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The p38 MAPK Inhibitor SB203580 Induces Cytochrome P450 1A1 Gene Expression in Murine and Human Hepatoma Cell lines through Ligand-Dependent Aryl Hydrocarbon Receptor Activation

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Abstract

We have previously shown that the p38 MAPK inhibitor SB203580 (SB) significantly induced the Cyp1a1 gene expression at the mRNA and activity levels, whereas dramatically inhibited the induction of Cyp1a1 by TCDD in murine hepatoma Hepa 1c1c7 cells. However, the molecular mechanisms involved were not investigated yet. Therefore, the current study aims to examine the capacity of SB to induce the constitutive *CYP1A1* gene expression in Hepa 1c1c7 and HepG2 cells and to explore the mechanisms involved. Our results showed that SB induced the Cyp1a1 mRNA, protein, and activity levels in a concentration-dependent manner in Hepa 1c1c7 cells. The increase in Cyp1a1 mRNA by SB was completely blocked by the transcriptional inhibitor, actinomycin D; implying that SB increased *de novo* RNA synthesis. In addition, lack of Cyp1a1 induction by SB in mutant AhR-deficient C12 cells and with co-treatment with AhR antagonist, α -naphthoflavone, clearly suggests an AhR-dependent induction. This was further supported by the ability of SB to induce Cyp1a1 independent from its effect on MAPKs and also it was able to bind to and activated AhR transformation and its subsequent binding to the XRE. This is the first demonstration that the p38 MAPK inhibitor, SB can directly bind to and activate AhR induce *Cyp1a1* gene expression in an AhR-dependent manner and represents a novel mechanism by which SB induces this enzyme.

Keywords

AhR; SB203580; Cyp1a1; p38 MAPKs; siRNA; gene expression

Introduction

The cytochrome P450 1A1 (CYP1A1) is a monooxygenase enzyme that is involved in a number of cellular functions such as metabolism of xenobiotics (1). CYP1A1 has been shown to be responsible for the bioactivation of a variety of environmental carcinogens such as polycyclic aromatic hydrocarbons (PHAs) to epoxide and diol-epoxide intermediates (2).

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The biochemical and carcinogenic effects of PAHs are primarily initiated by binding to and activation of a cytosolic ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR). Mechanistically, upon binding with its ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), AhR dissociates from its inhibitory proteins (3, 4) allowing it to translocate to the nucleus, where it heterodimerizes with a nuclear transcription factor protein called the AhR nuclear translocator (ARNT) (5). The heterodimeric AhR-ARNT complex then binds to specific DNA recognition sequences, GCGTG, within the xenobiotic responsive element (XRE) located in the promoter region of all AhR-dependent genes, including *CYP1A1* (6–8).

Although the classical AhR ligands and *CYP1A1* inducers such as PAHs are structurally similar and share several physiochemical properties, recent findings have demonstrated the structural diversity of *CYP1A1* inducers (9). Consequently, activation of AhR is not just restricted to these compounds, in that a large number of newly identified AhR ligands whose structures and physiochemical properties significantly differ from those of PAHs have been previously reported (10, 11). Although the majority of these non-classical AhR ligands are weak *CYP1A1* inducers and possess a low probability of human exposure, this list has expanded to include a number of widely prescribed drugs such as omeprazole (12), primaquine (13), and sulindac (14).

The AhR has been identified as a target of several signaling pathways that cross-talk with its own regulatory pathway, such as proteasomal degradation (15), redox-sensitive transcription factors (16), and the mitogen-activated protein kinases (MAPKs) (17). Among those MAPKs, p38 MAPKs are important enzymes involved in cellular signaling, apoptosis, carcinogenesis and in pathogenesis of variety of diseases (17). The pyridinyl imidazole SB203580 (SB) (Fig. 1) has been reported to be a potent and selective inhibitor of p38 MAPK and hence become the pharmacological inhibitor of choice for assessing the role of p38 MAPKs in mediating biological processes, including the AhR pathway (18–21). In this regard, several previous studies have investigated the effect of SB on the AhR-*CYP1A1* pathway. In particular, it has been reported that SB significantly suppressed *CYP1A1* gene induction by TCDD through p38 MAPK-independent pathway in different mammalian cell lines, such as murine hepatoma Hepa 1c17 (18, 20), human hepatoma HepG2 (18), and monkey fibroblast kidney COS-7 (19) cells. Unfortunately, none of these previous studies have examined the effect of SB on the constitutive expression of *CYP1A1* gene expression.

In the light of the background described above, we have recently reported that treatment of Hepa 1c17 cells with SB significantly induced the Cyp1a1 mRNA and activity levels (20). Therefore, the objectives of the current study were to investigate the potential effect of SB on the constitutive expression of Cyp1a1 in both Hepa 1c17 and HepG2 cells and to explore the underlying molecular mechanisms. The current manuscript provides the first evidence for the ability of SB to induce *CYP1A1* gene expression in murine and human cell lines through AhR-dependent mechanisms.

Materials and Methods

Materials

7-Ethoxyresorufin, Dulbecco's Modified Eagle's Medium (DMEM), anti-goat IgG peroxidase secondary antibody, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). 2,3,7,8-tetrachlorodibenzofuran (TCDF) and [³H]-TCDD (13 Ci/mmol) were obtained from Dr. Safe (Texas A&M University). Amphotericin B, and resorufin were purchased from ICN Biomedicals Canada (Montreal, QC). TRIzol

reagent and lipofectamine kits were purchased from Invitrogen Co. (Grand Island, NY). High Capacity cDNA Reverse Transcription kit and SYBR[®] Green PCR Master Mix were purchased from Applied Biosystem (Foster city, CA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 goat polyclonal primary antibody and goat anti-ARNT antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chemiluminescence Western blot detection kits were obtained from GE Healthcare Life Sciences (Piscataway, NJ). Actinomycin D (Act-D) was purchased from Calbiochem (San Diego, CA). Poly(dI.dC) were purchased from Amersham Canada (Oakville, ON). [γ -³²P]ATP was supplied by the DNA Core Services Laboratory University of Alberta (Edmonton, AB). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

Biohazard precaution

TCDD is toxic and a likely human carcinogen. All personnel were instructed as to safe handling procedures. Lab coats, gloves, and masks were worn at all times, and contaminated materials were collected separately for disposal by the Office of Environmental Health and Safety at the University of Alberta.

Animals and ethics

All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Hartley guinea pigs weighing 250–300 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were exposed to 12 h of light and 12 h of dark daily and given free access to food and water.

Cell culture and treatments

Murine hepatoma Hepa 1c1c7 wild-type (WT), AhR-deficient murine hepatoma (C12), and human hepatocellular carcinoma HepG2 cells (American Type Cell Cutler, Manassas, VA), were maintained in DMEM, without phenol red supplemented with 10% fetal bovine serum, 20 μ M L-glutamine, 50 μ g/ml amikacin, 100 IU/ml penicillin G, 10 μ g/ml streptomycin, and 25 ng/ml amphotericin B. Cells were grown in 75 cm² tissue culture flasks at 37 °C under a 5% CO₂ humidified environment.

The cells were seeded in 96- and 6-well cell culture plates in DMEM culture media for Cyp1a1 enzyme activity and RNA and protein assays, respectively. In all experiments, the cells were pretreated for the indicated time intervals in serum-free media with various concentrations of SB or 1 nM TCDD as indicated. Stock solutions of TCDD and SB were prepared in DMSO and stored at –20 °C. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

Cytotoxicity of SB

The effect of SB on Hepa 1c1c7 cell viability was determined by measuring the capacity of reducing enzymes present in only viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) to colored formazan crystals as described previously (22). Briefly, Hepa 1c1c7 cells were treated for 24 h with various concentrations of SB. Thereafter, media were removed and cells were incubated with MTT for 2 h. the color intensity in each well was then measured at wavelength of 550 nm using EL 312e 96-well microplate reader, Bio-Tek Instruments Inc (Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

RNA Extraction and cDNA Synthesis

After incubation with the test compound for the specified time periods, total cellular RNA was isolated using TRIzol reagent (Invitrogen®) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (>1.8). Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Briefly, 1.5 µg of total RNA from each sample was added to a mixture of 2.0 µl of 10x reverse transcriptase buffer, 0.8 µl of 25x dNTP mix (100 mM), 2.0 µl of 10x reverse transcriptase random primers, 1.0 µl of MultiScribe reverse transcriptase, and 3.2 µl of nuclease-free water. The final reaction mixture was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C.

Quantification of mRNA Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®). The 25-µl reaction mixture contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. Mouse primers and probes for *Cyp1a1* (forward: 5'-GGT TAA CCA TGA CCG GGA ACT-3', reverse: 5'-TGC CCA AAC CAA AGA GAG TGA-3'), Extracellular Regulated Kinase 1 (ERK1) (forward: 5'-TCC GCC ATG AGA ATG TTA TAG GC-3', reverse: 5'-GGT GGT GTT GAT AAG CAG ATT GG-3'), ERK2 (forward: 5'-CAG ACA TGA GAA CAT CAT TGG CA-3', reverse: 5'-TAA AGG TCC GTC TCC ATG AGG-3'), p38 (forward: 5'-GGA GAA GAT GCT CGT TTT GGA-3', reverse: 5'-TTG GTC AAG GGG TGG TGG-3'), β-actin (forward: 5'-TAT TGG CAA CGA GCG GTT CC-3', reverse: 5'-GGC ATA GAG GTC TTT ACG GAT GTC-3'); and human primers and probes for *CYP1A1* (forward: 5'-CTA TCT GGG CTGTGG GCA A-3'; reverse: 5'-CTG GCT CAA GCA CAA CTT GG-3'), and for β-ACTIN (forward: 5'-TAT TGG CAA CGA GCG GTT CC-3', reverse: 5'-GGC ATA GAG GTC TTT ACG GAT GTC-3') were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of *CYP1A1*, *ERK1*, *ERK2*, or *p38* genes between treated and untreated cells were corrected by the levels of β-ACTIN. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data were analyzed using the relative gene expression (i.e., ΔΔCT) method, as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen (23). Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene β-ACTIN and relative to a calibrator. The fold change in the level of *CYP1A1* or target genes between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change = $2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_{t(\text{target})} - C_{t(\beta\text{-actin})}$ and $\Delta(\Delta C_t) = \Delta C_{t(\text{treated})} - \Delta C_{t(\text{untreated})}$.

Protein Extraction and Western Blot Analysis

Twenty-four hours after incubation with the test compound, approximately 1.5×10^6 cells per six-well culture plates were collected in 100 µl lysis buffer (50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1mM EDTA, 10% glycerol (v/v), 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail) (24). Total cellular proteins were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortex mixing every 10 min, followed by centrifugation at 12,000× g for 10 min at 4°C.

Western blot analysis was performed using a previously described method (24, 25). Briefly, 25 µg of protein from each treatment group was separated by 10% sodium dodecyl sulfate

(SDS)-polyacrylamide gel electrophoresis (PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk powder, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were washed several times with TBS-Tween-20 before being incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature in TBS solution containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was carried out in blocking solution for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare, Mississauga, ON). The intensity of Cyp1a1 protein bands was quantified relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) protein, using ImageJ® image processing program (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>).

Determination of CYP1A1 enzymatic activity

Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was performed on intact, living Hepa 1c1c7, C12, and HepG2 cells, using 7-ethoxyresorufin (7ER) as a substrate and normalized for cellular protein content as described previously (24).

Transient Transfection and Luciferase Assay

Hepa 1c1c7 cells were transiently transfected with the XRE-luciferase reporter plasmid pGudLuc1.1 (26) by incubating cells with DNA-lipofectamine complexes in a serum-free and antibiotics-free medium. Following 16 h, the cells were treated for additional 24 h with increasing concentrations of test compounds in a fresh serum-free medium. After treatments, cells were washed with PBS, thereafter 200 µl of Passive Lysis Buffer (Promega®) was added into each well with continuous shaking for at least 20 min, and then the content of each well was collected separately in 1.5 ml microcentrifuge tubes. Enzyme activities were determined using a luciferase reporter assay system (Promega®), and quantified using a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA). Luciferase activities were reported as emitted light per well as a percent of control, vehicle treated cells.

Transfecting Hepa 1c1c7 cells with siRNA

Hepa 1c1c7 cells were plated onto 6- and 48-well cell culture plates. Each well of cells was transfected with ERK1, ERK2, or p38 siRNA at the concentration of 20 nM using INTERFERin reagent according to manufacturer's instructions (Polyplus). siRNAs against ERK1 (sense: CAA AUG CAG UAU UUU UGU Utt, and antisense: AAC AAA AAU ACU GCA UUU Gag), ERK2 (sense: CAA AUG CAG UAU UUU UGU Utt, and antisense: AAC AAA AAU ACU GCA UUU Gag), and p38 (sense: CAA AUG CAG UAU UUU UGU Utt, and antisense: AAC AAA AAU ACU GCA UUU Gag) were purchased from Integrated DNA technologies (IDT, Coralville, IA). Transfection efficiency was determined using RT-PCR to detect ERK1, ERK2, and p38 mRNA post-transfection up to 24 h. Therefore, cells were treated at 6 h post-transfection with SB (10 µM) for 6 h to determine ERK1, ERK2, p38, and Cyp1a1 mRNA levels, or 24 h to determine Cyp1a1 catalytic activity levels.

Electrophoretic Mobility Shift Assay (EMSA)

For preparation of guinea pig hepatic cytosol, freshly excised livers, from male Hartley guinea pigs (250–300 g), were homogenized in ice-cold HEGD buffer (25 mM HEPES, 5 mM EDTA, 10% glycerol, pH 7.4) using three passes with a Teflon-glass homogenizer. The resulting homogenate was centrifuged at 9000 × g for 20 min at 4°C followed by

centrifugation of the supernatant at $100,000 \times g$ for 60 min at 4°C . Aliquots of guinea pig liver cytosol ($100,000 \times g$ supernatant) were stored at -80°C until use.

To visualize the ability of SB to induce the transformation and subsequent DNA binding of the AhR, a complementary pair of synthetic oligonucleotides containing the sequence 5'-GAT CTG GCT CTT CTC ACG CAA CTC CG-3' and 5'-GAT CCG GAG TTG CGT GAG AAG AGC CA-3', corresponding to the XRE binding site, were synthesized and radiolabeled with $\gamma\text{-}^{32}\text{P}\text{-ATP}$ at the 5-end using T4 polynucleotide kinase, and used as a probe for EMSA reactions as described previously (8, 27). Hepatic cytosol of untreated guinea pig was incubated *in vitro* for 2 h with DMSO, SB (100 μM), or TCDD (20 nM). Aliquots of cytosolic protein (80 μg) were incubated for 30 min at room temperature in a reaction mixture (30 μl) containing 25 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), 1 μg spermidine, and 5 μg poly(dI,dC). Thereafter, ~ 1 ng (100,000 cpm) [^{32}P]-labeled XRE was incubated with the mixture for another 30 min before being separated through a 4% nondenaturing PAGE.

The specificity of binding was confirmed by competition experiments, cytosolic extracts were preincubated at room temperature for 20 min with a 100-fold molar excess of unlabelled XRE or 0.6 μg of anti-ARNT antibody (Santa Cruz Biotechnology, Inc.) before the addition of the labelled XRE. The gel was dried at 80°C for 1h, and AhR-XRE complexes formed are visualized by autoradiography (28).

Competitive Ligand Binding Assay

Ligand binding was performed using hydroxyapatite (HAP) assay as previously described (29) with slight modifications. Specifically, untreated guinea pig cytosolic protein was diluted to 2 mg/ml in MEDG buffer (25 mM 3-(N-morpholino) propanesulfonic acid, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiotreitol, 10% [v/v] glycerol). Aliquots of 100 μl were incubated in the presence of 2 nM [^3H]TCDD alone (total binding), 2 nM [^3H]TCDD and 200 nM 2,3,7,8-tetrachlorodibenzofuran (TCDF) (100 fold excess of competitor, non-specific binding) or 2 nM [^3H]TCDD in the presence of increasing concentrations of SB (20 μM , 100 μM , and 200 μM). All chemicals stocks were prepared in DMSO, in which DMSO content in reactions was adjusted to 2% (v/v) where necessary. After 1.5 h incubation at room temperature, reactions were further incubated with 250 μl of hydroxyapatite suspension for additional 30 min with gentle vortexing every 10 min. Thereafter, reactions were washed three times with 1 ml MEGT buffer (25 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 10% [v/v] glycerol, 0.5% [v/v] Tween 80). The HAP pellets were transferred to 4 ml scintillation vials, scintillation cocktail was added and reactions were counted in a scintillation counter.

Statistical Analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat[®] for Windows (Systat Software, Inc, CA). One-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when $p < 0.05$.

Results

Effect of SB on Hepa 1c1c7 Cell Viability

To determine the maximum non-toxic concentrations of SB to be utilized in the current study, Hepa 1c1c7 cells were exposed for 24 h to increasing concentrations of SB (1, 5, 10, 20, and 40 μM). The MTT assay showed that the concentrations ranging from 1 to 20 μM

did not affect cell viability. On the other hand, the concentration of 40 μM decreased cell viability by 30% (Fig. 2). Based on these findings, SB concentrations 1, 5, 10, and 20 μM were utilized in all subsequent experiments.

Concentration-dependent Induction of *Cyp1a1* Gene Expression by SB in Hepa 1c1c7 WT Cells

To determine the capacity of SB to alter the expression of *Cyp1a1* gene, we examined the effect of SB on *Cyp1a1* mRNA, protein, and activity levels in Hepa 1c1c7 cells. For this purpose, Hepa 1c1c7 WT cells were incubated for the indicated time points with increasing concentrations of SB (1, 5, 10, and 20 μM) or TCDD (1 nM), as a positive control. Figure 3A shows that SB induced *Cyp1a1* mRNA in a concentration-dependent manner as determined by RT-PCR. The submaximal induction was achieved at a concentration 10 μM (3-fold), whereas the highest concentrations tested, 20 μM , increased *Cyp1a1* mRNA by approximately 6-fold, which was approximately 50% of what was observed with TCDD (12-fold).

To further examine whether the induction of *Cyp1a1* mRNA in Hepa 1c1c7 WT cells in response to SB treatment is translated into functional protein and catalytic activity, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of SB or TCDD, thereafter, *Cyp1a1* protein and catalytic activity were determined by Western blot analysis and EROD assay, respectively. Figure 3B and C show that in a pattern similar to what was observed with mRNA, SB induced *Cyp1a1* protein and catalytic activity in a concentration-dependent manner (Fig. 3A). The maximal inductions of *Cyp1a1* protein and catalytic activity were observed at 20 μM (Fig. 4).

Induction of *CYP1A1* Gene Expression by SB in HepG2 Cells

In order to examine the relevance of SB-associated inductions of murine *Cyp1a1* gene to humans, HepG2 cells were incubated for indicated time points with only a single concentration of SB (10 μM) that showed submaximal induction in Hepa 1c1c7. *CYP1A1* mRNA and catalytic activity were then determined using RT-PCR and EROD assays, respectively. Figure 4 shows that SB induced human *CYP1A1* mRNA and catalytic activity by approximately 2- and 5-fold, respectively, in a manner similar to murine *Cyp1a1* mRNA levels (Fig. 3A and C). These results show that murine *Cyp1a1* was two times more sensitive to SB treatment than human *CYP1A1*.

Transcriptional Induction of *Cyp1a1* Gene by SB in Hepa 1c1c7 WT Cells

Initially, we questioned whether the induction of murine *Cyp1a1* by SB (Fig. 3) is regulated at the transcriptional level. Therefore, we tested the hypothesis that SB increases the *de novo* *Cyp1a1* RNA synthesis. For this purpose, Hepa 1c1c7 WT cells were treated for 6 h with a single concentration of SB (10 μM), which showed submaximal induction, in the presence and absence of 5 $\mu\text{g/ml}$ Act-D, a RNA synthesis inhibitor. Thereafter *Cyp1a1* mRNA expression was determined by RT-PCR. If SB increased the amount of *Cyp1a1* mRNA through increasing its *de novo* RNA synthesis, we would expect to observe a decrease in the content of *Cyp1a1* mRNA after the inhibition of its RNA synthesis.

Figure 5 shows that pretreatment of the cells with Act-D abolished the constitutive expression of *Cyp1a1* mRNA, and completely blocked SB-mediated induction of *Cyp1a1* mRNA. Furthermore, the induction of *Cyp1a1* mRNA by TCDD, which is known to be a transcriptional event, was completely prevented by Act-D. These results suggest that SB increases the *Cyp1a1* mRNA content at the transcriptional level by increasing its *de novo* RNA synthesis, in a manner similar to that observed with TCDD.

AhR-dependent Induction of Cyp1a1 by SB

The possibility that SB induces Cyp1a1 gene expression through an AhR-dependent mechanism was addressed by several approaches. Therefore, a series of independent experiments were conducted.

Induction of AhR-Dependent Reporter Gene Expression by SB in Hepa 1c1c7 WT Cells

—The ability of SB to induce the AhR-dependent gene expression was assessed using Hepa 1c1c7 cells transiently transfected with the XRE-driven luciferase reporter gene. Cells were incubated for 18 h with SB (1, 5, 10, and 20 μ M) or TCDD (2 nM), as a positive control. Figure 6 showed that treatment of Hepa 1c1c7 cells with SB significantly induced the reporter gene in a concentration-dependent manner; similar to what was observed with TCDD.

Lack of SB-mediated Induction of the Cyp1a1 Gene in Mutant AhR-deficient C12 Cells

—To confirm the AhR-dependent induction of *Cyp1a1* gene by SB, mutant AhR-deficient cells, C12, were treated for indicated time points with the same concentrations of SB used in WT cells (0, 1, 5, 10, and 20 μ M) or TCDD (1 nM), thereafter, Cyp1a1 mRNA and catalytic activity were determined using RT-PCR and EROD assay, respectively. Figure 7 shows that all tested concentrations of SB failed to induce Cyp1a1 mRNA and activity in C12 cells, suggesting that AhR is essential for SB-mediated induction of Cyp1a1.

Lack of SB-mediated Induction of the Cyp1a1 Gene by the AhR Antagonist—

To further confirm the AhR-dependent induction of Cyp1a1 gene by SB, we tested the effect of specific AhR antagonist, α -naphthoflavone (α NF) on SB-mediated induction of the Cyp1a1 activity in both Hepa 1c1c7 and HepG2 cells. For this purpose, the cells were pre-treated for 1 h with α NF before incubation with SB (10 μ M) for additional 24 h, thereafter, Cyp1a1 catalytic activity was determined by EROD assay. Figure 8A showed that treatment of Hepa 1c1c7 cells with α NF inhibited the basal activity of Cyp1a1 by approximately 50% and completely abolished SB-induced Cyp1a1 activity. On the other hand, using human HepG2 cells, α NF alone did not alter basal CYP1A1 activity, however dramatically blocked CYP1A1 induction by SB (Fig. 8B), in a pattern similar to what is observed in Hepa 1c1c7 cells.

Induction of Cyp1a1 Gene by SB is Independent of ERK1, ERK2 or p38 MAPKs

—We took a genetic approach to confirm whether or not MAPKs are involved in the SB-mediated induction of Cyp1a1 mRNA and catalytic activity levels. For this purpose, Hepa 1c1c7 cells were transfected with mouse ERK1, ERK2, p38 or Silencer negative control siRNA for 6 h, and then the cells were treated with SB (10 μ M). To test the selectivity of the siRNAs for ERK1, ERK2, and p38 we determined their mRNA levels in transfected cells. Our results show that ERK1, ERK2, and p38 siRNAs significantly decreased ERK1, ERK2, and p38 mRNA levels by 96%, 90%, and 60%, respectively as compared to control non-transfected cells (Fig. 9). On the other hand, cells transfected with ERK1, ERK2, p38, or silencer select negative control siRNAs, followed with SB treatment showed Cyp1a1 mRNA and catalytic activity levels similar to those of non-transfected cells (Fig. 9). Furthermore, ERK1, ERK2, p38, or silencer select negative control siRNAs did not affect the constitutive levels of Cyp1a1 mRNA and catalytic activity levels, eliminating the possibility that the inhibitory effects of ERK1, ERK2, or p38 siRNAs might have been due to any toxicity.

Activation of AhR Transformation and XRE Binding by SB—The ability of SB to directly interact with the AhR receptor molecule and activate its translocation to the DNA-binding form in the nucleus with the subsequent binding to the XRE was measured by

EMSA. Untreated guinea pig hepatic cytosol was pre-incubated, *in vitro*, for 2 h with SB (100 μ M) or TCDD (20 nM), a positive control for AhR transformation. Figure 10 shows that SB increased the AhR/ARNT/XRE complex formation (lane 2), as demonstrated by the intensity of the shifted band, as compared to control (lane 1), in a manner similar to TCDD effect (lane 3). The specificity of SB-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition assay using anti-ARNT antibody (lane 4) or 100-fold molar excess of unlabeled XRE (lane 5).

SB is a Direct AhR Ligand—To establish that SB is a direct ligand for the AhR, a ligand competition binding assay using hydroxyapatite was performed (Fig. 11). The total binding is the overall binding of [3 H]-TCDD to cytosolic protein. However part of this binding is non-specific, i.e. not through the AhR, or not through ligand-binding center of the AhR. To account for this non-specific binding, reactions are conducted in the presence of 100-fold excess of competitor. TCDF was chosen rather than TCDD because of its higher solubility as TCDD would not be soluble at 200 nM. Therefore, difference between total and non-specific binding is specific binding of [3 H]-TCDD to the AhR. Our results demonstrated that SB at the concentrations of 20 μ M, 100 μ M, and 200 μ M was able to significantly displace [3 H]-TCDD by 17%, 60%, and 80%, respectively (Fig. 11).

Discussion

In the present study we have demonstrated the first evidence that the specific inhibitor of p38 MAPK, SB, is a novel ligand and agonist of AhR and hence inducer of *CYP1A1* gene expression. This is supported by the following findings; a) SB induced murine and human *CYP1A1* gene expression at the transcriptional level as the RNA synthesis inhibitor, Act-D, completely blocked *Cyp1a1* induction by SB; b) The AhR-dependent induction of *Cyp1a1* by SB was evidenced by the blockade effect of AhR-antagonist, α NF, on *Cyp1a1* induction and by the lack of induction in mutant AhR-deficient cells; c) the *Cyp1a1* induction by SB is not a consequence of p38 or ERK inhibition as demonstrated by the lack of p38 and ERK gene silencing to attenuate SB effect; and d) SB as novel ligand and agonist of AhR is evidenced by the ability of SB to directly bind to and activate AhR *in vitro* to its DNA binding form and to displace [3 H]-TCDD specific binding to AhR.

The MAPKs are among the intracellular signaling networks that govern the AhR-CYP1A1 pathway (17). The three best characterized MAPK subfamilies, c-Jun N-Terminal kinase (JNK), ERK1/2, and p38, are the targets of pharmacological and genetic manipulations to uncover their roles. In this context, it has been reported that inhibitors of JNK and ERK MAPKs, SP600125 and U0126, respectively, are partial agonists of the AhR and inducers of CYP1A gene (30, 31). However, information reported in the literature on the effects of p38 MAPK inhibitor, particularly SB, on the constitutive CYP1A1 gene expression in cells from mammalian species remains unclear. In this context, recent work from our laboratory have shown that SB induced the *Cyp1a1* gene expression at the mRNA and activity levels, whereas dramatically inhibited the induction of *Cyp1a1* by TCDD in Hepa 1c1c7 cells (20). However the molecular mechanisms involved remain unknown. Therefore, the main objectives of the current work were to investigate the potentiality of SB to induce CYP1A1 gene expression in Hepa 1c1c7 and HepG2 cells, and to explore the molecular mechanisms involved.

Knowledge of the regulation of *Cyp1a1* gene expression shows that the activation of a cytosolic transcriptional factor, AhR, is the first step in a series of molecular events promoting *Cyp1a1* transcription and translation processes. To examine whether the regulation of *Cyp1a1* gene expression by SB is a transcriptional and/or posttranscriptional event, a series of experiments were carried out. Initially, we demonstrated here that SB

significantly increased the basal Cyp1a1 at the mRNA, protein, and activity levels in a concentration-dependent manner in murine Hepa 1c1c7 cells (Fig. 3). Such effect was not species-specific, in that human CYP1A1 was induced by SB in HepG2 cells (Fig. 4); however the induction capacity in Hepa 1c1c7 cells was higher than in HepG2 cells.

The transcriptional regulation of *Cyp1a1* gene expression by SB, in the current study, was demonstrated first by the ability of the transcription inhibitor, Act-D, to significantly block the newly synthesized Cyp1a1 mRNAs (Fig. 5) suggesting a requirement of *de novo* RNA synthesis for the induction of Cyp1a1 mRNA by SB in a manner similar to that obtained with TCDD (Fig. 4). Second, the increase of luciferase reporter gene expression that occurs only through the AhR activation (Fig. 6) suggests an AhR-dependent transcriptional control and excludes the possibility of any posttranscriptional mechanisms, such as mRNA stability (Pasco et al., 1988).

The direct evidence for the involvement of AhR in the transcriptional regulation of *Cyp1a1* by SB in the current study is strongly supported by several approaches. Firstly, SB induced *Cyp1a1* gene expression in Hepa 1c1c7 (WT) but not in the mutant AhR-deficient cells (C12) (Fig. 7). Of interest, the slight increase in Cyp1a1 mRNA observed in response to TCDD treatment (3-fold) could be attributed to the fact that C12 cells still exhibit a 5–10% AhR activity as reported previously (32–34). Secondly, the ability of the specific AhR antagonist, α NF, that is known to directly bind to the AhR receptor and elicit a protein conformation that has very low affinity for DNA (35), to completely block the SB-mediated induction of Cyp1a1 mRNA and catalytic activity (Fig. 8). Taken together, these observations strongly suggest the requirement of AhR in the SB-mediated induction.

At this stage, we suspect that inhibition of p38, ERK1, or ERK2 MAPK signaling pathways are directly or indirectly involved in Cyp1a1 modulation by SB. However, lack of influence of knocking down ERK1, ERK2, or p38 genes on the induction of Cyp1a1 mRNA and activity levels by SB excludes any role of these MAPKs in the induction of Cyp1a1 by SB (Fig. 9). This raises the question of whether or not SB is a ligand and agonist for the AhR. Therefore, we examined the ability of SB to directly bind to and activate AhR protein using EMSA. In the current study we have shown that SB was able to directly bind to and induce transformation of cytosolic AhR to its DNA-binding form *in vitro* (Fig. 10), which is extensively used to assess binding and affinity of ligands to the AhR (36). Perhaps the most interesting part in the current study was the ability of SB to displace [3 H]TCDD as assessed by ligand binding assay using hydroxyapatite method. These results indicate that SB is a novel AhR ligand that induces dissociation of the AhR from its HSP90 complex, translocation to the nucleus, and binding to the XRE, in a manner similar to that observed with the classical AhR ligand, TCDD (Fig. 11). In addition, activating and transforming AhR by SB into its DNA-binding form *in vitro* (Fig. 10) to a greater extent than its ability to induce the AhR-dependent gene expression in intact cells (Fig. 6) could possibly suggest the involvement of coactivator/corepressor initiation of gene transcription (36). Taken together these results not only suggest the AhR-dependent induction of CYP1A1 by SB, but also exclude, a possible role of p38 MAPK signaling pathway inhibition in the SB-mediated effect.

In conclusion, data presented in the current manuscript clearly demonstrated that SB is a novel AhR ligand that can directly activate the AhR and modulate the *Cyp1a1* gene expression at the transcriptional level through AhR-dependent and MAPK-independent mechanisms.

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List of Abbreviations

AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
SB	SB203580
Cyp1a1	cytochrome P450 1a1
DMSO	dimethylsulfoxide
EMSA	electrophoretic mobility shift assay
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
MAPK	mitogen-activated protein kinase
SEM	Standard Error of Mean
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
XRE	xenobiotic responsive element

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Fig. 1. Chemical structure of SB
4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole.

Fig. 2. Effect of SB on cell viability

Hepa 1c1c7 WT cells were treated for 24 h with various concentrations of SB (1, 5, 10, 20, and 40 μM). Cell viability was determined using MTT assay. Values are presented as % of the control (mean ± SEM, n=6). * $p < 0.05$ compared to control (0 μM).

Fig. 3. Effects of SB on Cyp1a1 mRNA (A), protein (B), and activity (C) levels in Hepa 1c1c7 cells

(A) Hepa 1c1c7 cells were treated for 6 h with various concentrations of SB (0, 1, 5, 10, and 20 μ M) or TCDD (1 nM) as a positive control. The amount of Cyp1a1 mRNA was quantified using RT-PCR and normalized to β -actin housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change $\pm$ SEM. (n = 4). +p < 0.05 compared with control (0 μ M). (B) Hepa 1c1c7 cells were treated for 24 h with the same concentrations of SB and TCDD, thereafter Cyp1a1 protein level was determined by Western blot analysis. CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of Cyp1a1 protein bands was quantified relative

to the signals obtained for Gapdh protein, using ImageJ®. One of the three representative experiments is shown. (C) Hepa 1c1c7 cells were treated for 24 h with the same concentrations of SB and TCDD. Cyp1a1 enzyme activity was measured in intact living cells using 7ER as a substrate. Values are presented as mean $\pm$ SEM, n=8. [†] $p<0.05$ compared to control (0 μ M).

Fig. 4. Effects of SB on CYP1A1 mRNA (A) and activity (B) levels in HepG2 cells
(A) HepG2 cells were treated for 6 h with SB (10 μM). Thereafter the amount of CYP1A1 mRNA was quantified using RT-PCR and normalized to β-ACTIN housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change ± SEM. (n = 4). +*p* < 0.05 compared with control. (B) HepG2 cells were treated for 24 h with the SB (10 μM), thereafter, CYP1A1 enzyme activity was measured in intact living cells using 7ER as a substrate. Values are presented as mean ± SEM, n=8. +*p* < 0.05 compared to control.

Fig. 5. Effects of RNA synthesis inhibitor Act-D on the induction of Cyp1a1 mRNA by SB
Hepa 1c1c7 cells were treated with 5 µg/ml Act-D, a RNA synthesis inhibitor, 30 min before exposure to either 10 µM SB or 1 nM TCDD for additional 6 h. The amount of Cyp1a1 mRNA was quantified using RT-PCR and normalized to β-actin housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change ± SEM.. (n = 4). +*p* < 0.05 compared with control; **p* < 0.05 compared to same treatment in the absence of Act-D.

Fig. 6. Effect of SB on XRE luciferase activity

Hepa 1c1c7 cells transiently transfected with XRE luciferase reporter gene were grown onto 12-well cell culture plates for 24 h. Thereafter, cells were incubated with increasing concentrations of SB (0, 1, 5, 10, and 20 μ M) or TCDD (1 nM) for an additional 12 h. Cells were lysed and luciferase activity was measured according to the manufacturer's instructions. The graph represents the mean $\pm$ SEM ($n = 4$). + $p < 0.05$ compared with control (0 μ M).

Fig. 7. Effects of SB on Cyp1a1 mRNA (A) and activity (B) levels in AhR-deficient C12 cells
(A) C12 cells were treated for 6 h with various concentrations of SB (0, 1, 5, 10, and 20 μ M) and TCDD (1 nM) as a positive control. The amount of Cyp1a1 mRNA was quantified using RT-PCR and normalized to β -actin housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change $\pm$ SEM. (n = 4). $^{+}p < 0.05$ compared with control (0 μ M). (B) C12 cells were treated for 24 h with the same concentrations of SB and TCDD. Cyp1a1 enzyme activity was measured in intact living cells using 7ER as a substrate. Values are presented as mean $\pm$ SEM, n=8. $^{+}p < 0.05$ compared to control (0 μ M).

Fig. 8. Effects of the AhR antagonist α NF on the induction of Cyp1a1 activity by SB in Hepa 1c1c7 (A) and HepG2 (B) cells

(A) Hepa 1c1c7 and (B) HepG2 cells were pretreated for 2 h with AhR antagonist, α NF, before co-treated with SB (10 μ M) for additional 24 h. The Cyp1a1 enzyme activity was measured in intact living cells using 7ER as a substrate. Values are presented as mean $\pm$ SEM, n=8. $^+p<0.05$ compared to control.

Fig. 9. Effects of ERK1, ERK2, and p38 siRNAs on ERK1, ERK2, and p38 mRNAs (A) the induction of Cyp1a1 mRNA (B) and catalytic activity (C) levels by SB in Hepa 1c1c7 cells (A and B) Hepa 1c1c7 cells were transiently transfected with 20 nM ERK1, ERK2, p38, or Silencer select negative control siRNAs (-ve siRNA) for 6 h, thereafter cells were treated with vehicle or SB (10 μ M) for another 6 h for mRNA. **(A) ERK1, ERK2, and p38 and (B) Cyp1a1 mRNAs** were quantified using RT-PCR and normalized to β -ACTIN housekeeping gene. Duplicate reactions were performed for each experiment and the values represent mean of fold change $\pm$ SEM. ($n = 4$). $*p < 0.05$ compared with control. **(C)**, Hepa 1c1c7 cells were transiently transfected with 20 nM ERK1, ERK2, p38, or Silencer select negative control siRNAs (-ve siRNA) for 6 h, thereafter cells were treated with vehicle or SB (10 μ M) for additional 24 h. Cyp1a1 activity was measured in intact living cells using 7ER as a substrate. Values are presented as mean $\pm$ SEM ($n = 8$). $*P < 0.05$, compared to corresponding siRNA alone.

Fig. 10. Effect of SB on AhR/ARNT/XRE binding

Cytosolic extracts (2 mg) from untreated guinea pig liver were incubated with DMSO, SB (100 μ M), or TCDD (20 nM) for 2 h. The cytosolic proteins were mixed with [γ - 32 P]-labeled XRE and the formation of AhR/ARNT/XRE complexes were analyzed by EMSA. The specificity of binding was determined by incubating TCDD-treated cytosolic extracts with 100-fold molar excess of cold XRE or anti-ARNT antibody. AhR/ARNT/XRE complex formed on the gel was visualized by autoradiography. This pattern of AhR activation was observed in three separate experiments, and only one is shown.

Fig. 11. SB is an AhR ligand

Untreated guinea pig hepatic cytosol (2 mg/ml) was incubated with 2 nM [³H]-TCDD alone (total binding), 2 nM [³H]-TCDD and 200 nM TCDF (100 fold excess of competitor) (non-specific binding), or 2 nM [³H]-TCDD in the presence of increasing concentrations of SB (20 μM, 100 μM, 200 μM) and the samples analyzed by the hydroxyapatite assay as described under Materials and Methods. Values were adjusted for non-specific binding and expressed as % specific binding relative to the absence of a competitor ligand. Values are presented as mean ± SEM, n=9. **p*<0.05 compared to [³H]-TCDD.