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JNK inhibitor SP600125 is a partial agonist of human aryl hydrocarbon receptor and induces CYP1A1 and CYP1A2 genes in primary human hepatocytes

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ABSTRACT

SP600125, a specific inhibitor of c-Jun-N-Terminal kinase (JNK), was reported as a ligand and antagonist of aryl hydrocarbon receptor (AhR) [Joiakim A, Mathieu PA, Palermo C, Gasiewicz TA, Reiners Jr JJ. The Jun N terminal kinase inhibitor SP600125 is a ligand and antagonist of the aryl hydrocarbon receptor. Drug Metab Dispos 2003;31(11):1279–82]. Here we show that SP600125 is not an antagonist but a partial agonist of human AhR.

SP600125 significantly induced CYP1A1 and CYP1A2 mRNAs in primary human hepatocytes and CYP1A1 mRNA in human hepatoma cells HepG2. This effect was abolished by resveratrol, an antagonist of AhR. Consistent with the recent report, SP600125 dose-dependently inhibited CYP1A1 and CYP1A2 genes induction by a prototype AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in human hepatocytes. Moreover, SP600125 displayed typical behavior of a partial agonist in HepG2 cells transiently transfected with a reporter plasmid containing two inverted repeats of the dioxin responsive element or with a plasmid containing 5′-flanking region of human CYP1A1 gene. SP600125 transactivated the reporter plasmids with EC_{50} of 0.005 and 1.89 μM, respectively. On the other hand, TCDD-dependent transactivation of the reporter plasmids was inhibited by SP600125 with IC_{50} values of 1.54 and 2.63 μM, respectively. We also tested, whether the effects of SP600125 are due to metabolism. Using liquid chromatography/mass spectrometry approach, we observed formation of two minor monohydroxylated metabolites of SP600125 in human hepatocytes,

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Abbreviations: AhR, aryl hydrocarbon receptor; CYP1A1/2, cytochrome P450 isoform 1A1/2; HepG2, human hepatoma cells; JNK, c-Jun-N-terminal kinase; RVT, resveratrol—an antagonist of AhR; SP600125, 1,9-pyrazoloanthrone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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human liver microsomes but not in HepG2 cells. These data imply that biotransformation is not responsible for the effects of SP600125 on AhR signaling.

In conclusion, we demonstrate that SP600125 is a partial agonist of human AhR, which induces CYP1A genes.

1. Introduction

c-Jun-N-terminal kinase (JNK) is an important enzyme involved in cellular signaling, apoptosis, carcinogenesis and in pathogenesis of diabetes [1]. Significant progress in the research of JNK biochemistry has been achieved due to discovery of its specific inhibitor 1,9-pyrazoloanthrone [2], which is often referred to as SP600125 (for structure see Fig. 1). Recently, Joiakim et al. reported that SP600125 is also an antagonist of aryl hydrocarbon receptor (AhR) in MCF10A human breast cancer cells and in murine hepatoma cells Hepa 1c1c7 [3]. AhR is a ligand-activated transcriptional factor belonging to the family of basic-helix–loop–helix transcription factors, which plays a key role in drug metabolism [4], chemically induced carcinogenesis [5], development, differentiation and many other essential physiological functions [6]. The inactivated form of the receptor exists in the cytoplasm in a complex with chaperone proteins. After binding a ligand, the AhR translocates to the nucleus, where it binds its dimerization partner, aryl hydrocarbon nuclear translocator (ARNT). The activated AhR/ARNT heterodimer complex binds to its cognate DNA sequences, termed xenobiotic response elements (XREs) and activates expression of AhR target genes such as CYP1A1 and CYP1A2 [7].

We observed that SP600125 induces CYP1A1 and CYP1A2 mRNAs in primary cultures of human hepatocytes, i.e. the opposite effect than expected for AhR antagonist. In order to explain this discrepancy, we tested three hypotheses: (i) the effects of SP600125 are AhR independent; (ii) CYP1A1 and CYP1A2 induction is caused by SP600125 metabolites; (iii) SP600125 is not an antagonist but a partial agonist of human AhR.

We employed gene reporter assays in HepG2 cells transiently transfected with a reporter plasmid containing two inverted repeats of the dioxin responsive element or with a plasmid containing 5′-flanking region of human CYP1A1 gene, CYP1A1/2 mRNAs expression analyses in human hepatocytes and HepG2 cells and HPLC/MS analyses to investigate SP600125 metabolism in human liver microsomes and HepG2 cells. Dose–response analyses using non-linear regression were performed to calculate the half maximal effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}) of SP600125 in gene reporter experiments. Finally, resveratrol, another AhR antagonist was used to examine interaction of SP600125 with AhR [8].

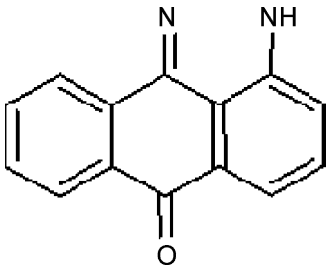

Fig. 1 – Chemical structure of 1,9-pyrazoloanthrone (SP600125).

We show that SP600125 is not an antagonist but a partial agonist of human AhR, which significantly induces CYP1A1 and CYP1A2 genes. In addition, we observed formation of two minor monohydroxylated metabolites of SP600125 in human hepatocytes, human liver microsomes but not in HepG2 cells. These data imply that biotransformation is not responsible for the effects of SP600125 on AhR signaling. Since SP600125 is considered as a specific JNK inhibitor, the major finding of the paper is that SP600125 interferes with AhR signaling, and its agonistic effect should be seriously taken in account in future studies.

2. Materials and methods

2.1. Materials

Collagen-coated culture dishes were purchased from BD Biosciences (Le Pont de Claix, France). Hyperfilm Trade Mark ECL and chemiluminescence-developing reagents were from Amersham (now GE LifeScience, United Kingdom). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was purchased from Ultra Scientific (N. Kingstown, RI). LightCycler FastStart DNA Master^{PLUS} SYBR Green I was purchased from Roche Diagnostic Corporation (Meylan, France). Oligonucleotide primers used in RT-PCR reactions were purchased from Invitrogen. SP600125 (1,9-pyrazoloanthrone) and resveratrol were from Sigma–Aldrich (St. Quentin Fallavier, France). Human liver microsomes were purchased as pooled, cryopreserved samples from Advancell (Barcelona, Spain) from five men and five women. Microsomes were obtained according to ethical rules of the country of origin (Spain). All other chemicals were of the highest quality commercially available.

2.2. Primary cultures of human hepatocytes

Hepatocytes were prepared from lobectomy segments, resected from adult patients for medical reasons unrelated to our research program. Human liver samples used in this study were obtained from four patients: FT 280 (man, 59 years); LH 18 (woman, 69 years); LH 19 (woman, 46 years); LH 20 (woman, 67 years). Tissue acquisition protocol was in accordance with the requirements issued by local ethical commissions in France and the Czech Republic. Hepatocytes

were isolated as previously described [9]. Following isolation, the cells were plated on collagen-coated culture dishes at a density 1.4×10^5 cells/cm^2. In addition, *Long-term human hepatocytes in monolayer* Batch HEP220221 (Biopredic International, Rennes, France) were used. Culture medium was as described previously [10] enriched for plating with 2% fetal calf serum FCS (v/v). The medium was exchanged for serum-free medium the day after and the culture was allowed to stabilize for an additional 48–72 h prior to treatments. Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. HepG2 cells

Human hepatoma cells HepG2 (ECACC no. 85011430) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Plasmids

pDRE-luc plasmid containing two inverted repeats of the xenobiotic responsive element (XRE) of mouse *cyp1a1* upstream of the thymidine kinase promoter and luciferase reporter gene [11,12] was kindly provided by Dr. L. Poellinger (Karolinska Institute, Stockholm, Sweden). p1A1-luc plasmid containing 5′-flanking region (−1566 to +73) of human CYP1A1 gene subcloned into the KpnI–HindIII double-digested pGL3-Basic vector (Promega, Madison, WI) upstream of the firefly luciferase reporter gene [13] was a generous gift from Dr. Robert Barouki (INSERM U490, Paris, France). For gene reporter assays, HepG2 cells were transiently transfected by lipofection (FuGENE 6) with 300 ng/well of pDRE-luc or p1A1-luc plasmid in 24-well plates. Following 16 h of stabilization, the cells were treated for 24 h with increasing concentrations of SP600125 in the presence or absence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM final concentration). After the treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein.

2.5. Western blotting

Following the treatments, total protein extracts were prepared as described elsewhere [14]. Protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL.). SDS-PAGE gels (8%) were run on a Hoefer apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was stained with Ponceau S for control of transfer and then saturated with 8% non-fat dried milk for 2 h at room temperature. Blots were probed with antibody against human CYP1A1 (CYP1A1 (G-18) goat polyclonal IgG; dilution 1/500) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit. Films were scanned and intensity of the bands was evaluated by densitometry.

2.6. mRNA determination and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIZOL Reagent (Invitrogen). cDNA was synthesized from 200 ng of total RNA using M-MLV Reverse Transcriptase (Invitrogen) at 37 °C for 50 min in the presence of random hexamers (Amersham Biosciences). One tenth was used for qRT-PCR amplification using the Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The following program was used: an activation step at 95 °C for 10 min was followed by 40 cycles of PCR (denaturation at 95 °C for 10 s; annealing of 7 s at 65 °C for CYP1A1/2 or 68 °C for GAPDH; elongation at 72 °C for 17 s). Primers were as follows:

- CYP1A1 (forward): 5′-TCCGGGACATCACAGACAGC-3′;
- CYP1A1 (reverse): 5′-ACCCTGGGGTTCATCACCAA-3′;
- CYP1A2 (forward): 5′-CATCCCCCACAGCACAACAA-3′;
- CYP1A2 (reverse): 5′-TCCCACTTGGCCAGGACTTC-3′;
- GAPDH (forward): 5′-CAAAGTTGTCATGGATGACC-3′;
- GAPDH (reverse): 5′-GGTCGGAGTCAACGGATTTGGTCG-3′.

The measurements were performed in duplicates. Expression of CYP1A1 and CYP1A2, mRNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by delta-delta method assuming Pfapfl coefficient to be 2.

2.7. Metabolism of SP600125

SP600125 (100 μM final concentration) or vehicle (DMSO; 0.1%, v/v, final concentration) were incubated for 2 h with primary cultures of human hepatocytes, cultured HepG2 cells or pooled human liver microsomes. For human liver microsomes, reaction mixture contained in 200 μL: 100 pmol CYP, NADPH-generating system (3.76 mM isocitrate, 1.04 mUnits of isocitrate dehydrogenase, 0.485 mM $NADP^+$, 5 mM $MgSO_4$), 100 μM SP600125 in 50 μM Tris/KCl buffer, pH 7.4. The reaction was stopped by addition of 20 μL of 70% $HClO_4$. Metabolites were extracted to dichlormethane, vortexed for 15 s and the organic extract was subsequently evaporated under gentle stream of nitrogen. Subsequently, 100 μL of the mobile phase was added to the sample. Similarly, 1 mL of culture media was deproteined by 100 μL of $HClO_4$ prior to the extraction with dichloromethane. As a positive control, SP600125 was added to the blank mixture/media post-denaturation to exclude possible non-enzymatic transformation of SP600125.

The extracts were subjected to high performance liquid chromatography (HPLC) analyses.

2.8. HPLC analyses

Separations were done using a HPLC method based on chromatography on the Merck RP-18e LiChrospher 100 column (Darmstadt, Germany) with a mobile phase consisting of acetonitrile/water/acetic acid mixture (40:59.9:0.1, v/v). A Shimadzu Class VP system (Tokyo, Japan) was used with detection at 200 nm and a flow rate of 1 ml/min. Two minor metabolites of SP600125 were found in 3.9 and 4.7 min (M1 and

M2; Fig. 6). These metabolites were isolated by HPLC (fractionation, post-column collection) and subjected to mass spectrometry (MS) analyses.

2.9. μLC/MS² analyses

Standard of SP600125 and fractionated metabolites M1 and M2 were analyzed by μLC/MS². Micro-liquid chromatograph CapLC XE was hyphenated with Q-TOF Premier mass spectrometer (Waters, Milford, USA). Microcolumn Gemini C-18, particle size of the stationary phase 5 μm (Phenomenex, USA, column dimensions: 150 mm × 300 μm i.d.) was used at a flow rate 5 μL/min. Binary gradient elution was performed. Mobile phase A was 5.7 mmol l^{-1} solution of acetic acid in water with 5% acetonitrile, mobile phase B was pure acetonitrile; gradient profile was as follows: 0–5 min 0–10% B, 5–25 min 10–50% B, 25–40 min 50–80% B, 40–45 min 80–100% B. Evaporated samples were simply dissolved in mobile phase A (150 μL) and centrifuged prior injection. Injection volume was 1 μL, sample was injected using autosampler.

Optimized parameters of electrospray were: capillary voltage +2.5 kV, sampling cone 40 V, source temperature 50 °C, desolvation temperature 200 °C, cone gas flow 20 L/h and desolvation gas flow 300 L/h. Data were obtained in a single V mode. Data were collected in cyclic repeated scan events covering MS and MS/MS data (collision induced dissociation of parent ion in collision cell, previously isolated in first quadrupole of mass analyzer) during chromatographic run. MS spectra used for interpretation were averages of scans over chromatographic peaks (baseline subtraction was used to filter impurities of mobile phase). Retention times of SP600125, M1 and M2 were 26.7, 20.8 and 22.6 min, respectively. Solution of Leucine–Enkephaline (20 μg/L) in mixture of water:methanol (1:1) was used for lock mass correction during exact mass measurement. Flow rate of the solution into the reference ESI probe of ion source was 2 μL/min.

2.10. Statistical analyses

One-way ANOVA followed by Dunnett's multiple comparison *post hoc* test was used for statistical analysis of differences between two groups using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Two-way ANOVA with interaction was used to compare induction profiles of *CYP1A1* and *CYP1A2* mRNAs in RT-PCR experiments. Data are plotted as means ± standard deviation. EC_{50} (xenobiotic concentration required to achieve half-maximum promoter activation) and IC_{50} (the concentration that is required for 50% inhibition) values were determined according to Hill's equation by non-linear regression analysis using GraphPad Prism Software from at least seven-point curves performed in triplicate.

3. Results

3.1. SP600125 induces CYP1A1 and CYP1A2 mRNAs expression in human hepatocytes

Initially, we investigated the effects of SP600125 on AhR-dependent expression of CYP1A1 and CYP1A2 mRNAs in primary cultures of human hepatocytes. For this purpose, we treated human hepatocytes with increasing concentrations of SP600125 and analyzed the levels of CYP1A1 and CYP1A2 mRNAs employing qRT-PCR. SP600125 caused statistically significant ($p < 0.05$) dose-dependent induction of CYP1A1 and CYP1A2 mRNAs (Fig. 2). The potency of SP600125 and TCDD to induce CYP1A1 and CYP1A2 varied between individual human hepatocytes cultures (Table 1). Maximal induction observed after treatment with SP600125 (1×10^{-4} M) was about 5–20% of the induction reached by typical AhR agonist TCDD (5×10^{-9} M). Two-way ANOVA analyses confirmed identical pattern in the induction of CYP1A1 and CYP1A2 genes in the experiments.

In addition, we used resveratrol (RVT), an antagonist of AhR [8], to test whether the effects of SP600125 are AhR-dependent. RVT (100 μM) strongly inhibited CYP1A1 and CYP1A2 mRNAs induction mediated by both SP600125 and TCDD (Fig. 2). These results indicate that SP600125 induces of CYP1A1 and CYP1A2 mRNAs via AhR and that SP600125 might be a partial agonist of

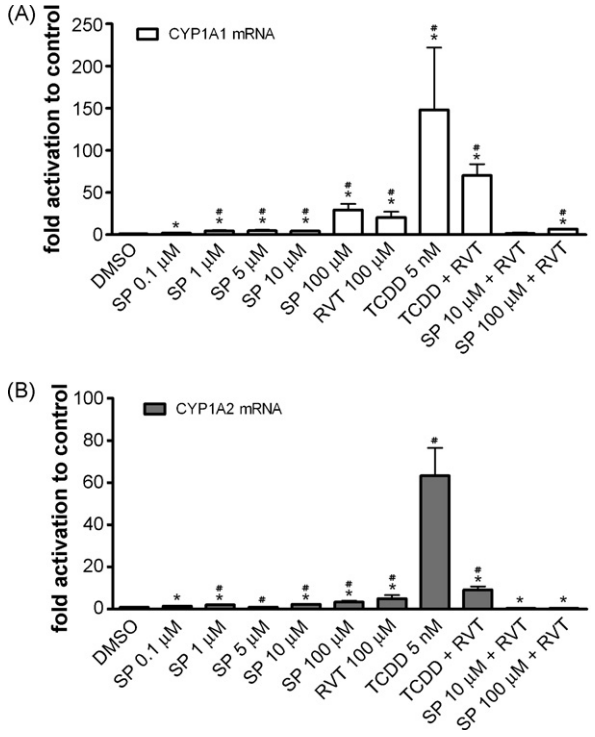

Fig. 2 – Effects of SP600125 on the expression of CYP1A1 and CYP1A2 mRNAs in human hepatocytes. Human hepatocytes were treated with increasing concentrations of SP600125 (0.1–100μM), TCDD (5 nM) and AhR antagonist resveratrol (RVT; 100 μM). As a control, hepatocytes were treated with vehicle (DMSO, 0.1%). Representative RT-PCR analyses of CYP1A1 (Panel A) and CYP1A2 (Panel B) mRNAs are shown. The data are mean ± S.D. from duplicate measurements and are expressed as fold induction over DMSO-treated cells. The data were normalized to GAPDH mRNA levels. Similar behavior was observed in three different human hepatocytes cultures. #Significantly different from DMSO-treated cells ($p < 0.05$); *significantly different from TCDD-treated cells ($p < 0.05$).

Table 1 – Primary human hepatocytes were treated with TCDD (5 nM) or SP600125 (10 μM) for 24 h and CYP1A1 and CYP1A2 mRNAs were analyzed with RT-PCR in three different primary cultures FT 280, LH19 and LH20

	Culture FT 280		Culture LH 19		Culture LH 20	
	CYP1A1	CYP1A2	CYP1A1	CYP1A2	CYP1A1	CYP1A2
SP600125 (10 μM)	195×	29×	79×	16×	4.3×	2.3×
TCDD (5 nM)	ND	ND	1271×	387×	148×	63×

The values indicate fold induction of CYP1A1 and CYP1A2 mRNAs by SP600125 and TCDD as compared to DMSO-treated hepatocytes. ND: not determined.

AhR since it induces AhR-controlled genes but with lesser efficacy in comparison with TCDD. Interestingly, RVT (100 μM) itself significantly ($p < 0.05$) induced CYP1A1 and CYP1A2 mRNAs, indicating partial agonistic effects of RVT on AhR.

3.2. SP600125 inhibits TCDD-mediated induction of CYP1A1 and CYP1A2 mRNAs and proteins in human hepatocytes

In principle, partial agonist partly (not fully) activates a receptor and at the same time it inhibits receptor activation by a full agonist. Thus, we tested the effects of SP600125 on TCDD-inducible expression of CYP1A1 and CYP1A2 mRNAs in human hepatocytes. We treated human hepatocytes with increasing concentrations of SP600125 in the presence of TCDD and we analyzed the levels of CYP1A1 and CYP1A2 mRNAs and their proteins. As expected, SP600125 caused statistically significant ($p < 0.05$) dose-dependent inhibition of CYP1A1 and CYP1A2 mRNAs induction by TCDD (Fig. 3A). Induction of both CYP1A1 and CYP1A2 proteins by TCDD was also abolished by SP600125 (Fig. 3B).

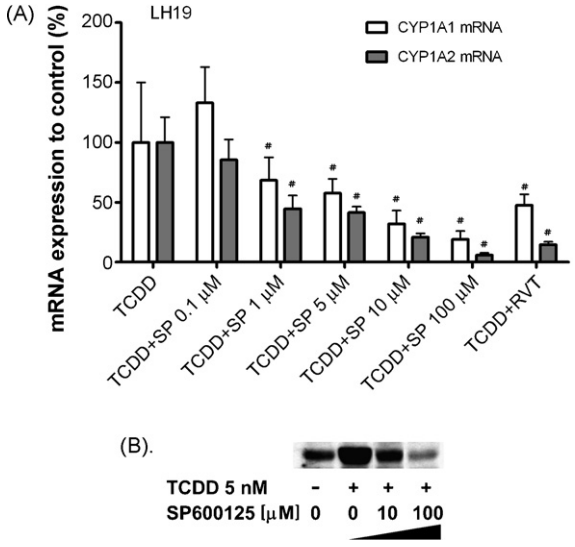

Fig. 3 – Effects of SP600125 on TCDD-inducible expression of CYP1A1 and CYP1A2 mRNAs in human hepatocytes. Human hepatocytes were treated with increasing concentrations of SP600125 (0.1–100 μM) in the presence of TCDD (5 nM) for 24 h. AhR antagonist resveratrol (RVT) was used at the concentration of 100 μM. As a control, hepatocytes were treated with vehicle (DMSO, 0.1%). (Panel A) Representative RT-PCR analyses of CYP1A1 and CYP1A2 mRNAs are shown. The data are mean ± S.D. from duplicate measurements and are expressed as % of induction attained by 5 nM TCDD (100% induction). Expression of tested genes was normalized to the mRNA levels of GAPDH housekeeping gene. Similar behavior was observed in two different human hepatocytes cultures. #Significantly different from TCDD-treated cells ($p < 0.05$). (Panel B) Representative western blotting analysis of CYP1A proteins is shown. For details see Section 2. Similar data were obtained from two different human hepatocytes cultures.

3.3. SP600125 induces CYP1A1 mRNA in HepG2 cells

We also tested whether SP600125 is capable to induce CYP1A1 mRNA in human hepatoma cells HepG2. Cells were treated with vehicle (0.1% DMSO), SP600125 and TCDD in the presence or absence of resveratrol. Both SP600125 and TCDD significantly ($p < 0.05$) induced CYP1A1 mRNA in HepG2 cells and these effects were abolished by AhR antagonist resveratrol (Fig. 4). These data confirm that SP600125 induces CYP1A genes via AhR not only in non-transformed cells but also in immortalized cancer cell line HepG2.

3.4. Examination of SP600125 effects on AhR in gene reporter assays in HepG2 cells

Next we examined the effects of SP600125 on AhR using gene reporter assays. For this purpose, HepG2 cells were transiently transfected with pDRE-luc plasmid containing two inverted repeats of the dioxin responsive element (DRE) or with p1A1-luc plasmid containing 5′-flanking region of human CYP1A1 gene. Thereafter, cells were treated with increasing concentrations of SP600125 in the presence or absence of TCDD. Both reporters are competent for monitoring AhR transcriptional activity. pDRE-luc contains only specific binding sites for AhR while p1A1-luc should be closer to physiological situation with respect to AhR-CYP1A signaling, since it contains 5′-flanking flanking region of CYP1A1 gene from −1566 to +73. SP600125 transactivated pDRE-luc and p1A1-luc reporter plasmids with an EC_{50} of 0.005 and 1.89 μM, respectively (Fig. 5). TCDD-dependent transactivation of pDRE-luc and p1A1-luc was inhibited by SP600125 with IC_{50} values of 1.54 and 2.63 μM, respectively (Fig. 5). These data clearly show that SP600125 is a partial agonist of human AhR. Additionally, activation of pDRE-luc and p1A1-luc by SP600125 and TCDD was significantly ($p < 0.05$) abolished by AhR antagonist resveratrol. RVT (100 μM) inhibited p1A1-luc activation by SP600125 and TCDD down to 11.8% and 6.8%, respectively (data not shown).

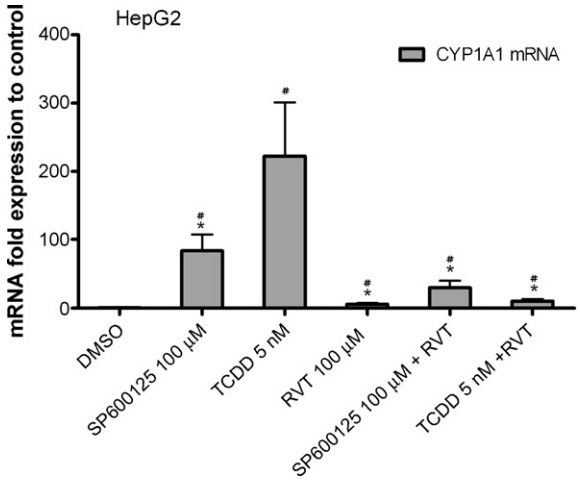

Fig. 4 – Effects of SP600125 on the expression of CYP1A1 mRNA in HepG2 cells. Human hepatoma cells were treated with vehicle (DMSO), SP600125 (100 μM) and dioxin (TCDD; 5 nM) in the presence or absence of AhR antagonist resveratrol (RVT; 100 μM) for 24 h. Representative RT-PCR analyses of CYP1A1 mRNA are shown. The data are mean ± S.D. from duplicate measurements and are expressed as fold induction over DMSO-treated cells. The data were normalized to GAPDH mRNA levels. Similar behavior was observed in three independent experiments. #Significantly different from DMSO-treated cells ($p < 0.05$); *significantly different from TCDD-treated cells ($p < 0.05$).

3.5. *SP600125 is metabolized in human hepatocytes, human liver microsomes but not in HepG2 cells*

Finally, we tested whether the activation of human AhR is caused by metabolites of SP600125. We incubated SP600125 (100 μM final concentration) for 2 h with primary cultures of human hepatocytes, cultured HepG2 cells or pooled human liver microsomes. Using HPLC/UV analyses, we detected two minor metabolites (M1 = 3.9 min; M2 = 4.7 min) of SP600125 in human hepatocytes and human liver microsomes, but not in HepG2 cells (Fig. 6A—only data from microsomes are shown). Since SP600125 activated AhR in both human hepatocytes (Fig. 2) and HepG2 cells (Figs. 4 and 5), it is likely that the effects of SP600125 on human AhR do not involve metabolic transformation of SP600125.

We show that SP600125 is not an antagonist but a partial agonist of human AhR, which significantly induces CYP1A1 and CYP1A2 genes. In addition, we observed formation of two minor metabolites of SP600125 in human hepatocytes, human liver microsomes but not in HepG2 cells. These data imply that biotransformation is not responsible for the effects of SP600125 on AhR signaling.

3.6. *Identification of SP600125 metabolites by μLC/MS2*

We isolated metabolites M1 and M2 by HPLC fractionation (post-column collection) and the obtained fractions were analyzed by micro-liquid chromatography/mass spectrometry ($\mu LC/MS^2$). Electrospray ionization (ESI) of SP600125 provided quasi-molecular ion $[M+H]^+$ with m/z 221 in first order mass spectra (MS). Ion m/z 237 dominated in the related spectra of both metabolites M1 and M2 after their chromatographic separation (Fig. 6B, spectra on the left). The mass difference $\Delta m/z = 16$ between maternal compound SP600125 and metabolites (M1 and/or M2) suggests incorporation of oxygen atom into the molecule. Taking in account polyaromatic structure of SP600125 (Fig. 1), the formation of two isomeric monohydroxylated metabolites is plausible.

We performed MS/MS experiment to elucidate the structure of metabolites. Characteristic elimination of carbon monooxide and/or nitrogen molecule (both $\Delta m/z = 28$) leading to ions of m/z 193 and m/z 165 (dominant fragment ion) was observed in MS/MS spectra of SP600126 (Fig. 6B, spectra on the right). The same fragmentation pattern (i.e. elimination CO and/or N_2) was observed in MS/MS spectra of M1 and M2 (fragment ions with m/z 209 and m/z 181) suggesting that studied metabolites have the same structural base as the original compound. Beside ion m/z 153 occurs in collision spectra which could be explained by the elimination of carbon

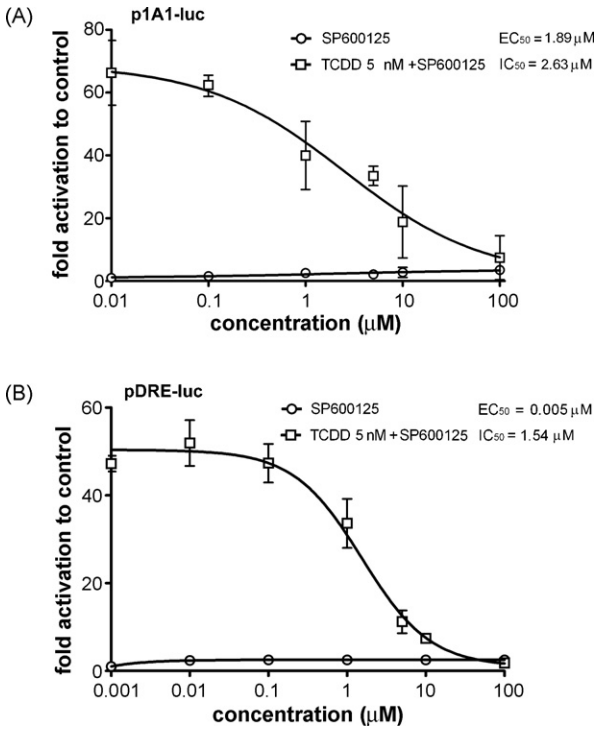

Fig. 5 – Effects of SP600125 on activation of (B) pDRE-luc and (A) p1A1-luc reporter plasmids in HepG2 cells. HepG2 cells were transiently transfected by lipofection (FuGENE 6) with 300 ng/per well of pDRE-luc or p1A1-luc plasmid. Following 16 h of stabilization, the cells were treated with increasing concentrations of SP600125 in the presence or absence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM final concentration) for 24 h. After the treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. The data are mean ± S.D. from triplicate measurements and are expressed as fold induction over DMSO-treated cells. The presented IC_{50} and EC_{50} values are the means from three independent transfection experiments.

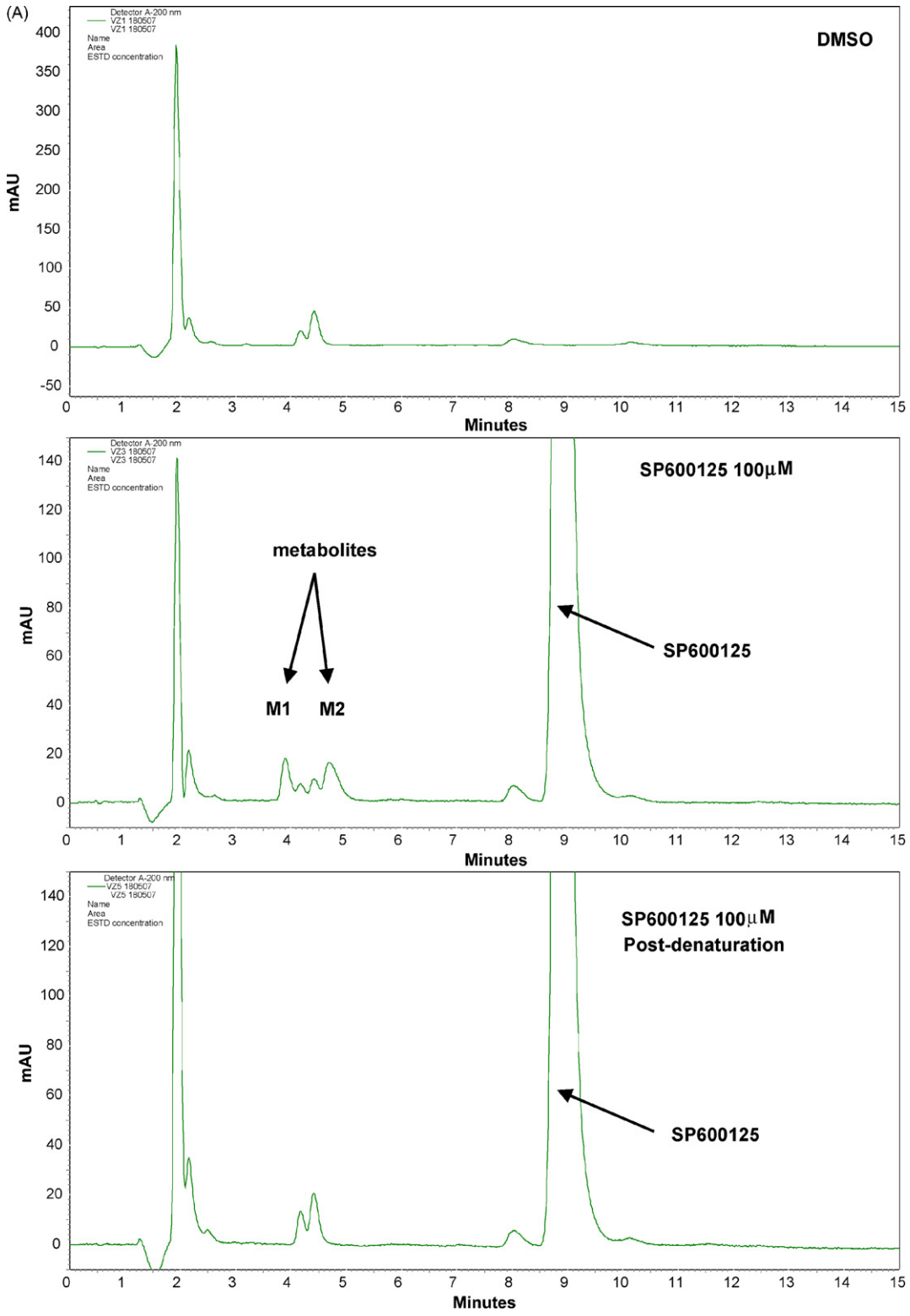

Fig. 6 – Metabolism of SP600125 in human liver microsomes. SP600125 (100 μM final concentration) or vehicle (DMSO; 0.1%, v/v, final concentration) were incubated for 2 h with human liver microsomes. Mixture was deproteined by $HClO_4$, as described in Methods section. As a positive control, SP600125 was added to the blank mixture/media post-denaturation. Following centrifugation (13,000 rpm/3 min), supernatants were subjected to HPLC analyses. (Panel A) Representative HPLC chromatograms (UV detection at 200 nm): upper panel—negative control (Blank; DMSO); middle panel—SP600125 and its metabolites M1 and M2; lower panel—positive control (Blank; SP600125 post-denaturation). (Panel B) Metabolites M1 and M2 shown in Panel A were isolated by fractionation (post-column collection). SP600125, M1 and M2 were subjected to μLC/MS^2 analyses. Representative ESI-MS and related MS/MS spectra of SP600125 and metabolites M1 and M2 are shown. For details see Sections 2 and 3.

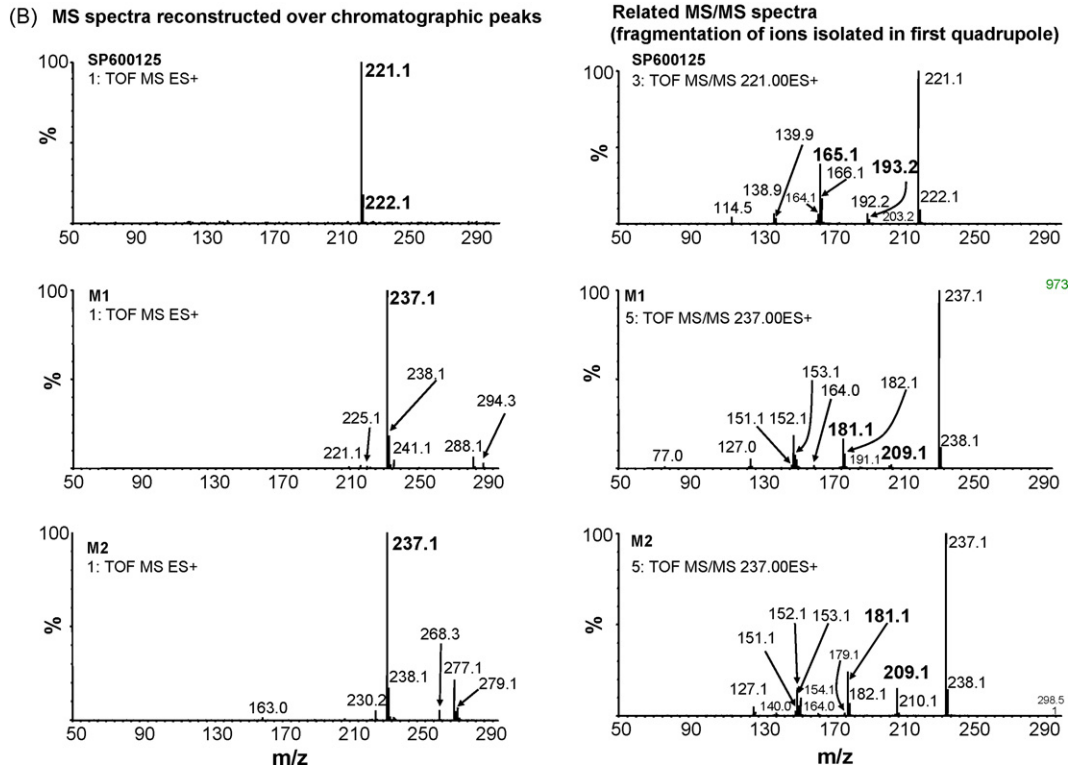

Fig. 6. (Continued).

monooxide from hydroxylated aromatic ring. Finally, abundant fragment ion *m*/*z* 127 can be formed by the cleavage of acetylene ($\Delta m/z = 26$) from ion *m*/*z* 153.

Exact mass measurement of parent ions of both metabolites supports the identification of monohydroxylated derivatives. Their calculated mass was *m*/*z* 237.0664. Measured value was *m*/*z* 237.0662 for M1 (Δ −0.8 ppm) and *m*/*z* 237.0670 for M2 (Δ +2.5 ppm), respectively. Mass spectrometry unambiguously identified metabolites M1 and M2 as monohydroxylated derivatives of SP600125, however, the position of hydroxy group in the molecule has not been determined. This is the objective of the next study.

4. Discussion

In the present paper we demonstrate that specific inhibitor of JNK kinase 1,9-pyrazoloanthrone (SP600125) is a partial agonist of human AhR. This is supported by the following findings: (i) SP600125 induced CYP1A1 and CYP1A2 mRNAs in human hepatocytes, but the magnitude of the induction did not correspond to full activation of AhR. In parallel, SP600125 inhibited CYP1A1 and CYP1A2 mRNAs induction in human hepatocytes by typical AhR agonist TCDD. Similar effect of SP600125 was observed in HepG2 cells on AhR-mediated CYP1A1 mRNA expression. (ii) SP600125 displayed typical behavior of a partial agonist in gene reporter assays performed in HepG2 cells transiently transfected with pDRE-luc or p1A1-luc plasmids. (iii) The effects of SP600125 occurred via AhR, because AhR antagonist resveratrol abolished induction of CYP1A genes by SP600125 in human hepatocytes and HepG2 cells. (iv) The effects of SP600125 on AhR-CYP1A signaling are not due to the metabolic transformation of SP600125.

Many papers report on the findings that compound ''X'' is an antagonist of the receptor ''Y''. However, the classical pharmacology strictly defines the properties of antagonist and this is often ignored or misinterpreted in the scientific literature. In fact, pure antagonists are very rare and the majority of so-called antagonists are partial agonists. Full agonist ligand is able to cause maximal effect. An example of AhR conventional agonist is dioxin (TCDD). On the other hand, a partial agonist shows intermediate effectiveness in response and does not produce full effect even at maximal concentrations. Partial agonist also can act as antagonist. When it binds to the receptor (and produces submaximal response even when receptors are fully occupied), it also occupies the drug binding site competitively with respect to a full agonist. An antagonist binds to the receptor, but initiates no effect itself. An antagonist inhibits the action of an agonist or a partial agonist by preventing the agonist from binding to the receptors [15,16]. The potency of the ligand (agonist, partial agonist, antagonist) is defined by its affinity (i.e. tendency to bind a receptor, EC_{50}) and efficacy (i.e. ability, once bound, to initiate the changes leading to effects). Even resveratrol, used in this study as ''golden antagonist of AhR'' [8], seems to be rather partial agonist than antagonist, since it weakly induced CYP1A genes in human hepatocytes and HepG2 cells (Figs. 2 and 4).

Joiakim et al. showed that SP600125 is a ligand of mouse AhR [3]. However, ligand binding assay employing a reference ligand in general does not distinguish between agonist, partial agonist or antagonist. The authors show by EMSA assay in

extracts from MCF10A cells that SP600125 slightly increases binding of human AhR to its consensus DNA sequence, at the same time. However, SP600125 blocks AhR binding in the extracts from TCDD-treated cells. This is typical behavior for a partial agonist. Consistently, we show here that the partial agonistic effects of SP600125 on CYP1A1/2 expression and pDRE-luc/p1A1-luc reporters occur via AhR, as confirmed by another AhR antagonist resveratrol.

The finding that JNK specific inhibitor SP600125 interferes with another signaling pathway is of great importance, since AhR plays pivotal role in drug metabolism [4], carcinogenesis [5], development, differentiation and many other essential physiological functions [6]. The impact of this finding depends on the design of the study employing SP600125 [3]. Thus, the data obtained in past in studies employing SP600125 should be eventually re-interpreted with respect to possible involvement of AhR pathway. Similarly, the interaction between SP600125 and AhR should be considered in future studies.

For instance, the role of JNK in AhR signaling is not entirely understood yet. The techniques such as transient or stable transfections of reporters, JNK dominant negative vectors or deletion of JNK by siRNA method were applied in studies on JNK role in AhR functions in proliferating cell lines [17]. However, the approach of chemical inhibition of JNK by SP600125 is not applicable due to interactions between SP600125 and AhR.

We conclude that SP600125 is a partial agonist of human AhR, which significantly induces CYP1A1 and CYP1A2 genes.

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