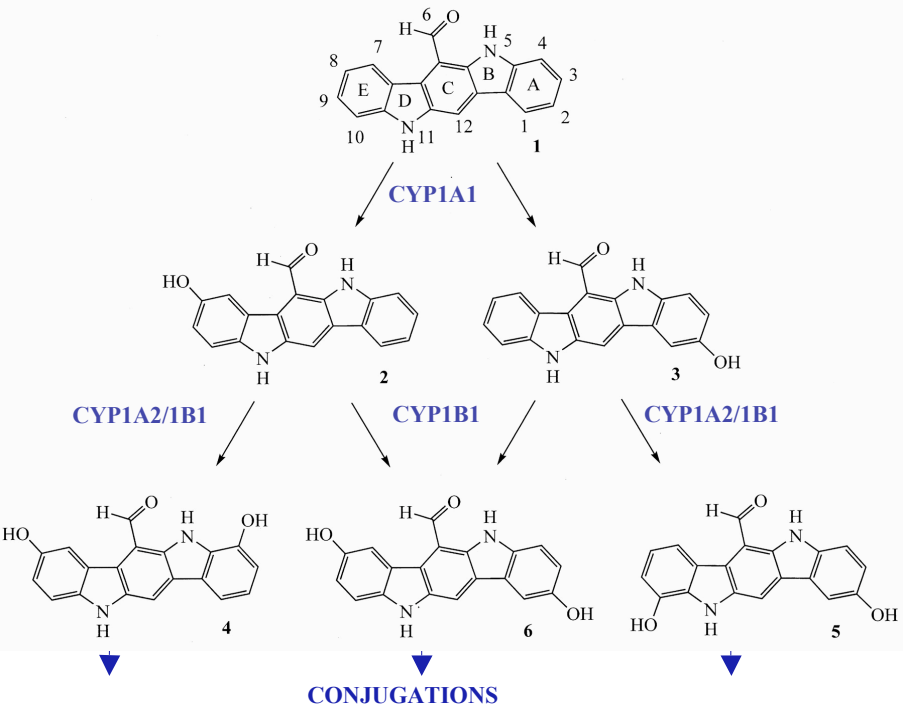
THE METABOLISM OF THE SUGGESTED ENDOGENOUS AH RECEPTOR LIGAND FORMYLINDOLO[3,2-b]CARBAZOLE

Ulf Rannug[1] Linda Bergander[1], Emma Wincent[1] and Agneta Rannug[2]

1 Department of Genetic and Cellular Toxicology, Stockholm University, Stockholm, Sweden
2 Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Introduction

The Aryl hydrocarbon receptor or Ah receptor (AhR) is a ligand-dependent transcription factor ubiquitously expressed in most mammalian cells. Several compounds or groups of compounds, representing a wide spectrum of chemical structures have been identified as agonists for AhR (for a recent review see Denison and Nagy[1]. Xenobiotics such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans, a range of polychlorinated biphenyls as well as polycyclic aromatic



hydrocarbons have been characterized as high affinity ligands for the AhR [2,3]. Physiological compounds with chemical properties different from the known xenobiotic high affinity ligands have been suggested as potential natural AhR ligands, such as tryptamine and indole acetic acid [4], bilirubin and biliverdin[5], lipoxinA_4[6] and prostaglandin[7]. An intriguing fact is the number of ligands identified as being formed from tryptophan. This group contains low affinity ligands like indirubin and indigo[8] and trypthantrin and malassezin[9,10] but also high affinity ligands of the indolo[3,2-*b*]carbazole type [11,12,13]. The tryptophan photoproducts constitute an important group among the

indolo[3,2-*b*]carbazole derivatives and 6-formylindolo[3,2-*b*]carbazole (FICZ), suggested as an endogenous ligand to the Ah receptor (AHR), has been studied in more detail. Our previous studies have demonstrated that FICZ is a rapid and transient inducer of CYP1A1 gene expression when present in low concentrations in several cell types in vitro[14]. The observed transient induction indicated a rapid metabolism of FICZ, which was confirmed by experiments using liver S9 fractions from Aroclor pretreated rats [14,15]. CYP1A1 is a prerequisite for the first step in the metabolism *in vitro* since mono-hydroxylated metabolites could not be detected in Hepa–1 cells defective in CYP1A1 activity.

Further metabolism studies were carried out to elucidate the metabolic fate of FICZ and to identify the enzymes responsible. Comparisons of the metabolism in Aroclor induced rat liver S9 with the metabolism in non-induced rat liver S9 and in human liver S9 demonstrate overlapping pathways but also metabolic steps more prominent in the human S9. Using Aroclor induced rat liver S9-mix five major metabolites of FICZ (**1,** in the metabolic scheme below), were identified by Bergander et al.[16]. The first step in the metabolic pathway is the formation of two monohydroxylated derivatives (fraction M3) 2-OH-FICZ (**3**) and 8-OH-FICZ (**2**), the minor and major regioisomer respectively. The metabolism is continued by the formation of 4,8-dOH-FICZ (**4**) from **2** and 2,10-dOH-FICZ (**5**) from **3**. The most polar metabolite peak detected, (M1) contains 2,8-dOH-FICZ (**6**) which is also formed from the monohydroxylated metabolites (M3).

Methods and Materials

Incubations of FICZ with Aroclor induced rat liver S9 were performed according to Wei et al.[15] with the following modifications. For studies on the involvement of the conjugating enzymes sulphotransferase and glucuronosyltransferase, the S9-mix (1.7 mg protein/ml) was supplemented with 1.2 mM adenosine-3´-phosphate-5´-phosphosulphat (PAPS) and 2 mM uridine-5´-diphosphoglucuonic acid (UDPGA), respectively. The concentration of FICZ was 6.7 μM. Higher concentrations of FICZ and protein (20 μM and 3.3 mg/ml, respectively) were used for the detection of minor metabolites. The incubations employing non-induced rat liver S9 or human liver S9 were basically carried out in the same way. Extractions and analyses by HPLC were performed as earlier described[15,16] except that the samples were dissolved in acetonitrile instead of in methanol. The HPLC analyses were performed with a Merck Hitatchi LaCrom® instrument equipped with a L-7100 pump, a L-7455 diode array detector and a Shimadzu RF-535 fluorescence HPLC monitor, excitation/emission 390/525 nm. A reverse phase Kromasil 100-5C18 column (250 x 4.6 mm) from Scantec, Sweden was employed for analysis and separation. The mobile phase as well as the gradient was applied according to Bergander et al.[16].

Results and Discussion

The formation of metabolites from FICZ employing human liver S9 as a metabolizing system is shown in fig. 1.

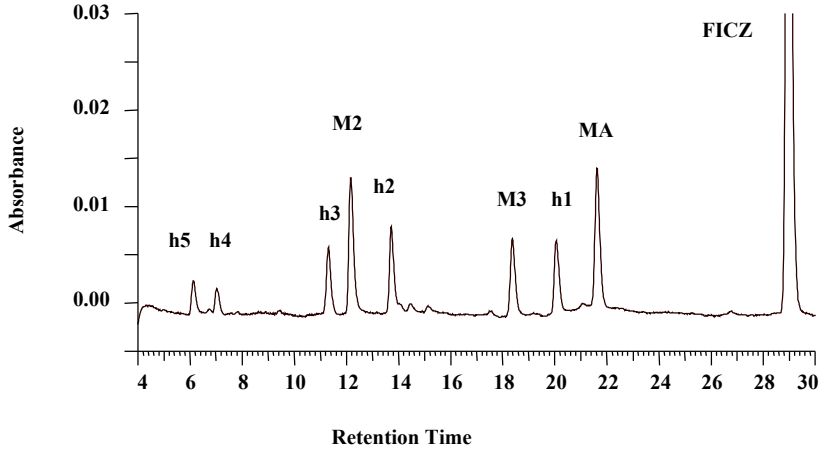


Figure 1: Reverse phase elution profile of metabolite formation from FICZ (20μM) using human metabolizing system.

The metabolite fractions M2 and M3, but not the third dihydroxylated metabolite (M1, **6**) identified after rat liver metabolism, were detected in incubations with human liver. Additionally, the human metabolism produced a series of metabolites (h1-h5), found only in minute amounts or not at all in rat liver S9. Formation of h1 did not require NADPH and seems to be related to the oxidation of the formyl group to the corresponding acid, 6-carboxyindolo[3,2-*b*]carbazole (**7**).

This indicates that apart from the cytochrome P450 dependent pathway humans can also employ other enzymes in the metabolism of FICZ, e.g. aldehyde dehydrogenases (ALDH). It therefore seems likely that the more polar human specific metabolites h2-h5 are mono- and dihydroxylated derivatives of **7** analogous to the precursor product relationship determined for the pathway FICZ - M3 - (M1,M2). The metabolite MA, which is slightly less polar than the monohydroxylated FICZ metabolites, constitutes a major metabolite in the human metabolism. MA is, however, formed only in small amounts using Aroclor induced S9 system and not detected after incubations with non-induced rat liver S9. The chemical structure of MA has not yet been determined but LC-MS analysis indicated a molecular weight of 300, in agreement with a monohydroxylated metabolite. Metabolites monohydroxylated at the position 4 and 10 should, most likely, also be formed when FICZ is metabolized. A hydroxyl group in the position close to the nitrogen should give rise to less polar metabolites compared to the 2 or 8-hydroxylated metabolites. It is therefore conceivable that MA represents these two mono-hydroxylated derivatives of FICZ.

1 **7**

To verify the involvement of the cytochrome P450 isoenzymes 1A1, 1A2 and 1B1 in FICZ metabolism by rat liver S9, the effects of different selective inhibitors have been analyzed (Bergander et al, manuscript). In brief the following results were obtained. Since the same major metabolites of FICZ were formed with the non-induced S9 as with the induced rat liver S9 although in different quantities one could anticipate an overlapping substrate specificity of CYP1A1/1A2. The formation of **2** and **3** are, most likely, formed by both CYP1A1 and 1A2, although with slower kinetics for 1A2 compared to 1A1. The formation of M2 (**4** and/or **5**) was slow but significant in the non-induced S9. Therefore, it is likely that CYP1A2 is an important enzyme for the hydroxylation in the 4 and 10 position subsequent to the formation of **2** and **3**. Interestingly, the mono-hydroxylated precursor metabolites (e.g. MA) were not detected in any substantial amount in the non-induced S9. This can be expected if the same enzyme catalyzes both reactions. No release of the mono-hydroxylated metabolites from the active site of the protein should then occur. CYP1A2 could, on the other hand, have a lower affinity for FICZ compared to the hydroxylated derivatives; a step preferentially performed by the CYP1A1 enzyme. An inhibitory effect of primaquine, known as a CYP1A2 inhibitor, on FICZ metabolism pointed to an involvement of CYP1A2 in the formation of **4** and **5**.

Cofactors for the conjugating (phase II) enzymes sulphotransferase and glucoronosyltransferase were added to the incubation mixture to investigate a possible participation of conjugation reactions in the further metabolism. The addition of either UDPGA or PAPS to both rat and human liver S9 resulted in a substantial reduction of **4** and **5**. When using the human S9, PAPS was most efficient. The human liver S9 supplemented with PAPS provided a substantial reduction also of the monohydroxylated metabolites. The amounts of the human specific metabolites were

also affected by the addition of both UDPGA and PAPS. MA could not be detected after the addition of PAPS but it was not substantially reduced by the addition of UDPGA. The same pattern could be seen for the metabolites h3 and h2, were the addition of PAPS more efficiently reduced the amount of the metabolites. However, h1 responded more to the addition of UDPGA compared to the addition of PAPS.

Conclusions

The tryptophan photoproduct FICZ, which is a high affinity ligand to the aryl hydrocarbon receptor is also a strong inducer of the Ah-gene battery. The induction is, however, transient mainly dependent on its fast metabolism. In contrast to TCDD, FICZ thus effectively induces its own metabolism.

Acknowledgements

This study was supported by the Swedish Radiation Protection Institute.

References

1. Denison, M.S. and Nagy, S.R. (2003) Annu Rev Pharmacol Toxicol, 43, 309.
2. Poland A. and Knutson, J.C. (1982 Annu. Rev. Pharmacol. Toxicol **22:**517.
3. Rannug, U., Sjögren, M., Rannug, A., Gillner, M., Toftgård, R., Gustafsson, J.-Å., Rosenkranz, H. and Klopman, G. (1991) Carcinogenesis, 12, 2007.
4. Heath-Pagliuso S, Rogers WJ, Tullis K, Seidel SD, Cenijn PH, Brouwer A and Denison MS (1998) Biochemistry 37:11508.
5. Phelan D, Winter GM, Rogers WJ, Lam JC and Denison MS. (1998) Arch Biochem Biophys 357:155.
6. Schaldach CM, Riby J, Bjeldanes LF. (1999) Biochemistry. 38(23):7594.
7. Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B and Denison MS. (2001) J Biochem Mol Toxicol 15:187
8. Rannug, U., Bramstedt, H. and Nilsson, U. (1992) Mutat Res, 282, 219.
9. Schrenk, D., Riebniger, D., Till, M., Vetter, S. and Fiedler, H.P. (1997) Biochem Pharmacol, 54, 165.
10. Wille, G., Mayser, P., Thoma, W., Monsees, T., Baumgart, A., Schmitz, H.J., Schrenk, D., Polborn, K. and Steglich, W. (2001) Bioorg Med Chem, 9, 955.
11. Bjeldanes LF, Kim JY, Grose KR, Bartholomew JC and Bradfield CA (1991) Proc Natl Acad Sci 88:9543.
12. Rannug A, Rannug U, Rosenkranz H.S, Winquist L, Westerholm R, Agurell E and Grafström A-K (1987) J Biol Chem 262:15422.
13. Rannug U, Rannug A, Sjöberg U, Li H, Westerholm R and Bergman J (1995) Chem Biol 2:841.
14. Wei, Y.D., Helleberg, H., Rannug, U. and Rannug, A. (1998) Chem Biol Interact, 110, 39.
15. Wei, Y.D., Bergander, L., Rannug, U. and Rannug, A. (2000) Arch Biochem Biophys, 383, 99.
16. Bergander, L., Wahlstrom, N., Alsberg, T., Bergman, J., Rannug, A. and Rannug, U. (2003) Drug Metab Dispos, 31, 233.