# THE PAH CALUX® BIOASSAY AS A PROMISING IN VITRO TOOL FOR DETECTION AND MONITORING OF THE CARCINOGENIC POTENCY OF PAH MIXTURES

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a widespread and diverse class of environmental toxicants that have been associated with mutagenic, carcinogenic and teratogenic effects. Due to the large number of different PAHs and their heterogenicity in terms of structure and toxicological potency, risk assessment of PAHs usually relies on the chemical quantification of a limited set of prioritized PAH congeners.
A central mediator with respect to PAH-induced toxicity in vertebrate species appears to be the aryl hydrocarbon receptor. Mechanistic studies in vertebrate models confirmed the role of this receptor in genotoxicity and carcinogenicity (14), cell cycle regulation (2; 11), and adverse developmental effects (5; 18). Therefore, reporter cell lines for the quantification of PAH-induced, AhR-mediated activity are obvious candidates for toxic potency estimation of PAH-mixtures. Monitoring of PAHs using the DR CALUX, a rat hepatoma-based reporter cell line for the luciferase-based quantification of AhR-mediated activity, have already been reported on several occasions for several matrices as well as pure compounds (e.g.: 1; 4; 8; 10).
In the current study a novel constructed cell line is evaluated for the detection of PAH-induced toxic potency. This cell line will be refered to as the PAH CALUX.

## Materials and Methods

### *Cell culture*

DR CALUX® (H4IIe-pGudLuc stable cell line (7) and PAH CALUX® cells (H4IIe-pDREtataLuc stable cell line (17) ) cells were cultured in α-MEM medium supplemented with 10% FCS.

### *Chemicals and reference samples*

All PAHs reported in this study were obtained from Ultra (Kingstown, USA). All PAHs were diluted in dimethylsulfoxide (DMSO; Acros, Geel, Belgium) and stored at -20°C.

### *CALUX® bioassays*

DR CALUX cells and PAH CALUX cells were plated in 96-well cell culture plates with α-MEM medium supplemented with 10% FCS at a volume of 100 µl per well (40.000 cells/well). The next day, 100 µl conditioned growth medium containing the compounds to be tested (dissolved in DMSO) was added to the cells in triplicate (0.8% DMSO). After the required exposure time (2, 4, 6 or 24 hr) the medium was removed, cells were washed with 100 µl PBS, lysed in 30 µl of Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 4 seconds per well.

### *Data analysis*

Luciferase activity per well was measured as relative light units. Fold induction was calculated by dividing the mean value of light units from exposed and nonexposed (solvent control) wells. Luciferase induction as a percentage of maximal benzo(a)pyrene activity was calculated by setting the highest fold induction of benzo(a)pyrene at 100%. Data are represented as mean values ± SEM from at least three independent experiments with each experimental point performed in triplicate. Dose–response curves were fitted using the sigmoidal fit y ¼ a0 þ a1/(1 þ exp(_(x _ a2)/a3)) in GraphPad

Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). The EC50 values were calculated by determining the concentration by which 50% of maximum activity was reached using the sigmoidal fit equation.

### *Calculation of (total) REP values and total $BEQ_{PAH}$ values*

Relative potencies (REPs) were obtained by dividing the EC50 values of the respective PAH congener, by the EC50 value of benzo(a)pyrene, which was used as reference compound. Total benzo-a-pyrene equivalents of PAHs ($BEQ_{PAH}$) values were obtained by multiplication of the concentrations (M) of the PAHs with their REP value and subsequent adding up of the individual relative potencies.

## Results and Discussion

### *Cell line selection and optimization of the exposure time*

Comparisons between the responses of both reporter cell lines for AhR-mediated activity by PAHs following different exposure times were performed. Both reporter construct and exposure time appeared of critical influence on the potential of this approach for PAH-toxic response detection. These tests were performed with concentration series of benzo(a)pyrene, dibenzo(a,h)pyrene, benzo(b)fluoranthene, and benzo(a)anthracene. The responses in both bioassays for benzo(a)pyrene are depicted in Fig. 1a & 1b.

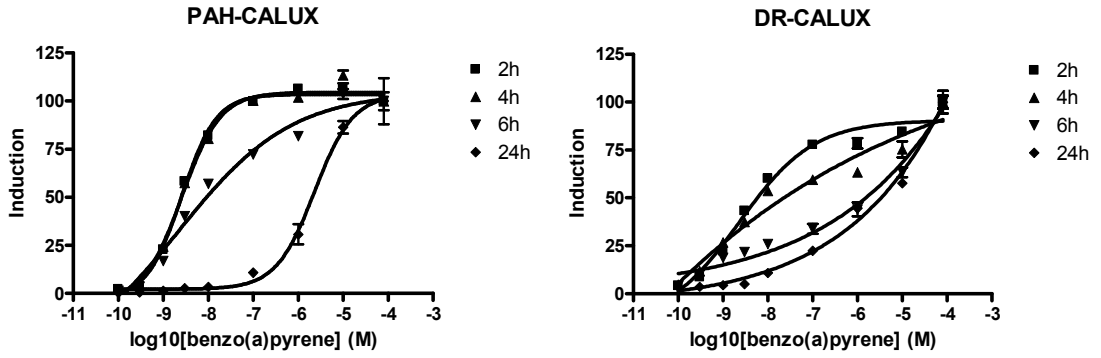


**Figure 1a&b.** Dose response curves for benzo(a)pyrene using the PAH CALUX cell line and the DR CALUX cell line at different exposure times.

Already after a 2 hour of exposure a good and steep sigmoidal dose responsive relation is observed in the PAH CALUX assay, while no clear maximum could be observed in the DR CALUX cells at this early time point. Upon increasing the incubation period, the PAH-mediated dose-response curves of the DR CALUX cells were becoming less steep and EC50 values more difficult to determine. For the PAH CALUX cells this decline occurred to some extent at 6 hours and clearly at 24 hours. In all cases the PAH CALUX line gave much more reliable EC50 estimates (data not shown). These observations are also representative for the other PAHs tested. We continued experiments with the PAH CALUX cell line using an exposure time of 4 hours.

Complete sigmoidal dose reponse curves could be obtained for most of these PAHs. This allowed us to determine their logEC50 values and to calculate their relative potencies (REP; Table 1). There was a clear correspondence with the IARC classification of carcinogenic compounds: Whereas PAHs that are not or very weak ligands for the AhR receptor are classified as probably not carcinogenic or not classifiable as carcinogenic, the PAHs that are strong ligands for the AhR receptor are classified as (probable / possible) carcinogens. Tested PAHs that appeared non- or weak ligands for the AhR

receptor were designated a toxicity equivalence factor (TEF; relative to benzo(a)pyrene) below 0.01 according to Nisbet and Lagoy (15).

**Table 1.** Comparison of the relative potencies of 16 EPA PAHs in the PAH-CALUX assay with their IARC classification (3) and TEF-values according to Nisbet and LaGoy (15)

| PAH | MW | Relative potency (PAH-CALUX) | IARC classification * | TEF |
|---|---|---|---|---|
| naphtalene | 128 | <0.001 | - | 0.001 |
| acenaphtylene | 152 | <0.001 | - | 0.001 |
| acenaphptene | 154 | <0.001 | 3 | 0.001 |
| fluorene | 166 | <0.001 | 3 | 0.001 |
| phenanthrene | 178 | <0.001 | 3 | 0.001 |
| anthracene | 178 | <0.001 | 3 | 0.01 |
| fluoranthene | 202 | <0.001 | 3 | 0.001 |
| pyrene | 202 | <0.001 | 3 | 0.001 |
| chrysene | 228 | 0.84 | 2B | 0.01 |
| benz(a)anthracene | 228 | 0.42 | 2B | 0.1 |
| benzo(b)fluoranthene | 252 | 13.9 | 2B | 0.1 |
| benzo(k)fluoranthene | 252 | 3.7 | 2B | 0.1 |
| benzo(a)pyrene | 252 | 1 | 1 | 1 |
| dibenz(a,h)anthracene | 278 | 1.7 | 2A | 5 |
| indeno(c,d)pyrene | 276 | 1.3 | 2B | 0.1 |
| benzo(ghi)perylene | 276 | <0.001 | 3 | 0.01 |

*) IARC classification (1=carcinogenic to human; 2A=probably carcinogenic to humans; 2B=possibly carcinogenic to humans; 3=not classifiable as carcinogenic to humans)

### *PAH measurement in mixtures*

Especially for PAHs, that seldomly occur as pure compounds, good performance of the assay in response to mixtures is essential. A dual approach was followed in order to validate the performance of the assay for quantification of the amount of $BEQ_{PAH}$ in mixtures. Firstly, eight synthetic mixtures consisting of 16 EPA-PAHs in equivalent ratios as have been reported for several soils in literature were prepared. The expected loads in terms of total $BEQ_{PAH}$ were calculated (Table 2) and compared with the measured values. The measured values for the synthetic mixtures were between 8 to 59% lower than the calculated values, which is quite a good performance considering the complexity of the mixtures.

**Table 2.** Overview of measured values of PAHs (in $BEQ_{PAH}$) in synthetic mixtures compared with calculated $BEQ_{PAH}$ based on relative potencies for the PAH-CALUX as reported in Table 1.

| Mixture (ref) | measurement 1 | measurement 2 | measurement 3 | Measured $BEQ_{PAH}$* | Calculated $BEQ_{PAH}$ | Deviation (%) |
|---|---|---|---|---|---|---|
| A | 0,008 | 0,008 | 0,007 | 0,008 | 0.011 | -27 |
| B | 0,018 | 0,015 | 0,013 | 0,015 | 0.028 | -47 |
| C | 0,011 | 0,010 | 0,012 | 0,011 | 0.012 | -8 |
| D | 0,008 | 0,010 | 0,008 | 0,008 | 0.018 | -55 |
| E | 0,011 | 0,010 | 0,011 | 0,011 | 0.024 | -55 |
| F | 0,014 | 0,011 | 0,080 | 0,011 | 0.025 | -56 |
| G | 0,006 | 0,004 | 0,005 | 0,006 | 0.010 | -42 |
| H | 0,009 | 0,016 | 0,008 | 0,009 | 0.022 | -59 |

*) Median value in M $BEQ_{PAH}$. Equivalent molar ratios of EPA PAHs were used as in soils reported in: A: Industrial site, Holmsund, Sweden (13); B: Roadside, Agra, India (12); C: Industrial site, France (16); D: Industrial site, Germany (16); E: Agricultural soil, The Netherlands (6); F: Urban Soil, United Kingdom (9); G: Industrial site, Portugal (16); H: Industrial site, Lulea, Sweden (13).

## Conclusion

The presented data demonstrate that the PAH CALUX is a promising bioassay for the detection of the carcinogenic potency *in vitro* of PAHs in mixtures.

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