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Short Communication

THE JUN N-TERMINAL KINASE INHIBITOR SP600125 IS A LIGAND AND ANTAGONIST OF THE ARYL HYDROCARBON RECEPTOR

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ABSTRACT:

Exposure of the immortalized human breast epithelial cell line MCF10A to the Jun N-terminal kinase (JNK) inhibitor anthra[1,9-cd]pyrazol-6(2*H*)-one (SP600125) suppressed, in a concentration-dependent manner (IC_{50} ~2 μM), the induction of *CYP1A1* by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Cotreatment with SP600125 also suppressed the accumulation of TCDD-induced nuclear aryl hydrocarbon receptor (AhR)-DNA complexes, as assessed by electrophoretic mobility shift assays. Concentrations of SP600125 ≤ 50 μM did not transform the AhR into a DNA-binding species when added to rat liver cytosol. However, addition of SP600125 to cytosol just before TCDD addition completely suppressed AhR transformation and DNA binding (IC_{50} ~7 μM). Sucrose gradient analyses using rat liver and murine hepatoma 1c1c7 extracts demonstrated that SP600125 competed with TCDD for binding to the AhR. These results suggest that SP600125 is an AhR ligand and functions as an AhR antagonist at concentrations used to pharmacologically inhibit JNK.

The aryl hydrocarbon receptor is a ligand-activated transcription factor. In its nonligand-bound form it is primarily found in the cytoplasm complexed with 90-kDa heat shock protein (hsp90), ARA9/Ah receptor-interacting protein/X-associated protein 2 and a 23-kDa cochaperone protein (Ma and Whitlock, 1997; Carver et al., 1998; Kazlauskas et al., 1999). Upon ligand binding, the AhR[1] complex undergoes a conformational change resulting in translocation to the nucleus, complex dissociation, and subsequent AhR heterodimerization with ARNT (Heid et al., 2000). The resulting AhR/ARNT complexes, in conjunction with other coactivating or corepressing proteins, interact with enhancer sequences in target genes designated dioxin-responsive elements (DREs), and either stimulate (Hankinson, 1995; Schmidt and Bradfield, 1996) or suppress (Dong et al., 1997; Sulentic et al., 2000) target gene transcription. To date, several genes involved in phase I metabolism (e.g., *CYP1A1*, *CYP1A2*, *CYP1B1*) and phase II metabolism (e.g., *NQO1* and *ALDH4*) have been shown to contain functional DRE sequences in their promoter regions and are transcriptionally activated by a variety of AhR agonists (Schmidt and Bradfield,1996).

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[1] Abbreviations used are: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DMSO, dimethyl sulfoxide; DRE, dioxin-responsive element; EMSA, electrophoretic mobility shift assay; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; JNK, Jun N-terminal kinase; PD98059, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; PBS, phosphate-buffered saline; SP600125, anthra[1,9-cd]pyrazol-6(2*H*)-one; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzo-furan.

Address correspondence to: John J. Reiners, Jr., Institute of Environmental Health Sciences, Wayne State University, 2727 Second Ave., Rm. 4000, Detroit, MI 48201. E-mail: john.reiners.jr@wayne.edu

Numerous chemicals are AhR ligands (Denison et al., 2002). In general, all share the structural features of being small, aromatic, and planar. However, they can differ markedly in how they affect AhR function. Ligands such as β-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) function as agonists and are potent activators of the AhR (Gasiewicz et al., 1996). Conversely, the flavonoid-related molecules LY294002 and 3′-methoxy-4-nitroflavone function as antagonists due to their ability to inhibit activation of the AhR (Henry et al., 1999; Guo et al., 2000). In some cases, the agonist versus antagonist function of an AhR ligand varies with its concentration. For example, at the lower end of the concentration range at which they bind to the AhR, both α-naphthoflavone and PD98059 function as AhR antagonists. At higher concentrations they function as agonists (Wilhelmsson et al., 1994; Reiners et al., 1998, 1999).

The anthrapyrazolone SP600125 has been reported to be a potent and selective inhibitor of JNK1, -2, and -3 (Bennett et al., 2001). Because of its specificity and effectiveness in both cultured cells and whole animals, SP600125 has become the pharmacological inhibitor of choice for assessing the role of JNKs in mediating biological processes (Bennett et al., 2001; Brint et al., 2002; Gee et al., 2002; Reuther-Madrid et al., 2002; Zhang et al., 2002). The current study was prompted by our observation that the induction of *CYP1A1* by the AhR agonist TCDD in human breast MCF10A-Neo cells was suppressed by cotreatment with 12-*O*-tetradecanoylphorbol-13-acetate, but accompanied by the activation of JNK1 and -2 (Guo et al., 2001; J. J. Reiners, unpublished data). Given the putative role of phosphorylation in regulating AhR function (Berghard et al., 1993; Li and Dougherty, 1997; Park et al., 2000), we used SP600125 to determine whether JNK activities played a role in the suppression. Unexpectedly, we learned that SP600125 by itself potently inhibited the induction of *CYP1A1* by TCDD and functioned as a ligand and antagonist of the AhR.

Materials and Methods

Materials. TCDD and TCDF were purchased from Midwest Research Institute (Kansas City, MO). Trypsin/EDTA, epidermal growth factor, peni-

cillin/streptomycin solution, and horse serum were purchased from Invitrogen (Carlsbad, CA). [^{3}H]TCDD was purchased from Chemsyn (Lenexa, KS). [*methyl*-^{14}C]bovine serum albumin was obtained from PerkinElmer Life Sciences (Boston, MA). SP600125 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell Culture. The MCF10A cell line was obtained from the Cell Lines Resource (Karmanos Cancer Institute, Detroit, MI). Cells were cultured in supplemented Dulbecco's modified Eagle's medium/Ham's F-12 medium as previously described (Guo et al., 2001). Subconfluent cultures were treated with various concentrations of TCDD and/or SP600125 dissolved/diluted in DMSO (absolute volume of solvent $\leq$ 0.1% of medium volume).

The murine hepatoma cell line Hepa 1c1c7 was grown in modified Eagle's medium containing 10% fetal bovine serum, sodium pyruvate, L-glutamine, and sodium bicarbonate. All cell lines were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

RNA Preparation and Northern Blot Analyses. Cultures used for RNA isolation were washed twice with calcium and magnesium-free PBS at the time of harvest and stored at −80°C. Total cellular RNA was isolated using commercially available TRIzol reagent. RNA was resolved on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes as described by Reiners et al. (1997). The probes used for the detection of 7S and human CYP1A1 RNAs and the conditions used for hybridization have been described in detail (Reiners et al., 1997).

Extract Preparation. Sprague-Dawley rats were euthanized with pentobarbital or by CO_2 inhalation. Livers were removed after in situ perfusion with HEG (25 mM *H*epes, pH 7.6, 1.5 mM *E*DTA, and 10% *g*lycerol) and subsequently homogenized in HEDG (*HEG* plus 1 mM dithiothreitol) supplemented with protease inhibitors, as described by Gasiewicz and Bauman (1987). The homogenate was sequentially centrifuged at 10,000*g* for 10 min and 100,000*g* for 1 h. The supernatant fluid was stored at −80°C.

Cultures of Hepa 1c1c7 cells were washed with PBS, covered with HEDG supplemented with 0.3 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 2 mg/ml aprotinin, and harvested by scraping with a rubber policeman. The resulting suspension was homogenized with a Dounce glass homogenizer and subsequently centrifuged at 100,000*g* for 45 min. Supernatant fluids were stored at −80°C.

MCF10A cells released from culture plates by trypsin/EDTA treatment were diluted with culture medium containing 10% fetal bovine serum, pelleted, and washed once with PBS. After centrifugation, nuclear extracts were prepared from the cell pellets by a procedure described by Osborn et al. (1989).

Electrophoretic Mobility Shift Assay. The conditions reported by Shen et al. (1991) were used for in vitro AhR transformation and EMSA. Nuclear extracts prepared from MCF10A cultures and in vitro transformed rat liver extracts were used in the EMSA. Complementary oligonucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCG-3′ (the single-core DRE recognition sequence is underlined) were annealed and used to detect activated AhR/ARNT complexes.

Velocity Sedimentation of the AhR. The specific binding of [^{3}H]TCDD to the AhR was measured using a modification of the velocity sedimentation/sucrose gradient protocol described by Tsui and Okey (1981). Rat liver or Hepa 1c1c7 cytosol was incubated for 2 h at room temperature with 1 nM [^{3}H]TCDD in the presence of DMSO, 150 nM TCDF, or varied concentrations of SP600125. Thereafter, the cytosols were incubated with 1 mg of dextran-coated charcoal for 5 min (charcoal/dextran, 10:1 w/w) per mg of protein. After removal of dextran-coated charcoal by centrifugation, cytosols were applied to 10 to 30% (w/v) sucrose gradients in Beckman Quick Seal centrifuge tubes (Beckman Coulter, Inc., Fullerton, CA) and centrifuged for 2 h at 372,000*g* in a Beckman VTI-80 rotor. Fractions of 0.2 ml were collected from the top and assayed for radioactivity. [*methyl*-^{14}C]bovine serum albumin (4.4S) was used as an internal sedimentation marker. The differences in dpm between the TCDD versus the TCDD + TCDF treatment group, and TCDD versus TCDD + SP600125 treatment groups were used to estimate TCDD specific binding to the AhR.

Results and Discussion

Effects of SP600125 on *CYP1A1* Induction by TCDD. Concentrations of SP600125 $\geq$ 1 μM have been reported to potently inhibit JNK-dependent processes in a variety of cultured cell types (Bennett et al., 2001; Brint et al., 2002; Gee et al., 2002; Reuther-Madrid et al., 2002; Zhang et al., 2002). Concentrations of SP600125 ranging from 0.1 to 20 μM were not cytostatic/cytotoxic to MCF10A cultures (data not presented) and did not induce the accumulation of CYP1A1 mRNA (Fig. 1A). However, the induction of *CYP1A1* by TCDD was strongly suppressed by cotreatment with SP600125 in a concentration-dependent fashion (Fig. 1A). Complete suppression could be achieved with 10 μM SP600125 (IC_{50} ~2 μM based on three experiments). To assess whether SP600125 altered the AhR activation pathway in cultured cells, we examined its effect on TCDD-elicited AhR binding to DNA. Nuclear extracts isolated from TCDD-exposed MCF10A cultures formed AhR/DNA complexes when examined by EMSA (Fig. 1B). AhR/DNA complex formation was completely suppressed in cultures cotreated with TCDD and $\geq$10 μM SP600125 (Fig. 1B).

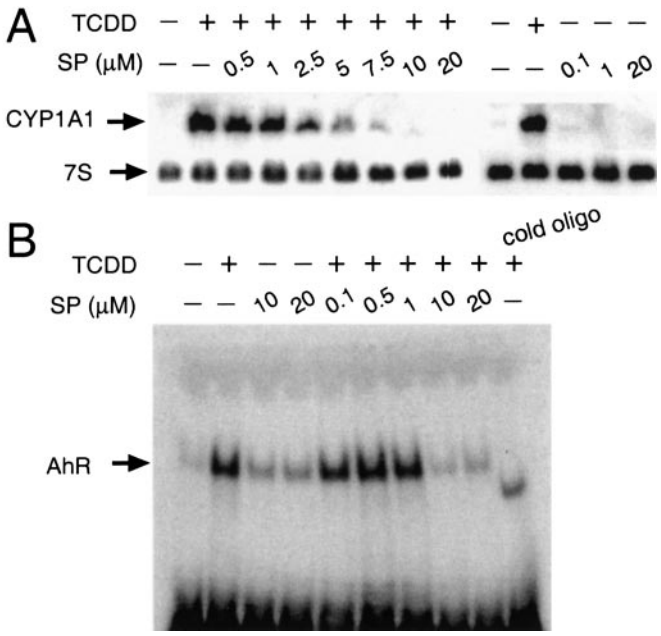

FIG. 1. *Suppression of CYP1A1 induction by SP600125.*

A, cultures of MCF10A cells were treated with DMSO, 10 nM TCDD, and/or varying concentrations of SP600125. SP600125 was added 0.5 h before TCDD. Cultures used for Northern blot analyses were harvested ~5 h after TCDD addition. B, cultures were treated with DMSO, SP600125, or 10 nM TCDD (± SP600125) for 2 h before the preparation of nuclear extracts, which were incubated with labeled DRE oligonucleotide and analyzed by EMSA. Data are representative of two to three independent experiments.

In Vitro AhR Transformation and DNA Binding. The inability of nuclear extracts isolated from cultures cotreated with TCDD and SP600125 to form AhR/DNA complexes, as assessed by EMSA, could reflect inhibitions of ligand binding, AhR translocation, AhR/ARNT complex formation, or AhR/ARNT/DNA complex formation. TCDD-mediated in vitro transformation of the AhR into a DNA-binding complex with ARNT is a well characterized process (Bank et al., 1992). Incubation of rat liver extracts with TCDD transformed the AhR so that it bound ARNT and formed a complex capable of binding to an oligonucleotide containing a DRE sequence (Fig. 2A). By itself, at concentrations ranging from 0.001 to 50 μM, SP600125 was unable to transform the AhR into a DNA-binding species (Fig. 2A). However, DNA-complex formation mediated by TCDD exposure was suppressed in a concentration-dependent fashion by coincubation with SP600125 (Fig. 2B, IC_{50} ~7 μM based on three experiments).

Competition by SP600125 with [^{3}H]TCDD for the AhR. Velocity sedimentation of the AhR on sucrose gradients was used to

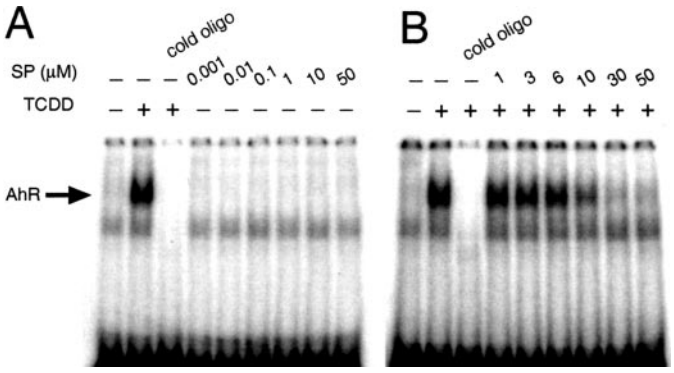

FIG. 2. *Analyses of SP600125 as an AhR agonist and antagonist in an in vitro transformation assay.*

Rat liver extracts were incubated with varied concentrations of SP600125 in the absence (A) or presence (B) of 5 nM TCDD before being incubated with labeled DRE oligonucleotide and analyzed by EMSA. Data are representative of three independent experiments.

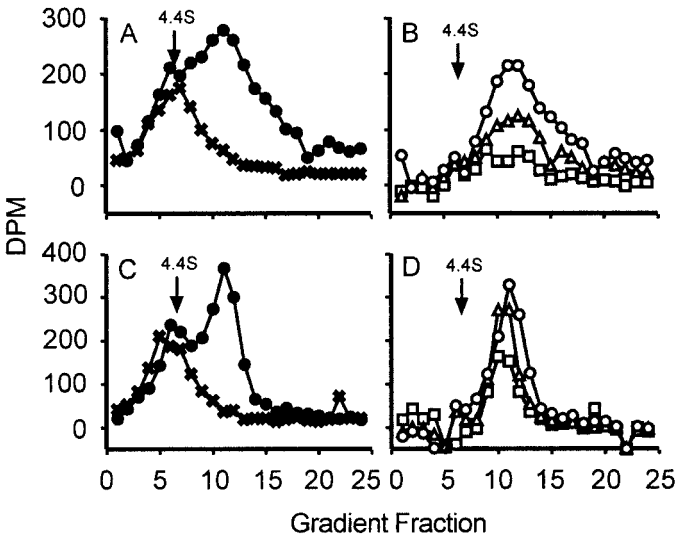

FIG. 3. *Sucrose gradient analyses of SP600125 suppression of TCDD binding to the AhR.*

Rat liver (A and B) or murine hepatoma 1c1c7 (C and D) extracts were incubated in vitro for 2 h with 1 nM [^{3}H]TCDD in the absence or presence of TCDF (150 nM) or varying concentrations of SP600125. At the end of 2 h the extracts were processed and subjected to velocity sedimentation on sucrose gradients as described under *Materials and Methods*. A and C, total [^{3}H]TCDD binding in the absence (●) and presence of TCDF (✕). B and D, specific binding of [^{3}H]TCDD to the AhR in the absence (○) and presence of 10 μM (△) and 50 μM (□) SP600125. The 4.4S marker corresponds to bovine serum albumin. Data are representative of two independent experiments.

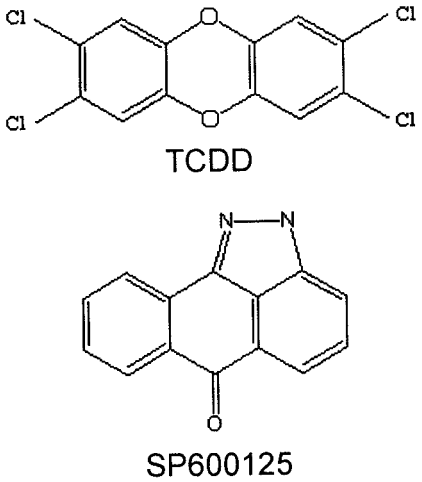

FIG. 4. *Structures of TCDD and SP600125.*

determine whether SP600125 could suppress the specific binding of TCDD to the AhR. Incubation of rat liver extract with TCDD led to the formation of TCDD/protein complexes (Fig. 3A). The portion of bound TCDD that could be competed with a 150-fold excess of TCDF represented AhR-specific TCDD binding (Fig. 3A). Coincubation of rat liver extract with SP600125 suppressed the formation of [^{3}H]TCDD/AhR complexes in a concentration-dependent fashion with a IC_{50} ~10 μM (Fig. 3B, and an additional experiment with concentrations ranging from 6–100 μM). Similar binding/competition studies were performed with extracts isolated from murine hepatoma 1c1c7 cells (Fig. 3, C and D). SP600125 also competed with TCDD for binding to the AhR in 1c1c7 extracts. However, it was less effective. Specifically, [^{3}H]TCDD binding to the AhR, in two studies using different extract preparations, was inhibited 21 or 27%, and 51 or 58%, in extracts cotreated with 10 or 50 μM SP600125, respectively (Fig. 3D and one additional study).

The ability of SP600125 to compete with TCDD for binding to the AhR, as scored by in vitro transformation/EMSA and velocity sedimentation assays, suggests that this anthrapyrazolone is an AhR ligand. Furthermore, its inability to transform the AhR found in rat liver extracts into a species capable of binding to a DRE-containing oligonucleotide suggests that it is an AhR antagonist. Given the structure of SP600125, it is not surprising that it is an AhR ligand (Fig. 4). Its dimensions are similar to TCDD, and it is aromatic and planar. Studies with synthetic flavones suggest that antagonist activity requires a substituent group with high electron density that might hydrogen-bond or have electrostatic interactions with sites in the AhR ligand-binding pocket (Henry et al., 1999). The pyrazole ring in SP600125 may function in this manner. Presumably, SP600125 suppressed the induction of *CYP1A1* in MCF10A cultures by TCDD and eliminated the ability of nuclear extracts to form AhR/DNA complexes by functioning as an AhR antagonist. Although we have found that SP600125 can also suppress the induction of *CYP1A1* in A549 cells in a human type II lung tumor cell line (J. J. Reiners, unpublished data), a recent report suggests that the efficacy of AhR antagonists may be species specific (Zhou et al., 2003).

The IC_{50} values for SP600125 competition with [^{3}H]TCDD for AhR binding in rat liver and 1c1c7 extracts, as assessed by velocity sedimentation analyses, differed ~5-fold. This difference may reflect our having used the same protocol for both extracts, which may not have been optimal for the murine extract. Alternatively, our data may reflect a species-specific difference in receptor affinity for SP600125. Velocity sedimentation analyses have shown subtle to marked differences in the binding of a variety of AhR ligands to the AhRs from different species (Denison et al., 1989; references within Denison et al., 2002).

The reported K_i for the in vitro interaction of SP600125 with recombinant JNK2 is 190 nM, whereas the IC_{50} for suppression of JNK activities in cultured cells varies between 5 and 10 μM (Bennett et al., 2001). A perusal of the existing literature using SP600125 for the inhibition of JNKs in cultured cells indicates that the compound is typically used at concentrations ranging from 10 to 50 μM. Based on the studies reported herein, we anticipate that concentrations of SP600125 sufficient to inhibit JNK activities in cultured cells and tissues would also suppress agonist activation of the AhR and those processes initiated by the activated AhR. The extent to which the AhR antagonist activity of SP600125 compromises its usefulness as a JNK inhibitor will be dependent on the nature of the study.

SP600125 joins the mitogen-activated protein kinase kinase inhibitor PD98059 (Reiners et al., 1998) and the phosphatidylinositol 3-kinase inhibitor LY294002 (Guo et al., 2000) as another small molecule used for the pharmacological inhibition of specific kinases, which also functions as an AhR antagonist. Although important as tools in biomedical research, our studies emphasize that small molecules touted as specific inhibitors often have other, potent biological activities.

Institute of Environmental Health Sciences (A.J., P.A.M., J.J.R.)
Wayne State University
Detroit, Michigan
and Department of Environmental Medicine (C.P., T.A.G.)
University of Rochester
Rochester, New York

ABY JOIAKIM
PATRICIA A. MATHIEU
CHRISTINE PALERMO
THOMAS A. GASIEWICZ
JOHN J. REINERS, JR.

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