Identification of Novel Ah Receptor Agonists Using a High-Throughput Green Fluorescent Protein-Based Recombinant Cell Bioassay†

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Received June 29, 2001

ABSTRACT: The Ah receptor is a ligand-dependent transcription factor that mediates the biological and toxic effects of polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin). Recent evidence also suggests a role for the AhR in normal physiology and development. Although a variety of structurally diverse chemicals are reported to bind to and activate the AhR, the full spectrum of structural chemical classes that can interact with the AhR remains to be elucidated. Large-scale analysis of the ligand binding specificity of the AhR requires the use of a high-throughput AhR bioassay system for chemical screening. We have utilized a recombinant mouse hepatoma cell line (H1G1.1c3) containing a stably integrated TCDD- and AhR-responsive enhanced green fluorescent protein (EGFP) reporter gene to screen a 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library consisting of 155 parental amines and up to 12 090 combinatorial products in less than 7 days for novel AhR agonists. These analyses have identified numerous parental amines as relatively potent inducers of EGFP (with EC_{50}s between 8 and 1000 μM) and also have revealed several novel products of the combinatorial chemical library synthesis with EC_{50}s between 10 and 100 μM. Overall, these results have not only allowed the identification of novel activators of the AhR but also demonstrate the utility of the recombinant H1G1.1c3 cell bioassay for high-throughput chemical screening.

The aryl hydrocarbon receptor (AhR)[1] is a ligand-dependent transcription factor that is activated by the high-affinity binding of a variety of halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs, respectively) (*1*, *2*). Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH, results in a variety of toxic and biochemical effects (*3*), the majority of which are mediated by the AhR (*4*−*6*). Ligand-dependent AhR activation results in its nuclear accumulation, dimerization with the AhR nuclear translocator (ARNT) protein, and binding of the ligand/AhR/ARNT dimer to DNA (*7*−*9*). The interaction of the AhR complex with its specific DNA recognition site, the dioxin response element (DRE), results in an increase in transcription of the adjacent gene, and numerous genes appear to be regulated by this mechanism (reviewed in *2*, *10*).

Over the past 20 years, numerous studies have examined the ligand binding specificity of the AhR with an emphasis on the analysis of HAHs and PAHs. These studies have provided important information with regard to the characteristics of AhR ligands and established key electronic, thermodynamic, and structural characteristics common to AhR ligands (*1*, *11*−*15*). Although these analysis have provided insight into the AhR ligand binding specificity, given the focus on HAHs and PAHs, it remains to be determined whether the derived characteristics are also relevant to other classes of ligands. The recent identification of numerous AhR ligands/agonists whose structural and physiochemical properties deviate significantly from those of the more "classical" HAH/PAH AhR ligands/agonists demonstrates that the AhR can be bound and activated by structurally diverse chemicals (*16*). Although the majority of these chemicals are relatively weak ligands when compared to HAHs and PAHs, identification and characterization of these novel AhR ligands are important for several reasons. Knowledge of the physiochemical and structural properties of these ligands not only will allow more accurate modeling of AhR ligands and the AhR ligand binding pocket but also will facilitate the development of more accurate approaches to predict AhR binding by unknown chemicals. Analysis of these "nonclassical" AhR ligands may also provide insights into the physiochemical characteristics of high-affinity endogenous ligand(s) for the AhR. Although numerous studies strongly suggest the existence of such a ligand(s), no endogenous physiological ligand has yet been identified.

Recent reports from our lab and others have described the development of several sensitive bioassay systems for the

† This research was supported by the National Institutes of Health (ES07685, ES04699, ES05707, AI41698) and by a National Institute of Environmental Health Sciences Environmental Toxicology Training Grant (ES07059).

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[1] Abbreviations: AhR, aromatic hydrocarbon receptor; ARNT, Ah receptor nuclear translocator; CYP1A1, cytochrome P4501A1; DRE, dioxin responsive element; DMF, *N*,*N*-dimethylformamide; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DTT, dithiothreitol; EC_{50}, effective concentration at 50%; EGFP, enhanced green fluorescent protein; EROD, ethoxyresorufin *O*-deethylase; HEDG, 25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

10.1021/bi011373v CCC: $22.00
Published on Web 12/22/2001

identification and characterization of AhR ligands/agonists, and these systems respond to AhR agonists with the induction of both endogenous and transfected reporter genes (*17*−*20*). These cell bioassays are very sensitive, and they have allowed the identification and characterization of several novel AhR agonists/ligands, including tryptamine, indoleacetic acid, carbaryl, bilirubin, biliverdin, lipoxin A4, prostaglandins, and others (*16, 21*−*26*). Characterization of the spectrum of chemicals that can bind to and activate the AhR will require a tremendous amount of chemical screening. High-throughput screening bioassays provide an avenue in which to rapidly screen large numbers of chemicals and chemical mixtures (i.e., combinatorial chemical libraries) for a desired activity. The screening of chemical libraries provides one avenue in which to generate the extensive amount of binding data needed to assess AhR ligand binding specificity. Although the currently available AhR-based cell bioassays are exquisitely sensitive, these systems have several characteristics (i.e., extensive sample preparation and analysis time, as well as relatively high reagent cost) which restrict their adaptation into high-throughput screening bioassays.

We have recently described the development of a novel high-throughput recombinant cell bioassay for the detection of AhR agonists (*27*). This bioassay employs mouse hepatoma (Hepa1c1c7) cells that have been stably transfected with a reporter plasmid containing the enhanced green fluorescent protein (EGFP) reporter gene under the AhR-dependent control of four dioxin-responsive elements (DREs). Exposure of these recombinant cells (H1G1.1c3) to TCDD and other AhR agonists results in an induction of EGFP in a dose-, time-, and chemical-specific manner (*27*). Because EGFP can be directly measured in intact cells without the addition of any necessary reagents, the H1G1.1c3 cells can be easily adapted to high-throughput applications. Here we demonstrate the utility of the recombinant H1G1.1c3 cells as a high-throughput bioassay through the rapid screening of a combinatorial chemical library containing up to 12 090 compounds as well as the 155 parental chemicals. These studies have resulted in the identification of several novel chemicals which can activate AhR-dependent EGFP gene expression.

MATERIALS AND METHODS

Materials. TCDD was a gift from Dr. Steven Safe (Texas A&M University). All of the parental amines, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), HPLC-grade *N*,*N*-dimethylformamide (DMF), and dimethyl sulfoxide were purchased from Aldrich. Many of these chemicals are extremely toxic and/or carcinogenic, and they were handled and disposed of as have previously described (*20*). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA), and Geneticin (G418) and all tissue culture media were from Life Technologies (Grand Island, NY).

Combinatorial Library Synthesis. Generation of the solution phase 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library was previously described in detail (*28*) and is depicted in Figure 1. DFDNB (5.0 g; 24.5 mmol) and 2.2 equiv of *N*,*N*-diisopropylethylamine were dissolved in 35.0 mL of DMF and further distributed into 155 individual wells of two 96-well polypropylene plates (Labnet Technologies, Edison, NJ; capacity 1.2 mL/well). For the primary reaction,

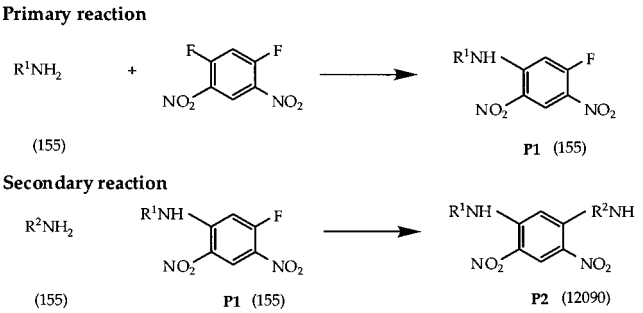

FIGURE 1: Synthetic scheme for the synthesis of the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library. 155 different amines were reacted with DFDNB to form 155 separate 1-alkylamino-2,4-dinitro-5-fluorobenzene compounds (P1). These were pooled in groups of 10 and reacted with the 155 amines for a second time to produce 12 090 1,5-dialkylamino-2,4-dinitrobenzene compounds (P2).

155 amines in DMF (0.35 mL, 1.0 M) were each added into individual wells containing the DFDNB solution. After sealing, the reactions were allowed to proceed at room temperature with strong agitation for 3 h. The contents of the wells (P1, Figure 1) were pooled in groups of 10, diluted to 69.75 mL, and distributed at 0.45 mL/well over 155 wells. Aliquots of the 155 amines (0.05 mL) were added to the corresponding wells of the 96-well plates, the plates were sealed, and the secondary reactions were allowed to proceed overnight with strong agitation. The final products (P2) at a predicted concentration of 1 mM were stored at 4 °C for future use. Because of the symmetrical nature of the 1,5-difluoro-2,4-dinotrobenzene molecule, the total number of predicted final products is 12 090, with 19 135 heterobifunctional and 155 homobifunctional compounds.

Cell Culture and Screening of the Combinatorial Chemical Library. H1G1.1c3 cells were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (50 units/mL each), and 968 mg/L G418. For microplate analysis of EGFP, cells were plated into black, clear-bottomed 96-well tissue culture dishes (Corning, San Mateo, CA) at 75 000 cells per well and allowed to attach for 24 h prior to chemical treatment. Treatment of cells with pure compounds or the combinatorial library involved adding 1.25 μL of each reaction mixture to 250 μL of MEM in a clean 96-well microplate (to a final concentration of 5 μM), and mixing by repeated pipetting. After removal of the medium from the cells, 100 μL of the medium containing the test chemicals was transferred from the dilution plate into the corresponding wells of a 96-well plate containing H1G1.1c3 cells. After incubation at 33 °C for 24 h [37 °C results in decreased EGFP activity (unpublished results)], EGFP levels were measured in the intact cells (without removal of medium) using a Fluostar microplate fluorometer (Phenix Research Products). Plates were read from the bottom of each well using an excitation wavelength of 485 nm (25 nm bandwidth) and an emission wavelength of 515 nm (10 nm bandwidth). To normalize fluorescence levels between experiments, the instrument fluorescence gain was adjusted so that the level of EGFP fluorescence induced by 1 nM TCDD produced a relative fluorescence of 9000 relative fluorescence units. Induction was expressed as a percentage of that attained with 1 nM TCDD (a maximal inducing concentration) after correction

for the background fluorescence in the solvent control samples.

Preparation of Cytosol. Male Hartley guinea pigs (250−300 g), obtained from Charles River Breeding Laboratories (Wilmington, DE), were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water. Hepatic cytosol was prepared in HEDG buffer [25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] as described (*29*) and stored at −80 °C until use.

Gel Retardation Analysis. A complementary pair of synthetic oligonucleotides containing the sequences 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATC-CGGAGTTGCGTGAGAAGAGCCA-3′ (corresponding to the AhR binding site of DRE3 and designated as the DRE oligonucleotide) were synthesized, purified, annealed, and radiolabeled with [^{32}P]ATP as described (*9*). Gel retardation analysis of cytosolic AhR complexes transformed in vitro with TCDD (20 nM) or the indicated compound was carried out as previously described (*21*) and protein−DNA complexes were visualized by autoradiography.

RESULTS

We have previously described the development of a rapid and sensitive AhR agonist-responsive EGFP-based recombinant H1G1.1c3 cell bioassay that has been optimized for use in a high-throughput 96-well microplate format (*27*). Although there is a significant level of constitutive EGFP expression in the H1G1.1c3 cells, the low degree of error (less than 4% between any two wells of the microplate for control values) and the high sensitivity of the assay [minimal detection limit of ∼1 pM and an EC_{50} of ∼18 pM (*27*)] allow for the identification of positive chemicals with a reasonable degree of confidence. Thus, the H1G1.1c3 cells provide us with a sensitive and high-throughput assay system for the detection of AhR agonists.

Screening of the 155 Parental Chemical Library Amine Reactants for AhR Agonist Activity. The synthesis of the selected combinatorial chemical library involves coupling of 155 different amines to DFDNB. Because pooled reactions of the library were to be screened without purification, we first needed to assess whether the parental amines as well as DFDNB could induce EGFP expression in H1G1.1c3 cells. In our initial studies, each chemical was added to cells at a final concentration of 10 mM. However, nearly all of the parental amines produced visible toxicity at this concentration. Each chemical, with the exception of compound 83, was subsequently tested at 1.0 and 0.1 mM final concentrations. Compound 83 (3,6-diaminoacridine) was not examined using H1G1.1c3 cells because it was inherently fluorescent at the same wavelength as EGFP. Subsequent analysis of compound 83 in our AhR-responsive luciferase cell bioassay (*20*) demonstrated that this chemical was inactive as an inducer. Of all 155 parental amines used to create the combinatorial library, 27 induced EGFP expression to between 20 and 140% of that induced by 1 nM TCDD (Table 1). DFDNB itself did not induce EGFP expression to any significant extent at any concentration tested (data not shown). The structures of the 11 parental amines that induced EGFP activity to a level greater than 70% of that induced by 1 nM TCDD are shown in Figure 2. Dose−response analysis of the most active compounds (parental amines 22, 66, 117, 142, and 143) in the H1G1.1c3 cells revealed that these chemicals were relatively potent inducers (Figure 3). The EC_{50}s for EGFP induction by these chemicals were between 9 and 140 μM (Table 2), approximately 5×10^5- to 1×10^7-fold lower that that of TCDD (18 pM). A

Table 1: Relative Potency of Selected Parental Amines as Inducers of EGFP Activity in H1G1.1c3 Cells

amine no.	name	relative activity[a] 0.1 mM	1.0 mM
12	1-aminonaphthalene	22.7	N[b]
22	(1*S*,2*R*)-(−)-*cis*-1-amino-2-indanol	117.1	91.5
23	6-aminoindazole	18.9	31.5
43	2-amino-1-phenylethanol	16.4	24.6
54	benzylpiperidine	28.0	N
55	benzylamine	24.6	2.1
57	benzylhydrazine dihydrochloride	136.7	35.5
58	3-benzyloxyaniline	70.8	0.6
64	2-chloroaniline	6.5	23.8
65	3-chloro-*p*-anisidine	52.6	54.9
66	3-chlorophenylhydrazine hydrochloride	140.5	N
71	(*R*)-(−)-1-cyclohexylethylamine	21.8	N
75	1,2-diaminocyclohexane	24.9	N
78	1,5-diaminonaphthalene	70.9	38.0
79	1,8-diaminonaphthalene	88.9	N
87	3,5-dimethoxybenzylamine	29.0	19.8
103	2-iodoaniline	35.9	18.5
112	2-(methylmercapto)aniline	85.3	80.1
114	3,4-(methylenedioxy)aniline	41.4	N
117	1-methyl-1-phenylhydrazine	82.6	76.7
126	1,2-phenylenediamine	37.8	17.9
134	piperonylamine	13.6	27.7
140	2-(trifluoromethyl)phenylhydrazine	48.6	19.0
141	4-(trifluoromethoxy)phenylhydrazine hydrochloride	112.7	N
142	1,2,3,4-tetrahydro-1-naphthylamine	128.2	84.4
143	1,2,3,4-tetrahydroisoquinoline	104.3	75.2
149	5,6,7,8-tetrahydro-1-naphthylamine	26.6	12.3

[a] Induction is expressed as the mean percent of 1 nM TCDD from two replicates. [b] N: Values were less than control.

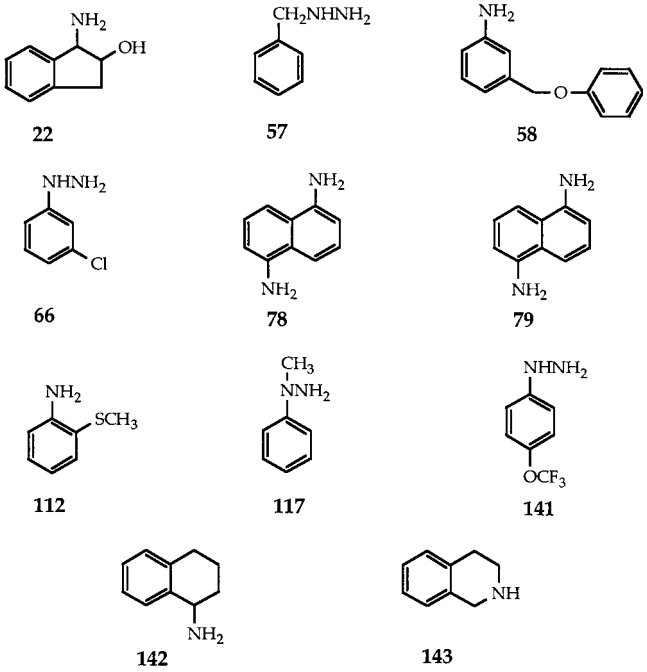

FIGURE 2: Structures of selected parental amines used in the synthesis of the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library that induced EGFP fluorescence in H1G1.1c3 cells to a level of ≥70% of that obtained with 1 nM TCDD.

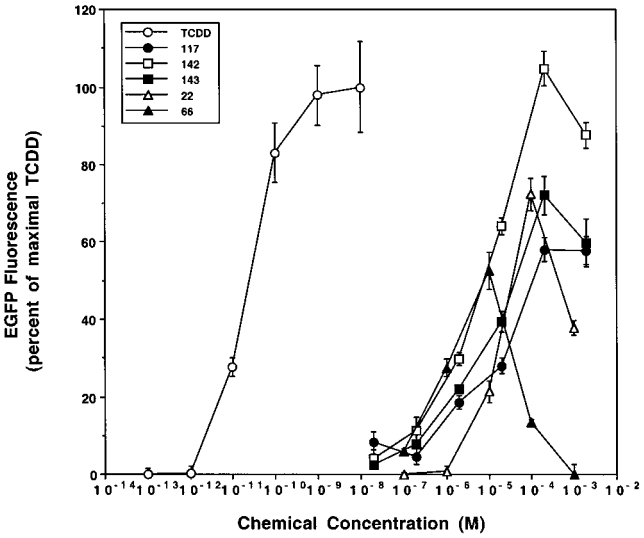

FIGURE 3: Dose−response relationships for induction of EGFP fluorescence in H1G1.1c3 cells by TCDD and selected parental amines. H1G1.1c3 cells were treated with the indicated chemicals for 24 h at 33 °C, and the EGFP activity was measured as described under Materials and Methods. Results are expressed as the mean ± SD of triplicate determinations relative to that induced by 1 nM TCDD. Data in each dose−response curve are significantly different from control ($p < 0.01$) as determined by the Student's *t*-test at concentrations equal to or greater than 1×10^{-11} M for TCDD, 2.5×10^{-6} M for P1:109, 2.5×10^{-6} M for P1:109,NH_2, and 2.5×10^{-6} M for P2:109,109.

Table 2: EC_{50}s and Relative Potencies for Induction of EGFP in H1G1.1c3 Cells

chemical	EC_{50} (μM)	relative potency[a]
TCDD	0.018	1
amine 22	60.4	3×10^{-7}
amine 66	9.0	2×10^{-6}
amine 117	139.5	1×10^{-7}
amine 142	12.6	1.5×10^{-6}
amine 143	78.3	2×10^{-7}

[a] Values are expressed relative to the EC_{50} for EGFP induction by TCDD.

decrease in EGFP activity by all of the amines was observed at 1−2 mM, and this correlated with visible cell toxicity/death observed at higher concentrations. To confirm that the inducing activity of these chemicals is due to their ability to activate the AhR, we examined the ability of these chemicals to stimulate AhR transformation and DNA binding in vitro using gel retardation analysis. Incubation of guinea pig hepatic cytosol with amines 22, 66, 117, 143, or 143 induced formation of a protein−DNA complex similar to that induced by TCDD (Figure 4). Previously studies have demonstrated that this induced protein−DNA complex represents the DNA-bound AhR complex (*2, 16*). These results indicate that the ability of these chemicals to induce EGFP expression is consistent with their ability to activate the AhR signal transduction pathway.

Screening of the Combinatorial Library. After synthesis, the combinatorial library was distributed over a total of 26, 96-well microplates in a total of 2494 wells, with each well containing the pooled groups of 10 predicted final products at an estimated concentration of 1 mM. In addition to the final products, each well could also contain unreacted chemicals and reaction intermediates. For library screening

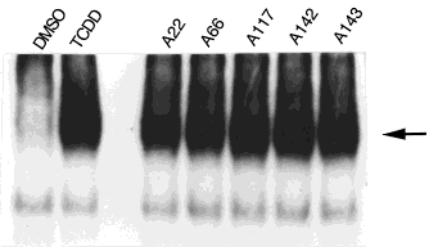

FIGURE 4: Parental amines stimulate AhR transformation and DNA binding in vitro. Guinea pig hepatic cytosol (16 mg/mL) was incubated with DMSO, 20 nM TCDD (in DMSO), or 4 mM of the indicated parental amine (in DMSO) for 2 h at 20 °C. Aliquots of each sample were mixed with [^{32}P]DRE, and protein−DNA complexes were resolved by gel retardation analysis as described under Materials and Methods. Only the protein−DNA complexes are shown, and the arrow indicates the position of the inducible AhR−DRE complex.

experiments, H1G1.1c3 cells grown in 96-well microplates were treated with an aliquot of each library reaction mixture (to a predicted final concentration of 5 μM in MEM, 0.5% DMF) for 24 h at 33 °C. DMF was not visibly toxic to the cells at this solvent concentration and did not inhibit induction of EGFP by TCDD (data not shown).

Typical results from four plates of our screening of the combinatorial library are shown in Figure 5. Each graph within the figure represents the induction results obtained for a single plate of 960 predicted final products. The results for each sample are the average of 2 separate determinations and represent EGFP reporter gene induction by a mixture of 10 different predicted final products containing 10 different R1 groups, a common R2 group, plus any reactants and intermediates of the library synthesis present in the final reaction mixture that was added to the well. Comparison of EGFP induction in several plates revealed not only that some well positions were consistently positive (i.e., induction was observed) but also that the magnitude of induction was very similar (e.g., compare wells B11, F6, G6, G7, and G11 in Figure 5A,B). The experimental design and layout of the library were such that each identical well position in this group of plates had a common R2 alkylamino group. Wells B11, F6, G6, and G7 contained as R2 amines 23, 66, 78, and 79, respectively, each of which was shown to induce EGFP expression in H1G1.1c3 cells by themselves (Table 1), while well G11 contained a parental amine (#83, 3,6-diaminoacridine) which was inherently fluorescent at the same wavelength as EGFP. As mentioned above, amine 83 was inactive as an inducer in the AhR-responsive luciferase bioassay, and this well was not analyzed further. In contrast to the results shown in Figure 5A,B, there was little or no induction of reporter gene expression for the majority of the samples tested (Figure 5C). Since many of these wells contained parent amines that were shown to induce EGFP in H1G1.1c3 cells by themselves (Table 1), the low level or lack of induction indicates that most of the combinatorial library conjugation reactions were relatively efficient and that the reaction products were poor inducers.

To demonstrate the utility of this rapid screening approach, a well that was positive only in one of the screened plates of library compounds [well H1 (Figure 5D)] was further analyzed. AhR agonist activity in this well would logically be assumed to result from a final product, as possible intermediates for these reactions would also be present in

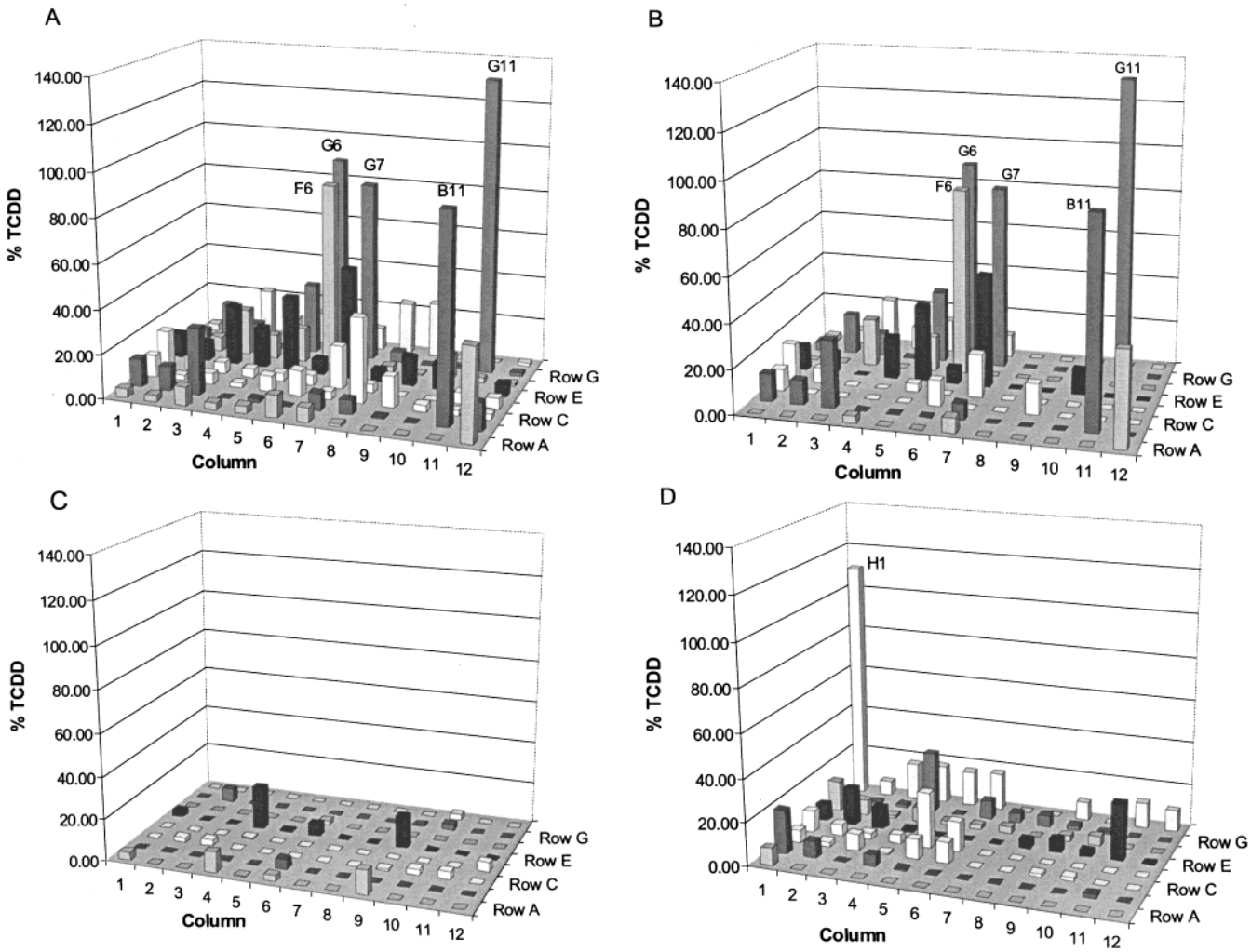

FIGURE 5: Typical 96-well screening results for the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library in H1G1.1c3 cells. The results from four typical plates (A−D) are presented. Each 96-well plate containing the combinatorial chemical library was screened for AhR agonist activity as described under Materials and Methods at an estimated final concentration of 5 μM. Each bar represents the average of two determinations.

other wells where EGFP was not induced (data not shown). Based on the reaction scheme, the products in this specific well would all have amine 109 in the R2 position and would contain amines 65−74 (Figure 6) in the R1 position. Although amines 65, 66, and 71 were shown to have AhR agonist activity, the low level or lack of activity in the other wells in this group that also contained these amines as an R1 substituent (rows G and H, Figure 5) indicated that the primary reaction with the DFDNB molecule was very efficient and little of the parental amines remained to induce EGFP. To examine the activity of the individual products in this well, each was synthesized and tested for their ability to induce EGFP in H1G1.1c3 cells. It should be noted that reaction amines 65 and 66 were not used in these experiments because of low reaction efficiency in the secondary reaction [based on HPLC analysis of the library synthesis reactions (data not shown)]. As shown in Figure 7, reactions containing amine 109 as the R2 conjugate and amines 68 and 74 in the R1 position were able to induce EGFP expression to 24 and 15% of maximal TCDD induction, respectively, at a concentration of 5 μM. In contrast, P2 products with amine 109 in the R2 position and amines 67 and 69−73 in the R1 position were relatively poor inducers, as compared to TCDD. Similar to the screening of the entire combinatorial library, these reactions were tested without purification of the final products, and as such, it was still possible that synthesis intermediates present in the final reaction mixture contributed to or were responsible for the ability to activate AhR. To examine this possibility further, products from the initial reaction (P1) that contained amines 109, 68, or 74 inserted onto the R1 position were tested for their ability to induce EGFP expression in H1G1.1c3 cells. As shown in Figure 8, the P1 compound containing amine 109 in the R1 position was able to induce EGFP expression to a level comparable to the reaction containing the P2:109,68 product (Figure 7), while the P1 products containing amine 68 or 74 in the R1 position were essentially negative.

In a final analysis of the AhR activity of products containing the parental amine 109, the relative potencies of P1:109, P1:109,NH_2 (a compound where the fluorine leaving group from P1:109 was replaced with an amino group), and the homobifunctional P2:109,109 were examined (Figure 9). At 25 μM, P1:109 induced EGFP activity to ~60% of that obtained by 1 nM TCDD (Figure 10), while P2:109,109 had essentially the same ability to activate the AhR as did P1:109. Interestingly, treatment of H1G1.1c3 cells with P1:109,NH_2 resulted in a significant increase in EGFP induction to 90% of that induced by TCDD. The reason for

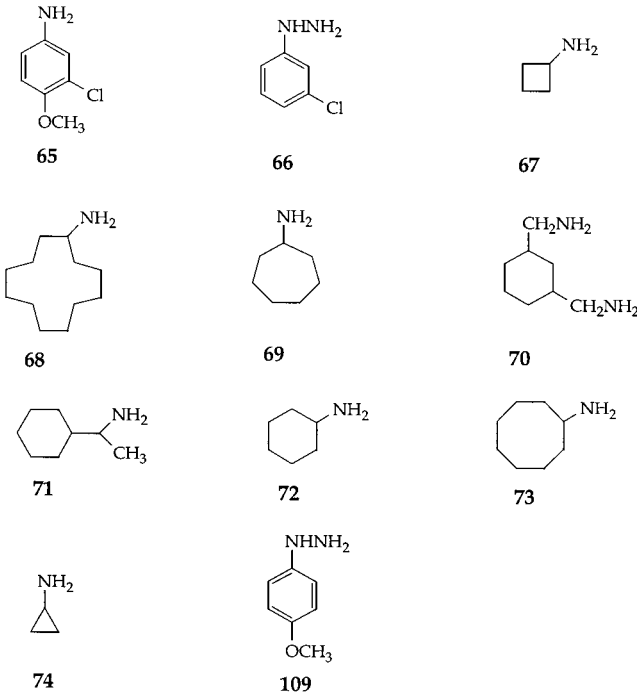

FIGURE 6: Structures of the parental amines used in the synthesis of the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library compounds present in well H1 in Figure 5D.

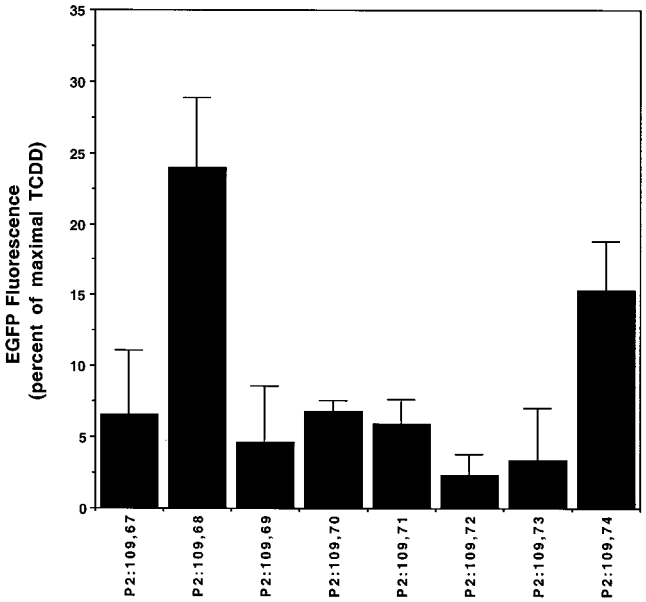

FIGURE 7: Induction of EGFP in H1G1.1c3 cells by individual compounds from a single well of the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library. The 10 compounds predicted to be present in well H1 of the plate shown in Figure 5D were synthesized individually and tested at 5 μM for their ability to induce EGFP expression in H1G1.1c3 cells as described under Materials and Methods. Each bar represents the mean ± the range from two separate determinations.

this significant change in inducibility remains to be determined.

DISCUSSION

We have recently developed a recombinant mouse hepatoma cell line, H1G1.1c3, that responds to AhR agonists with the induction of EGFP in a time-, dose-, and AhR-dependent manner (27). The cell bioassay is sensitive (minimal detection limit of ~1pM and an EC_{50} of 18 pM) and has a 300-fold dynamic response range, comparable to that of our previously described AhR−luciferase cell bioassays. However, given the numerous advantages of the EGFP reporter system including its rapidity (a 96-well plate is read in less than 1 min), simplicity (there are no washing, lysis, or reagent addition steps), ease of measurement (cell fluorescence is measured directly in intact cells in each well of the plate without the removal of media), low cost (no reagent costs), and analysis of the samples in the 96-well format, EGFP is our reporter gene of choice for high-throughput analysis. Because of the above-mentioned advantages, one person was able to screen the entire 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library of over 12 090 compounds in less than 7 days. From a comparative point of view, complete analysis of the library using the luciferase cell

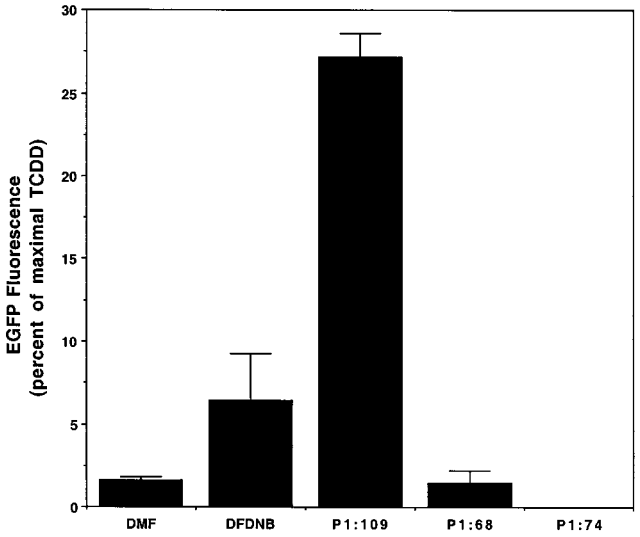

FIGURE 8: Induction of EGFP in H1G1.1c3 cells by P2:109,68 and P2:109,74 intermediates of the combinatorial chemical library. Possible intermediates of the combinatorial library synthesis (P1:109, P1:68, and P1:74) were tested at 5 μM for their ability to induce EGFP expression in H1G1.1c3 cells as described under Materials and Methods at an estimated final concentration of 5 μM. Each bar represents the mean ± the range from two separate determinations.

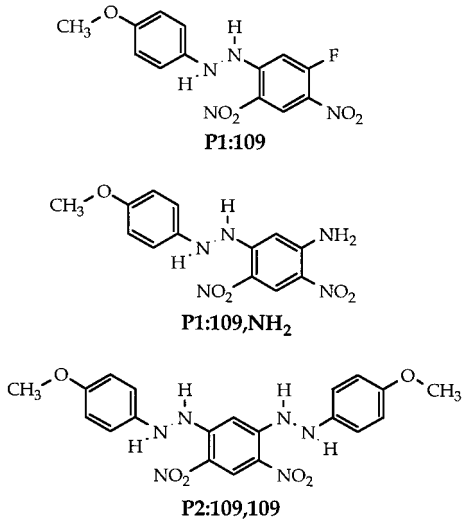

FIGURE 9: Structures of the 1,5-dialkylamino-2,4-dinitrobenzene combinatorial chemical library products P1:109, P1:109,NH_2, and P2:109,109.

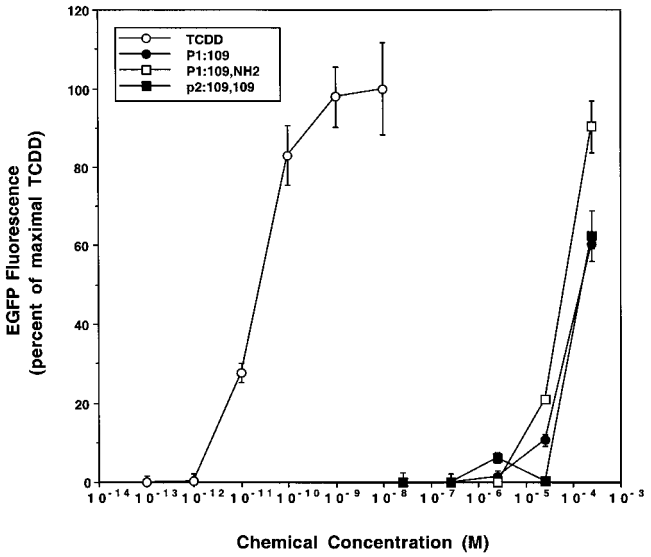

FIGURE 10: Dose−response relationships for the induction of EGFP by the mono- and dialkylamino-2,4-dinitrobenzene compounds P1:109, P1:109,NH_2, and P2:109,109. H1G1.1c3 cells were treated with the indicated chemicals for 24 h at 33 °C, and the EGFP activity was measured as described under Materials and Methods. Values are expressed as the mean ± SD of triplicate determinations relative to that induced by 1 nM TCDD. Data in each dose−response curve are significantly different from control ($p < 0.01$) as determined by the Student's *t*-test at concentrations equal to or greater than 1×10^{-11} M for TCDD, 1×10^{-5} M for compound 22, 1×10^{-6} M for compound 66, 2×10^{-6} M for compound 22, 2×10^{-6} M for compound 142, and 2×10^{-7} M for compound 143.

bioassay would have taken an additional 60 h of labor and an additional cost of $1245 for supplies.

In our initial experiments, we demonstrated the feasibility of rapidly screening 155 pure chemicals, and of the 27 that could induce EGFP expression, dose−response analysis revealed that the more active chemicals (amines 22, 66, 117, 142, 143) had EC_{50} values in the micromolar range. Although we were initially surprised by the inducing potency of the monocyclic compounds as activators of AhR-dependent gene expression, a subsequent review of the literature revealed several previously reported monocyclic aromatic amine inducers of CYP1A1. Cheung et al. (*30*) reported that 2,3-diaminotoluene and to a lesser extent 2,4-diaminotoluene not only could induce hepatic CYP1A1 activity and EROD activity in rats but also could competitively bind to the AhR. More recently, Degawa et al. (*31*) demonstrated that 4-methoxy-2-nitroaniline and to a lesser extent 2-methoxy-5-nitroaniline and 2-methoxy-4-nitroaniline could induce CYP1A1 and EROD in rat liver. However, the ability of these chemicals to bind directly to and activate the AhR was not examined. Given these published results and our gel retardation analysis experiments which revealed the ability of these amines to stimulate AhR transformation and DNA binding in vitro, it is expected that our monocyclic inducers and likely the nitroanisidines of Degawa et al. (*31*) are AhR ligands. Direct competitive AhR ligand binding by these compounds is currently being examined. In addition to these monocyclic amines, a recent study has also demonstrated the ability of several diaminonaphthalenes (1,5-, 1,8-, and 2,3-) not only to induce CYP1A1 expression and EROD activity in rat liver but also to competitively bind to the rat hepatic AhR (*30*). Our identification of both 1,5- and 1,8-diaminonaphthalenes (amines 78 and 79) as inducers not only confirms these previous results but also demonstrates the utility of our assay to accurately identify AhR agonists/ligands from a large group of compounds.

Knowledge of the inducing potency of the starting parental amines and the exact layout of the combinatorial chemical library was crucial to interpreting the library screening results and in the selection of the final reaction mixtures from which to test compounds individually. Screening compounds in groups of 10, without purification, required a certain amount of inferences to be drawn in order to determine the significance of positive results from a well. For instance, we chose not to individually test reactions from several well positions that were consistently positive in the screening, many of which contained amines that were positive themselves. Accordingly, to demonstrate the utility of the screening approach, we chose one of several wells that was not located in a consistently positive well position to increase the likelihood of identifying a novel P2 product that was an AhR activator. These experiments led to the identification of several novel compounds (P1:109, P1:109,NH_2, p2:109,109, and P2:109,68) that could activate the AhR signaling pathway. Since P1:109 produced a similar degree of EGFP induction as did P2:109,68, it is possible that the activity in the P2:109,68 reaction mixture was due to residual P1:109 in the final reaction mixture or that the effect of the R1 alkylamino substituent opposite the 109 group (amine 68) had no effect on AhR agonist activity. However, given that the primary and secondary reactions that produced these compounds were shown to be highly efficient by HPLC (data not shown), little residual P1:109 would be present in the final reaction mixture and responsible for the induction response. The fact that other R1 groups besides 68, 74, and 109 produced compounds that were essentially inactive (i.e., R1 groups 67, 69−73) indicates that the R group directly opposing amine 109 is indeed important in determining AhR agonist activity of the product. Interestingly, the parental amines were substantially more potent than any of the P2 products examined. Our failure to identify a final product of the combinatorial library synthesis (P2 compound) that was a potent AhR activator may be reflective of the larger size of these compounds as compared to the ligand binding pocket of the AhR. We have observed that the addition of bulky substituents in key positions in chlorinated dioxin congeners results in a drastic decrease in their ability to activate the AhR (data not shown). Also, no attempt was made in these studies to determine either the stability of these compounds, their antagonist activity, or their ability to penetrate cell membranes, all of which could greatly affect a chemical's ability to activate the AhR in this cell bioassay.

In conclusion, using the recombinant H1G1.1c3 cells, we have been able to rapidly and inexpensively screen 155 pure compounds and a combinatorial chemical library consisting of up to 12 090 compounds to identify several novel chemicals that could activate AhR-dependent gene expression. The primary advantages of the EGFP bioassay over previously described AhR-responsive bioassays are its high-throughput nature and lower cost. Accordingly, this cell line can be used for screening large numbers of chemicals in order to define AhR ligand specificity using quantitative structure−activity relationship analysis to identify natural AhR ligands. In addition, since previous studies (*32*−*34*) have described

several AhR agonists for use in clinical treatment of breast cancer [due to the antiestrogenic activity of AhR ligands (*35*, *36*)], H1G1.1c3 cells can be used as a high-throughput screen to identify additional therapeutic agents. Overall, the H1G1.1c3 cells provide an avenue for rapid and extensive routine screening to identify AHR agonists and antagonists.

ACKNOWLEDGMENT

We thank Drs. Martin Privalsky and Robert Rice for critical reading of the manuscript.

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BI011373V