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Novel Stably Transfected Gene Reporter Human Hepatoma Cell Line for Assessment of Aryl Hydrocarbon Receptor Transcriptional Activity: Construction and Characterization

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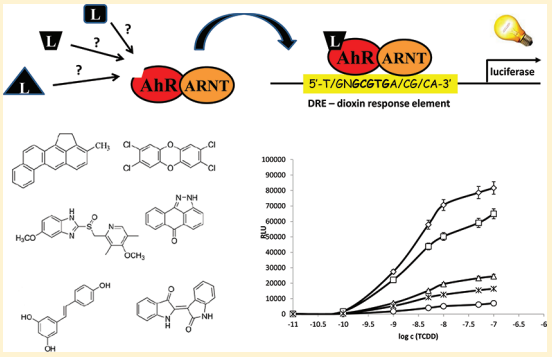
ABSTRACT: We constructed stably transfected gene reporter cell line AZ-AHR, allowing measurement of aryl hydrocarbon receptor (AhR) transcriptional activity. Human hepatoma HepG2 cells were transfected with a construct containing several AhR binding sites upstream of luciferase reporter gene. We prepared 12 clones and we characterized the best five in responsiveness to TCDD. Dose−response analyses were performed for various AhR ligands, including TCDD, 3-methylcholanthrene, indirubin, resveratrol, omeprazole, and SP600125. The EC_{50} values were similar in all tested clones. Induction of luciferase was time-dependent, and treatment for 6 h with 5 nM TCDD was sufficient to evaluate AhR transcriptional activity in 96-well plate format (8−24 fold induction). Response to AhR ligands of cryopreserved cells after thawing was not significantly different from that of fresh cells. Cell line remained fully responsive to AhR ligands over 15 passages and 30 days in culture without significant alterations. Overall, we have developed novel human luciferase reporter cell line AZ-AHR for monitoring AhR transcriptional activity. The sensitivity of the assay allows high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h of incubation, which has potential implication for studies of cytotoxic compounds.



1. INTRODUCTION

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor. It is activated by a variety of exogenous ligands, including synthetic compounds (polyaromatic amines, polyaromatic hydrocarbons, polychlorinated aromatic compounds, dioxins, SP600125 = 1,9-Pyrazoloanthrone) natural compounds (resveratrol, berberine, flavonoids) and drugs (omeprazole, lansoprazole, TSU-16). Endogenous AhR ligands are bilirubin, biliverdin, indirubin, indole, and tryptophan derivatives and arachidonic acid derivatives.[1] AhR regulates genes involved in drug metabolism such as CYP1A1, CYP1A2, CYP1B1, GSTs, UGTs, and NADHP reductase, but also many other genes, for example, AhRR, TGFβ, p27, IL-1β, Jun, and Bax. AhR is pivotal factor in regulation of drug metabolism, but it plays various endogenous functions such as regulating the cell cycle and proliferation, immune response, circadian rhythm and tumor promotion.[2]

In its resting state, AhR is sequestered in the cytosol in multiprotein complex with chaperones. Upon the binding of a lipophilic ligand, AhR translocates to the nucleus, where it forms heterodimer with AhR-nuclear translocator (ARNT). Heterodimer AhR/ARNT binds to promoter sequence called dioxin responsive element (DRE) (consensus sequence 5′-T/GNGCGTGA/CG/CA-3; core sequence 5′-GCGTG-3′) and triggers the expression of target genes.[1]

Given the roles and functions of AhR described above, this is of topical interest to have a potent and reliable tool for identification of AhR ligands and activators, with respect to environmental applications, drug−drug interactions and chemically induced carcinogenesis. Indeed, endogenous tumor-promoting AhR ligand was just identified and published in Nature.[3] Classical approach is the measurement of expression of AhR target genes, for example, CYP1A1 at the level of mRNA, protein and catalytic activity. However, it is rather approach for studying the mechanisms of AhR-CYP1A1 signaling pathway than fast and robust assessment of AhR activation by xenobiotics. A sophisticated technique is gene reporter assay, usually carried out in cancer cell lines transiently transfected with appropriate reporter plasmid harboring AhR response sequences.

Alternatively, cell lines can be stably transfected with reporter plasmid, usually using selection pressure by antibiotics. Construction of stably transfected cell line is time and material consuming process, but experimentation with stably transfected cells has several advantages: (i) There is no need to transfect cells

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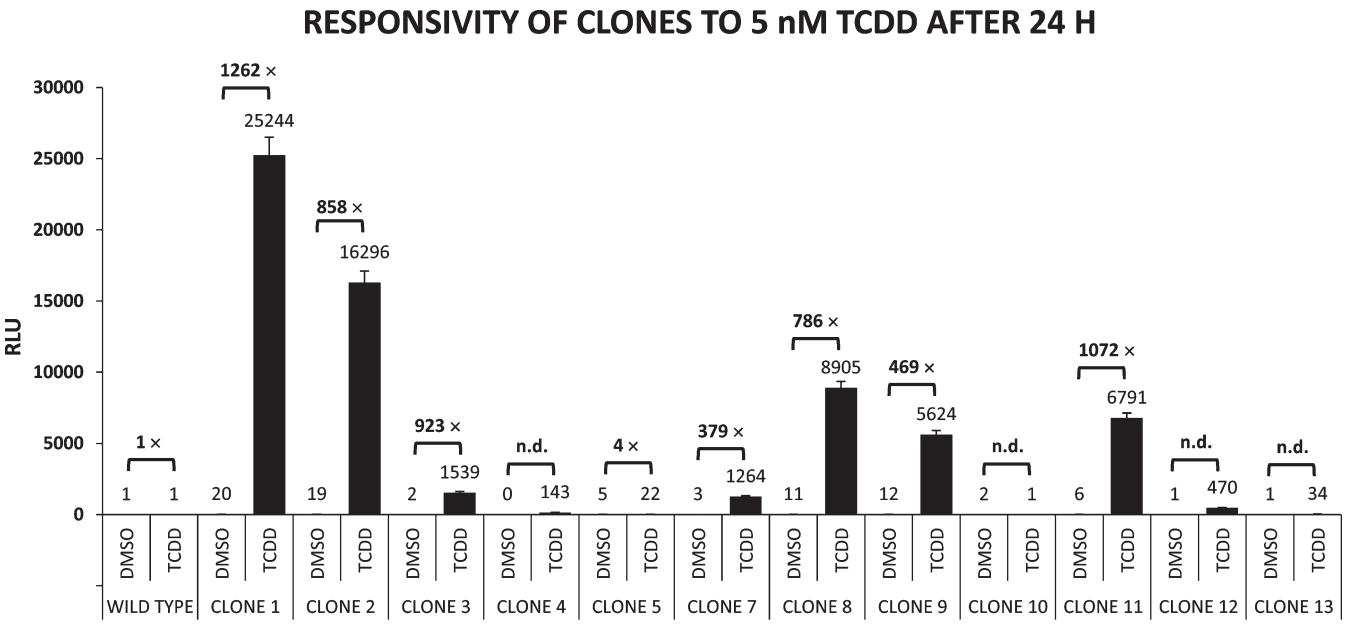

Figure 1. Response of AZ-AHR cells to TCDD. Parent HepG2 cells and clones of AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with TCDD (5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). For each clone was calculated fold induction for TCDD over DMSO treated cells, and the value is indicated above the pair DMSO-TCDD in the bar graph. Similar data were obtained from two independent experiments (two passages).

before each experiment, which saves time and materials; (ii) There is no need to normalize data per β-Gal due to the variable transfection efficiency; hence, the data are more homogeneous (iii) There is much higher sensitivity of the assay, because all the cells are transfected; (iv) Taking together points i−iii, stable cell lines allow high through-put measurements in 96-well plate format.

The first attempt to develop the stable reporter system for assessment of AhR transcriptional activity was performed by Postlind et al who transfected reporter plasmid pLUC1A1 containing CYP1A1 promoter to HepG2 cells. The resulting cell line referred as 101 L, was used to establish dose−response relationships for TCDD and polycyclic aromatic hydrocarbons.[4] Cell line 101 L was also used as high-volume screening system for identifying CYP1A inducers, such as flavonoids, resverarol, apigenin, curcumin, kaempferol, quercetin, etc.[5] Soon after 101 L cells, an in vitro bioassay called CALUX (chemical-activated luciferase expression) was invented. A rat hepatoma (H4IIE) cell line was stably transfected with a construct containing DRE sequence and the luciferase reporter gene.[6] The assay was developed for detection of AhR active compounds in sediments and pore water, but it was modified for many other applications.[7,8] Similar high-throughput reporter-gene system based on H4IIE cells stably transformed with luciferase gene under control of CYP1A1 promoter was later developed by others.[9] Stable cell line called DR-EcoScreen was derived from mouse hepatoma cells Hepa1c1c7 transfected with a reporter plasmid containing seven copies of DRE.[10] This line was used for sensitive, rapid and simple identification of AhR agonists among a large number of environmental chemicals.[10] Sato et al. constructed the stable hepatoma reporter cell line to simultaneous assessment of the transcriptional activation of CYP1A1 and CYP1A2 genes. They incorporated dual reporter plasmid containing the 23-kb region between CYP1A1 and CYP1A2 genes in human hepatoma cells HepG2.[11] Very recently, third generation of CALUX cell line derived from mouse hepatoma cells Hepa1c1c7 stably transfected with reporter plasmid containing multiple DRE was described.[12]

In the current paper, we describe construction and characterization of stably transfected human reporter cell line AZ-AHR, responsive to transcriptionally active AhR. Cells were responsive to various AhR ligands, yielding dose- and time- dependent induction of luciferase activity. The assay was very sensitive allowing high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing to AhR ligands was not significantly different from that of fresh cells.

2. MATERIALS AND METHODS

2.1. Materials. Lipofectamine 2000 transfection reagent were from Invitrogen (California). 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island). DMSO, 1,9-pyrazoloanthrone (SP600125), resveratrol (RVT), omeprazole (OME), indirubin (IND), 3-methylcholanthrene (3MC) and hygromycin were from Sigma-Aldrich (Prague, Czech Republic). Luciferase lysis buffer was from Promega (Hercules, CA). All other chemicals were of the highest quality commercially available.

2.2. Plasmid. Reporter plasmid pGL-4.27-DRE was constructed as follows: Two copies of F site sequences and one copy of B site and D site sequences of mice *Cyp1a1*gene[13] were synthesized and inserted using *Kpn*I-XhoI enzymes into the multiple cloning region of pGL4.27 [luc2P/minP/Hygro] vector (Cat. Nuber E8451) from Promega (Hercules, CA), upstream of the minimal promoter and *luc2*P reporter gene sequence.

2.3. Human Hepatoma HepG2 Cells. Human caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430)

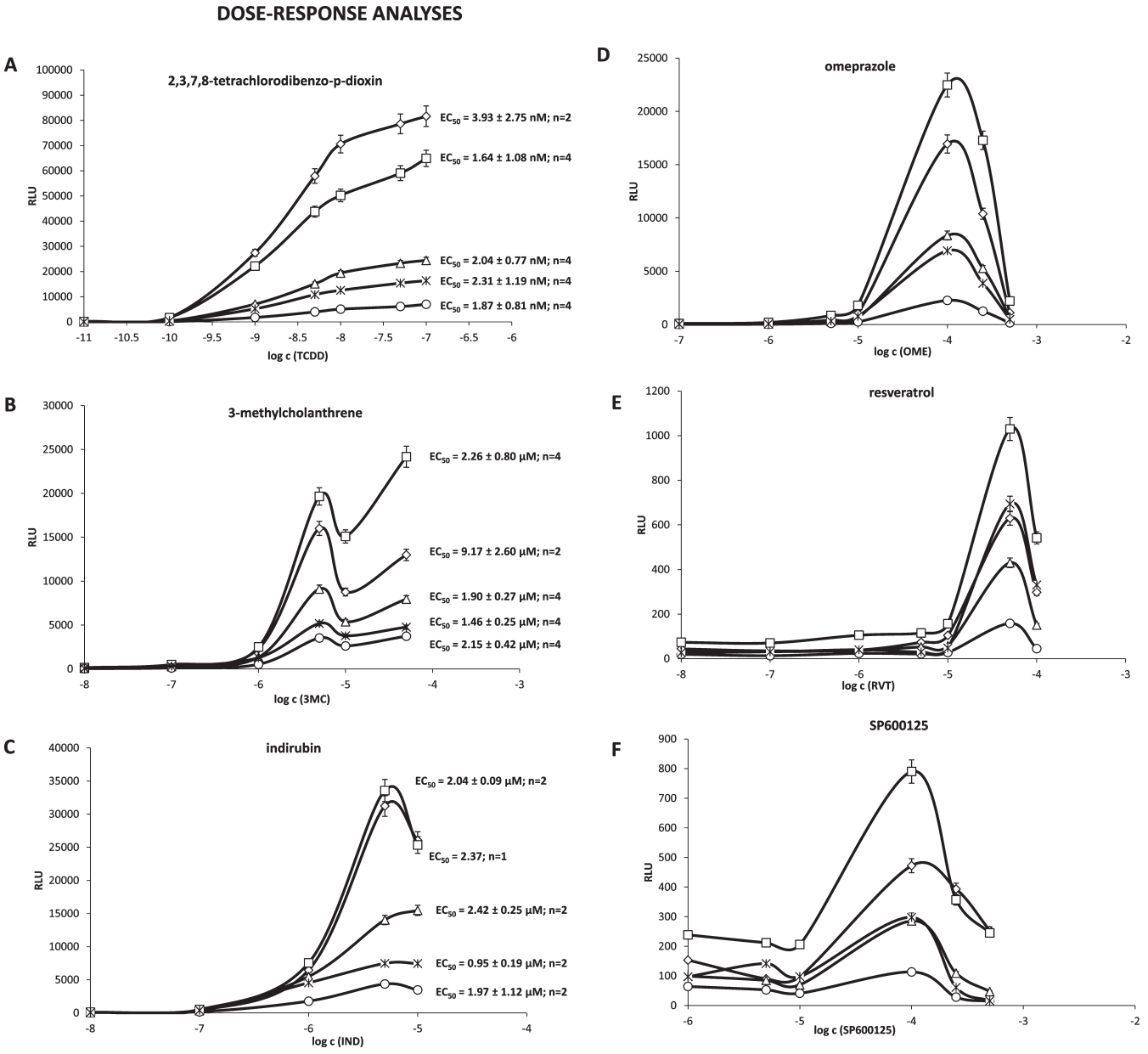

Figure 2. Dose−response analyses of AZ-AHR cells treated with AhR activators. Clones 1, 2, 8, 9, and 11 of AZ-AHR cells were treated for 24 h with: Panel A: TCDD (0.01−100 nM); Panel B: 3MC (0.01−50 μM); Panel C: IND (0.01−10 μM); Panel D: OME (0.1−500 μM); Panel E: RVT (0.01−100 μM); Panel F: SP600125 (1−500 μM). The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). Number of independent experiments (cell passages) for each clone treated with TCDD, 3MC and IND is indicated in graph legend. EC_{50} values are expressed as mean ± SD. Analyses of SP600125, RVT, and OME were performed in three independent experiments for each clone. Legend to plots: Clone 1 (◊); Clone 2 (□); Clone 3 (Δ); Clone 4 (○); Clone 5 (×).

were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Transfection of HepG2 Cells and Selection Process. HepG2 cells were seeded at the density of 1.3×10^6 cells in 60 mm culture dishes in 5 mL of the DMEM culture medium and transfected with pGL-4.27-DRE (10 μg) reporter plasmid using Lipofectamine 2000 reagent. After 36 h of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin (0.6 mg/mL). The medium was renewed every 3−4 days for the period of 9 weeks, until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500−1000 cells and cultured for additional 4 weeks under hygromycin resistance until small colonies were visible. Twelve colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations (termed AZ-AHR cells). Hygromycin resistant clones were analyzed for response to AhR ligands, including TCDD, 3-MC, IND, RVT, OME, and SP600125. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref 91997/ENV/10).

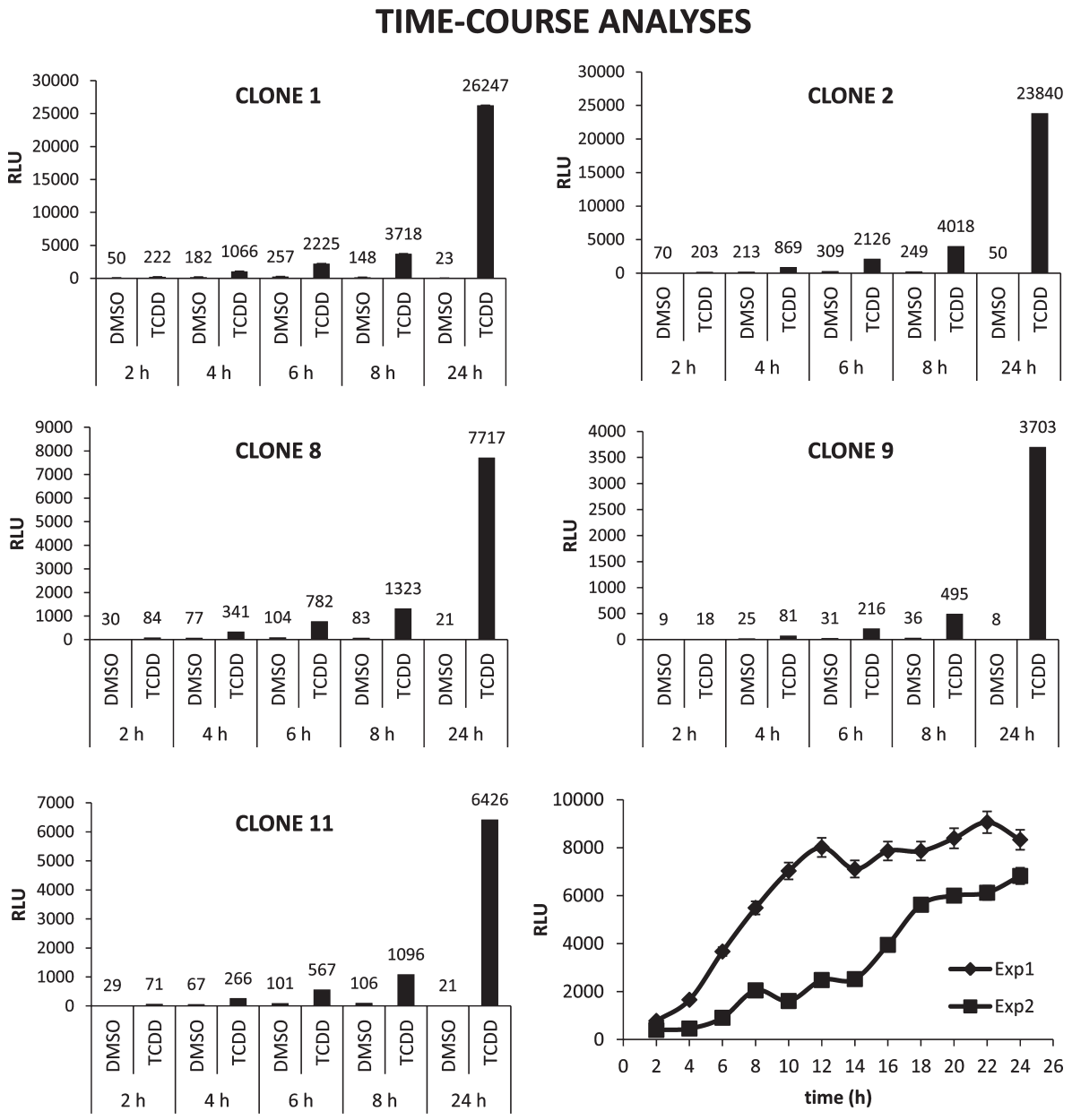

Figure 3. Time-course analyses of AZ-AHR cells treated with TCDD. Bar graphs: Clones 1, 2, 8, 9, and 11 of AZ-AHR cells were treated for 2, 4, 6, 8, and 24 h with 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V). Experiments were performed in three independent passages for each clone. Bottom right plot: Clone 2 was treated for 2 h, 4, 6, 8, 10, 12, 14, 18, 20, 22, and 24 h with TCDD and/or DMSO. Showed are data from two independent experiments. The data are mean from triplicate measurements and are expressed as relative luciferase units.

2.5. Gene Reporter Assay. AZ-AHR cells were seeded on 96-well plates in density 20 000 cells per well. Following 16 h of incubation, cells were treated with tested compounds as described in detail in figure legends. After the treatments, cells were lysed by reporter lysis buffer (Promega) and luciferase activity was measured in 96-well plate format, using Tecan Infinite M2000 and commercial reagents (Promega).

2.6. Cytotoxicity Testing. AZ-AHR cells were treated with the tested compounds for 24 h, using multiwell culture plates of 96 wells. In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal (i.e., positive control) and maximal (i.e., negative control) cell damage, respectively. The MTT assay was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The treatments were performed in quadruplicates and in two independent cell passages.

2.7. Statistical Analyses. Results were expressed as mean ± standard deviation. In all measurements, SD were lower than 5% of the data value, which is indicated in the graphs. Student's pair test was applied. The values of EC_{50} were determined using freeware program BioDataFit 1.02: Data Fit For Biologists (http://www.changbioscience.com/stat/ec50.html)

3. RESULTS

3.1. Response of AZ-AHR Cells to TCDD. Human hepatocellular carcinoma cells HepG2 were transfected with pGL-4.27-DRE luciferase reporter plasmid responsive to transcriptionally

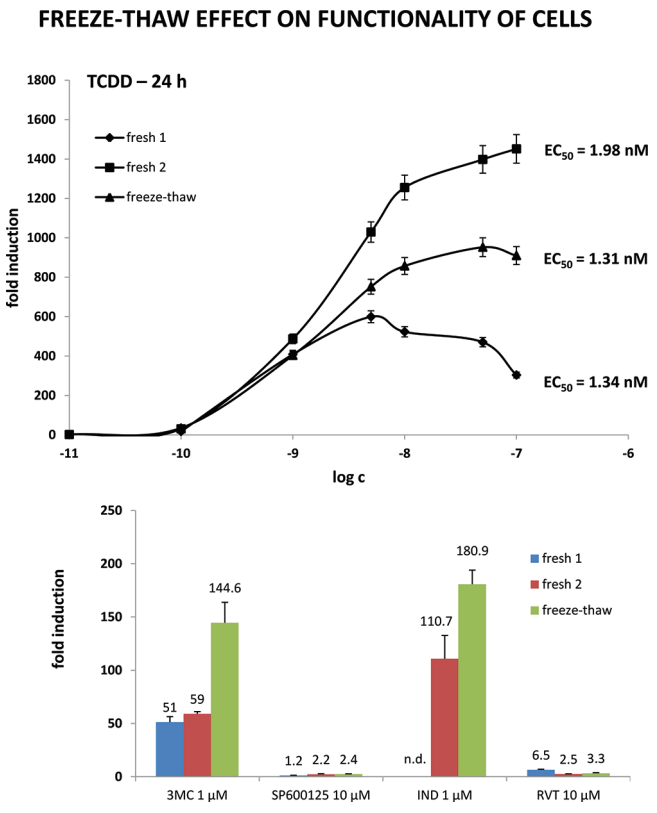

Figure 4. Response of AZ-AHR cells to AhR ligands after freeze−thaw cycle. Experiments were performed in clone 1 of AZ-AHR fresh cells (8th passage = *fresh 1* and 12th passage = *fresh 2*), and in clone 1 of AZ-AHR cells (freeze−thaw) after cryopreservation. Cells were treated with TCDD (0.01−100 nM), 3MC (1 μM), SP600125 (10 μM), RVT (10 μM), IND (1 μM) and DMSO (0.1% V/V) for 24 h. The data are mean from triplicate measurements and are expressed as fold inductions over DMSO-treated cells. Upper panel: dose−response curves for fresh and cryopreserved AZ-AHR cells treated with TCDD; Lower panel: Effects of 3MC, SP600125, RVT, IND on luciferase activity in fresh and cryopreserved AZ-AHR cells.

active aryl hydrocarbon receptor AhR (for details see Sections 2.2. and 2.4.). We isolated 12 clones of AZ-AHR hygromycin-resistant cells. We tested response of AZ-AHR clones to model AhR ligand TCDD. For this purpose, parent HepG2 cells and AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with 5 nM TCDD and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in 96-well format. There was no induction of luciferase activity in parent HepG2 cells. Out of 12 clones examined, significant induction of luciferase activity was reached in eight clones (1, 2, 3, 5, 7, 8, 9, 11), with fold induction ranging from 4× to 1262× . For further analyses, the most responsive clones (1, 2, 8, 9, 11), yielding both high relative luciferase units (RLU) and high fold induction over the vehicle-treated cells, were used (Figure 1).

3.2. Dose−response Analyses of AZ-AHR Cells Treated with AhR Activators. In the first series of functional tests, we performed dose−response experiments in five clones (1, 2, 8, 9, 11) of AZ-AHR cell line. Cells were treated in 96-well plates with increasing concentrations of TCDD, RVT, 3MC, SP600125, OME, IND, and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in cell lyzates. In parallel, we have tested cytotoxicity of AhR activators using standard MTT assay after 24 h of treatment. The values of IC_{50} were 10.9 μM and 398 μM for IND and OME, respectively, whereas IC_{50} were not reached within the tested concentration range for TCDD, RVT, 3MC, and SP600125.

Treatment of AZ-AHR cells with TCDD (10 pM to 100 nM), the most potent AhR ligand, yielded typical sigmoid logarithmic dose−response curves for each tested clone. With exception of clone 1 (EC_{50} = 3.93 nM), the values of EC_{50} were homogeneous between the clones, ranging from 1.64 to 2.31 nM (Figure 2A). Sigmoid-shaped curves were also obtained in all clones of AZ-AHR cells treated with 3MC (10 nM to 50 μM), with EC_{50} values ranging from 1.46 to 2.26 μM. Similarly as in case of TCDD, clone 1 yielded higher EC_{50} (9.17 μM) as compared to other clones. Interestingly, we have systematically observed local minimum in 10 μM concentration of 3MC (18 independent dose−response experiments), indicating more than one mechanism of 3MC action involved in signal transduction in high concentrations of 3MC, such as activation of Nrf2-ARE pathway[14] or AhR-independent induction of p21[15] (Figure 2B).

Indirubin, an endogenous ligand of AhR, induced luciferase activity in dose dependent manner in all tested clones with EC_{50} ranging from 0.95 to 2.42 μM. In concentrations of IND 10 μM and higher, the activity of luciferase descended, suggesting inhibition of luciferase catalytic activity, cytotoxicity (IC_{50} 10.9 μM) or feed-back mechanisms in supra-physiological concentrations (Figure 2C). The efficacy of TCDD, 3MC and IND were of the same order of magnitude (Figure 2A−C).

Omeprazole, a drug inhibiting proton pump, is an activator of AhR and inducer of CYP1A genes, but it does not bind to AhR in vitro and the mechanism of AhR activation by OME is unknown.[16] In gene reporter assays with 5 clones of AZ-AHR cells, OME displayed atypical dose−response profile of U-shaped curve, with maximum induction of luciferase activity (efficacy comparable to 3MC) at 100 μM concentration of OME (Figure 2D). Further decrease in luciferase activity could be due to the OME cytotoxicity (IC_{50} = 398 μM). We have also tested the effects of SP600125 (specific inhibitor of JNK) and RVT (natural antioxidant) on luciferase expression in AZ-AHR cells, since both compounds were described as partial agonists AhR.[17] Both RVT (Figure 2E) and SP600125 (Figure 2F) caused dose-dependent induction of luciferase activity in all tested clones, with maximal luciferase activity at 50 μM and 100 μM, respectively. Further increase in SP600125 and RVT concentrations caused decrease of luciferase activity, probably due to the interactions with other cell targets and inhibition of luciferase activity.

Collectively, we observed dose-dependent induction of luciferase activity in five clones of AZ-AHR cells by six structurally different AhR activators.

3.3. Time-Course Analyses of AZ-AHR Cells Treated with TCDD. In the next series of functional tests, we examined time effects of TCDD on luciferase expression in AZ-AHR cells. Clones 1, 2, 8, 9, and 11 were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 2 , 4, 6, 8, and 24 h with TCDD (5 nM) and DMSO (0.1% V/V). In all clones tested, TCDD caused time-dependent increase of luciferase activity. The assay was very sensitive and significant induction of luciferase activity was attained after 6 h of treatment with TCDD, with fold induction ranging from 8 to 24 fold. This implies potential application of AZ-AHR cell line in testing cytotoxic compound which may kill cells in longer time periods. There were not substantial differences between individual clones,

Table 1. Maintenance of Luciferase Inducibility by 5 nM TCDD

passage number	days in culture	clone 1		clone 2		clone 8		clone 9		clone 11	
		RLU	fold	RLU	fold	RLU	fold	RLU	fold	RLU	fold
3	1	16565	1273	16088	567	11570	788	9578	522	6307	787
5	7	25244	1262	16296	858	8905	786	5624	469	6791	1072
7	12	40016	599	5451	149	3608	1503	11750	746	9065	339
8	14	19815	417	21605	483	12709	643	4693	335	13803	746
9	16	nd	nd	36338	689	18931	653	5567	445	7629	254
12	22	57912	1059	43785	431	15131	496	3975	370	10827	274
13	26	27544	599	16428	345	7423	244	2817	352	8359	237
14	28	28698	2870	17583	440	6920	706	2368	592	9084	966
15	30	26247	944	23840	475	7717	371	3703	411	6426	300

with exception of clone 1 that displayed much higher fold induction of luciferase after 24 h as compared to other clones (Figure 3, bar graphs). In clone 2, we performed more detailed analysis, where the induction of luciferase was measured every 2 h in the period of 24 h. The maximal luciferase activity by TCDD was attained between 12 and 18 h of the treatment (Figure 3, bottom right plot).

3.4. Response of AZ-AHR Cells to AhR Ligands after Freeze−thaw Cycle. We tested functionality of AZ-AHR cells (clone 1) after cryopreservation. We treated fresh cells (8th passage = *fresh 1* and 12th passage = *fresh 2*) with TCDD (0.01−100 nM), 3MC (1 μM), SP600125 (10 μM), RVT (10 μM), IND (1 μM), and DMSO for 24 h. Thereafter, cells were frozen according to a general procedure, using fetal calf serum and DMSO as cryoprotectant. Cells were stored in liquid nitrogen for three weeks. After the thawing, the treatments (vide supra) were repeated in thawed cells.

Dose−response curves and EC_{50} values for TCDD were similar for fresh cells and cells after freeze−thaw cycle (Figure 4, top). There were not substantial differences between fold induction of luciferase activity by AhR ligands (3MC, IND, RVT, SP600125) in fresh and cryopreserved cells (Figure 4, bottom). Collectively, novel reporter AZ-AHR cell line is fully functional and responsive to AhR ligands after the cryopreservation process.

3.5. Maintenance of Luciferase Inducibility in AZ-AHR cells by TCDD. Finally, we tested the ability of five clones (1, 2, 8, 9, 11) of AZ-AHR cell line to respond to TCDD in long-term period. We checked response of cells to TCDD (5 nM; 24 h) after each passage of the cells. The induction of luciferase activity by TCDD was stable during 30 days of AZ-AHR cells in culture, which corresponds to 15 passages. Even though there was a variability between passages, there was no systematic decline or decrease in luciferase induction in both absolute RLU values and fold induction magnitude (Table 1).

DISCUSSION

Stably transfected reporter cell lines are mainly used for large scale detection of various activators, based on the plasmid transfected. The construction of stable cell line is time and material consuming process, but in long-term perspective, this experimental approach saves time and expenses, because there is no further need of transfection procedure before experimentation. In the population of cells selected on antibiotic, each cell (100%) is transfected with reporter plasmid. Therefore, this is not necessary to deal with transfection efficiency, and the assays are highly sensitive.

Several stably transfected gene reporter cells lines were developed for an assessment of transcriptional activity of AhR. The majority of these lines were derived from rodent cancer cells. Probably the most known system is an in vitro bioassay CALUX (chemical-activated luciferase expression), which is rat hepatoma H4IIE cell line stably transfected with a construct containing DRE sequence and the luciferase reporter gene.[6] The assay was used for environmental applications, but it was modified for many other purposes.[7,8] Rat cells H4IIE were also used by others.[9] The examples of stable cell lines derived from mouse hepatoma cells Hepa1c1c7 are DR-EcoScreen cells[10] and third generation of CALUX.[12] Since there are substantial differences between AhR signaling in rodent and human cells, for example the different effects of glucocorticoids on AhR signaling,[18] this is of great interest to establish cell line based on human cells, responding to AhR activation. The examples of reporter cell lines derived from human hepatoma HepG2 cells are 101 L cells[4] and cells allowing simultaneous assessment of the transcriptional activation of CYP1A1 and CYP1A2 genes.[11]

In the current paper, we describe construction and characterization of stably transfected human gene reporter cell line allowing assessment of AhR activators. This AZ-AHR cell line was prepared from HepG2 transfected with reporter plasmid pGL-4.27-DRE, containing two copies of F site sequences, one copy of B site and one copy of D site sequences.[13] Resulting cells yielded dose- and time- dependent induction of luciferase activity in response to treatment with various AhR ligands, including TCDD, 3MC, IND, RVT, OME and SP600125. The assay was very sensitive allowing high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h, which allows to test highly toxic compounds. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing to AhR ligands did not decline. This line is suitable for various environmental applications and screenings and in drug discovery.

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NOMENCLATURE

AhR	aryl hydrocarbon receptor
AhRR	AhR repressor
ARNT	AhR-nuclear translocator
CYP	Cytochrome P450
DRE	dioxin responsive element
GST	glutathione-S-transferase
HepG2	human hepatocellular carcinoma cells
IL-1β	interleukin 1 beta
IND	indirubin
3MC	3-methylcholanthrene
n	number of repeat experiments
OME	omeprazole
RVT	resveratrol
SP600125	
1,9-pyrazoloanthrone	
TCDD	2,3,7,8- tetrachlorodibenzo-*p*-dioxin
TGFβ	tumor growth factor beta
UGT	UDP-glucuronyl transferase

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