Suppression by p38 MAP Kinase Inhibitors (Pyridinyl Imidazole Compounds) of Ah Receptor Target Gene Activation by 2,3,7,8-Tetrachlorodibenzo-p-dioxin and the Possible Mechanism*

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Cytochrome P-450 1A1 (CYP1A1) is known to be induced by aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), through activation of the aryl hydrocarbon receptor (AhR). We found that p38 MAP kinase inhibitors (SB203580 and SB202190; 40 μM each) suppressed CYP1A1-mRNA induction by TCDD (2 nM) in mouse hepatoma Hepa-1 cells and in human hepatoma HepG2 cells, and also suppressed CYP1B1-mRNA induction by TCDD (2 nM) in human breast adenocarcinoma MCF7 cells. An analogue compound, SB204274, which does not inhibit p38 MAP kinase, also suppressed CYP1A1-mRNA induction by TCDD. Moreover, overexpression of a dominant-negative gene for p38 MAP kinase in Hepa-1 cells did not suppress Cyp1a1 reporter gene induction by TCDD. Therefore, the suppression of Cyp1a1 transcription by pyridinyl imidazole compounds is not because of their inhibition of p38 MAP kinase activity. Because SB203580 did not inhibit in vitro AhR transformation by TCDD, this compound was not acting as a simple AhR antagonist. SB203580 decreased TCDD-induced histone acetylation levels in the region of the Cyp1a1 gene promoter, especially around the TATA box sequence. This result suggests the possibility that pyridinyl imidazole compounds suppress the recruitment of some co-activator that has the histone acetyltransferase activity necessary for CYP1A1-mRNA transcription.

Polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene, are widespread environmental pollutants with many cytotoxic and biological effects. Immunosuppression, tumor promotion, and teratogenesis are well known toxic effects of these compounds in vertebrates (1–4).

The aryl hydrocarbon receptor (AhR) is a ligand-activated cytosolic protein that is a member of the family of the basic-helix-loop-helix-Per/ARNT/Sim transcription factors, and it regulates the induction of the Cyp1a1 gene (5). In the unstimulated state, AhR forms a complex with two molecules of heat shock protein 90 and the immunophilin homologue protein, XAP-2 (6, 7). When ligand (for example, TCDD) binds to the ligand-binding domain of AhR, a chaperone protein is dissociated from the complex, and AhR can then translocate into the nucleus. Once in the nucleus, AhR forms a heterodimer with AhR nuclear translocator (ARNT), and recognizes its target sequence, a xenobiotic responsive element (XRE) was found upstream of the CYP1A1, Ia2, IBl, and NQO1 genes (5, 8). The involvement of AhR in cytotoxicity is evidenced by AhR-null mice being resistant to the toxic effects of TCDD and other aromatic hydrocarbons (9). Because AhR is a central molecule in the action process of TCDD toxicity, the induction of cytochrome P-450 1A1 (CYP1A1) has been used as a model system for the study of the molecular mechanisms underlying the toxicity of TCDD.

The induction of CYP1A1 by TCDD is suppressed by pretreatment of immune cells with lipopolysaccharide (LPS), interleukin-1, or tumor necrosis factor (10, 11). The mechanisms involved in the intracellular signaling cascades induced by LPS have been analyzed in detail, and are known to induce activation of the MAP kinase (extracellular signal-regulated kinase, JNKs, p38s) pathway through its membrane-bound receptor, Toll-like receptor (12). LPS also activates the NF-κB pathway. Tian and co-workers (10) showed that activated AhR can interact with NF-κB subunit p65 (RelA), and that this interaction results in a suppression of the transcription of its target gene. Introduction of the oncogene ras into cultured human breast cancer cells suppresses the transcriptional activation by TCDD of several members of the AhR battery of genes (13). Puga and co-workers (14) showed that TCDD treatment induced a transient activation of transcription factors, especially AP-1, in Hepa-1 cells, and that this activation was AhR-dependent (14). They also reported that TCDD affected the expression level of various genes, including those for members of Ras/MAPK-related signaling pathways in HepG2 cells (15). These data suggest that the AhR pathway and the MAP kinase pathway may cross-talk with each other, but the detailed mechanism remains controversial (for example, how LPS suppresses CYP1A1 and the extent of the contribution of the MAP kinase pathway to induction by TCDD).

In the light of the background described above, we decided to focus on the p38 MAP kinase pathway, which is strongly acti-
Inhibitors of p38 MAP Kinase Suppress CYP1A1-mRNA Induction

Sequence (from /H11002 probe. A fragment of the (99.1% purity) from Calbiochem-Novabiochem Corp. (San Diego, CA). and SB202474 (99.6% purity) and SB202190

In this paper, we describe possible mechanisms by which pyridinyl imidazole compounds may suppress CYP1A1-mRNA induction by TCDD. Our data suggest that these widely used p38 MAP kinase-specific inhibitors may have another target molecule(s), besides p38 MAP kinase.

MATERIALS AND METHODS

Cell Culture—HepG2 (HB 8065) and MCF7 (HTB 22) cells were purchased from the American Type Culture Collection (Rockville, MD). The Hep-1 cell line, derived from mouse hepatoma, was kindly provided by Dr. D. Tsuda (Tohoku University). The cells were maintained in Dulbecco’s minimal essential medium (Invitrogen) containing 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin G in humidified 95% air, 5% CO2 at 37°C.

Chemicals—TCDD was purchased from Cambridge Isotope Laboratories, Inc. (St. Paul, MN). Labeled compound [32P]dCTP using Prime-It (Stratagene), and purified on a Sephadex G-50 spin column (Amersham Biosciences). Then, the 32P-labeled XRE was purified on a Sepadex G-50 column (Amersham Biosciences). In the experiment involving in vitro transformation of rat cytotoxic protein, 800 μg of protein was incubated with 10 μl TCDD, either alone or together with 20–160 μl SB203580 or 40 μl PB90859, for 3 h at 22°C. One-tenth of the incubated protein (80 μg protein) was then incubated for 20 min at 22°C with 225 ng of poly(dI-dC) containing 95 μM NaCl and 1× protease inhibitor (Complete™; Roche Diagnostics), 1 mM dithiorthiol). Protein (50 μg) was electrophoresed on 4% polyacrylamide in 0.5× TBE buffer for 90 min at 150 V. After the gel had been dried, the radioactive signal was detected and quantified using a Bioimage analyzer, BAS2000.

Western Blotting—Cells were transfected with expression vector, incubated for 48 h, then extracted with 50 μl of cell lysis buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1× protease inhibitor (Complete™; Roche Diagnostics), 1 mM dithiorthiol). Protein (50 μg) was electrophoresed on 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. The membrane was immunostained with anti-FLAG antibody (1000-fold dilution), and developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was carried out essentially by the method of Wang and Hancock (25). Hep-1 cells were grown to 80% confluence in two plates in a 10-cm dish. The cells were washed twice with phosphate-buffered saline (−), cross-linked with 1% formaldehyde at 37°C for 10 min, then collected using a cell scraper. They were then rinsed twice with ice-cold washing buffer (phosphate-buffered saline (−), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM pepstatin), suspended in 1 μl lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl, and 1× protease inhibitor (Complete™; Roche Diagnostics)), and incubated on ice for 10 min. Then, the cell suspension was sonicated for 15 s using a Branson sonifier (model W185 with micro-probe) at power setting 4, followed by centrifugation for 10 min at 4°C, so that the DNA fragment size was 200–600 bp. Supernatant (120 μl) was collected and diluted in ChIP dilution buffer (1080 μl of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitor (Complete™; Roche Diagnostics)) followed by immunoclearing with 2.5 μg of shared salmon sperm DNA and Protein A buffer (60 μl of 50% slurry Protein A-agarose (Amersham Biosciences) in ChIP dilution buffer for 2 h at 4°C. Immunoprecipitation was performed for 16 h at 4°C with 6 μl (200-fold dilution) anti-AhR antibody (SA-210; BIOMOL, Plymouth Meeting, PA). After immunoprecipitation, 60 μl of Protein A buffer was added, and the incubation was continued for another 1 h at 4°C. Precipitates were washed sequentially in low-salt buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 1× protease inhibitor (Complete™; Roche Diagnostics)) followed by high-salt buffer (25 mM KCl, 150 mM NaCl, 5 mM EDTA, 1% SDS, 0.1 M NaHCO3). Eluates were pooled and incubated at 65°C for 10 min to reverse the formaldehyde cross-linking. The remaining DNA was quantified after incubation at 45°C for 10 min with 10 μl of 0.5% EDTA, 20 μl of 1× Tris-HCl, pH 6.5, and 2 μl of Proteinase A (10 mg/ml), DNA fragments were purified by phenol-chloroform extraction, and precipitated using 2 volumes of ethanol. Precipitates were then suspended in 20 μl of TE buffer. For PCR, a 1×-
Pyridinyl imidazole compounds (p38 MAPK inhibitors)

CYP1A1-mRNA Induction by TCDD—A series of pyridinyl imidazole compounds (Fig. 1) was developed, and their individual abilities to suppress the synthesis of inflammatory cytokines were assessed. The IC_{50} concentration of SB203580 for p38 MAP kinase is below 1 μM and that of SB202190 is below 0.5 μM, whereas SB202474, which has no ability to inhibit p38 MAP kinase activity, is generally used as a negative control compound in p38 MAP kinase studies (16). The effects of these compounds on CYP1A1-mRNA induction by TCDD were tested by Northern hybridization (Fig. 2E). SB202190 and SB202474, analogues of the p38 MAP kinase inhibitor SB203580, suppressed CYP1A1-mRNA induction by TCDD in the same way as SB203580 itself (lanes 3–16). Furthermore, it shows that this effect is not because of inhibition of the p38 MAP kinase activity, because SB202474, which does not inhibit p38 MAP kinase activity at all, suppressed CYP1A1-mRNA induction by TCDD (lanes 6–8).

The p38 MAP Kinase Pathway Is Not Involved in the Induction of CYP1A1-mRNA by TCDD—To evaluate the contribution made by the p38 MAP kinase pathway to the induction of CYP1A1-mRNA by TCDD, we used dominant-negative constructs of p38 MAP kinases. MKK3 and MKK6 are upstream molecules of p38 MAP kinase. MKK3/6 dominant-negative constructs were transfected into Hepa-1 cells, and their effects were assessed on the activation of the Cyp1a1 reporter gene by TCDD (Fig. 4A). As shown in Fig. 4A, no significant suppression of reporter gene expression was observed with any combination. Furthermore, a p38 MAP kinase dominant-negative construct was also tested in the same way as MKK3/6 (Tables I). The “fold induction” (that is, the luciferase activity of the reporter gene in the presence of TCDD divided by that in the absence of TCDD) was not significantly different between the wild-type p38 MAP kinase and the dominant-negative p38 MAP kinase construct at 2 μg of plasmid DNA. The inhibitory function of dominant negative p38 MAP kinase in Hepa-1 cells was confirmed using the constitutively activated MKK6 and ATF-2 systems (Table II) (19). The left and right panels in Fig. 4B show the expression levels (detected by Western blotting) of the dominant-negative constructs for MKK3/6 and p38 MAP kinase, respectively. These results, like those described above, imply that the p38 MAP kinase pathway is not involved in the induction of CYP1A1-mRNA by TCDD.

SB203580 Does Not Suppress in Vitro AhR Transformation by TCDD—The flavonoid derivative, PD98059 (which was developed as a specific MEK inhibitor), strongly suppresses CYP1A1 induction by TCDD. Reiners and co-workers (26) revealed that this suppression was because of an antagonist effect of PD98059 on AhR. This compound strongly suppresses in vitro AhR transformation by TCDD. We performed an in vitro AhR transformation assay using SB203580, with PD98059 as a positive control. As shown in Fig. 5 (lane 3) a rat cytosolic AhR activated by 10 nM TCDD formed a complex with a 32P-labeled XRE sequence, and the complex formation was completely suppressed by the addition of a 200 times excess of cold XRE (lane 2). Co-incubation with PD98059 completely inhibited AhR activation by TCDD (lane 13), but co-incubation with SB203580 did not (lanes 8–11). Although at a concentration of 160 μM SB203580, AhR transformation was slightly decreased (about 13%, lane 11), this concentration was 4 times higher than that needed to suppress CYP1A1-mRNA induction by 75–90% (Fig. 2A). On treatment with 160 μM SB203580 alone, no AhR transformation was observed (lane 7). These results suggest that SB203580 does not act as a simple AhR antagonist, as PD98059 does.

SB203580 Suppresses CYP1A1-mRNA Induction by Omeprazole—Kikuchi et al. (27) showed that omeprazole induced...
CYP1A1-mRNA in a ligand-independent manner, and that the gene(s) that mediate its induction are present on human chromosome 10p (27). The induction of CYP1A1-mRNA by omeprazole was not suppressed by the AhR antagonist PD98059, suggesting the existence of a ligand-independent signaling pathway (Fig. 6, lane 9). However, PD98059 did suppress the ligand-dependent induction of CYP1A1-mRNA by TCDD (Fig. 6, lane 7). Omeprazole indirectly activates AhR, and the downstream mechanism is the same as that for TCDD. Hence, if SB203580 could be shown to suppress CYP1A1-mRNA induction by omeprazole, we would be able to locate the target molecule(s) of SB203580 to a site downstream of AhR signal transduction. As expected, SB203580 did suppress CYP1A1-mRNA induction by omeprazole (Fig. 6, lane 8). Because the p38 MAP kinase pathway is not involved in CYP1A1-mRNA induction by omeprazole, we would be able to locate the target molecule(s) of SB203580 to a site downstream of AhR signal transduction. As expected, SB203580 did suppress CYP1A1-mRNA induction by omeprazole (data not shown), these data suggest that SB203580, and its group of pyridinyl imidazole compounds, may suppress some molecule(s) downstream of AhR signal transduction.

**Fig. 2.** Effects of the p38 MAP kinase inhibitors on TCDD-induced CYP1A1- and CYP1B1-mRNAs. A and C, dose-dependent effect of SB203580 (SB) on the induction of CYP1A1-mRNA by TCDD. Hepa-1 (A) or HepG2 (C) cells were pretreated with various doses of SB203580 for 1 h before the addition of 2 nM TCDD. B, dose-dependent effect of TCDD on CYP1A1-mRNA, and its suppression by SB203580. Hepa-1 cells were pretreated with 40 μM SB203580 for 1 h before the addition of various doses of TCDD. D, effect of SB203580 on the induction of CYP1B1-mRNA by TCDD. MCF7 cells were pretreated with 40 μM SB203580 or 10 μM PD98059 (PD) for 1 h before the addition of 2 nM TCDD or solvent (DMSO). The membrane was hybridized with a CYP1B1-mRNA specific probe. In all experiments, cells were harvested 22 h after TCDD treatment. E, effect of SB202474 and SB202190 on CYP1A1 induction. Hepa-1 cells were pretreated with various doses of SB202474 and SB202190, analogues of pyridinyl imidazole, for 1 h before the addition of 2 nM TCDD. After incubation with TCDD for 22 h, cells were harvested, and Northern hybridization was performed using a Cyp1a1-cDNA probe. Upper panel of A shows quantification of the CYP1A1-mRNA signal using a Bioimage analyzer, BAS-2000 (Fujiﬁlm Co.). Each data point indicates the mean from three independent experiments, with the bar showing the standard deviation. The intensity of CYP1A1-mRNA was normalized with respect to that of β-actin.

**Fig. 3.** Suppression of Cyp1a1 reporter gene by SB203580. Hepa-1 cells were transfected with 0.5 μg of a reporter gene, the Cyp1a1-promoter region (−1642 to +57) ligated with pGL3Basic vector, together with 0.2 μg of pCAGGS-lacZ vector. After 16 h incubation, cells were treated with various doses of SB203580 for 1 h before the addition of 2 nM TCDD. Cells were incubated for a further 22 h, then harvested and luciferase activity was measured. Luciferase activity was normalized with respect to β-galactosidase activity. Asterisk (*) indicates p < 0.01 versus TCDD-treated sample (Student's t test).

CYP1A1-mRNA in a ligand-independent manner, and that the gene(s) that mediate its induction are present on human chromosome 10p (27). The induction of CYP1A1-mRNA by omeprazole was not suppressed by the AhR antagonist PD98059, suggesting the existence of a ligand-independent signaling pathway (Fig. 6, lane 9). However, PD98059 did suppress the ligand-dependent induction of CYP1A1-mRNA by TCDD (Fig. 6, lane 7). Omeprazole indirectly activates AhR, and the downstream mechanism is the same as that for TCDD. Hence, if SB203580 could be shown to suppress CYP1A1-mRNA induction by omeprazole, we would be able to locate the target molecule(s) of SB203580 to a site downstream of AhR signal transduction. As expected, SB203580 did suppress CYP1A1-mRNA induction by omeprazole (Fig. 6, lane 8).
stream of AhR signal transduction that are necessary for CYP1A1-mRNA transcription.

SB203580 Does Not Suppress Translocation of AhR to the Nucleus by TCDD—The activated AhR is translocated from the cytosol to the nucleus within a few hours after ligand treatment. To examine the possibility that SB203580 suppresses AhR translocation to the nucleus, we performed an EMSA using nuclear protein from Hepa-1 cells. As shown in Fig. 7, a specific shifted band was detected following treatment with 10 nM TCDD (lane 3), and this was completely suppressed by use of a 200-fold excess of cold XRE (lane 2). A shifted band of the same intensity was observed when Hepa-1 cells were pre-treated with 40 μM SB203580 before the addition of 10 nM TCDD (lane 4). This result indicates that SB203580 did not influence the translocation to the nucleus of the AhR activated by TCDD.

SB203580 Suppresses the Chromatin Acetylation Induced by TCDD—Tian and co-workers (10) showed that TCDD induces histone acetylation in the Cyp1a1 promoter region, and that this acetylation is involved in CYP1A1-mRNA transcription (10). Fig. 8 shows the result of our ChIP assay using AhR- and histone H4-specific antibodies. Panel A shows the time course of the AhR recruitment to the XRE region after TCDD treatment. At 1.5 h after the treatment, AhR had accumulated in the XRE region, and this effect then declined. As expected, there was no signal in the TATA box region in the immunoprecipitates generated using anti-AhR antibody (Fig. 8A). With regard to the histone acetylation level, the TATA box region was significantly acetylated at 2 h after TCDD treatment (i.e. 0.5 h after the peak of the AhR accumulation in the XRE region) (Fig. 8B). The histone in the XRE region was also slightly acetylated at 2 h after the treatment (Fig. 8B). These data suggest that the histone acetylation state really is changed in the course of CYP1A1-mRNA induction by TCDD. Next, we examined the effect of SB203580 on the histone acetylation level induced by TCDD. Pretreatment with 40 μM SB203580 at 1 h before treatment with 10 nM TCDD significantly suppressed histone H4 acetylation around the TATA box region (Fig. 8C), whereas pretreatment with 10 μM PD98059, an AhR antagonist, completely suppressed it (Fig. 8C). Under the same conditions, CYP1A1-mRNA induction, too, was significantly suppressed by SB203580 and completely suppressed by PD98059 (Fig. 2D).

SB203580 Suppresses TSA Augmentation of CYP1A1-mRNA Induction by TCDD—Trichostatin A is known to inhibit the activity of histone deacetylase. Xu and co-workers (28) showed that the 7-ethoxyresorufin O-deethylase activity induced by TCDD in rat hepatocytes was augmented by TSA treatment. Using these drastic conditions, we examined the effect of SB203580 on CYP1A1-mRNA induction and on the histone acetylation state in Hepa-1 cells. As shown in Fig. 9A, the histone H4 acetylation level was significantly increased by treatment with either TCDD alone or TSA alone. The CYP1A1-mRNA level induced by TCDD was also augmented by TSA pretreatment (Fig. 9B, lanes 7–9), although at a high TSA concentration the augmentation was slightly weaker (possibly because of the cytotoxicity of TSA). Following pretreatment with 40 μM SB203580 for 1 h before treatment with TCDD, CYP1A1-mRNA induction was strongly suppressed (Fig. 9B, lanes 11–13). It is not clear whether the effect of pyridinyl imidazole (PI) compounds is to inhibit AhR activity in some way that would decrease acetylation or whether the compounds influence acetylation by some other method. This could be addressed in part by examining the effects of PI compounds on TSA-mediated acetylation that should allow an independent effect on acetylation to be distinguished from an inhibition of AhR activity. The right panel of Fig. 9A shows that SB203580 did not influence the acetylation of histone H4 in the region of Cyp1a1 gene mediated by TSA.

DISCUSSION

PI compounds, exemplified by SB203580, are widely used as p38 MAPK chemical inhibitors. SB203580 has been shown to bind competitively to the ATP-binding pocket of p38 MAPK, and to inhibit its activity in a specific manner (29). We observed here that PI compounds inhibited CYP1A1-mRNA induction by TCDD (Fig. 2). At first, we suspected that the p38 MAPK pathway might be involved in CYP1A1-mRNA induction by TCDD. However, p38 MAPK dominant-negative and MKK3/6 dominant-negative constructs did not suppress the induction of Cyp1a1 reporter gene activity by TCDD (Tables I and II and Fig. 4). Furthermore, SB202474, which has no ability to inhibit p38 MAPK activity and is generally used as a negative control compound in p38 MAPK studies (16), also suppressed CYP1A1-mRNA induction by TCDD. From these results, we conclude that the p38 MAPK pathway is not involved in CYP1A1-mRNA induction by TCDD, and that PI compounds have another target molecule(s), which might be important for CYP1A1-mRNA transcription by TCDD, besides p38 MAPK.

Several compounds are known to suppress CYP1A1-mRNA induction by TCDD; for example, α-naphthoflavone (30, 31) and the MEK inhibitor PD98059 (2’-amino-3’-methoxyflavone) (26), which has a flavonoid structure, like the naphthoflavones. Previous studies have demonstrated that these compounds act as AhR antagonists. For instance, resveratrol, which is a mem-
ber of the polyphenol compounds, suppresses CYP1A1-mRNA induction by TCDD by acting as a simple AhR antagonist (32). Therefore, there was a possibility that PI compounds also directly inhibit this induction by acting as an antagonist to AhR. However, the results of our in vitro AhR transformation and EMSA using nuclear extracts showed that SB203580 did not suppress either AhR activation (Fig. 5) or its nuclear translocation (Fig. 7) by TCDD. Therefore, PI compounds are not acting as simple AhR antagonists. Kikuchi et al. (27, 33) have shown that omeprazole, an inhibitor of H^+/K^-ATPase, activates AhR in a ligand-independent way. Our Fig. 6 shows that PD98059, an AhR antagonist, potently suppressed CYP1A1-mRNA induction by TCDD, but did not suppress that by omeprazole. However, SB203580 did suppress CYP1A1-mRNA induction by omeprazole.

### Table I

| Transfected plasmid DNA          | p38MAPK.D.N. (μg) | Wild (μg) |
|---------------------------------|------------------|-----------|
| -TCDD (Luc/β-galactosidase)     | 0.115 ± 0.011    | 0.283 ± 0.001 |
| +TCDD (Luc/β-galactosidase)     | 0.730 ± 0.058    | 0.534 ± 0.051 |
| -Fold Increase                  | 6.35             | 6.43 |

### Table II

| Lane No. | p38 MAPK.D.N. | p38 MAPK (Wild) |
|----------|---------------|----------------|
| MKK6.C.A | pFA-ATF2      | pFA-ATF2       |
| 0.002 ± 0.001 | 0.059 ± 0.002 | 0.025 ± 0.001 |

### Fig. 5. Effect of SB203580 on in vitro AhR activation by TCDD.

Rat liver extracts (800 μg) were transformed in vitro for 3 h using 10 nM TCDD in the presence of various concentrations of either SB203580 (20, 40, 80, or 160 μM) or 40 μM PD98059. Subsequently, the extracts were incubated for 20 min at 22°C with 1 ng of [32P]-labeled XRE probe, and analyzed on 4% polyacrylamide native gel. To confirm the specificity of XRE binding to AhR, a 250-fold excess of cold XRE was added (lane 2). The data shown are the most representative of those obtained in three independent experiments.

### Fig. 6. Effect of SB203580 on CYP1A1-mRNA induction by omeprazole.

HepG2 cells were pretreated for 1 h with 40 μM SB203580 (SB) or 10 μM PD98059 (PD), then treated for a further 22 h with 2 nM TCDD, 200 μM omeprazole (OP), or solvent (DMSO). Cells were harvested, and Northern hybridization was performed using a Cyp1a1-cDNA probe.

### Fig. 7. Effect of SB203580 on EMSA using nuclear protein.

Hepa-1 cells were pretreated for 1 h with 40 μM SB203580, then treated for a further 1.5 h with 10 nM TCDD, 200 μM omeprazole (OP), and solvent (DMSO). Cells were harvested, and nuclear protein was prepared. Then, 5 μg of nuclear protein was incubated with 1 ng of [32P]-labeled XRE probe, and analyzed on a 4% polyacrylamide native gel. To confirm the specificity of XRE binding to AhR, a 200-fold excess of cold XRE was added (lane 2). The positions of the nonspecific band (N.S.), specific band, and free probe are shown by thick bars at the side of lane 4.
Omeprazole is not a ligand for AhR (34), but instead activates AhR through a tyrosine kinase-dependent pathway, and it induces CYP1A1-mRNA in human HepG2 cells (33). Therefore, the above data again suggest that PI compounds are not simple AhR antagonists, and may instead act on the downstream process involved in AhR signal transduction.

Tan and co-workers (35) reported that SB202190 suppressed both JNK activation and the induced expression of CYP1A1 protein by TCDD. We used the method of reporter gene assay to examine the possible involvement of the JNK pathway in CYP1A1-mRNA induction by TCDD using a JNK dominant-negative expression vector. However, in our system there was no significant activity change in the reporter gene, which was driven by a Cyp1a1 regulatory sequence, between the sample obtained using control vector and that obtained using a dominant-negative JNK expression vector (data not shown). Therefore, the suppression effect on CYP1A1-mRNA exerted by SB202190 may not be because of a decrease in the activity of JNK.

Recently, it has been shown that nitrobenzylthioinosine-sensitive equilibrative nucleoside transporter 1 is another target molecule for PI compounds, and that it potently suppresses the differentiation of K562 cells induced by cytarabine (Ara C) (36). We checked the possibility that CYP1A1-mRNA suppression by PI compounds may be attributable to a suppression of equilibrative nucleoside transporter 1. However, nitrobenzylthioinosine, an equilibrative nucleoside transporter 1 inhibitor, did not suppress CYP1A1-mRNA induction by TCDD (data not shown). This implies that there is another target molecule(s) for PI compounds, besides equilibrative nucleoside transporter 1.

A previous study has shown that LPS, a bacterial cell-wall component, which can activate the immune system through its receptor (Toll-like receptor 4), suppresses CYP1A1-mRNA induction by TCDD (3). Recently, Ke and co-workers noted that histone acetylation of the Cyp1a1 promoter, especially the TATA box region, was suppressed by treatment with LPS (11). There is much evidence that histone molecules tend to be acetylated in the chromatin of the promoter region of the target gene before the transcription of its mRNA, and the subsequent alteration in the chromatin structure gives transcription factors easy access to the promoter region (37). In the case of

![Figure](https://example.com/figure8.png)

**Figure 8.** Effect of SB203580 on TCDD-induced chromatin acetylation. Hepa-1 cells were treated with 10 nM TCDD, then harvested at the times indicated. A ChIP assay was performed using (A) an AhR- or (B) an acetylated histone H4 (AcH4)-specific antibody. To identify the immunoprecipitated sequence, XRE- and TATA-box-specific primers, as shown at the top of the panel, were used for semiquantitative PCR. C. Hepa-1 cells were pretreated for 1 h with 40 μM SB203580 (SB) or 10 μM PD98059, then treated for a further 1.5 h with either 10 nM TCDD or solvent (DMSO). Cells were harvested, and the ChIP assay was performed using AcH4-specific antibody.

![Figure](https://example.com/figure9.png)

**Figure 9.** Effect of SB203580 on TSA augmentation of CYP1A1-mRNA induction by TCDD. Hepa-1 cells were pretreated for 1 h with various concentrations of TSA (0.5, 1, or 2 μM), then treated for 1 h with 10 μM SB203580 (SB). Subsequently, cells were divided into two groups. A. Cells were incubated for 1.5 h with 10 nM TCDD, then harvested for the ChIP assay using histone H4-specific antibody and TATA box-specific primers (−285 to +66 bp). B. The remainder of the cells were incubated for 22 h in the presence of either 2 nM TCDD or solvent. Cells were harvested, and Northern hybridization was performed using a Cyp1a1-cDNA probe. Upper panel shows quantification of CYP1A1 signals (performed as described in the legend to Fig. 2). The radioactivity of CYP1A1 was normalized with respect to that of β-actin.
CYP1A1 mRNA induction by TCDD, histone acetylation and chromatin remodeling are important. Basic transcription factors, such as p300, SRC-1, and cAMP-response element-binding protein, which have histone acetyltransferase activity, are necessary for CYP1A1 mRNA transcription (38, 39). Furthermore, Brahma/SWI2-related gene-1, which is an ATPase subunit of chromatin remodeling factor SWI-SNF complexes, is critical for CYP1A1 mRNA transcription by TCDD (25). For that reason, we thought that PI compounds might affect the chromatin modification process. The results of our ChIP assay showed that AhR binding to the XRE sequence (1.5 h after TCDD treatment) was followed by histone acetylation of TATA box chromatin (2–3 h after TCDD treatment) (Fig. 8). This histone acetylation by TCDD was suppressed by pretreatment with SB203580 (Fig. 8C). This result suggests that PI compounds may suppress the recruitment to the Cyp1a1 gene promoter region of some coactivator that is necessary for mRNA transcription.

The augmentation of CYP1A1 mRNA induction by pretreatment with TSA (Fig. 9) also indicated that histone acetylation is important in this process. The TSA treatment may increase the global acetylation of the gene by TSA treatment may increase the global acetylation of the gene by TSA may increase the recruitment of this factor on this site.

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