A GREEN FLUORESCENT PROTEIN BASED RECOMBINANT CELL BIOASSAY FOR THE DETECTION OF ACTIVATORS OF THE ARYL HYDROCARBON RECEPTOR: APPLICATION FOR SCREENING OF A 1,5-DIALKYLAMINO-2,4-DINITROBENZENE COMBINATORIAL CHEMICAL LIBRARY

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Introduction

Ligands of the aryl hydrocarbon receptor (AhR) are persistent and widespread environmental contaminants and include 75 polychlorinated dibenzo-p-dioxins (PCDDs), 135 polychlorinated dibenzofurans (PCDFs), and 209 polychlorinated biphenyls (PCBs)[1], as well as many other endogenous and exogenous compounds[2] These compounds, said to have dioxin-like activity, cause a spectrum of toxic effects including tumor promotion, birth defects and immune suppression[3]. AhR ligands also induce gene expression, with the majority if work focusing on a battery of detoxification enzymes such as cytochrome P4501A1 (CYP1A1). Because the toxicity of these compounds and the induction of CYP1A1 are both mediated by AhR, there is a high degree of correlation between toxicity and enzyme induction[1]. Taking advantage of this correlation, we have developed several recombinant cell bioassays that consist of cell lines stably transfected with a luciferase reporter gene that is under the control of the dioxin responsive domain from the mouse CYP1A1 gene. These bioassays have been used to measure the activity of compounds and of mixtures of chemicals to induce AhR-dependant gene expression[4,5].

Here we describe a dioxin responsive cell line that uses the enhanced green fluorescent protein (EGFP) as the reporter gene. EGFP has numerous advantages over luciferase, including low cost, ease of measurement, and lack of a requirement for reagent addition. These advantages have allowed us to optimize the bioassay for the screening of a 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library theoretically consisting of 23870 products for AhR activation.

Materials and Methods

Generation of the recombinant cell line The cell line Hepa-EGFP1.1 was created by the stable transfection of Hepa1c1c7 cells with the dioxin responsive reporter construct pEGFP1.1. This plasmid has the enhanced green fluorescent protein gene under the control of the four dioxin responsive elements from the mouse CYP1A1 gene.

Chemical treatment and measurement of fluorescence Hepa-EGFP1.1 cells were plated into black clear-bottomed 96-well microplates (Corning) at 75,000 cells per well. After 24 hours, the media was replaced with nonselective media containing the chemical to be tested or DMSO as a

control. EGFP was measured without the removal of the media at the indicated time points on a Fluostar microplate fluorometer (Molecular Dynamics) with an excitation maximum 485 nm (25 nm bandwith) and an emission wavelength of 515 (10 nm bandwith). The gain was adjusted for each experiment so that incubation with 10^{-9} M TCDD resulted in a relative fluorescence of 9000 units. Samples were run in triplicate and wells containing media were used as blanks and subtracted as background.

Screening of the combinatorial chemical library The 1,5-dialkylamino-2,4-dinitrobenzene library consists of 23870 possible compounds in groups of 10 at an estimated concentration of 0.01M. Each group of chemical products as well as all the starting materials were screened in duplicate at a final concentration of 50 uM, and the results expressed as a percent of 10^{-9} TCDD. Positive groups were identified and the compounds were resynthesized and tested individually.

Results and Discussion

The recombinant cell line Hepa-EGFP1.1 consists of the mouse hepatoma cell line, Hepa1c1c7, which has been stably transfected with a dioxin responsive green fluorescent protein reporter gene. This reporter gene has the advantage of being able to be read directly in living cells without the need for cell disruption and reagent addition. Hepa-EGFP1.1 cells respond to treatment of TCDD with reporter gene expression in a dose dependant fashion, with an EC_{50} of 3 x 10^{-11} and a limit of detection of 1 x 10^{-12} (Figure 1). Induced green fluorescent protein expression becomes significantly higher than control at 6 hours and continues to increase linearly for at least 48 hours. Hepa-EGFP1.1 cells exhibit similar selectivity for AhR ligands as previously described in other dioxin responsive cell lines[4].

Because of the relative ease of reporter gene measurement, a combinatorial library of 23870 possible 1,5-dialkylamino-2,4-dinitrobenzene compounds was screened by one person over the course of 1 week (Figure 2). The screening resulted in the identification of numerous positive wells, compounds and starting materials as having AhR agonist activity. One negative of this screening approach is that chemicals which are inherently fluorescent at the same wavelengths as EGFP may interfere with the assay and their activity can not be assessed.

Overall we feel that this bioassay can serve as a sensitive and convenient method of detecting dioxin-like activity. The ease of reporter gene measurement as well as adaptation to a 96 well format will make it a method of choice wherever large numbers of samples need to be measured.

Acknowledgments
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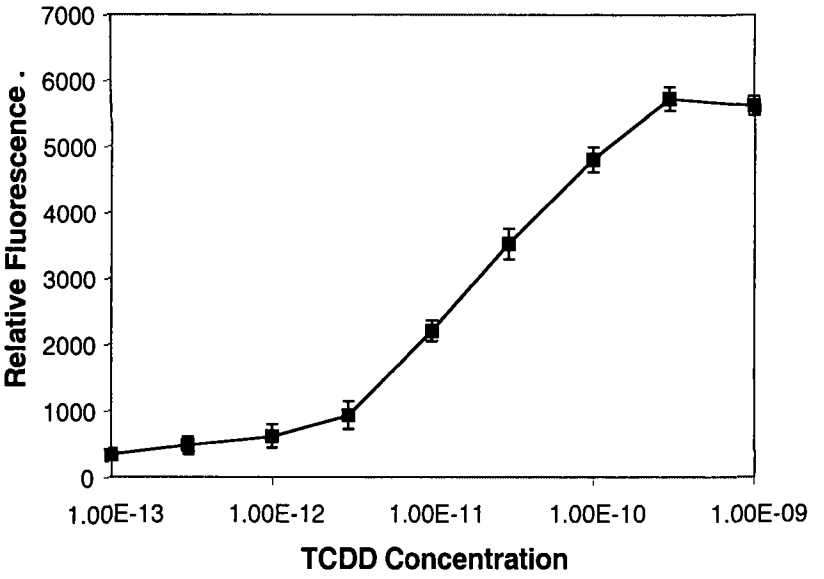

Figure 1 Concentration-response relationship for TCDD in Hepa-EGFP1.1 cells

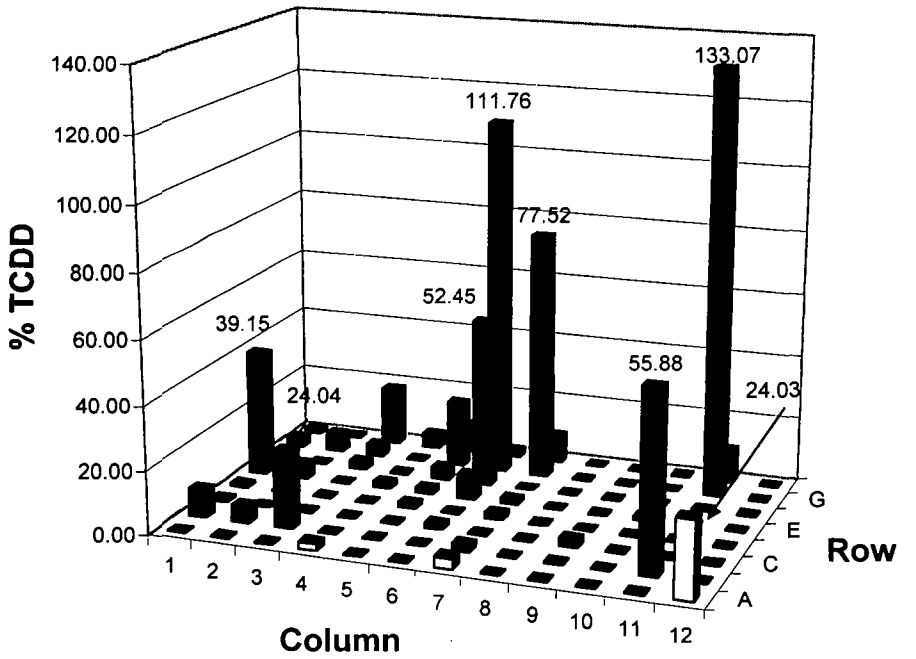

Figure 2 Screening of a 1,5-dialkylamino-2,4-dinitrobenzene library for dioxin like activity. Each bar represents the activity from a mixture of 10 compounds.