Effects of oxfendazole on the Ah receptor-mediated induction of ethoxyresorufin-O-deethylase and luciferase activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin in Hepa-1c1c7 and H4IIE cell-lines

Ron L.A.P. Hoogenboom and Astrid R.M. Hamers
State Institute for Quality Control of Agricultural products (RIKILT-DLO), Bornsesteeg 45, 6708 PD Wageningen, The Netherlands

1. Introduction

Biological tests for detecting the presence of dioxins and dioxin-like compounds in food or environmental samples are a promising alternative for the often expensive and time-consuming analytical methods. Recently, new cell-lines have been developed expressing a luciferase-gene in response to the binding of a suitable substrate with the Ah-receptor and subsequently the dioxin-responsive elements in the DNA[1]. It is expected that these cell-lines are more specific and more sensitive than the more classically used models aiming at an induction of cytochrome P450 1A related enzyme activities in the cells. This has been confirmed by the results of tests with dioxins and related compounds[1].

In our laboratory, the suitability of these biological models for testing extracts of milk samples is under investigation. These studies include the possible interference of residues other than dioxins and PCBs with the test result. Aim of the present study was to investigate the interaction between 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and oxfendazole, an anthelmintic drug belonging to a class of widely used benzimidazole compounds. Treatment of rats[2] and rabbits[3] with these type of compounds has been shown to result in an induction of cytochrome P450 1A related enzyme activities in the liver. Furthermore, oxfendazole has been shown to act as a non-competitive inhibitor of cytochrome P450 1A related enzyme activities in the case of the rabbit [3].

2. Materials and Methods

Materials
Wild type or pGudluc 1.1-transfected Hepa-1c1c7 and H4IIE cells were obtained from the department of Toxicology from the Agricultural University in Wageningen. Fetal calf serum (FCS) was purchased from Gibco BRL (Breda, The Netherlands), 2,3,7,8-tetrachlorodibenzo-p-dioxin from Schmidt BV (Amsterdam, The Netherlands), ethoxyresorufin from Pierce (Oud Beijerland, The Netherlands), resorufin, sulfatase (Helix pomatia), α-MEM medium, EMEM medium without phenol-red, penicillin/streptomycin, bovine serum albumins from Sigma (St. Louis, USA) and dimethylsulfoxide (Uvasol) from Merck (Darmstadt, FRG).

Exposure of cells
Wild type or pGudluc 1.1-transfected Hepa 1c1c7 and H4IIE cells were cultured in α-MEM medium supplemented with 10% (v/v) FCS, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells

were seeded in 24 multiwell plates at a density of about 0.15 x10^6/well. Exposure of the cells to the test compounds was started at confluency by replacing the medium with 200 μl of medium containing different concentrations of oxfendazole and 50 μl of medium containing different concentrations of TCDD. The final concentration of dimethylsulfoxide (DMSO), which was used to dissolve both compounds in the medium, was 1%.

Determination of 7-ethoxy-resorufin deethylase (EROD) activity

The exposure to the test compounds after 24 hrs was ended by aspiration of the medium. Cells were washed twice with EMEM without phenol-red (EMEM-PR$^-$), incubated for 15 min in EMEM-PR$^-$, which was subsequently replaced by a solution of 5 μM ethoxyresorufin in EMEM-PR$^-$ (prepared from a stocksolution of 2.5 mM in DMSO). After incubation for 15, 30 or 60 min, the medium was aspirated and stored at -20°C untill analysis. The multiwell-plates containing the cells were stored at -20°C for a protein determination.

Before the quantification of the formed resorufin, media samples were incubated with sulfatase/glucuronidase for deconjugation of resorufin conjugates. Therefore, an aliquot of 100 μl of a medium sample was mixed with 100 μl of a solution of sulfatase (2 mg/ml) in sodium-acetate buffer (0.2 M/ pH 5) and incubated for 1 hr at 37°C. Following the addition of 350 μl of Tris-HCl (0.15 M, pH 7.8), the fluorescence was immediately determined in an LS 50 fluorescence spectrofotometer (Perkin-Elmer), at λ_{ex} 510 nm and λ_{em} 586 nm. The concentration of resorufin was calculated by comparison with a standard curve.

For determining the protein content, cells were dissolved in 0.5 ml sodium dodecyl sulphate/ NaOH (5% /0.4 N) and diluted with 0.5 ml water. The protein content was determined with the Biorad protein assay[4], by mixing 10 μl with 25 μl reagens A and 200 μl reagens B in a microtiter plate. The absorption at 650 nm was subsequently determined in a microplate reader and compared with a standard curve of BSA.

Determination of luciferase activity

Following exposure, cells were homogenized and the luciferase activity and protein content determined in these cell homogenates as described previously[1].

3. Results

Exposure of wild-type Hepa-1c1c7 and H4IIE cells to TCDD resulted in a dose related increase in the deethylation of 7-ethoxyresorufin (data not shown). Incubation of cells with oxfendazole alone also resulted in an increased EROD-activity, especially in the case of the Hepa 1c1c7 cells (Figure 1A and B).

When H4IIE cells were exposed to TCDD and increasing concentrations of oxfendazole, the dioxin related increase in the EROD-activity was not observed at higher levels of the benzimidazole drug (Figure 1B). Quite differently, the combination effect appeared to be additive instead of antagonistic in the case of Hepa-1c1c7 cells (Figure 1A).

Exposure of pGudluc 1.1-transfected H4IIE cells to TCDD also resulted in an increased EROD-activity (Figure 2A), as well as a marked increase in the intracellular concentration of luciferase (Figure 2B). A slight induction in both the EROD-activity and the luciferase concentrations was observed after exposure of the cells to oxfendazole (Figure 2). Coexposure to both compounds again resulted in a decreased EROD-activity at higher concentrations of oxfendazole (Figure 2A). However, this was not accompanied by a decrease in the dioxin related elevation of the luciferase concentration.

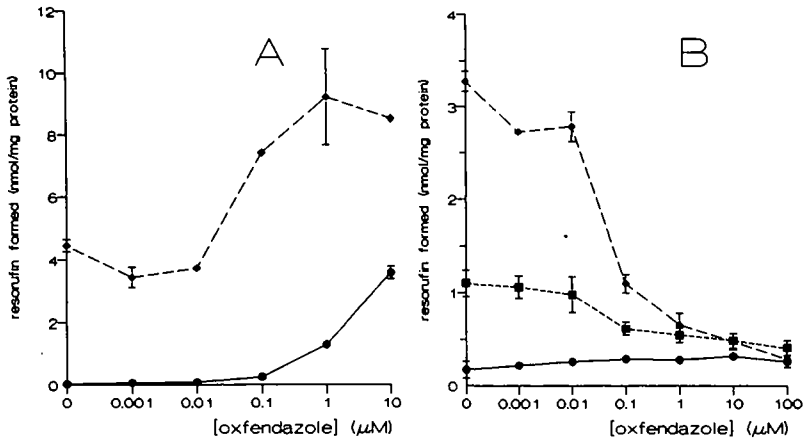

Figure 1. Effect of oxfendazole on the induction of EROD-activity by TCDD in Hepa 1c1c7 (A) or H4IIE (B) cells. Cells were incubated with 0 (●), 10 (■) or 100 (♦) pM TCDD and various concentrations of oxfendazole for 24 hrs, prior to the incubation with ethoxyresorufin for 30 (Hepa 1c1c7) or 15 min (H4IIE). Results are expressed as the mean ± SD for n=2 (Hepa 1c1c7) or n=3 (H4IIE).

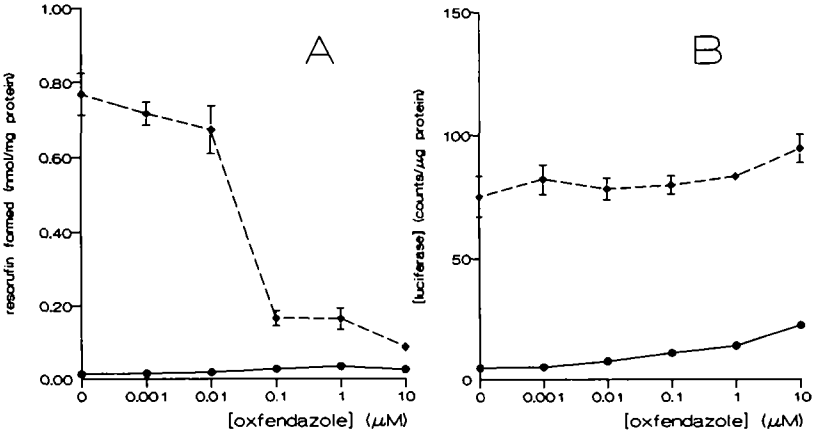

Figure 2. Effect of oxfendazole on the induction of EROD-activity (A) and luciferase concentration (B) by TCDD in pGudluc 1.1-transfected H4IIE cells. Cells were incubated in the absence (●) or presence (♦) of 100 pM TCDD and various concentrations of oxfendazole for 24 hrs, prior to the incubation with ethoxyresorufin for 60 min or the collection of the cells. Results are expressed as the mean ± SD for n=3.

Exposure of pGudluc 1.1-transfected Hepa-1c1c7 cells to either TCDD or oxfendazole resulted in an increased EROD activity as well as an increased luciferase concentration in the cells. Again, the dioxin induced levels in either enzyme activity were not decreased by oxfendazole (data not shown).

4. Discussion

The negative effect of oxfendazole on the TCDD induced EROD-activity in H4IIE cells, is in line with reports from previous studies with rabbits, showing that oxfendazole can act as a non-competitive inhibitor of cytochrome P450 1A catalyzed enzyme activities[3]. The lack of effect of oxfendazole on TCDD-induced luciferase activities in pGudluc 1.1-transfected H4IIE cells further supports the idea that the drug does not interfere with the transcriptional side of the process[3], or the binding of TCDD to the Ah receptor[5]. The clear differences observed between the rat and mouse cell-line might be related to the difference in the structure of the P450 1A enzymes.

The present data do not support the idea that the same mechanism, *i.e.* binding of oxfendazole or a metabolite to cytochrome P450 1A enzyme(s), underlies the induction of EROD activity by the drug. First of all this effect is not only observed in the H4IIE cells, but also in Hepa-1c1c7 cells, which appear to be insensitive to the inhibitory effects of the drug. Secondly, exposure of pGudluc 1.1-transfected Hepa-1c1c7 and H4IIE cells resulted not only in an induction of EROD-activity but also in the intracellular concentration of luciferase, indicating that the effect is related to the transcriptional side of the induction process.

The present data further support the use of newly developed cell-lines, like the pGudluc 1.1-transfected cells, for the biological detection of dioxin-like compounds. First of all, in comparison with the EROD-activity, an increased luciferase activity was observed at 3-10 fold lower concentrations of TCDD (data not shown). Secondly, these cells might be less sensitive to interferences of other non-dioxin-like compounds, like *e.g.* benzimidazole compounds. Furthermore, the present data further support the use of specially developed cell-lines for studying the mechanistic sides of effects.

5. References

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