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MOLECULAR TOXICOLOGY

# Sunitinib, a tyrosine kinase inhibitor, induces cytochrome P450 1A1 gene in human breast cancer MCF7 cells through ligand-independent aryl hydrocarbon receptor activation

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**Abstract** Sunitinib (SUN) is a new multi-targeted oral tyrosine kinase inhibitor that has both anti-angiogenic and anti-tumor activities. However, information reported in the literature on the effects of SUN on the constitutive expression of cytochrome P450 1A1 (*CYP1A1*) gene in cells from mammalian species remains unclear. Therefore, the main objectives of the current work were to investigate the potentiality of SUN to induce *CYP1A1* gene expression in human breast cancer MCF7 cells and to explore the molecular mechanisms involved. Our results showed that SUN induced the CYP1A1 mRNA, protein, and activity levels in a concentration-dependent manner in MCF7 cells. The increase in CYP1A1 mRNA by SUN was completely blocked by the transcriptional inhibitor, actinomycin D; implying that SUN increased de novo RNA synthesis. Furthermore, the ability of SUN to increase luciferase reporter gene expression suggests an aryl hydrocarbon receptor (AhR)-dependent transcriptional control and excludes the possibility of any posttranscriptional mechanisms. In addition, blocking of AhR activation by resveratrol, a well-known AhR antagonist, prevented the SUN-induced *CYP1A1* gene expression, further confirms the involvement of AhR. Interestingly, this was associated with the inability of SUN to directly bind to and induce transformation of cytosolic AhR to its DNA-binding form in vitro, suggesting that the effect of SUN does not involve direct binding to AhR. The current manuscript provides the first evidence for the ability of SUN to induce *CYP1A1* gene expression in MCF7 cells through AhR ligand-independent mechanisms.

**Keywords** Sunitinib · AhR · CYP1A1 · MCF7 · XRE

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## Introduction

The cytochrome P450 1A1 (CYP1A1) is a monooxygenase enzyme that is involved in a number of cellular functions such as metabolism of xenobiotics (Walisser et al. 2005). CYP1A1 has been shown to be responsible for the bioactivation of a variety of environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs) to epoxide and diol-epoxide intermediates (Shimada and Fujii-Kuriyama 2004). 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, Fig. 1), AhR dissociates from its inhibitory proteins (Denison et al. 1986; Sogawa and Fujii-Kuriyama 1997) allowing it to translocate to the nucleus, where it heterodimerizes with a nuclear transcription factor protein called the AhR nuclear translocator (ARNT) (Whitelaw et al. 1994). 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-regulated genes, including *CYP1A1* (Denison et al. 1989; Korashy and El-Kadi 2006; Nebert et al. 2004).

The AhR has been identified as a target of several signaling pathways that cross-talk with its own regulatory pathway, such as proteasomal degradation (Pollenz

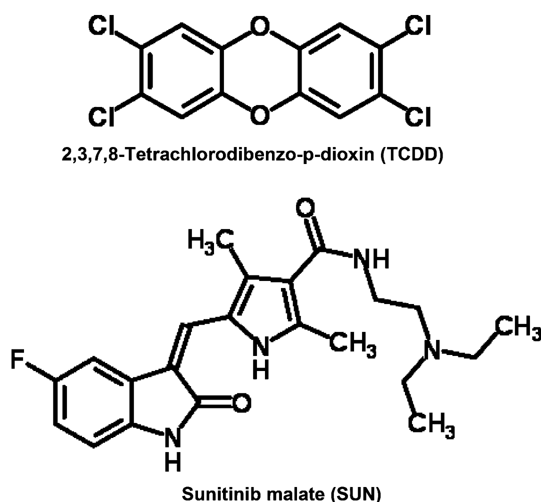


**Fig. 1** Chemical structures of TCDD and SUN

and Buggy 2006), redox-sensitive transcription factors (Zordoky and El-Kadi 2009), and the mitogen-activated protein kinases (Henklova et al. 2008). Among these signaling pathways, tyrosine kinase receptors, which play an important role in tumor growth, pathologic angiogenesis, and metastatic progression of cancer through the phosphorylation of target proteins (Lemmon and Schlessinger 2010), have been shown to differentially modulate the expression of CYP1A1 and AhR activation. In this regard, it has been reported that genistein, a tyrosine kinase inhibitor (TKI), potentiated the induction of CYP1A1 by TCDD but inhibited omeprazole-dependent CYP1A1 induction in rat hepatoma H4IIE cells (Backlund et al. 1997). In addition, Matsuoka-Kawano and co-worker have demonstrated the ability of TSU-16, a TKI, to induce human CYP1A1 and CYP1A2 through the activation of AhR (Matsuoka-Kawano et al. 2010). Interestingly, recent study has demonstrated that gefitinib, a clinically used TKI, induces the CYP1A1 mRNA and activity in epidermal growth factor receptor (EGFR)-wild type non-small cell lung cancer cell lines (Alfieri et al. 2011).

Among TKIs, sunitinib malate (SUN, Fig. 1) is a new multi-targeted oral anti-angiogenic and anti-tumor drug that has been recently approved against gastrointestinal stromal tumors and advanced renal cell carcinoma (Kassem et al. 2012). Although SUN is structurally similar to gefetinib, very little information has been reported in the literature on the effects of SUN on the constitutive *CYP1A1* gene expression in cells from mammalian species. Therefore, the present study was designed to investigate the capacity of SUN to induce *CYP1A1* gene expression in MCF7 cells and explore the molecular mechanisms involved. The current manuscript provides the first evidence for the ability of SUN to induce *CYP1A1* gene expression in MCF7 cells through AhR ligand-independent mechanisms.

## Materials and methods

### Materials

Sunitinib malate ((Z)-*N*-(2-(diethylamino) ethyl)-5-((5-fluoro-2-oxoindolin-3-ylidene) methyl)-2, 4-dimethyl-1H-pyrrole-3-carboxamide) was obtained from LC Laboratories (Woburn, MA). 7-Ethoxyresorufin, Dulbecco's Modified Eagle's Medium (DMEM), anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, 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). Amphotericin B and resorufin were purchased from ICN Biomedicals Canada (Montreal, QC). TRIzol reagent, T4 polynucleotide kinase, 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 Biosystems (Foster city, CA). Actinomycin D (Act-D) was purchased from Calbiochem (San Diego, CA). Nitrocellulose membrane was obtained 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). [$\gamma$-$^{32}$P]ATP was supplied by Perkin Elmer (Boston, MA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

### Cell culture and treatments

Human breast cancer MCF7 cells (American Type Cell Cutler, Manassas, VA) were maintained in DMEM, with phenol red supplemented with 10 % fetal bovine serum, 20 μM L-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin G, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B. Cells were grown in 75 $cm^2$ tissue culture flasks at 37 °C under a 5 % $CO_2$ humidified environment.

The cells were seeded onto 96-, 12-, and 6-well cell culture plates in DMEM culture media for CYP1A1 enzyme activity, mRNA, and protein assays, respectively. In all experiments, the cells were treated for the indicated time intervals in serum-free media with various concentrations of SUN as indicated. Stock solutions of SUN were prepared in DMSO and stored at −20 °C. In all treatments, the DMSO concentration did not exceed 0.05 % (v/v).

### Cytotoxicity of SUN

The effect of SUN on MCF7 cell viability was determined by measuring the capacity of reducing enzymes present in

only viable cells to convert MTT to colored formazan crystals as described previously (Korashy et al. 2011). Briefly, MCF7 cells were treated for 24 h with various concentrations of SUN; 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 using the following formula: cell viability = $(A_{\text{treated}})/(A_{\text{control}}) \times 100$ %.

### Total 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 and as described previously (Korashy et al. 2011). Briefly, 1.5 µg of total RNA from each sample was added to a mixture of 2.0 µl of 10× reverse transcriptase buffer, 0.8 µl of 25× dNTP mix (100 mM), 2.0 µl of 10× 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 Fast RT-PCR 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. Human primers 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′) (Korashy and El-Kadi 2012) were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of *CYP1A1* gene between treated and untreated cells was 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., ΔΔ CT) method, as described and explained previously (Livak and Schmittgen 2001). 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 target genes between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(\text{target})} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(\text{treated})} - \Delta Ct_{(\text{untreated})}$.

### Protein extraction and western blot analysis

Twenty-four hours after incubation with the test compound, approximately $1.5 \times 10^6$ 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, 1 mM EDTA, 10 % glycerol (v/v), 1 % Triton X-100, and 5 µl/ml of protease inhibitor cocktail). 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 (Korashy and El-Kadi 2004). 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 [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 (7ER) O-deethylase (EROD) activity was performed on intact living MCF7

cells using 7ER as a substrate (Kennedy et al. 1993). After incubation of the cells with increasing concentrations of SUN for 24 h, 100 µl of 2 µM 7ER in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8) was then added to each well. Immediately, an initial fluorescence measurement ($t = 0$) at excitation/emission (545 nm/575 nm) followed by additional set of fluorescence measurements of the wells was recorded every 5 min for 20 min interval using Baxter 96-well fluorometer (Deerfield, IL). The amount of resorufin formed in each well was determined by comparison with a standard curve of known concentrations and normalized to protein levels determined using a modified fluorescent assay (Lorenzen and Kennedy 1993). The rate of resorufin formation was expressed as percentage of control.

### Transient transfection and luciferase assay

MCF7 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 µg of the XRE-driven luciferase reporter plasmid pGudLuc 1.1 (Nagy et al. 2002), generously provided by Dr. M.S. Denison (University of California at Davis), using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen®). Luciferase assay was performed according to the manufacturer's instructions (Promega®) as described previously (Korashy et al. 2007). 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 phosphate-buffered saline; 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.

### Electrophoretic mobility shift assay (EMSA)

XRE complementary oligonucleotides, 5′-GGAGTTGCGT GAGAAGAGCC-3′ and 5′- GGCTCTTCTCACGCAACT CC-3′, were synthesized, annealed, and labeled with $\gamma$-$^{32}$P-ATP at the 5-end using T4 polynucleotide kinase and used as a probe for EMSA reactions as described previously (Denison et al. 1989; Korashy et al. 2007). Aliquots of the guinea pig liver cytosolic protein (2 mg) 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 (vol/vol), and 400 ng 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 % non-denaturing PAGE. For the competition assay, proteins were preincubated at room temperature for 20 min with either 0.6 µg anti-ARNT antibody (Santa Cruz Biotechnology, Inc.) or a 100-fold molar excess of unlabeled (cold) XRE before the addition of the [$^{32}$P]-labeled XRE. The gel was dried at 80 °C for 1 h, and AhR-XRE complexes formed were visualized by autoradiography (El Gendy and El-Kadi 2010; Gharavi and El-Kadi 2005).

### 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 SUN on MCF7 cells viability and proliferation

To determine the maximum non-toxic concentrations of SUN to be utilized in the current study, MCF7 cells were exposed for 24 h to increasing concentrations of SUN (0, 1, 2.5, 5, 10, and 20 µM) using MTT assay. Our results showed that all concentrations ranging from 1 to 5 µM did not significantly affect cell viability (Fig. 2). However, SUN 10 and 20 µM significantly decreased the cell viability to approximately 80 and 30 %, respectively. Based

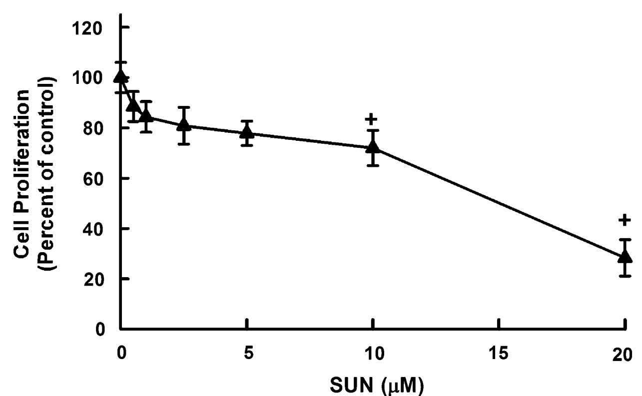


**Fig. 2** Effect of SUN on MCF7 cell viability. MCF7 cells were treated for 24 h with various concentrations of SUN (0, 1, 2.5, 5, 10, and 20 µM). Cell viability was determined using MTT assay. Values are presented as % of the control (mean ± SEM, $n = 6$). $^{+}p<0.05$ compared to control (0 µM)

on these findings, SUN concentrations 2.5, 5, and 10 μM were utilized in all subsequent experiments in MCF7 cells (Fig. 2).

## Induction of *CYP1A1* gene expression by SUN in MCF7 cells

To determine the capacity of SUN to alter the expression of *CYP1A1* gene, MCF7 cells were incubated for 6 h with increasing concentrations of SUN (2.5, 5, and 10 μM); thereafter, CYP1A1 mRNA levels were determined by RT-PCR. Figure 3a shows that SUN-induced CYP1A1 mRNA expression in a concentration-dependent manner. The submaximal induction was achieved at a concentration 5 μM (3-fold), whereas the highest concentrations tested, 10 μM, increased CYP1A1 mRNA by approximately 5-fold (Fig. 3a).

To further examine whether the induction of CYP1A1 mRNA in MCF7 cells in response to SUN treatment is translated into functional protein and catalytic activity, MCF7 cells were treated for 24 h with the same concentrations of SUN; thereafter, CYP1A1 protein and catalytic activity were determined by Western blot analysis and EROD assay, respectively. Figure 3b, c show that SUN induced CYP1A1 protein and catalytic activity in a concentration-dependent manner in a pattern similar to what was observed with mRNA. The maximal inductions of CYP1A1 protein and activity observed at 10 μM were approximately 5.5- and 2.5-fold, respectively.

## Transcriptional induction of *CYP1A1* gene by SUN in MCF7 cells

To explore the molecular mechanisms involved in the induction of CYP1A1 by SUN, initially we questioned whether the induction of human CYP1A1 by SUN (Fig. 3) is regulated at the transcriptional level; we tested the hypothesis that SUN increases the de novo CYP1A1 RNA synthesis. For this purpose, MCF7 cells were treated for 6 h with a single concentration of SUN (10 μM), which showed maximal induction, in the presence and absence of 5 μg/ml Act-D, a RNA synthesis inhibitor. Thereafter, CYP1A1 mRNA expression was determined by RT-PCR. If SUN 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 4 shows that pretreatment of the cells with Act-D alone completely blocked the constitutive expression of CYP1A1 mRNA. Importantly, the SUN-induced CYP1A1 mRNA level was markedly blocked by the RNA synthesis

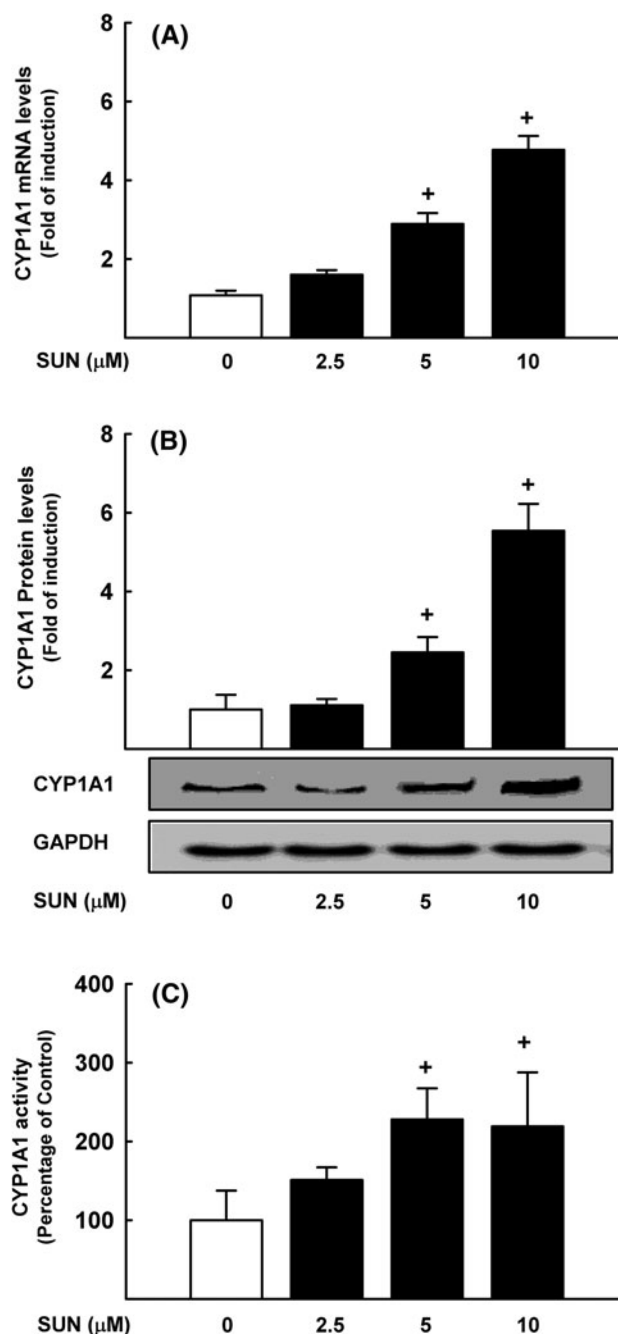


**Fig. 3** Effects of SUN on CYP1A1 mRNA (**a**), protein (**b**), and activity (**c**) levels in MCF7 cells. **a** MCF7 cells were treated for 6 h with various concentrations of SUN (0, 2.5, 5 and 10 μM). Total RNA was isolated using TRIzol reagent, and CYP1A1 mRNA was quantified by RT-PCR. Duplicate reactions were performed for each experiment, and the values represent mean of fold change ± SEM. ($n = 6$). **b** MCF7 cells were treated for 24 h with the same concentrations of SUN; thereafter, CYP1A1 protein level was determined by Western blot analysis. One of the three representative experiments is shown. **c** MCF7 cells were treated for 24 h with the same concentrations of SUN. 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 (0 μM)

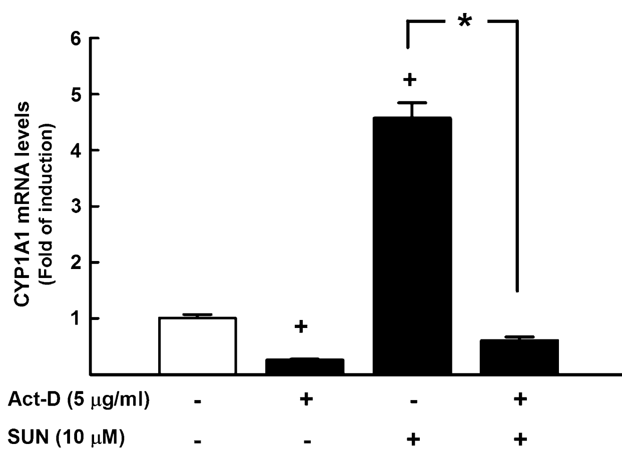


**Fig. 4** Effects of RNA synthesis inhibitor Act-D on the induction of CYP1A1 mRNA by SUN. MCF7 cells were treated with 5 μg/ml Act-D, a RNA synthesis inhibitor, 30 min before co-exposure to 10 μM SUN for additional 6 h. Total RNA was isolated using TRIzol reagent, and the amount of CYP1A1 mRNA was 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. ($n = 6$). $^{+}p < 0.05$ compared with control; $^{*}p < 0.05$ compared to same treatment in the absence of Act-D

inhibitor, Act-D, indicating that SUN increased the CYP1A1 mRNA content at the transcriptional level by increasing its de novo RNA synthesis.

### AhR-dependent induction of CYP1A1 by SUN

The possibility that SUN 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 SUN in MCF7 cells*

First, we questioned whether the induction of CYP1A1 mRNA by SUN is attributed to an increase in the expression of AhR-dependent reporter gene. Therefore, MCF7 cells, transiently transfected with the XRE-driven luciferase reporter gene, were incubated for 18 h with SUN (10 μM). Figure 5a shows that treatment of MCF7 cells with SUN significantly induced (2-fold) the reporter gene which is known to occur only through the AhR activation.

#### *Blocking of the SUN-mediated induction of CYP1A1 mRNA by the AhR antagonist resveratrol*

To further confirm the AhR-dependent induction of the *CYP1A1* gene by SUN, we tested the effect of the AhR antagonist, resveratrol (RES) (Beedanagari et al. 2009; Imig et al. 2002), on SUN-induced CYP1A1 mRNA. For this purpose, MCF7 cells were treated with 20 μM RES in the presence and absence of 10 μM SUN for an additional 6 h. Thereafter, CYP1A1 mRNA expression was quantified by RT-PCR. Figure 5b shows that induction of CYP1A1 mRNA in response to 10 μM SUN was completely prevented by RES, suggesting that AhR is essential for SUN-mediated induction of CYP1A1.

### Ligand-independent activation of AhR

The ability of SUN to directly interact with AhR molecule and activate its translocation to the DNA-binding form in the nucleus with the subsequent binding to the XRE was determined by EMSA. Untreated guinea pig hepatic cytosol was preincubated, in vitro, for 2 h with SUN (50 μM) or TCDD (20 nM), a positive control for AhR transformation. Figure 5c shows that TCDD markedly induced the AhR/ARNT/XRE complex formation (lane 3) as compared to control (lane 1). However, SUN was not able to activate the AhR/ARNT/XRE complex formation (lane 2). The specificity of AhR/ARNT heterodimer binding to XRE was confirmed by the competition assay using anti-ARNT antibody, supershift (lane 4) and a 100-fold molar excess of unlabeled XRE (lane 5).

## Discussion

The present study demonstrates the first evidence that tyrosine kinase inhibitor, SUN, induces *CYP1A1* gene expression at the transcriptional levels in human breast cancer MCF7 cells through ligand-independent AhR activation. This is supported by the following findings; (a) induction of *CYP1A1* gene by SUN at the mRNA, protein, and activity levels in a concentration-dependent manner; (b) blocking of the SUN-induced CYP1A1 expression by the RNA synthesis inhibitor, Act-D; (c) increase XRE luciferase reporter gene expression; (d) inhibition of SUN-induced CYP1A1 mRNA by the AhR antagonist, RES; and (e) inability of SUN to bind to and activate AhR.

Recent findings have demonstrated that activation of AhR and induction of CYP1A1 are not just restricted to PAHs (the classical ligands) and that a large number of newly identified CYP1A1 inducers whose structures and physiochemical properties significantly differ from those of PAHs have been previously reported (Gharavi and El-Kadi 2005; Seidel et al. 2000). 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 (Lemaire et al. 2004), primaquine

(Werlinder et al. 2001), and sulindac (Ciolino et al. 2006). Prior to commencing the current experiments, the effects of SUN on the CYP1A1 expression from mammalian species were not known. Hence, the objective of the current study was to evaluate the effect of SUN on the modulation of CYP1A1 in vitro using human breast cancer MCF7 cell lines.

The in vitro MCF7 cell model was utilized in the current study for several reasons. First, MCF7 cells are extensively used as an in vitro model for investigating the AhR activation and CYP1A1 induction by several xenobiotics. This is because of MCF7 cells showed higher CYP1A1 expression levels in response to TCDD and other PAHs in comparison with human hepatocellular carcinoma HepG2 cells, a well-studied in vitro model for AhR activation (Jorgensen and Autrup 1996; Skupinska et al. 2009). Second, the presence of a cross-talk between AhR and estrogen receptor (ER), which is only expressed in MCF7 cells, in that pretreatment of MCF7 cells with TCDD caused a rapid decrease in nuclear ER binding activity and immunoreactive protein (Labrecque et al. 2012; Liu et al. 2006). Third, several recent studies have demonstrated a possible chemotherapeutic role of SUN against breast cancer (Fratto et al. 2011; Yardley et al. 2012). The in vitro concentrations of SUN used in the current study were maintained within the therapeutic range of plasma concentration reported in human. For example, human subjects given 80 mg SUN for the treatment of advanced renal cell carcinoma had mean plasma concentrations range from 0.5 to 1 μM (Hasinoff et al. 2008). In addition, in vitro study on primary neonatal rat cardiomyocytes to determine the cardiotoxicity of SUN has utilized 1 and 4 μM (French et al. 2010). Moreover, the chronic use of SUN, its prolonged half-life, and its high tissue distribution expected with repeated dosing each collectively provides a high degree of in vivo relevance to the results arising from the concentrations of SUN (1–5 μM) used in the presently described in vitro experiments.

Initially, we demonstrated here that SUN significantly increased the basal CYP1A1 expression at the mRNA, protein, and activity levels in a concentration-dependent manner in MCF7 (Fig. 3). The current knowledge of the

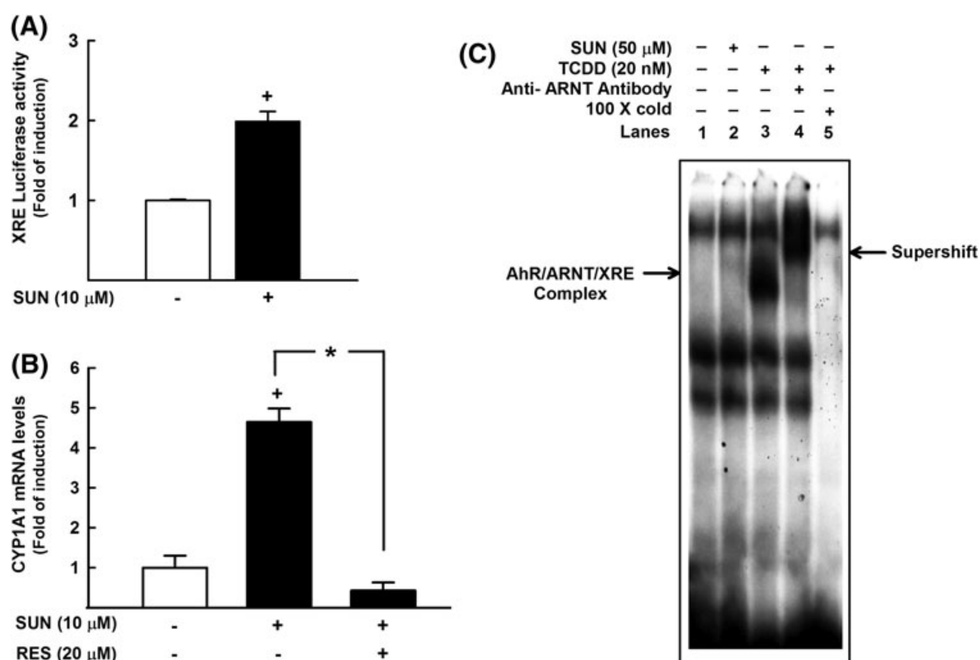


**Fig. 5** Effect of SUN on XRE luciferase activity (**a**), the AhR antagonist RES (**b**), and AhR/ARNT/XRE binding (**c**). **a** MCF7 cells transiently transfected with XRE luciferase reporter gene were grown onto 12-well cell culture plates for 24 h. Thereafter, cells were incubated with increasing SUN (10 μM) for an additional 12 h. Cells were lysed and luciferase activity was measured according to the manufacturer's instructions. The graph represents the mean ± SEM ($n = 4$). $^{+}p < 0.05$ compared to control (0 μM). **b** MCF7 cells were pretreated for 2 h with 20 μM RES prior to co-exposure with 10 μM SUN for additional 6 h. Total RNA was isolated using TRIzol reagent, and 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 ($n = 6$). $^{+}p < 0.05$ compared with control; $^{*}p < 0.05$ compared to same treatment in the absence of RES. **c** Cytosolic extracts (2 mg) from untreated guinea pig liver were incubated in vitro with SUN (50 μM, *lane* 2) or TCDD (20 nM, *lane* 3) 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 anti-ARNT antibody (*lane* 4) and 100-fold molar excess of cold XRE (*lane* 5). 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

mechanism of CYP1A1 induction by PAHs such as TCDD, the most potent CYP1A1 inducer tested to date, clearly suggests a transcriptional regulation, in which activation of a cytosolic transcriptional factor, AhR, is the first step in a series of molecular events promoting CYP1A1 expression. The transcriptional regulation of *CYP1A1* gene expression by SUN, in the current study, was demonstrated first by the ability of the transcription inhibitor, Act-D, to significantly block the newly synthesized CYP1A1 mRNA (Fig. 4) suggesting a requirement of de novo RNA synthesis for the induction of CYP1A1 mRNA by SUN (Fig. 4).

The AhR involvement in the SUN-mediated induction of CYP1A1 in MCF7 cells was evidenced first by the ability of SUN to increase XRE-dependent luciferase reporter gene expression that occurs only through the AhR activation (Fig. 5a) suggests an AhR-dependent transcriptional control and excludes the possibility of any post-transcriptional mechanisms, such as mRNA stability (Pasco et al. 1988). Second, blocking of the SUN-induced *CYP1A1* gene expression by RES, a naturally occurring polyphenolic compound, that has been shown to block AhR and inhibit the expression of several CYP enzymes particularly CYP1A1 (Beedanagari et al. 2009; Imig et al. 2002), suggests a direct role of AhR in the expression of *CYP1A1* gene (Fig. 5b).

In light of the information described above, our results suggested a direct evidence for the involvement of AhR in the transcriptional regulation of *CYP1A1* by SUN. This raises the question of whether or not SUN is a ligand and agonist for the AhR. Therefore, we examined ability of SUN to directly bind to and activate AhR protein using EMSA in guinea pig cytosol model, which showed the greatest degree of AhR transformation in response to AhR ligand (Bohonowych and Denison 2007), an assay which is extensively used to assess binding and affinity of ligands to the AhR (Jeuken et al. 2003). Perhaps the finding of greatest interest in the current study was the observation that SUN did not directly bind to and induce transformation of cytosolic AhR to a DNA-binding form in vitro (Fig. 5c), a property exerted by traditional AhR ligands, implying that SUN is not an AhR ligand. Importantly, the inability of SUN to directly bind to and induce transformation of cytosolic AhR to its DNA-binding form in vitro was associated with an increase in luciferase reporter gene expression that occurs only through AhR activation, which suggests that AhR shuttles between the cytosol and nucleus in the absence of exogenous ligand. Taken together, these results strongly suggest a ligand-independent AhR activation process.

Ligand-independent AhR activation has been reported by several drugs and chemicals. For example, omeprazole has been shown to induce the AhR-dependent gene expression such as CYP1A1 without direct binding to the AhR (Backlund and Ingelman-Sundberg 2004; Lemaire et al. 2004). In addition, benzimidazole derivatives are potent inducers of CYP1A1 in rabbit and human hepatocytes, but apparently do not bind the AhR (Lesca et al. 1995). Although the exact mechanisms governing the ligand-independent activation of AhR are still not clear, it has been suggested that metabolic activation of these compounds into AhR ligands or their abilities to stimulate endogenous AhR ligand could play a role (Heath-Pagliuso et al. 1998; Schaldach et al. 1999; Sinal and Bend 1997). Moreover, it has been reported that activation of the cyclic adenosine monophosphate (cAMP) mediator (Oesch-Bartlomowicz et al. 2005) or mitogen-activated protein kinases (MAPKs) signaling pathways (Ikuta et al. 2004) increases AhR translocation in a manner somehow similar to, but functionally different from, TCDD-mediated mechanisms. Another situation in which CYP1A1 can be induced in the absence of ligand is through oxidative stress-mediated effects or induction of cell differentiation that parallels an increase in the AhR transcript (Delescluse et al. 2000).

In conclusion, the current manuscript provides the first evidence for the ability of SUN to induce *CYP1A1* gene expression in MCF7 cell line at the transcriptional level through AhR ligand-independent mechanisms.

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