Metabolites of biphenyls and halogenated biphenyls: reactivity of dihydroxylated biphenyls *in vitro* and *in vivo*

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Introduction

Several non-halogenated and halogenated biphenyls are suspected to be carcinogenic by being metabolized to dihydroxy-biphenyls or their quinones. For example, 2,5-dihydroxybiphenyl (2,5-BP) is a major metabolite of the fungicide *ortho*-phenylphenol that induces bladder cancer in rats (1). The leukemogene benzene is metabolized to phenol and further by peroxidases to 4,4'-BP and 2,2'-BP, making both of them and their quinones posssible candidates as the ultimate carcinogens of benzene (2). Halogenated biphenyls, hydroxylated to dihydroxy metabolites with the hydroxyl groups in one ring either *ortho* or *para* to each other (3), may be further oxidized to (semi)quinones that bind to DNA (4) and nucleotides *in vitro* (5), again raising concern about their possible involvement in cancer induction. Other potential dihydroxy metabolites of halogenated biphenyls have the dihydroxyl groups in 4,4' position. Very little is known about these compounds.

In order to learn more about the chemical and biological reactivity of such dihydroxybiphenyls, and to determine structure activity relationships, the influence of the position of the hydroxyl groups and of the number, position and kind of halogens in the molecule were of interest. This information should lay a foundation for prediction of the reactivity of similar compounds and thus helpful for risk assessment and new research initiatives. We therefore selected biphenyls with the hydroxyl groups in the positions 2,5- (2,5-BP), 3,4- (3,4-BP), 4,4'-(4,4-BP), and 2,2'-(2,2-BP) as well as the following halogenated dihydroxy biphenyls, 4'-chloro-2,5- (4-C-2,5-BP), 3,5,3',5'-tetrachloro-4,4' (TCBP), and 3,5,3',5'-tetrabromo-4,4'-biphenol (TBBP). We measured *in vitro* stability, spontaneous and enzymatic oxidation and binding of the oxidized products to glutathione. We also determined cytotoxicity of these compounds to cells in culture and depletion of intracellular glutathione.

Materials and Methods

Synthesis and Characterization

2,5-BP, 3,4-BP, 4,4'-BP and 2,2'-BP were purchased from Aldrich and used without further purification. The synthesis of 4-C-2,5-BP from the corresponding quinone was described earlier (6). TCBP and TBBP were synthesized as follows: Four equivalents of chlorine or bromine were added slowly to a solution of 4,4'-BP in glacial acetic acid heated under reflux. The reaction was allowed to reach room temperature after all elemental chlorine or bromine was consumed. A white precipitate formed that was filtered off and recrystallized from glacial acetic acid. During the recrystallization process we observed that TCBP was less stable in an acetone, ethanol and acetic acid solution than TBBP. The melting points of both compounds are in agreement with the literature values (7). In addition, both compounds were characterized by ^{1}H and ^{13}C NMR, FT-IR,

MS and combustion analysis. The IR spectra were obtained using a Nicolet Magna-IR 560 Spectrometer E.S.P. The NMR spectra were recorded on a Varian Gemini-200.

TCBP: mp = 235-236°C (Lit.: 233°C). ^{1}H-NMR (DMSO-d6, 200 MHz) δ 3.35 (br s, 2 OH), 7.71 (s, 4 Ar-H). IR [cm^{-1}]: 3401 (ν(OH)), 1565, 1472, 1347, 1266, 1222, 1205, 1188, 854, 807. MS m/z (rel Int.): 322 (80), 324 (100) and 326 (49) (all $M^{+\bullet}$). Elemental Analysis Calcd. for $C_{12}H_6Cl_4O_2$: C 44.73, H 1.88; found: C 44.64, H 1.91.

TBBP: mp = 269°C (Lit.: 264°C). ^{1}H-NMR (DMSO-d6, 200 MHz) δ 3.31 (br s, 2 OH), 7.85 (s, 4 Ar-H). IR [cm^{-1}]: 3441 (ν(OH)), 1550, 1458, 1327, 1263, 1165, 853, 748. MS m/z (rel Int.): 498 (15), 500 (65), 502 (100), 504 (64) and 506 (14) (all $M^{+\bullet}$). Elemental Analysis Calcd. for $C_{12}H_6Br_4O_2$: C 28.93, H 1.21; found: C 28.77, H 1.19.

Measurement of stability, spontaneous and enzymatic oxidation and GSH binding

The stability of the compounds in aqueous solution was tested by incubating 100 nmoles of compound in 1 ml PBS at 37° C in the dark. After 6 hrs, the spectrum of the incubation solution was plotted over the spectrum of a fresh solution of the same compound. A decrease of the dihydroxy-indicating peak at about 250-300 nm (depending on the compound tested), and an increase in a quinone-indicating peak at about 320-400nm was interpreted as oxidation of the test compound. For measurement of enzymatic oxidation 100 nmoles of compound were incubated in 1 ml PBS with horseradish or lactoperoxidase and increasing concentrations of H_2O_2. Again the spectrum of the compound after enzymatic incubation was plotted over the spectrum of fresh compound and changes in the peaks analyzed. Non enzymatic binding of the oxidized test compound to GSH was measured indirectly, by first plotting the spectrum after enzymatic oxidation before addition of GSH and after equimolar or 2-4x excess of GSH was added.

Measurement of cytotoxicity

For *in vivo* studies the human leukemia cell line HL-60 was used. These cells have considerable myeloperoxidase activity (8), which could be influential in oxidizing the compounds to the corresponding quinone. Cells were grown in RPMI1640, supplemented with 5% fetal calf serum, glutamin, penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Toxicity of the compounds was determined by seeding $4x10^4$ cells into wells of 96 well microtiter plates with 200 µl medium contained the test compounds in two-fold dilution steps. Toxicity was determined after 1 day exposure by measuring ^{3}H-thymidine incorporation or by counting the cells per well after 3 days of exposure. All experiments were done in triplicate. Data are expressed as percent of solvent only control.

Determination of intracellular glutathione depletion

Intracellular glutathione was determined by incubation of cells with monobromobimane (MBB). This compound is able to enter living cells and becomes fluorescent upon binding to GSH. Briefly, $3x10^6$ HL-60 cells in 3 ml medium were exposed to the test compounds at different concentrations. After 4, 6, or 24 hrs of exposure 40 µl cell suspension was removed and cell viability analyzed by determined the number of tryphanblue negative cells per ml. MBB was added to the remaining cells, which were incubated for an additional 30 min at 37° C in the dark. Cells were placed on ice in the dark. Fluorescence of the cell suspension was determined immediately at an emission wavelength of 395 nm and an excitation wavelength of 470 nm.

Results and Discussion

Measurement of stability and spontaneous and enzymatic oxidation in aqueous environment

With 2,5-BP the peak at 300nm had disappeared after 6 hrs incubation in PBS. No change in the spectrum was seen with 3,4-BP, 2,2'-BP, 4,4'-BP, TCBP and TBBP. With most of the test compounds addition of peroxidase/H_2O_2 resulted in an immediate change of the color of the incubation mixtures, which turned from clear to bright yellow-red. This is indicative of a highly conjugated structure and was reflected in changes in the spectra with all compounds tested with the exception of 2,2'-BP, which showed only minor changes in the spectrum.

Non-enzymatic binding to glutathione

Addition of GSH to the oxidized compounds resulted in further changes. With TCBP, 4-C-2,5-BP, 2,5-BP 3,4-BP, and 4,4'-BP the color and the quinone-peak at 320-400 nm disappeared immediately. The new spectra resembled more the old dihydroxy spectra, without being exactly the same. We conclude that a dihydroxy-glutathionyl adduct was formed by Michael addition of GSH to the quinone metabolites. With TBBP the spectrum of the (red) solution did change slightly, but the red color did not disappear. This observation suggests that the quinoid compound reacted with GSH, but no adduct formation under re-aromatization to a thioether occurred. This phenomenon will be further investigated.

Measurement of cytotoxicity

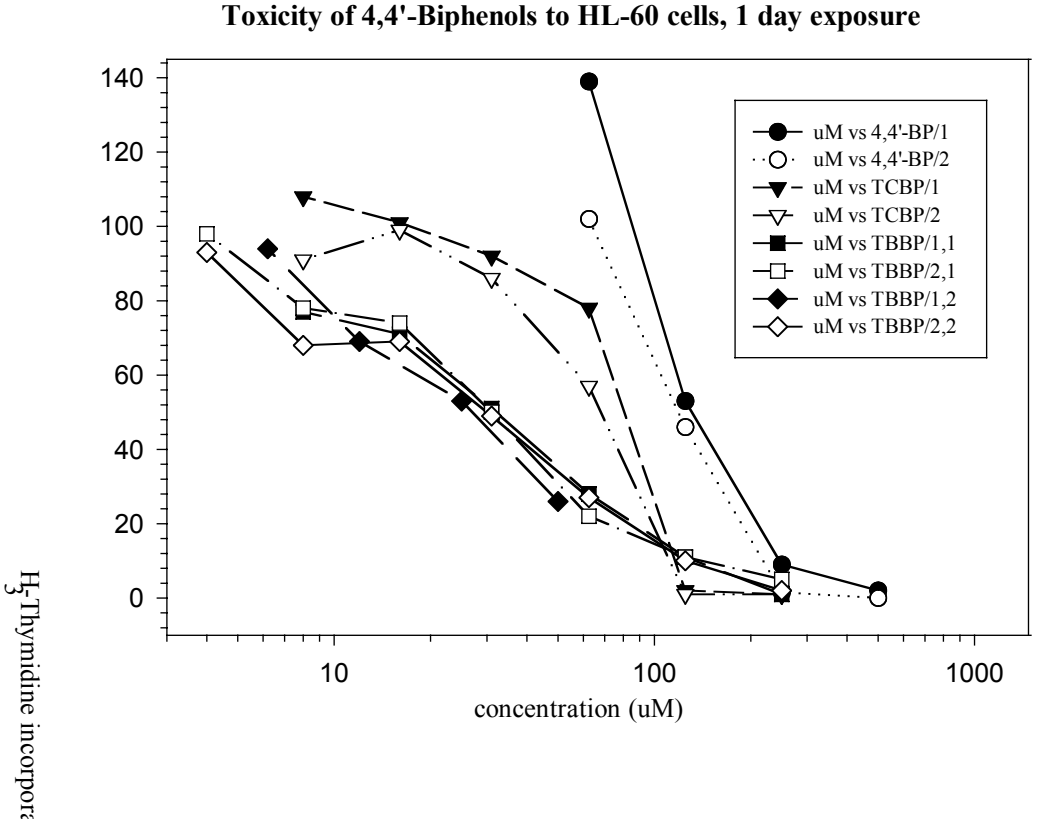
Exposure of HL-60 cells to the non-halogenated biphenols for 3 days resulted in a decrease in cell number from 100% of control to about 10% of control in 4 concentration doublings, equivalent of about 1 order of magnitude increase in concentration. The most toxic compound was 2,5-BP, followed by 3,4-BP, 4,4'-BP and 2,2'-BP. The later one was about 2 orders of magnitude less toxic then the first one. This indicates that the position of the dihydroxy groups has indeed a major influence on the cytotoxicity of these compounds. A comparison of the three compounds with 4,4'-dihydroxys but different/no halogenation resulted in increased toxicity from 4,4'BP < TCBP < TBBP, with about 1 order of magnitude in concentration difference between the first and the last compound (Figure).

Microscopic inspection of cells after 4-6 hrs of exposure to high concentrations of the test compounds revealed that dead cells due to 4,4'-BP and TBBP were small, whereas tryphanblue positive cells in the 2,5-BP and 3,4-BP group had normal size. We speculate that different mechanisms of cell death may be involved. This is further supported by the observation that the cell size was significantly increased after 1 day treatment with sublethal (10 μM) concentrations of 4,4'-BP, but not with 2,5-BP.

Intracellular glutathione depletion

Treatment of HL-60 cells for 4-6 hrs with high concentrations of the compounds resulted in toxicity with 2,5-BP > 3,4-BP > TBBP > 4,4'-BP (TCBP was not yet tested in this assay). Since cell death was observed after few hours, it is probably due to acute toxicity by GSH depletion or other mechanisms and not caused by apoptosis.

Exposure to 20 μM of 2,5-BP or 3,4-BP for 4 hrs resulted in a 35-50% decrease in intracellular GSH, but little toxicity. Since both compounds bind GSH avidly *in vitro* after oxidation we assume that this phenomenon is responsible for the GSH depletion. With 4,4'-BP and TBBP the number of living cells and intracellular GSH decreased in parallel with increasing concentrations and therefore may just reflect the number of surviving cells. After 24 hrs exposure



to these 2 compounds the percent of intracellular GSH was higher then the percent of living cells. This may indicate increased GSH synthesis after exposure to 4,4-BP or TBBP. Further studies are needed to confirm these preliminary results.

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