Carcinogenesis vol.28 no.5 pp.1052–1057, 2007
doi:10.1093/carcin/bgl248
Advance Access publication December 20, 2006

A novel synthetic analogue of a constituent of *Isodon excisus* inhibits transcription of CYP1A1, -1A2 and -1B1 by preventing activation of the aryl hydrocarbon receptor

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We investigated the effect of a novel synthetic analogue of a constituent from the Chinese medicinal herb *Isodon excisus*, 3-(3-methoxy-phenyl)-*N*-(3, 4, 5-trimethoxy-phenyl)-acrylamide (compound 343), on the carcinogen activation pathway mediated by the aryl hydrocarbon receptor (AhR) in human hepatoma HepG2 cells. We found that compound 343 inhibited the upregulation of cytochrome P-450 (CYP) enzyme activity in cells treated with the AhR ligands and potent carcinogens, dimethylbenz[a]-anthracene (DMBA) or 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Compound 343 also inhibited the DMBA- or TCDD-induced increase in CYP1A1, -1A2 and -1B1 mRNA levels. Carcinogen-induced transcription of CYP genes was also suppressed by compound 343, as measured by a reporter gene controlled by the xenobiotic-responsive element (XRE). This was confirmed by measuring the amount of carcinogen-induced CYP1A1 heterogeneous nuclear RNA. Compound 343 blocked the DMBA- or TCDD-induced activation of the AhR DNA-binding capacity for the XRE, as measured by a chromatin immunoprecipitation assay. Compound 343 also inhibited CYP enzyme activity in microsomes isolated from DMBA- or TCDD-treated cells, as well as the activity of recombinant CYP1A1, -1A2 and -1B1, indicating that compound 343 directly inhibits CYP enzymes. These results indicate that compound 343 is both a potent inhibitor of carcinogen-induced CYP enzyme expression, as well as a direct inhibitor of CYP enzymes.

Introduction

The Chinese medicinal herb *Isodon excisus* has been used for a long time as a therapeutic agent for the treatment of cancer and inflammation in China and Korea. However, the mechanism of action has not been fully understood. Recently, several studies demonstrated the effects of *I.excisus* in cancer cells. The diethyl ether extract of the whole *I.excisus* plant was shown to potently inhibit aromatase activity (1), which leads to the inhibition of estrogen biosynthesis in breast cancer cells (2). Synthetic analogues of the isolates from the whole plant *I.excisus* were found to inhibit growth and induce apoptosis in different malignant cell lines, including melanoma and leukemia cells (3). It was further demonstrated that the molecular mechanism of the proapoptotic effect on lymphoma cells induced by 3-(3-methoxy-phenyl)-*N*-(3, 4, 5-trimethoxy-phenyl)-acrylamide (compound 343, Figure 1), a novel synthetic analogue of an *I.excisus* constituent, was mediated through arrest in the G_2/M phase of the cell cycle (4).

The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix family of transcription factors that regulates the expression of a number of genes involved in the activation and detoxification of carcinogens (5). The receptor is a ubiquitous cytosolic protein that, once bound by a ligand, translocates to the nucleus, where, with its protein partner, the aryl hydrocarbon nuclear translocator, it forms a transcription factor, which triggers the induction of transcription of a number of genes. These genes contain within their promoters the sequences known as xenobiotic-responsive elements (XRE) to which the activated receptor complex binds. The best characterized of the AhR-mediated genes include *CYP1A1*, *CYP1A2* and *CYP1B1* (6,7), which encode CYP1A1, CYP1A2 and CYP1B1 enzymes that catalyze the oxidative catabolism of the parent carcinogens, generating a variety of genotoxic metabolites (8). These metabolites may covalently bind DNA, introducing adducts that can cause mutations leading to cellular transformation, or may be conjugated by detoxification enzymes and eliminated (9). The AhR and the pathway it mediates are therefore central to carcinogenesis induced by many environmental carcinogens. These carcinogens include polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 7, 12-dimethylbenz[a]anthracene (DMBA) as well as their halogenated derivative 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent ligand of the receptor.

In the present study, we investigated the effects of a novel synthetic analogue of an *I.excisus* constituent, compound 343, on the carcinogen activation pathway mediated by the AhR in human hepatoma HepG2 cells. We demonstrate that compound 343 suppresses the carcinogen-activating pathway in two ways: by inhibiting AhR activation, thereby blocking carcinogen-induced upregulation of cytochrome P-450 (CYP) gene expression, and by directly inhibiting CYP enzyme activity. These results indicate that compound 343 is a promising preventive agent against polycyclic aromatic hydrocarbons-induced tumorigenesis.

Materials and methods

Materials

Human hepatoma HepG2 cells were from American Type Culture Collection (Rockville, MD). RPMI-1640, glutamine, fetal bovine serum and phosphate-buffered saline (PBS) were from Biosource (Rockville, MD). DMBA, dimethyl sulfoxide (DMSO), ethoxyresorufin, salicylamide, phenylmethylsulfonylfluoride, ethylenediaminetetraacetic acid (EDTA), leupeptin, aprotinin and Pepstatin A were from Sigma (St Louis, MO). TCDD was from the Midwest Research Institute (Kansas City, MO). Human recombinant CYP1A1 and -1B1 supersomes and CYP1A2 microsomes were from BD Gentest (Woburn, MA). RNeasy Mini, Omniscript and polymerase chain reaction (PCR) purification kits were from Qiagen (Valencia, CA). Random primers for cDNA synthesis were from Stratagene (La Jolla, CA). TaqMan Universal PCR Master Mix and real-time reverse transcription–polymerase chain reaction (RT–PCR) primers for CYP1A1, CYP1A2, CYP1B1 and 18S were 'assays on demand' from Applied Biosystems (Foster City, CA). Tris–borate, Tris–borate running buffer, high-density sample buffer and LipofectAMINE were from Invitrogen (Carlsbad, CA). Luciferase assay system was from Promega Corp. (Madison, WI). Primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were from Clontech (Palo Alto, CA). Protease inhibitor tablets were from Roche (Indianapolis, IN). Anti-AhR rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A/G Sepharose/Salmon Sperm DNA (50% slurry) was from Upstate (Charlottesville, VA).

Cell culture

HepG2 human hepatoma cells were grown with RPMI-1640 supplemented with 2 mM glutamine and 10% fetal bovine serum in a 5% CO_2 humidified

Abbreviations: AhR, aryl hydrocarbon receptor; compound 343, 3-(3-methoxy-phenyl)-*N*-(3, 4, 5-trimethoxy-phenyl)-acrylamide; CYP, cytochrome P-450; DMBA, dimethylbenz[a]anthracene; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EROD, ethoxyresorufin-*O*-deethylase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; hnRNA, heterogeneous nuclear RNA; NADPH, nicotinamide adenine dinucleotide phosphate reduced; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; SDS, sodium deodecyl sulfate; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element.

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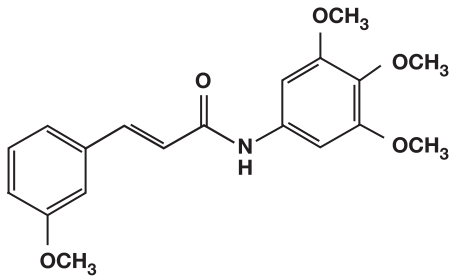

Fig. 1. Chemical structure of compound 343.

incubator at 37°C. For all the treatment compounds, DMSO was used as the vehicle and did not exceed 0.1%.

Chemistry

The synthesis of compound 343 was reported previously (4).

Microsomal CYP enzyme activity

Cells in 175 cm^2 flasks were treated with 1 μM DMBA or 250 pM TCDD for 24 h to induce CYP-associated ethoxyresorufin-*O*-deethylase (EROD) activity. Microsomes were isolated as follows: the cells were washed twice with PBS, trypsinized and pelleted by centrifugation at 500*g* for 5 min at 4°C. The pellet was washed in PBS, repelleted and resuspended in 0.25 M sucrose/10 mM Tris–HCl, pH 7.5, with protease inhibitors (100 μg/ml phenylmethylsulfonylfluoride, 300 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin and 0.7 μg/ml Pepstatin A). The cells were sonicated for 30 s on ice using a Branson Sonifier at setting 2. The sonicate was then centrifuged at 10 000*g* for 10 min at 4°C and the supernatant was centrifuged at 500 000*g* for 15 min at 4°C. The resulting microsomal pellet was resuspended in the above buffer, and the protein was assayed by the Bradford (10) method. Aliquots of microsomes were stored at −80°C. CYP enzyme activity was measured by EROD assay in the following manner: 2.5 μg of microsomes were brought up to 100 μl with PBS (pH 7.2) and 400 nM ethoxyresorufin was added, along with DMSO and the indicated concentrations of compound 343. The reaction was initiated by the addition of 500 μM nicotinamide adenine dinucleotide phosphate reduced (NADPH). The reaction mixture was transferred to a 96-well plate, and EROD activity was quantified. Increasing fluorescence as a result of the conversion of ethoxyresorufin to resorufin by CYP enzymes was measured using a CytoFluor multiwell plate reader (Applied Biosystems), with an excitation of 530 nm and emission at 590 nm. The reaction was allowed to run for 30 min. EROD activity was linear for time (30 min) and protein concentration.

Recombinant CYP1A1, -1A2 and -1B1 activity

Recombinant CYP1A1 supersomes (1.2 μM), CYP1A2 microsomes (12 μM) or CYP1B1 supersomes (1.2 μM) were incubated with 0.4 μM ethoxyresorufin and indicated concentrations of compound 343 in a final volume of 100 μl of PBS, pH 7.2. The reaction was initiated by the addition of 500 mM NADPH. The reaction mixture was transferred to a 96-well plate, and EROD activity was determined in a CytoFluor multiwell plate reader as described above.

CYP enzyme activity in intact HepG2 cells

The ability of compound 343 to affect CYP enzyme activity was determined in intact HepG2 cells by measurement of EROD activity. The cells, in 24-well plates, were treated with 1 ml of growth medium containing 1 μM DMBA or 250 pM TCDD for 12 h in the presence of the indicated concentrations of compound 343. At the end of the incubation, the medium was removed, and the wells were washed once with PBS. Then, 5 μM ethoxyresorufin in growth medium as a substrate was added. Additionally, the growth medium was supplemented with 1.5 mM salicylamide in order to inhibit conjugating enzymes (11). EROD activity was determined in a CytoFluor multiwell plate reader as described above.

Real-time RT–PCR

In 6-well plates, HepG2 cells were treated with 1 μM DMBA or 250 pM TCDD and the indicated concentrations of compound 343, for 12 h. Cells were washed twice with PBS, and total RNA was isolated with the RNeasy Mini kit according to manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using random primers and the Omniscript kit according to manufacturer's instructions. Real-time PCR was performed in the reaction mixture containing 12.5 μl TaqMan Universal PCR Master Mix, 8.75 μl diethylpyrocarbonate (DEPC) water, 1.25 μl of primers and 2.5 μl of cDNA on a Bio-Rad iCycler Real-Time Detection System. Primers for CYP1A1, CYP1A2, CYP1B1 and 18S mRNA had a FAM™ reporter dye at the 5′ end and a non-fluorescent quencher at the 3prime; end of the probe. Amplification conditions were one cycle at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min and ended with one cycle at 60°C for 1 min. All samples to be compared in the same experiments were run in the same plate. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. The Bio-Rad iCycler Real-Time Detection System quantitation protocol was used to determine cycle time (CT) for each gene, to amplify to a specified threshold; 18S was used as an internal control for all reactions. Normalization to 18S was done by subtracting the CT for 18S from the CT of each CYP gene, yielding $dCT_t = CT_t(\text{gene}) - CT_t(18S)$ (t = time). To calculate fold change (FC) in transcript level, the equation $FC = 2^{-ddCT_t}$ was used.

Transcription of CYP1A1

The transcription of the *CYP1A1* gene was determined by measuring the level of heterogeneous nuclear RNA (hnRNA) by semiquantitative RT–PCR as described by Elferink and Reiners (12). In 6-well plates, cells were treated with 1 μM DMBA or 250 pM TCDD and various concentrations of compound 343, for 12 h. RNA isolation and cDNA synthesis were performed as described above. Primer sequences and conditions for hnCYP1A1 were as described by Guigal *et al.* (13). The level of hnCYP1A1 was normalized to the level of G3PDH. The optimum cycle number that fell within the exponential range of response for hnCYP1A1 (25 cycles) and G3PDH (17 cycles) was used. This assay has been well characterized as a valid substitute for nuclear run-on experiments as a measure of transcription rates (14).

Luciferase assay

HepG2 cells were seeded onto 6-well plates at 400 000 cells/well. After 24 h, the cells were transiently transfected with a luciferase reporter vector (1.5 μg/well) containing three repeats of the XRE and a β-galactosidase vector (pCMV-β-gal, 0.6 μg/well) with the use of LipofectAMINE, according to manufacturer's protocol. After 6 h, transfected cells were treated with indicated concentrations of compound 343 in the presence of 25 μM DMBA or 10 nM TCDD, for 24 h. Luciferase activity was measured using a Microlumat LB 96P luminometer (EG and G Berthold, Germany). β-Galactosidase activity was determined using a standard *o*-nitrophenyl-β-D-galactopyranoside colorimetric assay described by Rosenthal (15).

ChIP assay

ChIP assay was performed using a method described by Matthews *et al.* (16), with slight modifications. In 75 cm^2 flasks, cells were treated with 10 nM TCDD and indicated concentrations of compound 343, for 1.5 h. Cells were washed with warm PBS and protein–DNA complexes were cross-linked with 1% formaldehyde for 10 min. Cross-linking was quenched by adding 125 mM glycine, and cells were washed with PBS, harvested and resuspended in 300 μl lysis buffer (50 mM Tris–HCl, pH 8.1, 10 mM EDTA, 1% sodium deodecyl sulfate (SDS), protease inhibitor tablet) and sonicated three times for 30 s each time (setting 3, 50% duty cycle). The soluble chromatin was collected by centrifugation, and 100 μl of the supernatants were diluted with 900 μl dilution buffer (1.1% Triton X-100, 1.1 mM EDTA, 16.7 mM Tris, 167 mM NaCl, protease inhibitor tablet). An aliquot of the diluted sample (20 μl) was put aside and represented the input fraction. The supernatants were incubated with 40 μl of protein A/G Sepharose/Salmon Sperm DNA (50% slurry) under gentle agitation for 2 h at 4°C. The supernatants were transferred to new tubes, 1 μg of anti-AhR antibody was added to each one of them and the tubes were incubated overnight on a tilt board at 4°C. Protein A/G Sepharose/Salmon Sperm DNA (30 μl of a 50% slurry) was then added, and incubation was continued for 1.5 h. Samples were spun down, and the resulting pellets were washed for 5 min in 1 ml of low-salt buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS], 1 ml of high-salt buffer [20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS], 1 ml of LiCl wash buffer [20 mM Tris–HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% Na-deoxycholate] and two times in 1 ml of tris-EDTA (TE) buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA]. Protein–DNA complexes were eluted by adding 250 μl of fresh elution buffer (1% SDS, 100 mM $NaHCO_3$) for 30 min with rotation, and the cross-links were reversed by overnight incubation at 65°C. DNA was purified using a PCR purification kit and eluted in 50 μl of elution buffer provided by the manufacturer. ChIP DNA was amplified by RT–PCR with conditions and primer sequences for the XRE present in CYP1A1 enhancer region described by Matthews *et al.* (16). The results were normalized using input samples.

Statistical analysis

Statistical analyses were performed with STATVIEW Statistical Analysis Software (SAS Institute, San Francisco, CA). Differences between group mean values were determined by a one-factor analysis of variance, followed by Fisher protected least significant difference *post hoc* analysis for pairwise comparison of means.

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Results

Effect of compound 343 on DMBA- or TCDD-induced microsomal CYP enzymes activity

The cellular capacity for enzymatic activation of procarcinogenes was measured by EROD assay, which is specific for the CYP1A1, -1A2 and -1B1 enzyme family. The activity of CYP enzymes was measured in microsomes freshly isolated from HepG2 cells that had been incubated with 1 μM DMBA or 250 pM TCDD for 24 h. DMBA treatment of cells resulted in an increase in CYP enzyme activity from non-detectable to 0.3 pmole/min/μg protein. Co-incubation with compound 343 caused a concentration-dependent decrease in activity, with an interpolated IC_{50} of 2.7 μM. Likewise, TCDD incubation resulted in a profound increase in CYP enzyme activity (to 5 pmole/min/μg) that was inhibited by compound 343 with an interpolated IC_{50} of 4.0 μM (Figure 2A).

Effect of compound 343 on recombinant CYP enzyme activity

The inhibition of EROD activity mediated by compound 343 was further examined using recombinant CYP1A1, CYP1B1 and CYP1A2 to test whether this event was due to a direct effect on CYP enzyme activity. Compound 343 caused a concentration-dependent decrease in the EROD activity (Figure 2B) of CYP1A1 (IC_{50} = 2 μM) and CYP1B1 (IC_{50} = 6.5 μM). There was only a modest inhibition of CYP1A2 activity.

We further analyzed the kinetics of recombinant CYP1A1 enzyme inhibition evoked by compound 343. There was an increase in K_m with increasing compound 343 concentrations, while V_{max} remained unchanged. These results are presented in the form of a Hanes–Woolf plot (Figure 2C), which demonstrates that CYP1A1 inhibition occurred in a competitive and concentration-dependent manner.

Effect of compound 343 on DMBA- or TCDD-induced CYP enzyme activity in intact HepG2 cells

Treatment of intact HepG2 cells with 1 μM DMBA or 250 pM TCDD resulted in an increase in EROD-specific activity from non-detectable levels in controls to 2 pmol/min/well and 12 pmol/min/well, respectively. Compound 343 caused a concentration-dependent inhibition of EROD activity, with an interpolated IC_{50} of 7.0 and 2.5 μM, respectively (Figure 2D).

Effect of compound 343 on CYP1A1, -1A2 and -1B1 mRNA expression

DMBA (1 μM) caused a 30.5-, 11- and 3.1-fold increase in CYP1A1, CYP1B1 and CYP1A2 expression in HepG2 cells, respectively, compared with DMSO control. TCDD (250 pM), the most potent ligand of AhR, induced the expression of CYP1A1, CYP1B1 and CYP1A2 mRNA to 84.4-, 67- and 17.5-fold, compared with DMSO controls, respectively. Co-treatment with compound 343 caused a concentration-dependent inhibition of both DMBA- and TCDD-induced mRNA expressions for all three CYP isoforms (Figure 3).

Effect of compound 343 on XRE-controlled luciferase reporter activity

HepG2 cells were transiently transfected with a luciferase reporter vector containing three copies of the XRE. DMBA (1 μM) induced luciferase activity by 7.2-fold, and TCDD (250 pM) by 174-fold above control levels. The addition of compound 343 resulted in a concentration-dependent decrease in luciferase activity (Figure 4).

Effect of compound 343 on the transcription of the CYP1A1 gene

The transcription of the *CYP1A1* gene was measured by quantifying the level of hnRNA by RT–PCR. Incubation of HepG2 cells with DMBA (1 μM) or TCDD (250 pM) caused a profound increase in CYP1A1 hnRNA, by 8- and 24-fold above control levels, respectively.

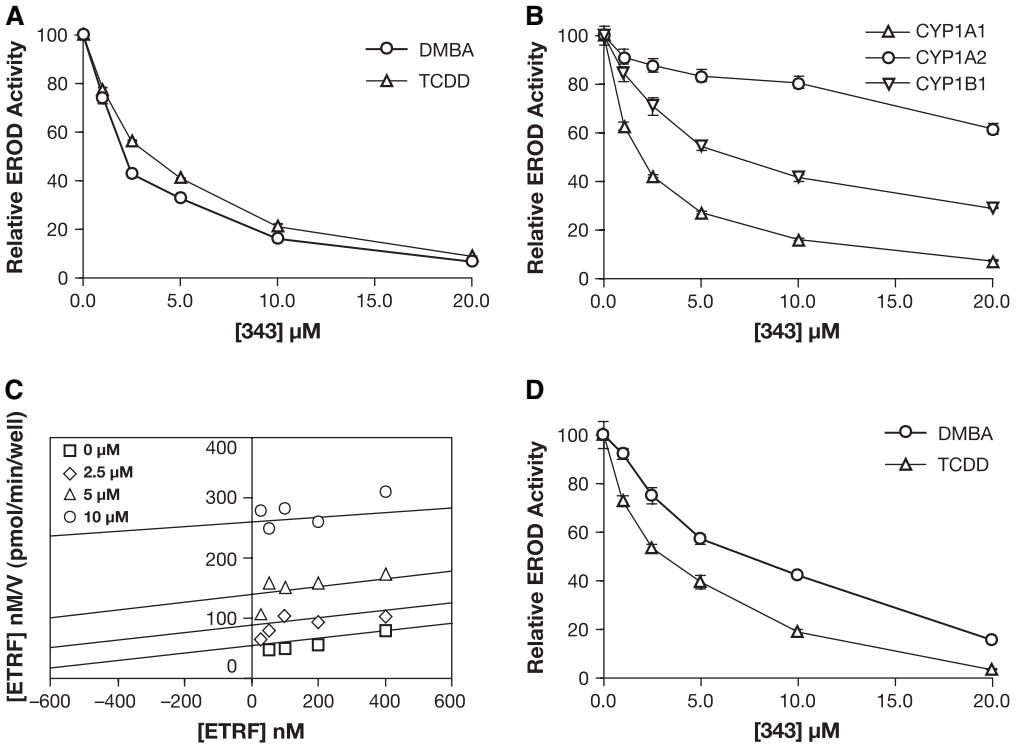

Fig. 2. Effect of compound 343 on microsomal CYP enzyme activity. (**A**) Microsomes were isolated from HepG2 cells that had been treated with 1 μM DMBA or 250 pM TCDD for 24 h to induce CYP enzyme activity. EROD activity of 2.5 μg of microsomal protein was measured in the presence of DMSO (control) or the indicated concentrations of compound 343, $n = 4 \pm$ SE. (**B**) EROD activity of 1.2 μM CYP1A1 supersomes, 12 μM CYP1A2 microsomes or 1.2 μM CYP1B1 supersomes was measured in the presence of DMSO (control) or the indicated concentrations of compound 343 ($n = 4 \pm$ SE). (**C**) EROD activity of 1.2 μM CYP1A1 supersomes was measured in the presence of different concentrations of both compound 343 and ethoxyresorufin (substrate). Results are shown in the form of a Hanes–Woolf plot, $n = 4$. (**D**) EROD activity was measured in intact cells that had been treated with 1 μM DMBA or 250 pM TCDD and indicated concentrations of compound 343 for 12 h, $n = 4 \pm$ SE.

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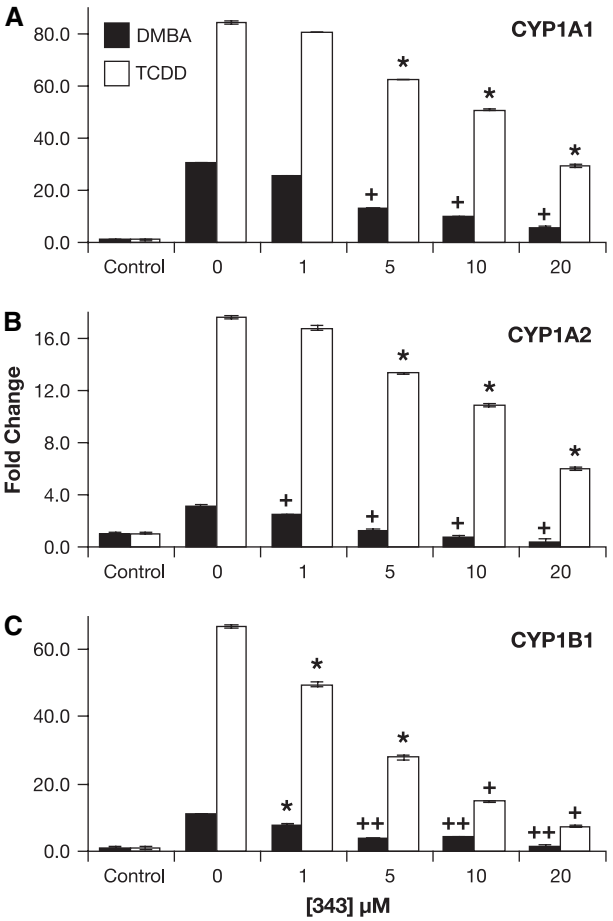

Fig. 3. Effect of compound 343 on CYP mRNA levels. HepG2 cells were treated with 1 μM DMBA or 250 pM TCDD and the indicated concentrations of compound 343 for 12 h. Levels of (**A**) CYP1A1, (**B**) CYP1A2 and (**C**) CYP1B1 mRNA were measured by real-time RT–PCR and normalized to that of 18S mRNA; $n = 3 \pm$ SE ($^{*}P < 0.05$; $^{+}P < 0.001$; $^{++}P < 0.0001$).

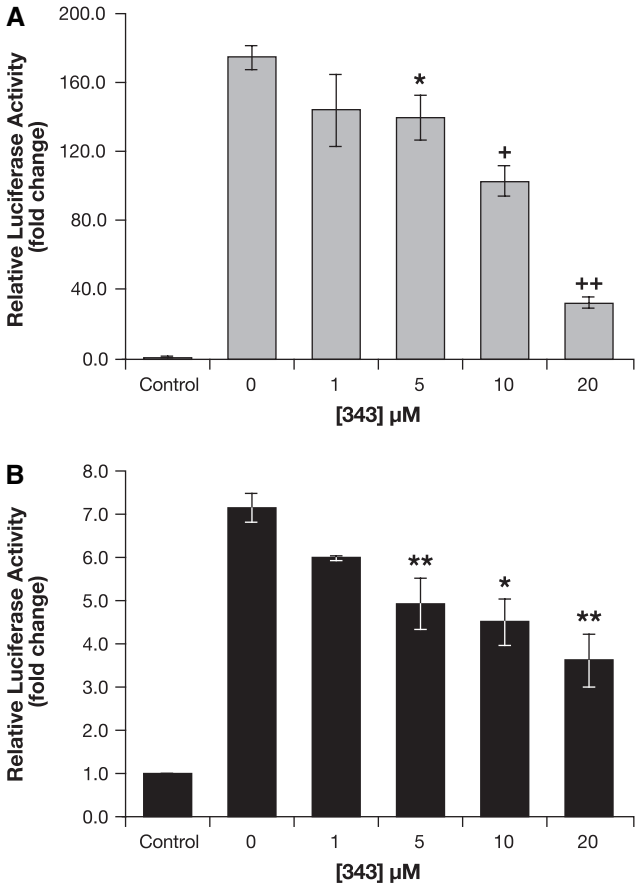

Fig. 4. Effect of compound 343 on XRE-controlled transcription. HepG2 cells transfected with an XRE-controlled luciferase reporter vector and a vector expressing β-galactosidase were treated with (**A**) 10 nM TCDD or (**B**) 25 μM DMBA for 24 h in the presence of DMSO (control) or indicated concentrations of compound 343. The amount of luciferase was normalized to β-galactosidase levels, $n = 3 \pm$ SE ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{+}P < 0.001$; $^{++}P < 0.0001$).

The stimulatory effect by DMBA or TCDD on the transcription of the *CYP1A1* gene was diminished significantly by the addition of compound 343 (Figure 5).

Effect of compound 343 on AhR activation

The effect of compound 343 on TCDD-induced activation of the XRE-binding capacity of the AhR was measured by ChIP assay. Treatment of the cells with TCDD (10 nM) caused a significant increase in AhR translocation to the nucleus and in consequent binding to the XRE. This was abrogated in cells treated with compound 343 (Figure 6).

Discussion

Constituents of many Chinese herbs have been increasingly used as complementary medicines in the treatment of breast, prostate, liver, colon, spleen and lung cancers in the USA and Asian countries. Of all anticancer agents approved for use, 30–40% are derived from plant sources and/or natural products, e.g. paclitaxel from the Pacific yew tree (*Taxus brevifolia*), irinotecan from the bark of the *Camptotheca accuminata* tree, *Vinca* alkaloids from the periwinkle plant (*Catharanthus roseus*) and etoposide from the mandrake plant (*Podophyllum peltatum*). Our laboratory has identified phytochemicals that modulate the activity of the AhR and affect *CYP* gene transcription and enzyme activity, e.g. quercetin (17), diosmin and diosmetin (18), curcumin (19), dibenzoylmethane (20) and resveratrol (21). Although herbal medicines have been used for thousands of years, the biochemical and molecular mechanisms have yet to be defined. Recently, Dothager *et al.* (4) presented a novel synthetic analogue of a constituent from the Chinese herb *I.excisus*, compound 343 that induces apoptosis in lymphoma cells through G_2/M cell cycle arrest. Several other reports demonstrated that G_1 cell cycle progression may be controlled by the AhR and CYP1A1 (22,23). Considering these data, we hypothesized that the AhR-mediated carcinogen activation pathway may represent the molecular target for compound 343.

In our study, we investigated two carcinogens: DMBA and TCDD. DMBA is a well-established model compound that induces mammary gland tumors in rodents (24). TCDD is a major environmental contaminant, generated by a variety of industrial processes. The primary source of human exposure to TCDD is food, particularly through dairy products, meat and fish (25). TCDD is detectable in human serum and is known to accumulate in adipose tissue of humans and animals (26). It also reaches significant levels in human breast milk, providing a pathway for infant exposure (25). We chose HepG2 cells for our experiments because these cells have been extensively used in studies of polycyclic aromatic hydrocarbons metabolism and have a well-characterized AhR pathway (27).

We studied the effects of compound 343 on CYP-associated EROD activity in microsomes isolated from HepG2 cells that had been treated with DMBA or TCDD, and we found that compound 343 directly inhibited microsomal CYP enzyme activity in a concentration-dependent manner (Figure 2A). However, microsomal EROD assay measures the combined activity of CYP1A1, CYP1A2 and CYP1B1. In order to

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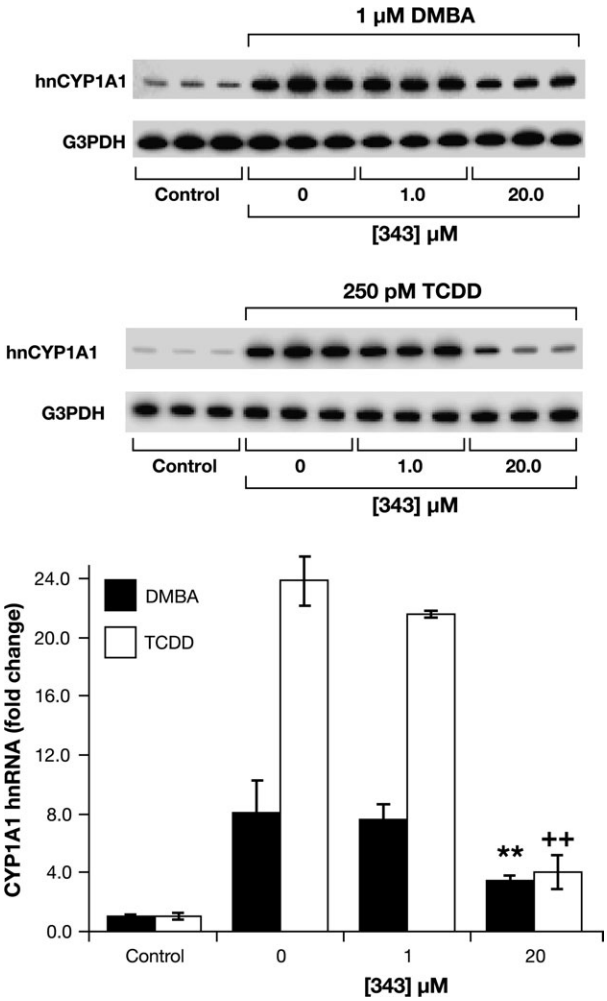

Fig. 5. Effect of compound 343 on CYP1A1 gene transcription. HepG2 cells were treated with 1 μM DMBA or 250 pM TCDD and DMSO (control) or the indicated concentrations of compound 343 for 12 h. CYP1A1 hnRNA levels were measured by RT–PCR and normalized to that of G3PDH, $n = 3 \pm$ SE ($^{**}P < 0.01$; $^{++}P < 0.0001$).

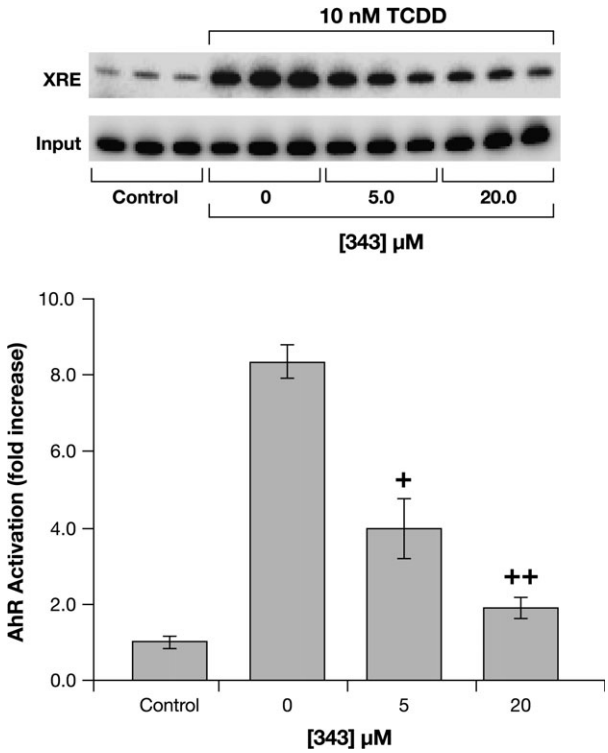

Fig. 6. Effect of compound 343 on AhR activation. HepG2 cells were incubated with DMSO (control) and 10 nM TCDD alone or together with indicated concentrations of compound 343 for 1.5 h. Chromatin immunoprecipitation assay was performed with polyclonal antibody against AhR. RT–PCR from immunoprecipitated fractions was performed using primers specific to the XRE present in the promoter region of CYP genes. The results were normalized using input fractions, $n = 3 \pm$ SE ($^{+}P < 0.001$; $^{++}P < 0.0001$).

determine specifically the effect of compound 343 on individual CYP isozymes, we performed EROD assays employing recombinant CYP1A1 and -1B1 supersomes and CYP1A2 microsomes. These studies showed that compound 343 predominantly inhibits CYP1A1-associated EROD activity and to a lesser extent CYP1B1 and CYP1A2 activity (Figure 2B). Furthermore, the analysis of the kinetics of recombinant CYP1A1 enzyme inhibition by Hanes–Woolf plot demonstrated that the inhibition is competitive; the K_m value for the substrate was increased in the presence of compound 343, but the V_{max} value remained unchanged (Figure 2C). To test whether compound 343 would also inhibit cellular CYP enzyme activity, we measured the EROD activity in intact HepG2 cells. The treatment of cells with DMBA or TCDD caused an increase in EROD activity that was inhibited by compound 343 in a concentration-dependent manner (Figure 2D). Compound 343 treatment of HepG2 cells in the absence of carcinogen did not induce EROD activity (data not shown). These results demonstrate that compound 343 is capable of entering the cells and interacting with the enzyme *in situ*. Although the decrease in DMBA- or TCDD-induced CYP1A1, -1A2 and -1B1 enzymatic activity in intact cells may be due to the direct inhibition, it may also result from modulation of the AhR-mediated pathway. Therefore, we examined the effects of compound 343 on *CYP1A1*, *-1A2* and *-1B1* expression induced by the hydrocarbon DMBA or halogenated hydrocarbon TCDD. The increases in mRNA levels of CYP1A1, -1A2 and -1B1 induced by both carcinogens were inhibited by compound 343 in a concentration-dependent manner (Figure 3). Consistent with the lack of effect on EROD activity in intact cells, treatment with compound 343 alone did not affect basal CYP1A1, -1A2 and -1B1 mRNA expression (data not shown). Our results suggest that compound 343 administered alone does not act as an agonist of AhR, in contrast to some other inhibitors of aryl hydrocarbon-induced CYP expression, such as dibenzoylmethane (20), galangin (28), curcumin (19) or dimethoxyflavone (29).

To determine whether the inhibitory effects of compound 343 on DMBA- or TCDD-induced CYP mRNA expression occurred at the transcriptional level, we transfected HepG2 cells with a luciferase reporter vector containing three repeats of the XRE, present in the promoter regions of CYP genes. Treatment of the transfected cells with either DMBA or TCDD resulted in a significant increase in XRE-controlled transcription that was diminished by compound 343 in a concentration-dependent manner (Figure 4). We further confirmed the ability of compound 343 to specifically inhibit the transcription of *CYP1A1* gene by measuring the level of hnRNA in the cells co-treated with DMBA or TCDD and compound 343. Since hnRNA levels, unlike mRNA levels, are not changed by processing or transport, they reflect gene transcription rate (12). In agreement with the CYP enzyme activity, mRNA levels and XRE-controlled luciferase activity, both DMBA- or TCDD-induced *CYP1A1* gene transcriptions were significantly inhibited by co-treatment with compound 343 (Figure 5). Since gene transcription is mediated by the translocation of the ligand-activated AhR to the nucleus, where it binds to the XRE of target genes, we employed ChIP assays to measure the effect of compound 343 on translocation and DNA binding of the AhR. As shown in Figure 6, compound 343 inhibited the binding of the TCDD-activated

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AhR to the XRE. These data indicate that the decrease in TCDD-stimulated transcription and CYP mRNA levels caused by compound 343 (Figures 3–5) was a consequence of preventing the binding of activated AhR to XRE. The ChIP assay is somewhat less sensitive than the other assays performed in this study, requiring 10 nM TCDD (versus 250 pM in other assays) for a statistically significant increase in band intensity. DMBA, being far less potent an AhR ligand than TCDD, could not, therefore, be examined in this assay.

This is the first demonstration of compound 343 as a potent inhibitor of the AhR pathway that, as a result, can block signal transduction initiated by carcinogens such as DMBA or TCDD.

This may be the critical mechanism that results in its previously demonstrated effect of cell cycle arrest in tumor cells. Furthermore, compound 343, by blocking the upregulation of CYP enzymes, may inhibit the metabolic activation of xenobiotics to genotoxic species. This would decrease the carcinogenicity of many significant environmental carcinogens, preventing the initiation of cancer. Compound 343 is an easily synthesized analogue of a natural product that has been used for centuries, suggesting that it would be safe to use. These results indicate that compound 343 is a promising agent for further chemoprevention research.

Acknowledgements

This research was supported, in part, by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. P.J.H. thanks the National Science Foundation for partial support of this work (NSF-CAREER award CHE-0134779).

Conflict of Interest Statement: The authors have no financial conflict of interest.

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Received August 15, 2006; revised November 8, 2006; accepted December 7, 2006

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