Development of an *in vitro* rainbow trout cell bioassay for AhR-mediated toxins

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Abstract

A recombinant rainbow trout cell line was developed by stably transfecting the RTH-149 cell line with the pGudLuc1.1 plasmid. The new cell line, designated Remodulated Lightning Trout (RLT), contains the luciferase reporter gene under the control of the aromatic hydrocarbon receptor (AhR) and dioxin responsive enhancers (DREs). The RLT bioassay is relevant to fish and can be compared directly with similar bioassays derived from other species. It has several advantages over assays for endogenous AhR-inducible genes such as ethoxyresorufin-o-deethylase (EROD). Luciferase can be detected at levels as low as 10^{-20} M, the luciferase gene is controlled only by the AhR, and luciferase does not recognize dioxin-like compounds as substrates.

In the RLT cell line, luciferase induction increases linearly with time up to four days of exposure. Luciferase activity has a linear relationship with protein concentration throughout the range tested. Luciferase induction increases with the dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Luciferase activity was significantly greater than background at 0.1 pM TCDD.

Introduction

Halogenated aromatic hydrocarbons (HAHs), including dioxins, dibenzofurans, and PCBs, are persistent toxic chemicals. Because each class of HAH includes many congeners with different toxicities, the toxicity of HAH mixtures found in the environment is difficult to assess using chemical analysis alone. Bioassays provide an integrative measure of the toxicity of complex mixtures of HAHs. Most current bioassays use mammalian cell lines and measure EROD activity as an index of *CYP1A1* induction. The results of bioassays derived from mammalian cell lines may not be applicable to fish, since fish respond differently than mammals to many HAHs[1]. The objectives of this study are to develop and characterize a bioassay relevant to fish using the luciferase reporter gene as an index of AhR-activation. This bioassay can be compared directly with bioassays using the same reporter system but derived from cell lines of different species, such as the mouse hepa1c1c7 cell line developed by Denison, et al.[2] Luciferase activity can be detected at levels as low as 10^{-20} M, and

has no background activity in vertebrate cell lines. Unlike EROD activity, luciferase activity is controlled only by DREs, and is not inhibited by HAHs[3].

Methods

Cell Lines
RTH-149 (rainbow trout hepatoma, ATCC 1710) was derived from an aflatoxin-induced trout liver tumor[4,5]. The Ah receptor has been detected in the RTH-149 cell line[6]. RTH-149 cells were grown in basal Eagle's medium supplemented with 10 % fetal bovine serum at 21 °C and harvested with trypsin or cell lysis buffer (Promega).

Plasmids
The plasmid pGudLuc1.1 contains the long terminal repeat region from the mouse mammary tumor virus (MMTV-LTR), and four DREs taken from the 5′ region of the mouse *CYP1A1* gene (Figure 1). Preliminary studies have shown that the MMTV promoter supports transcription in fish cells, and that fish AhR recognizes the mouse DREs.

Transfections
Stable transfections used the polybrene method developed by Kawai and Nishizawa[7]. We co-transfected cells with pGudLuc1.1 and pSV_2neo. Stably transfected clones were selected with geneticin (G418). A clone with low background luciferase activity and high induced luciferase activity was selected for use in all subsequent experiments.

Luciferase Assays
Cells were dosed with TCDD in 1 μL dimethyl sulfoxide (DMSO) per mL medium, harvested in cell lysis buffer (Promega), and analyzed for protein content using a dye-binding assay (Bio-Rad)[8]. The cell lysates were then incubated with luciferase assay reagent (Promega), which contains the substrates luciferin and coenzyme A[9]. Light production was quantified with a Turner luminometer, and expressed as arbitrary luminescence units.

Results and Discussion

The relationships between luciferase induction and time, protein concentration, and dose were investigated. The time course and protein concentration experiments used a dose of 1000 pM TCDD. Luciferase induction increased in a linear relationship with time up to four days of exposure (Figure 2). Since four days of exposure produced induction with little variability, four days of exposure was used to describe the concentration-response relationship. The relationship between luciferase induction and protein concentration was linear over the entire range tested in both induced cells and solvent controls (Figure 3). This indicates that normalizing luminescence to protein concentration will give an accurate measure of luciferase induction. The dose-response relationship showed luciferase activity significantly higher than the control at all doses tested, including the lowest dose tested, 0.1 pM TCDD. Luciferase induction increased with dose over the entire range tested (Figure 4).

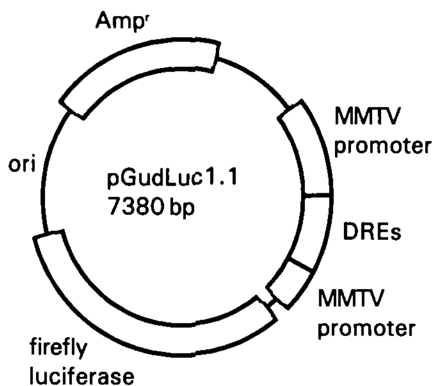

Figure 1: pGudLuc1.1 plasmid structure.

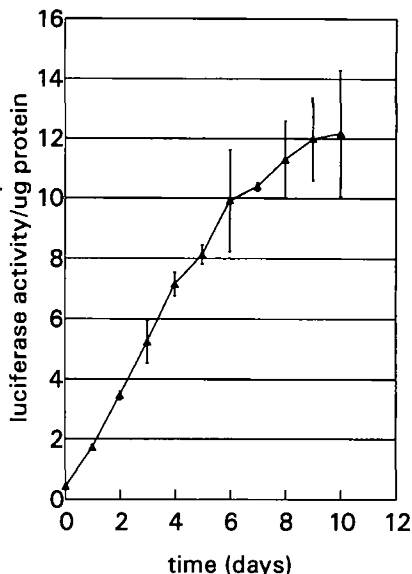

Figure 2: Luciferase induction vs time of exposure to 1000 pM TCDD.

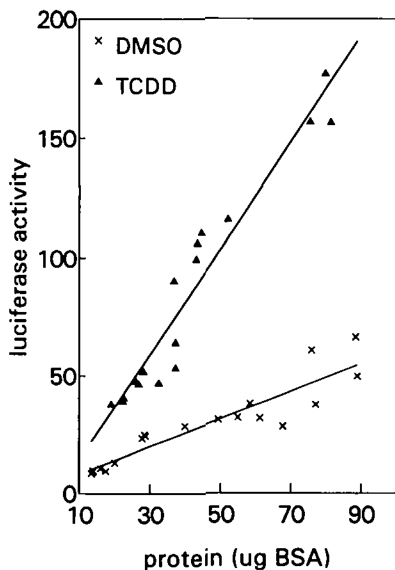

Figure 3: Relationship between protein concentration and luciferase induction after 1 day of exposure to 1000 pM TCDD or 0.1 % DMSO.

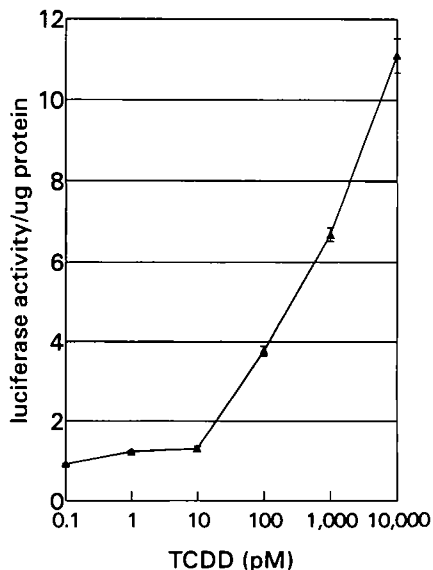

Figure 4: Relationship between TCDD dose and luciferase activity after 4 days of exposure. All treatments were significantly greater than control.

Conclusions

The RLT luciferase bioassay is a useful tool in determining the potency of HAHs to fish. After it is calibrated to the responses of whole fish, it can be used to develop TCDD equivalence factors (TEFs) relevant to fish. It will be useful in studies of interactions among HAH congeners. The RLT bioassay can be compared with chemical analyses of mixtures to check for undefined sources of toxicity. It can be used in fractionation studies to identify sources of AhR-mediated toxicity.

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