Effects of Hydroxylated PCB Metabolites on the Gap-Junctional Intercellular Communication between Mouse Hepa1c1c7 Cells.

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Abstract.

3,3',4,4'-Tetrachlorobiphenyl (3,3',4,4'-TCB) and its metabolites 2-OH-3,3',4,4'-TCB (2-OH-TCB), 4-OH-3,3',4',5-TCB (4-OH-TCB), 4,4'-$(OH)_2$-3,3',5,5'-TCB (4,4'-OH_2-TCB) and 5-OH-3,3',4,4'-TCB (5-OH-TCB) were tested on their effects on the intercellular communication (IC) between hepa1c1c7 cells. The effects on IC were most severe after exposure to 3,3',4,4'-TCB, its metabolites followed the order 2-OH-TCB > 5-OH-TCB > 4,4'-OH_2-TCB > 4-OH-TCB. IC effects were compared to induction of

EROD activity as an Ah-receptor mediated effect. Except for the parent compound and for 2-OH-TCB IC respons in hepa1c1c7 cells was not paralleled by the EROD induction in the same cell type but had a different structure activity relation.

Introduction.

3,3',4,4'-TCB is one of the most toxic compounds of the polychlorinated biphenyls. Toxic responses of this co-planar PCB are similar to those of 2,3,7,8-TCDD, including induction of ethoxy-resorufin-O-deethylase (EROD)[1], dermal toxicity, immunotoxicity, teratogenicity and carcinogenicity[2]. Most of the toxic responses of these polyhalogeneted hydrocarbons (PAHs) are thought to be mediated by the aryl hydrocarbon receptor (AhR). After ligand-receptor binding and complex formation with dioxin responsive elements on the DNA, increased transscription and translation of genes occur that may be responsible for the many toxic effects of these compounds. Recently we reported the involvement of this AhR in the process of tumour promotion by means of the *in vitro* uncoupling of IC between hepa1c1c7 cells[3,4] which was paralleled by EROD induction.
3,3',4,4'-TCB is metabolized rather quickly *in vivo*[5] and *in vitro*[6] thus it is important to determine whether effects of 3,3',4,4'-TCB are due to the parent compound and/or its metabolite(s). In this report we compare the effects of 2-OH-TCB, 4-OH-TCB, 4,4'-OH_2-TCB and 5-OH-TCB to the effects of the parent compound 3,3',4,4'-TCB on IC between hepa1c1c7 cells. Furthermore the response on IC inhibition was related to the induction of EROD activity in order to determine the possible importance of the AhR in the *in vitro* tumour promoting effects of these compounds.

Material&Methods.

Chemicals. 2-OH-3,3',4,4'-TCB (2-OH-TCB), 4-OH-3,3',4',5-TCB (4-OH-TCB), 4,4'-$(OH)_2$-3,3',5,5'-TCB (4,4'-OH_2-TCB) and 5-OH-3,3',4,4'-TCB (5-OH-TCB) were kindly provided by Dr. E. Klasson-Wehler from Department of Environmental Chemistry, Wallenberg Laboratory, University of Stockholm, purity was 99.5% by GC. 3,3',4,4'-TCB was purchased from Schmidt BV (Amsterdam, The Netherlands). Dimethyl sulfoxide (DMSO) was from Janssen Chimica (Geel, Belgium, 99.9% pure). Media for tissue culture were from Life Technologies (Breda, The Netherlands), Lucifer Yellow CH was from Sigma Chemicals (St. Louis, USA).

Microinjection. For microinjection experiments cells were plated 48 h prior to the measurement at a density of 5.10^5 cells per 2 cm petri dish (Greiner B.V., Alphen aan de Rijn, The Netherlands). After 24 h medium was decanted and exposure medium was added. Concentrations of DMSO never exceeded 0.5 %, which was used as control. After 24 h exposure medium was replaced by culture medium (α-MEM, 10% FCS) and microinjection was performed as described earlier[3]. From dose-respons curves EC_{50} values and maximal responses were calculated by Scatchard analysis as described before[4].

Ethoxyresorufin-O-deethylase (EROD) assay. EROD activity assays were performed according to a method kindly provided by Prof. J.P.Giesy, Michigan State University Department of Aquatic Toxicology. Briefly, after exposure for 24 h in 96 well plates the exposure medium was decanted and cells were rinsed twice with 0.5xPBS, cells were lysed using a 15 min incubation at room temperature with 20 μl nanopure water and rapid freezing at -80°C for at least 10 min. After freezing 100 μl Tris buffer (6.05 g Tris (hydroxymethyl)-aminomethane and 68.45 g sucrose per litre) was added supplemented

with 2 ml 2mM dicoumarol (3,3'-Methylene-bis (hydroxycoumarin), 0.067 g in 100 ml 0.1 N NaOH) per 100 ml Tris buffer. After addition of 50 μl 10 μM ethoxyresorufin cells were preincubated in an incubator (Dijkstra Ver., Amsterdam, The Netherlands) at 37°C. Reaction was started with the addition of 50 μl 0.5 mM NADPH in Tris-Dicoumarol buffer, reaction time was one hour. Ethoxyresorufine conversion to resorufin was linear in this time range (data not shown). After incubation plates were read with a cytofluor 2350 fluorimetric plate reader (Millipore, Etten-Leur, The Netherlands).

Mathematics. To calculate relative potencies EC_{50} values were used, which were determined regarding the maximal respons (ΔA_{max}) of the congener tested ΔA_{max} was calculated using Scatchard analysis. ΔA/concentration of the ligand tested was plotted against ΔA in as well the dose respons curves for GJIC as for EROD induction. A straight line was fitted using the "method of least squares", with the computer programm QuattroPro 4.0 (borland International, Inc.). The intercept on the x axis and the slope of the fitted line give values of the maximal inducable effect (ΔA_{max}) and the EC_{50} ($1/Kf$), as indicated by the following equation:

$$\Delta A/[X] = \Delta A_{max} K_f - \Delta A K_f$$

ΔA being the change in the respons e.g. inhibition of GJIC or induction of EROD, K_f being the formation constant of the ligand receptor complex, [X] is the concentration of the ligand tested. Using the standard error of X coefficient and the standard error of Y estimation ranges, calculated after linear regression of the dose-response curve, of the EC_{50} values and ΔA_{max} could be determined.

Results.

3,3',4,4'-TCB inhibited IC between hepa1c1c7 cells with the highest potency compared to its metabolites. EC_{50} and maximal responses on IC were determined (table 1) using Scatchard transformation of the dose response curves. Maximal IC inhibition was highest when the cells were exposed to the parent compound, its metabolites followed the order 2-OH-TCB > 5-OH-TCB > 4,4'-OH_2-TCB > 4-OH-TCB. EC_{50} value was lowest for 3,3',4,4'-TCB , i.e. 0.6-0.8 nM the OH-metabolites were one to two orders of magnitude less potent.

Table 1: Maximal inhibition of IC and EC_{50}values of 3,3',4,4'-TCB and four of its metabolites calculated using Scatchard analysis of the dose response courses.

Congener	Maximal GJIC (%)	EC_{50}value (nM)
3,3',4,4'-TCB	62-76	0.6-0.8
2-OH-TCB	50-60	7-20
4-OH-TCB	15-30	17-100
4,4'-OH_2-TCB	33-51	20-40
5-OH-TCB	34-65	3-12

For EROD activity maximal induction and EC_{50} values were also derived from Scatchard analysis of dose respons curves and are presented in table 2. 3,3',4,4'-TCB had the highest potency to induce EROD in hepa1c1c7 cells as can be concluded from the EC_{50} values. From the metabolites only 2-OH-TCB gave a significant amount of EROD induction of 25 ± 10 pmol/min*mg protein. 4-OH-TCB gave only a slight induction of 7 ± 3 pmol/min*mg protein which was significant from the DMSO control. Both 5-OH-TCB and 4,4'-OH_2-TCB did not show significant induction of EROD.

Table 2: Maximal inhibition of IC and EC_{50}values of 3,3',4,4'-TCB and four of its metabolites calculated using Scatchard analysis of the dose response courses.

Congener	Maximal EROD pmol/min*mg protein	EC_{50} values (nM)
3,3',4,4'-TCB	16-21	0.7-0.72
2-OH-TCB	15-35	1000-4000
4-OH-TCB	5-10	2000-4000
4,4'-OH_2-TCB	n.d	>4000
5-OH-TCB	n.d	>4000

n.d.; not determined.

Discussion.

From these results it is clear that for the co-planar 3,3',4,4'-TCB inhibition of IC between hepa1c1c7 cells is paralleled by EROD induction in the same cell type as was found in previous investigations[4]. Earlier we concluded that for the co-planar PCBs as for dioxins the IC respons is probably mediated by products of the AhR gene battery which become increased in the cytosol[3] after exposure to these compounds.

The 2-OH-TCB metabolite was most potent in both assays though EC_{50} value for IC inhibition differed markedly from EC_{50} values for EROD induction in hepa1c1c7 cells. 4-OH-TCB, 4,4'-OH_2-TCB as well as 5-OH-TCB were not able to induce EROD activity. 2-OH-TCB is the only phenolic metabolite tested so far with the hydroxy group on the ortho position. Although the relative EROD induction potency of 2-OH-TCB is at least 1000 fold less than the parent compound it is in the range of several mono-ortho chlorinated parent compounds, such as 2,3,3',4,4'-PCB. AhR affinity of this 2-OH-TCB does not differ that much from the AhR affinity of the other metabolites[7] and is only a factor 2 lower than the parent compound, 3,3',4,4'-TCB. The more than 1000 fold difference in the potency of EROD induction is striking compared to the much less difference in AhR binding affinity between OH-TCBs and the parent compound. The reason for this is not clear but may involve both a less efficient ligand-AhR-DRE binding and induction of *CYPIA1* gene expression or may be a result of extensive metabolism of phenolic metabolites in hepa1c1c7 cells.

Inhibition of GJIC follows the order of 2-OH-TCB > 5-OH-TCB > 4,4'-OH_2-TCB > 4-OH-TCB. Potency differences are not as striking as when metabolites were compared to the parent compound regarding EROD induction. Since EROD induction after exposure to these metabolites in hepa1c1c7 cells hardly occurs it is unlikely that IC responses are caused after AhR binding and transcription of the AhR gene battery. Inhibition of IC between hepa1c1c7 cells seems to have a different structure activity relationship than EROD induction and AhR binding. Potency to inhibit IC followed the same order as potency to uncouple mitochondrial respiration[8]. Uncoupling of respiration was thought to be resulting from clusters[9] of non-planar PCBs which can disrupt the inner membrane of mitochondrion. One can envision that a similar disruption may result in increased fluidity of the cell membrane which can cause the connexin proteins, the building blocks of gap-junctions, to disperse in the membrane. The position of the hydroxy group may influence the possible conformation of the molecule thus influencing its ability to form clusters which increase membrane fluidity. Though 5-OH-TCB is a planar molecule its effect on IC between hepa1c1c7 cells is severe. It might be that not only the planarity of the molecule is of importance but also the ability to form a cluster of molecules which can be stabilized through hydrogen bonds. Whether or not this mechanism is of importance in the inhibition of IC between hepa1c1c7 cells is not clear yet.

In conclusion.

The hydroxymetabolites (2-OH-TCB, 4-OH-TCB, 4,4'-OH_2-TCB and 5-OH-TCB) tested are much less potent in inducing EROD activity than their parent compound but are able to inhibit IC between hepa1c1c7 cells indicating that IC inhibition does not have the same structure activity relationship as EROD induction.

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