ZQ). Standard curves generated by serially diluting known concentrations of drug in control tissue extracts and Waters MassLynx 4.1 software were used to calculate tissue levels (19). All samples were within the range of the standards.

In vivo studies with aflatoxin

For the AFB₁ adduct studies, male F344 rats (Harlan, Indianapolis, IN) were gavaged with TBES or CDDO-Im dissolved in a vehicle of 10% DMSO, 10% cremophor, and 80% PBS. Forty-eight h later, rats were gavaged with AFB₁ (25 $\mu\text{g}/\text{rat}$) in DMSO. Rats were sacrificed 2 h after treatment with AFB₁, and DNA from flash frozen livers was isolated and analyzed for levels of AFB₁-DNA adducts by liquid chromatography-mass spectrometry as described (20). To examine hepatic foci, rats were gavaged with TBE-31 or CDDO-Im on Monday, Wednesday, and Friday morning for 3 successive weeks. Beginning on the second week, AFB₁ (25 $\mu\text{g}/\text{rat}$) was gavaged each Monday through Friday afternoon for 2 weeks. Five weeks after the final doses of drug and AFB₁, rats were sacrificed, and multiple sections of the left lateral lobe of the liver were fixed in acetone (4°C) and embedded in paraffin. Liver sections were stained for expression of glutathione S-transferase P (GST-P) positive foci and analyzed as previously published (20).

Statistical analysis

Linear regression was used to determine if there was a trend in the results with increasing doses of TBE-31. A Student's t-test (Stata Corporation, College Station, Texas) was used to compare the relative efficacy of TBE-31 and CDDO-Im for reduction of tumor burden.

RESULTS

TBEs inhibit the production of iNOS and ROS and upregulate phase 2 enzymes

Because chronic inflammation can drive carcinogenesis (7;21), our primary screen for testing new chemopreventive agents is their ability to block the production of inducible nitric oxide synthase (iNOS). In RAW264.7 macrophage-like cells stimulated with IFN- γ ,

0.1–0.3 μM of all of the TBEs reduced iNOS protein, but TBE-31 is clearly the most potent TBE and is comparable in potency to CDDO-Im (Fig. 2A), one of the most potent known semi-synthetic derivatives of oleanolic acid (17;20). The TBEs also effectively reduced iNOS protein levels in RAW cells stimulated with LPS (data not shown). Low nanomolar concentrations of the TBEs and CDDO-Im also induced the phase 2 cytoprotective proteins heme oxygenase-1 (HO-1, Fig. 2B) and NAD(P)H-quinone oxidoreductase (NQO1, Fig. 2C) in a variety of cells; TBE-31 is the most potent compound in the NQO1 assay. An expected outcome of the activation of phase 2 enzymes is a reduction in reactive oxygen species (ROS). In U937 cells treated with TBEs or CDDO-Im for 24 hrs and then challenged with tert-butyl hydroperoxide (tBHP) to produce ROS, all of the TBEs reduced ROS levels by 45–67% compared to control cells (Fig. 2D). TBE-31 and CDDO-Im are at least ten-fold more potent than the other TBEs in this assay, as TBE-31 and CDDO-Im were effective at 0.01–0.1 μM , while 1 μM concentration of TBE-9, –28, and –30 were needed to reduce ROS levels.

TBE-31 induces differentiation, blocks proliferation, and induces apoptosis in U937 leukemia cells

Because CDDO-Im is a potent inducer of monocytic differentiation (16), we treated U937 human leukemia cells with TBE-31 and CDDO-Im for 4 days and then measured expression of the CD11b (CR3 complement receptor) cell surface marker. As shown in Fig. 3A, CDDO-Im increased expression of CD11b 2–4 fold, but TBE-31 increased CD11b expression more than 10 fold compared to the vehicle-treated controls. Both TBE-31 and CDDO-Im also decreased protein levels of Id1 (Fig. 3B), a key protein that inhibits differentiation in leukemia cells (22;23), at the same concentrations that increased CD11b expression. Neither TBE-31 nor CDDO-Im reduced Id2 protein levels. TBE-31 and CDDO-Im at 0.1 μM also significantly reduced proliferation of both human U937 leukemia and RPMI 8226 myeloma cells (Fig. 3C). At even higher concentrations of TBE-31, PARP cleavage (Fig. 3D) and increased annexin V staining (data not shown) were observed in U937 cells. In the PARP assay, TBE-31 was slightly less active than CDDO-Im, as PARP cleavage occurred with 0.3 μM of CDDO-Im but not with TBE-31. TBE-31 also inhibited proliferation and induced apoptosis in a variety of other human cancer cells, including Jurkat leukemia cells, 8226 myeloma cells, MCF-7 breast cancer cells, and A549 lung cancer cells, although higher concentrations of TBE-31 were needed to inhibit proliferation and induce apoptosis in the MCF-7 and A549 adherent epithelial cells than in leukemia and myeloma cells grown in suspension (data not shown).

Targets of TBE-31

In Fig. 2 and Fig. 3, we showed that TBE-31 blocked the induction of the iNOS inflammatory protein, activated phase 2 enzymes such as HO-1 and NQO1, inhibited proliferation, and induced apoptosis. The multifunctional nature of TBE-31 is similar to CDDO and its analogues (9), and both TBE-31 and CDDO contain α,β -unsaturated carbonyl groups in their A and C rings. These functional groups suggest that TBE-31 may undergo Michael addition with nucleophilic targets containing reactive sulfhydryl residues. Indeed, TBE-31 directly interacts with DTT (Fig. 4A); adding DTT to a 100 μM solution of TBE-31 at pH 8 caused both an increase in the wavelength of maximum absorption from 242 nm to 268 nm and hypochromism, a decrease in the height of the absorption peak. Interestingly, the wavelength increase at maximum absorption was only 18 nm with TBE-9 (data not shown) vs. 26 nm with TBE-31 under identical conditions. Spectroscopic examination of the reaction of TBE-31 with murine recombinant Keap1 (data not shown) revealed a virtually identical difference spectrum, strongly suggesting that TBE-31 reacts with cysteine residues of Keap1. Currently, detailed investigations of the structure of Michael adducts of TBE-31 with protein targets are in progress. Because the TBEs can form Michael adducts, it is likely

they will interact with multiple protein targets and signal transduction pathways, as has been found with the oleanane triterpenoids (9). Although IKK is a direct molecular target of CDDO-Im and CDDO-Me (24) (25), the concentrations of CDDO-Im and TBE-31 that inhibited iNOS and activated phase 2 enzymes (Fig. 2) did not block the degradation of I β in RAW cells (Fig. 4B). However, pretreatment with TBE-31 at concentrations of at least 1 μ M inhibited the degradation of I β in both RAW (Fig. 4B) and HepG2 hepatocellular carcinoma cells (Fig. 4C) challenged with TNF α . Another known target of CDDO-Im is phospho-STAT3 (15), and TBE-31 reduced constitutive phospho-STAT3 levels in HepG2 cells (Fig. 4D) after only 2 h of treatment. In contrast, pSTAT3 levels were not affected by CDDO-Im treatment within 2 hrs (Fig. 4D), although by 24 h, pSTAT3 levels were markedly lower with both TBE-31 and CDDO-Im (data not shown).

Tissue distribution and stability studies

The potent induction of phase 2 enzymes and the reduction in iNOS protein (Fig. 2) by TBE-31 suggested it could be a useful drug for the prevention of cancer. To determine appropriate target organs for chemoprevention studies, 1 μ mol doses of TBE-9, TBE-31 and CDDO-Im were administered by gavage to male CD-1 mice (Fig. 5A). After 6 h, blood and tissues were harvested and analyzed by liquid chromatography-mass spectroscopy. The highest levels of TBE-31 were detected in the liver (0.7 μ mol/kg) and pancreas (0.5 μ mol/kg), but only low levels were found in the lung and brain. The levels of TBE-31 in blood were much higher (1 μ M) than with CDDO-Im (0.04 μ M). When TBE-31 and CDDO-Im were incubated at 37°C in human plasma, only 41% of the original CDDO-Im sample was present after 4 h and only 16% could be detected 24 h later (Fig. 5B). In contrast, 75% and 32% of the original TBE-31 sample remained after 4 and 24 h, respectively. Although CDDO-Im was converted to CDDO, no new degradation products were detected from TBE-31. To determine the optimal route of administration, mice were given TBE-31 by gavage, i.v. or i.p. injection. TBE-31 markedly increased expression of HO-1 protein in the liver with both i.p. injection and gavage (Fig. 5C), and because oral administration is preferable to i.p. injection for delivery of a drug, TBE-31 was given orally in an *in vivo* carcinogenesis study.

TBE-31 reduces formation of aflatoxin-DNA adducts and carcinogenesis in the livers of rats

Because of the similar potency of TBE-31 and CDDO-Im (Fig. 2–Fig. 4), the ability of CDDO-Im to suppress hepatic tumorigenesis induced by AFB $_1$ (20), and the high levels of TBE-31 found in the liver after gavage (Fig. 5A), we first tested whether TBE-31 could inhibit the formation of hepatic aflatoxin-DNA adducts. Pretreatment with TBE-31 for 48 h significantly ($P < 0.05$) reduced formation of aflatoxin- N 7 -guanine adducts in a dose-dependent manner, up to 85% at the highest dose used (Fig. 6A). Both TBE-31 and CDDO-Im also significantly ($P < 0.05$) increased enzyme activity of quinone reductase in the livers of rats treated with AFB $_1$ (data not shown). TBE-31 was more potent than CDDO-Im in this assay, which historically underestimates chemopreventive potential against hepatic pre-neoplastic lesions induced by aflatoxin (20;26). In a short-term model of hepatic tumorigenesis, both TBE-31 (at doses up to 60 μ mol/kg) and CDDO-Im (10 μ mol/kg) were well-tolerated, as there was no weight loss in any of these groups compared to the aflatoxin-treated rats. Both the number ($P = 0.001$) and diameter ($P = 0.024$) of GST-P positive foci in liver were significantly decreased with increasing doses of TBE-31, and the focal volume percent of foci (analogous to tumor burden) was also significantly ($P < 0.005$) reduced in a dose-dependent manner in all treatment groups. At the highest doses of TBE-31 (30 and 60 μ mol/kg), no foci were seen (Fig. 6B). Even at the lowest dose of 0.3 μ mol TBE-31/kg, the total tumor burden of preneoplastic GST-P positive foci was reduced over 90%, from an average of 1.73 in the control group to 0.15 in the treated group. At 10 μ mol/kg, TBE-31 and

CDDO-Im were equipotent ($P = 0.6$) and almost completely prevented the development of foci with reductions of preneoplastic lesions by more than 99%.

DISCUSSION

We have shown that low nanomolar concentrations of TBE-31, a new TBE with an acetylene group at C-8a, inhibit the induction of the inflammatory protein, iNOS; induce the phase 2 enzymes HO-1 and NQO1 and thus block the production of ROS; induce differentiation and suppress proliferation in U937 leukemia cells. At low micromolar concentrations, TBE-31 prevents the degradation of I κ B, reduces constitutive STAT3 phosphorylation, and induces apoptosis. TBE-31 also blocks the ability of aflatoxin, a known human carcinogen, to form adducts with DNA and reduces the burden of aflatoxin-induced preneoplastic foci in liver by 90–100%. It is equivalent to, or even slightly more potent than, CDDO-Im, one of the most active compounds ever tested in these assays, and is more stable in human plasma.

Activation of the phase 2 enzyme system to detoxify potential carcinogens and to reduce inflammation and oxidative stress is an emerging strategy for preventing cancer in both animals and humans (27;28). This cytoprotective response is regulated by transcription factor Nrf2 (NFE2-related factor-2) which, under quiescent conditions, is retained and targeted for degradation through interaction with its cytosolic repressor Keap1 (kelch-like ECH-associated protein 1). In response to environmental stimuli or small molecules that can react with sulfhydryl groups, Keap1 loses its ability to repress Nrf2, which subsequently undergoes nuclear translocation and binds to antioxidant-response elements (AREs) in the promoter regions of target genes, resulting in their transcription. Although all of the TBEs induce the phase 2 enzymes HO-1 and NQO1 (Fig. 2), TBE-31 is clearly the most potent TBE *in vitro* and *in vivo* (Fig. 6), and it significantly reduced formation of both AFB₁-DNA adducts and preneoplastic foci in the livers of rats challenged with aflatoxin (Fig. 6B). The potency of TBE-31 is similar to or better than that of the oleanane triterpenoid CDDO-Im for inhibiting hepatic tumorigenesis induced by aflatoxin, and this chemoprevention is dependent on activation of Nrf2 (20). Notably, both CDDO-Im and TBE-31 are 100 times more potent than has been reported for oltipraz, which inhibits activation of aflatoxin in humans (29).

Essentially all known inducers of phase 2 enzymes modify sulfhydryl groups (30), and covalent interactions with specific cysteine residues on Keap1 activate Nrf2 (31). As shown in Fig. 4A, TBE-31 directly interacts with DTT. However, Keap1 is only one of many regulatory proteins in the cell that contains reactive cysteines (32). Proteins in the NF- κ B and Jak-Stat pathway are also regulated by key cysteine residues, and TBE-31 prevents the degradation of I κ B and reduces levels of constitutive pSTAT3 in HepG2 hepatocellular carcinoma cells (Fig. 4). There are undoubtedly other cellular targets of the TBEs, as TBE-31 also is a potent inducer of U937 leukemia cell differentiation and apoptosis (Fig. 3); the protein targets that regulate these processes are not known. Targeting critical protein networks regulated by reactive cysteines is required for other multifunctional compounds such as 15-deoxy- (12,14)-prostaglandin J₂ (15d-PGJ₂) and is emerging as a powerful strategy for developing chemopreventive drugs (32–34). To identify the multiple targets of TBE-31, a biotinylated TBE, such as has been described for the oleanane triterpenoids (24;35) should also be synthesized. The present studies also suggest that TBE-31 should be tested in additional preclinical animal models to determine whether it should be considered for further clinical development as a chemopreventive or chemotherapeutic agent.

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TBE-9: $R = \text{CH}_3$

TBE-28: $R = \text{CH}_2\text{CH}_3$

TBE-30: $R = \text{CH}=\text{CH}_2$

TBE-31: $R = \text{C}\equiv\text{CH}$

CDDO: $R = \text{CO}_2\text{H}$

CDDO-Me: $R = \text{CO}_2\text{Me}$

Fig. 1. Structures of tricyclic bis-enones (TBE) and the oleanane triterpenoids CDDO, CDDO-Methyl ester and CDDO-Imidazole

Fig. 2. TBEs block the induction of iNOS and induce phase 2 cytoprotective proteins
RAW264.7 mouse macrophage-like cells (A and B) or U937 cells (B) were treated with TBEs or CDDO-Imidazole (Im) and IFN- γ (10 ng/ml) for 18 h (A) or with compounds alone for 6 h (B), and cell lysates were immunoblotted with iNOS, HO-1 or tubulin antibodies. Hepa1c1c7 cells were grown for 24 h and then treated with serial dilutions of compounds for 48 h. The concentration required to double (CD) the specific enzyme activity of NQO1 was used to quantify inducer potency (C). In (D), U937 cells were treated with TBEs or Im for 24 h. H₂DCFDA was added for 30 minutes, and then the cells were challenged with 250 μ M tert-butyl hydroperoxide for 20 minutes to induce the formation of reactive oxygen species. The mean fluorescence intensity of 10,000 cells per group was detected by flow cytometry, and results are expressed as percent of control.

Fig. 3. Increasing concentrations of TBEs induce differentiation, inhibit proliferation, and activate apoptosis in U937 leukemia cells

U937 cells were treated with various concentrations of TBE-31 and CDDO-Im for 4 days and CD11b expression was analyzed by flow cytometry (A) or were treated with 100 nM TBE-31 or 30 nM CDDO-Im (Im) for 8–24 h and Western blots of cell lysates were probed with Id1 and Id2 antibodies (B). To measure proliferation, cells were treated with TBEs or Im for 3 days and then evaluated using a [³H]thymidine incorporation assay (C). U937 cells also were treated for 24 h and Western blots were probed with PARP (cPARP, cleaved PARP) and tubulin antibodies to verify the induction of apoptosis (D). A = anisomycin (positive control, 10 μg/ml).

Fig 4. TBE-31 directly interacts with cysteine residues of Keap1 and blocks both the degradation of I κ B and constitutive STAT3 phosphorylation in HepG2 hepatocellular carcinoma cells
Spectrophotometric analysis (A) of 0.1 mM TBE-31 before (gray line) and after (black line) the addition of 1 mM DTT. (B) RAW cells and (C) HepG2 cells were pretreated with TBE-31 or CDDO-Im for 24 or 2 h, respectively, stimulated with TNF α for 15 min, and then immunoblotted with I κ B and tubulin antibodies. (D) HepG2 cells were also treated for 2 h and then immunoblotted with pSTAT3 and tubulin antibodies.

Fig. 5. Tissue levels (A) and stability of TBE-31

(B) TBE-31 and CDDO-Im were incubated in human plasma at 37°C, and samples were collected at 4 and 24 h and then analyzed by LCMS. (C) In an independent experiment, TBE-31 (1 μmol) was dissolved in propylene glycol (V = vehicle) and administered by i.v. or i.p. injection or by gavage to male CD-1 mice (3–4 per group). After 6 h, livers were harvested and homogenized, and Western blots of tissue lysates were probed with HO-1 and tubulin antibodies.

Fig. 6. TBE-31 reduces both the formation of aflatoxin-DNA adducts and pre-neoplastic lesions in the livers of rats injected with aflatoxin

(A) Rats were gavaged with TBEs or CDDO-Im. Forty-eight h later, rats were gavaged with 25 $\mu\text{g}/\text{rat}$ of AFB₁. Rats were sacrificed 2 h after treatment with AFB₁, and DNA from flash frozen livers was isolated and analyzed for levels of AFB₁-DNA adducts by liquid chromatography-mass spectrometry. * $P < 0.05$ compared to vehicle. Values are the mean $\pm$ SE ($n=4$). (B) Rats ($n = 5$) were gavaged with TBE-31 or CDDO-Im on Monday, Wednesday, and Friday mornings for 3 successive weeks. Beginning on the second week, AFB₁ (25 $\mu\text{g}/\text{rat}$) was gavaged each Monday through Friday afternoon for 2 weeks. Five weeks after the final doses of drug and AFB₁, rats were sacrificed, and multiple sections of the left lateral lobe of the liver were fixed and embedded. Liver sections were stained for expression of GST-P positive foci and analyzed for preneoplastic tumor burden ("volume percent").