9-Hydroxy Ellipticine Exerts Both Agonist and Antagonist Properties at the Ah (Dioxin) Receptor

Kurl RN, Mondi M, and Olnes M.
Program in Clinical Pharmacology and Graduate Program in Pathobiology, Brown University School of Medicine, Providence, RI 02912, U.S.A.

Ellipticine, an alkaloid of plant origin, has antitumor properties[1], and is used clinically in the treatment of breast cancer. The structure of ellipticine resembles the geometry of a purine-pyrimidine base pair and the pyridocarbazole structure of this alkaloid makes it intercalate with DNA[2]. Ellipticine (E) is known to cause DNA damage, primarily double strand breaks, by trapping the "cleavable complex" of topoisomerase II bound to DNA. 9-Hydroxy ellipticine (9-OHE), shown in Fig. 1, is the major metabolite of E, and is more active than the parent compound both in cytotoxicity and antitumorigenic studies.

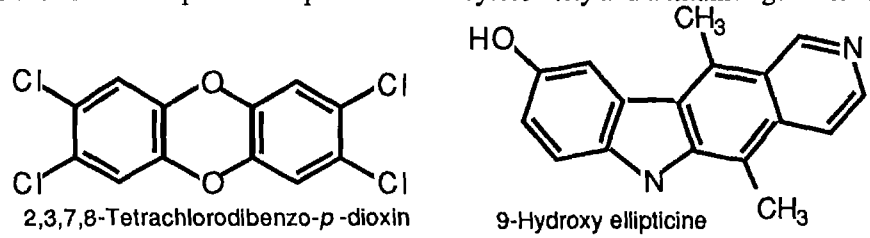

Figure 1. *Chemical structures of TCDD and 9-OHE.*

Both 9-OHE and E have the ability to inhibit aryl hydrocarbon hydroxylase (AHH) activity[3], but the mechanism has not been delineated. 9-OHE has also been reported to bind to the Ah receptor in rat lung cytosol[4]. It is now known that induction of AHH involves binding of ligand to the cytoplasmic Ah receptor, followed by binding of the ligand-receptor complex to enhancer sequences known as xenobiotic responsive elements (XRE) located upstream of the cytochrome P450IA1 (CYPIA1) gene thereby leading to increased transcription[5]. In this extended abstract we report that 9-OHE inhibits [^{3}H]-TCDD binding to the Ah receptor in rat hepatic cytosol and at high concentrations 9-OHE is unable to transform the receptor to a form that binds to XRE.

Materials and Methods

Chemicals: [1,6-^{3}H]-TCDD (30Ci/mmol) was obtained from Cambridge Isotope Laboratories (Woburn, MA). Benzo(a)pyrene was obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]-ATP (7,000 Ci/mmol) was from ICN (Irvine, CA). Poly [d(I-C)] and herring sperm DNA were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were of molecular biology grade.

Preparation of hepatic cytosol.. Male Sprague-Dawley rats (70-100g) from Taconic Farms (NY) were sacrificed under anaesthesia and the livers were perfused *in situ* with phosphate buffered saline. Hepatic tissue was finely minced and homogenized in buffer containing HEPES (25mM, pH 7.6), EDTA (2.0mM), 2-mercaptoethanol (2mM), phenylmethylsulfonylflouride (PMSF, 1mM) and 10% (v/v) glycerol. The homogenate

was centrifuged 105,000 x g for 60 min. and the supernatant free from lipid layer was frozen at -80°C in aliquots.

Sucrose gradient analysis. Cytosol (6mg/ml) was incubated for 2 h at 22°C with [^{3}H]-TCDD in the absence or presence of 9-OHE. Because of the low solubility of TCDD in aqueous solutions, cytosol was incubated with benzo(a)pyrene to test the specificity of binding. The gradients were centrifuged in a Beckman rotor (SW55) at 45,000 rpm for 18 h at 4°C. Ten drop fractions were collected by piercing the bottom of the tubes and assaying for radioactivity using 5ml of cytoscint ES (ICN, Irvine, CA).

Gel shift assays. The complementary oligodioxyribonucleotides corresponding to the XRE-3 region of the CYPIA1 gene were synthesized, annealed, and [γ^{32}P]-labelled at the 5' ends using T-4 polynucleotide kinase. Cytosol was initially incubated for 2 h at 22°C with either TCDD, 3-Methylcholanthrene (3-MC), or 9-OHE. Thereafter, aliquots corresponding to 85µg cytosolic protein were mixed with gel shift buffer containing either herring sperm DNA (500ng, sonicated) or poly d[I-C] (200ng) for 10 min. ^{32}P-labelled XRE-3 was added and the incubation continued for 20 min at 22°C and was analyzed by non-denaturing gel electrophoresis (4% acrylamide, 95V/4 h in buffer containing Tris-boric acid and EDTA). The gel was dried and exposed to XAR-5 film at -80°C.

Results

Displacement of [^{3}H]-TCDD binding in rat hepatic cytosol by 9-hydroxy ellipticine. Previously it was reported that TCDD specifically binds a protein in rat hepatic cytosol which sediments at 8-9S in sucrose density gradient centrifugation[6], and can be competed for in a concentration dependent manner with unlabelled Ah receptor ligands such as benzo(a)pyrene, and 3-methylcholanthrene. To examine whether or not 9-OHE displaces specific [^{3}H]-TCDD binding activity in rat hepatic cytosol, sucrose density gradients were performed. As shown in figure 2, the ability of 9-OHE to compete for binding to the Ah receptor is concentration dependent, with maximum displacement at 190µM competitor

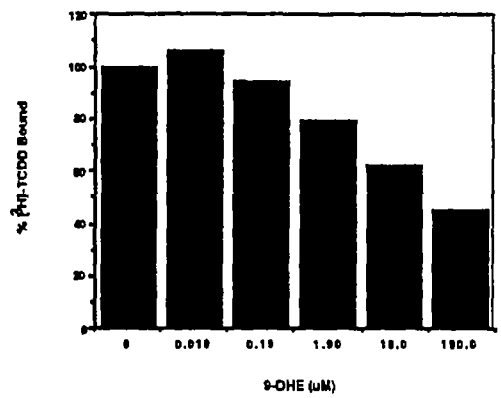

Figure 2. *Concentration dependent competition of [^{3}H]-TCDD in rat hepatic cytosol by 9-hydroxy ellipticine.* Cytosol was treated as described in materials and methods with 2nM [^{3}H]-TCDD in the presence or absence of the indicated concentrations of 9-hydroxy ellipticine and analyzed by sucrose gradient analysis. Specific binding was determined by calculating the area under the 8-9S peak for each concentration.

Gel retardation assay analysis. To examine the effects of 9-OHE binding on transformation of the Ah receptor to the DNA binding form, a gel retardation assay was performed. Rat hepatic extracts were incubated with Ah receptor agonists 3-methylcholanthrene (3-MC) and TCDD, increasing concentrations of 9-OHE, or vehicle, and DNA binding of the Ah receptor was determined by gel retardation assay using [^{32}P]-labelled XRE-3 probe. As shown in Fig. 3, treatment of the extracts with 3-MC and TCDD induces a high molecular weight protein-XRE complex indicated by the arrow. The specificity of this complex is indicated by diminished intensity of the band in the presence of a 15-fold molar excess of unlabelled XRE-3 probe (lane 5). Low concentrations of 9-

OHE induce formation of this complex (Fig. 3, lanes 6-8), while concentrations of 19μM and 190μM diminish or obliterate the Ah receptor-XRE band (lanes 9,10).

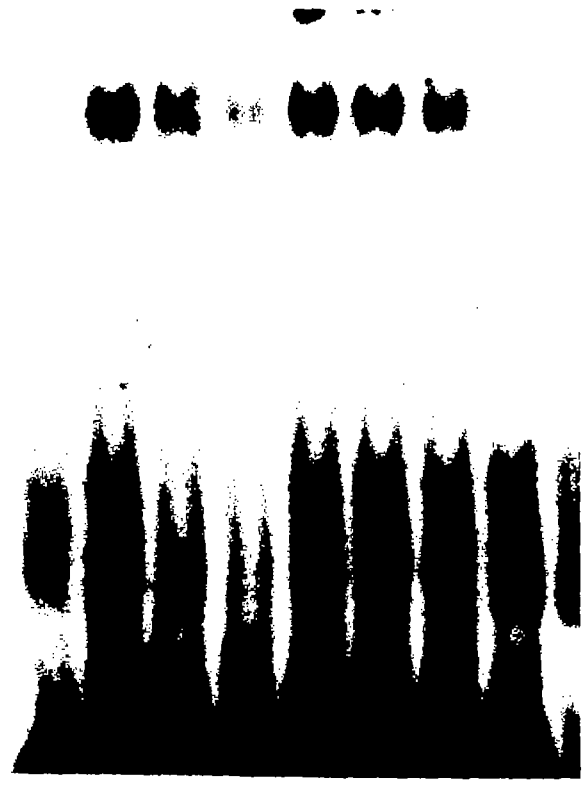

Figure 3. *9-Hydroxy ellipticine inhibits Ah-receptor-XRE-3 complex formation in rat hepatic cytosol.* Extracts were incubated as described in materials and methods with 20μl/ml DMSO (lane 2), 50μM 3-MC (lane 3), 20nM TCDD (lanes4,5), 19nM 9-OHE (lane 6), 190nM 9-OHE (lane 7), 1.9μM 9-OHE (lane 8), 19μM 9-OHE (lane 9), and 190 μM 9-OHE (lane 10), and analyzed by gel retardation assay using labelled XRE-3 probe. Arrow indicates Ah receptor agonist inducible complex, and probe was run off the gel. Lane 1 contained free probe and a 50x molar excess of unlabelled XRE-3 was incubated with extract in lane 5.

Discussion

Ellipticine has been previously reported to bind to the Ah receptor in murine hepatic cytosol, but with reduced affinity as compared to TCDD[7]. Similarly, 9-OHE has been shown to bind to the the Ah receptor in rat lung cytosol with greater affinity than the parent compound[7]. In this study we demonstrate that 9-OHE competes with radioactively labelled TCDD for binding to the Ah receptor in rat liver cytosol.

It is currently believed that binding of agonists to the Ah receptor results in dissociation of an oligomeric complex consisting of the Ah receptor, a monomer or dimer of heat shock protein-90kD, and other less characterized accessory proteins such as the Ah receptor nuclear translocator (Arnt) gene product[8]. This ligand-induced dissociation event

results in formation of an Ah receptor dimer which has the ability to bind XRE with high affinity and specificity. At low concentrations (1.9μM), 9-OHE appears to act as an agonist with respect to transformation of the Ah receptor. However, at concentrations which exert maximum displacement of [^{3}H]-TCDD for binding to the Ah receptor, 9-OHE inhibits Ah receptor-XRE complex formation. While this study was in progress, Gasiewicz and Rucci reported that α-naphthoflavone acts as an antagonist of TCDD by forming a complex with the Ah receptor which is incapeable of binding to XRE[9]. However, this inhibition could be overcome by increasing the concentration of α-naphthoflavone[9]. In the present study, we found that 9-OHE induced inhibition of Ah receptor-XRE band formation is not reversible in the presence of excess (190μM) ligand. These differences may represent variations in how particular ligands interact with the putative 6.8x13.7 Angstrom binding pocket of the Ah receptor[10].

The antagonistic effects of 9-OHE and related compounds on AHH activity in rat hepatic microsomes have been previously described, and reported to result from a direct interaction of 9-OHE with the enzyme[3]. Here we present direct evidence that 9-OHE acts as antagonist of the Ah receptor, suggesting an alternative mechanism involving transcriptional downregulation of AHH.

Acknowledgements

This work was supported in part by National Institutes of Health Grant ES05303.

References

1 Kohn KW, Ross WE, and Glanbiger D. In: Hahn FE, ed. *Antibiotics*. New York, NY: Springer-Verlag, 1979: 195-213.
2 Kohn KW, Waring MJ, Glaubinger D, and Friedman CA. Intercalative binding of ellipticine NSC 71714 to DNA. *Cancer Res.* 1975; 35: 71-6.
3 Lesca P, Lecointe P, Paoletti C, and Mansuy D. Ellipticines as potent inhibitors of aryl hydrocarbon hydroxylase: their binding to microsomal cytochromes P450 and protective effect against benzo(a)pyrene mutagenicity. *Biochem. Pharmacol.* 1978; 27: 1203-9.
4 Kurl RN, Chaudhary KC, and Villee CA. Characterization and conrol of cytosolic binding proteins for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the rat lung. *Pharmacol.* 1986; 33: 181-9.
5 Jones PBC, Galeazzi DR, Fisher JM, and Whitlock JP. Control of cytochrome P1-450 gene expression by dioxin. *Science*. 1985; 226: 1499-1502.
6 Poland A, and Knutson J. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol.* 1982; 22: 517-54.
7 Bigelow SW, and Nebert DW. The Ah regulatory gene product. Survey of nineteen polycyclic aromatic compounds and fifteen benzo(a)pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicol. Letts.* 1982; 10:109-18.
8 Hoffman EC, et al. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* 1991; 252: 954-58.
9 Gasiewicz TA, and Rucci G. α-Naphthoflavone acts as an antagonist of 2,3,7,8-tetrachlorodibenzo-p-dioxin by forming an inactive complex with the Ah receptor. *Mol. Pharmacol.* 1991; 40: 607-12.
10 Gillner M, et al. Interactions of indoles with specific binding sites for 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat liver. *Mol. Pharmacol.* 1985; 28:357-63.