Dissertation submitted to obtain the degree of Master of Science in Chemistry

CALUX BIOASSAY FOR THE ESTIMATION OF BAP, 7-METHYLBENZO[A]PYRENE, ANTHRACENE AND LIQUID PARAFFIN ACTIVITY

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Science & Bio-Engineering Sciences
Abstract

The potency of benzo[a]pyrene (BaP), anthracene, 7-methylbenzo-[a]-pyrene and liquid n-paraffin mix towards the aryl hydrocarbon receptor (AhR) was tested by using the chemical-activated luciferase expression (CALUX) bioassay. The H1L7.5c1 cell line was exposed to different (dilution) doses of the tested chemicals, and for different exposure times (2.5 and 24 hours). BaP and 2,3,7,8 TCDD were used as reference ligands for both the 2.5 and 24 exposure times. Strong induction and clear dose-response curves were obtained for BaP, anthracene and 7-methylbenzo-a-pyrene under 2.5h exposure time. The n-Paraffin mix did not give any response whatever the exposure time considered. In addition, the dose-response curve of 7-methylbenzo-[a]-pyrene was close to that observed for BaP for an exposure time of 2.5 h. The potencies, calculated based on 20%,50% and 80% effective concentrations of 7-methylbenzo-[a]-pyrene and anthracene were found to be approximately twice and 0.01 times the reference BaP for a 2.5h exposure time. However, these potencies are both 10 million times lower than the TCDD reference for a 24h exposure time. By contrast, the BaP potency under 24h exposure time was around 100 times lower than the potency of the TCDD reference. This study showed also that the methylated polycyclic aromatic hydrocarbons (PAHs) are more persistent (less degraded) than the traditional PAHs over short exposure times.
Keywords

Polycyclic aromatic hydrocarbons (PAHs)
CALUX
In vitro assay
REP
Benzo(a)pyrene (BaP),
Anthracene
7-methylbenzo(a)pyrene
n-Paraffin mix
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Finally, I would like to give my special thanks to my parents for supporting and encouraging me all the time.
## Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BaP</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>CALUX</td>
<td>Chemically activated luciferase gene expression</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>TEF</td>
<td>Toxicity equivalent factors</td>
</tr>
<tr>
<td>REP</td>
<td>Relative effect potency</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzodioxin</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>a-MEM</td>
<td>Alpha-minimal essential medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>7M-BaP</td>
<td>7-methylbenzo-[a]-pyrene</td>
</tr>
</tbody>
</table>
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1. Introduction

1.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing only carbon and hydrogen which are composed of two or more fused aromatic (benzene) rings. PAHs are ubiquitously occurring globally and are verified to have toxic effects on animals and human. Incomplete combustion of organic materials is the primary origin of PAHs. Major sources of PAHs arise from human activity, including urban and industrial resources, vehicular emissions, residential heating, tobacco smoking; coal gasification, lubricants production plants, coal-tar plants, coke, asphalt pitch and aluminum plants and catalytic cracking production in petroleum industries (Hyötyläinen T, 2004) (Laane, 2014). Moreover, some PAHs are from natural origins and include natural losses, forest fires or leakage of coal or petroleum deposits and volcanic activities (S.M.Mansourb, 2016). Afterwards, their fates are determined by their physicochemical properties. Because of non-polarity, hydrophobicity and low vapor pressure properties for most PAHs, PAHs from industrial and nature sources, especially for petroleum industry, prefer to be absorbed on particles and accumulated in nearby sediments (Tolosa I, 2004), whereas PAHs from urban activity occur into the air, including outdoor urban atmosphere and indoor air (Sakai R, 2002).

The difference of molar weight for PAHs leads to different physical properties in the environment. Low molar weight PAHs (two to three aromatic rings) are mainly found in the vapor phase at room temperature, while high molar weight multi-ringed PAHs (not less than five rings) are predominantly bound to particles (K.Srogi, 2007). Four aromatic rings PAHs are in intermediate phases which are intersected between vapor and particulate phase, based on the ambient temperature (S.M.Mansour, 2016). Amongst all PAHs, those consisting of five to six aromatic rings predominantly occur in particulates, mostly due to their low volatility and higher molar weights (Klara
Slezakova, 2012). As a result, particle-bound PAHs (pPAHs) are treated as very toxic to animal and human health and are being intensively studied.

The U.S. Environmental Protection Agency (EPA) and EU Scientific Committee for Food (SCF) each have, respectively, categorized 16 PAHs and 15 PAHs as priority pollutants (table 1.), which are both considered in environmental monitoring studies and risk assessments of PAH-involved sites. However, recent research showed that alkylated PAHs are more potent than PAHs themselves in biological assay (Yue Sun, 2014). Accordingly, the traditional priority status of 16 EPA standards was questioned and ought to be updated in recent years. Specifically, larger and highly relevant PAHs, those containing heteroatoms and alkylated PAHs, should be taken into account in future studies and possibly even legislation (Andersson JT, 2015).
Table 1. Priority PAHs of The U.S. Environmental Protection Agency (EPA) & EU Scientific Committee for Food (SCF)

<table>
<thead>
<tr>
<th>List</th>
<th>Common Name</th>
<th>Structure</th>
<th>List</th>
<th>Common Name</th>
<th>Structure</th>
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<tr>
<td>EPA, SCF</td>
<td>Benzo[a] pyrene</td>
<td></td>
<td>EPA, SCF</td>
<td>Dibenz[a,h] anthracene</td>
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<tr>
<td>EPA</td>
<td>Acenaphthene</td>
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<td>SCF</td>
<td>Dibenzo [ae]pyrene</td>
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<td>EPA</td>
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<tr>
<td>EPA, SCF</td>
<td>Benzo[b] fluoranthene</td>
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<td>EPA</td>
<td>Fluoranthene</td>
<td></td>
</tr>
<tr>
<td>SCF</td>
<td>Benzo[j] fluoranthene</td>
<td></td>
<td>EPA</td>
<td>Fluorene</td>
<td></td>
</tr>
<tr>
<td>EPA, SCF</td>
<td>Benzo[k] fluoranthene</td>
<td></td>
<td>EPA, SCF</td>
<td>Indeno[1,2,3-cd]pyrene</td>
<td></td>
</tr>
<tr>
<td>EPA, SCF</td>
<td>Benzo[ghi] perylene</td>
<td></td>
<td>SCF</td>
<td>5-Methyl chrysene</td>
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<tr>
<td>EPA, SCF</td>
<td>Chryrsene</td>
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<td>EPA</td>
<td>Naphthalene</td>
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<tr>
<td>SCF</td>
<td>Cyclopenta [cd]pyrene</td>
<td></td>
<td>EPA</td>
<td>Phenanthrene</td>
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</tr>
<tr>
<td>EPA</td>
<td>Pyrene</td>
<td></td>
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</table>
1.2 Estimation of PAH toxicity

Previous knowledge showed that PAHs have carcinogenic effects on laboratory animals (S.Hecht, 1988), and epidemiologic studies of factory workers who were highly exposed in aluminum smelters and coke ovens, have shown distinct excesses of lung cancer and extremely connotative excesses of bladder cancer (Ben Armstrong, 2004). The results of biological experiments showed that PAHs increased incidences of lung, bladder, skin, liver and stomach cancers. PAHs can also affect the lymphoreticular and immune systems and moreover can have neurologic, reproductive and developmental effects (Blanton, 1988) (N.Harper, 1996). In total, the toxic analysis of PAHs contamination comprises more than 100 PAHs compounds (EPA, 2012). Benzo[a]pyrene (BaP) is considered as a marker for total exposure to carcinogenic PAHs, because the contribution of BaP to the total carcinogenic effect is comparatively high (WHO, 2000).

Toxicity equivalency factors (TEF) were originally developed for analyzing and expressing the toxicity of dioxin-like compounds since the mid-1980s. Moreover, these dioxin like compounds showed similar structural properties and displayed a common cellular mechanism of action which induces comparable biological and toxic response. As a result, assumptions towards the toxic effects of different dioxin and dioxin like mixtures have been considered to be additive (Safe S, 1985). Due to this contribution, the notion of toxic equivalency came up. The toxicity of a mixture can be expressed in a single number, the toxic equivalency (TEQ). It employs the relative effect potency (REP) determined for individual PCDD, PCDF and PCB compounds for producing toxic or biological effects relative to a reference compound, normally 2,3,7,8-TCDD (Barnes D, 1991). The total toxic equivalent (TEQ) is established by the sum of the products of the concentration of each dioxin like compound multiplied by its TEF value, and, as such, represents an estimate of the total 2,3,7,8-TCDD-like activity of the mixture (see equation (1)). The World Health Organization (WHO) has organized international level expert meetings with the aim to harmonize the toxic equivalency
factors (TEFs) for dioxin and dioxin-like compounds, therefore suggesting to national regulatory authorities since the early 1990s (Martin van den Berg, 2006).

For PAHs, a similar approach was developed since the early 1990s (Ian C.T.Nisbet & Peter K, 1992). The TEF value of 16 EPA priority PAHs congeners are shown on table 2.

Table 2. TEF value of EPA 16 priority PAHs. (EPA, 2012)

<table>
<thead>
<tr>
<th>Congener</th>
<th>TEF</th>
<th>Congener</th>
<th>TEF</th>
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<tr>
<td>Benzo(a)pyrene</td>
<td>1</td>
<td>Anthracene</td>
<td>0.01</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>1</td>
<td>Naphthalene</td>
<td>0.001</td>
</tr>
<tr>
<td>Benz(a)anthracene</td>
<td>0.1</td>
<td>Acenaphthylene</td>
<td>0.001</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>0.1</td>
<td>Acenaphthene</td>
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</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>0.1</td>
<td>Fluorene</td>
<td>0.001</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>0.1</td>
<td>Phenanthrene</td>
<td>0.001</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0.01</td>
<td>Fluoranthene</td>
<td>0.001</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>0.01</td>
<td>Pyrene</td>
<td>0.001</td>
</tr>
</tbody>
</table>

To estimate the biological and toxic potency of different PAHs, two approaches are commonly used. The traditional method is to implement the chemical analytical data to roughly calculate the total carcinogenic potency by using Toxicity equivalency factors (TEFs) evaluation (Lee, 2010). Toxicity equivalency concentrations (TEQs) are calculated by the result of summing up the values obtained by TEF values and concentrations of PAHs, equation as shown below:

$$\text{TEQ} = \sum C_i \times TEF_i$$  \hspace{1cm} (Equation 1)

The PAHs hazard evaluation by this pure chemical analysis method, however, was questioned to underestimate the real AhR-mediated toxic potency of PAHs mixtures in mammal cells by some studies (Schneider, Roller, Kalberlah, & Schuhmacher-Wolz, 2002). PAHs are (rapidly) metabolized to electrophilic derivatives by several cytochrome P450 proteins which can mutate DNA, therefore activate proto-oncogenes or inactivate tumor suppressing genes (Hankinson, 1995). As such, instead of using the
chem-TEQ derived values, the other approach for PAHs potency estimation is to implement an in vitro bioassay which employs specific bio-chemical pathways, aryl hydrocarbon receptor (AhR) pathways, which are essential in the onset of toxic response induced by different PAHs (Mimura & Fujii-Kuriyama, 2003). Using these in vitro gene expression and reporter assays, the AhR-potency is shown as bioassay-derived BaP equivalents (bio-TEQs) (Peter A. Behnisch, 2001). Typically, benzo[a]pyrene was chosen as the reference PAH as it has a relatively high carcinogenic effect. In earlier studies, 2,3,7,8 TCDD was also chosen as a reference compound for PAHs (WHO, 1998) (Delistraty D., 1997). Using this technique, the relative potencies (REP) values can be calculated by looking at responses elicited by pure compounds relative to BaP/TCDD. REP values of the analyzed compounds were determined by a ratio of EC$_{50}$, EC$_{20}$ and EC$_{80}$ of the reference compound TCDD or BaP (REP=$\frac{(EC_{xx})_{\text{reference}}}{(EC_{xx})_{\text{sample}}}$) (Equation 1) (M. Van der Berg, 1998) (Maria Larsson, 2014).

\[
\text{REP} = \frac{(EC_{xx})_{\text{reference}}}{(EC_{xx})_{\text{sample}}} 
\]

(Equation 2)

Where,
REP: Relative potency factors
EC$_{xx}$: EC$_{20}$ or EC$_{50}$ or EC$_{80}$
Reference: TCDD or BaP
Sample: analyzed compounds
1.3 Chemically Activated Luciferase Gene Expression (CALUX)

1.3.1 Aryl hydrocarbon Receptor (AhR)

PAHs have carcinogenic effects on animal cells and bind to the aryl hydrocarbon receptor which mediates the toxicity of PAHs. The Aryl hydrocarbon Receptor (AhR) is a soluble intracellular protein which can bind with dioxin and dioxin like compounds and afterwards goes through transformation leading to transcriptional activation of AhR responsive genes. PAHs were chemically inert for themselves and are activated by cytochrome P450 (CYP) enzymes to become more reactive epoxide metabolites. These PAHs epoxides then become highly reactive diol-epoxides by epoxide hydrolase. These diol-epoxides can exhibit carcinogenic effect in animals. Theses metabolites-AhR complexes move into the nucleus and further affect gene expression. By this mechanism, the ligand-activated AhR-dependent induction of gene expression is commonly treated as a biomarker for PAHs toxicity (Peter A. Behnisch, 2001) (Isabelle Windal, 2005).

1.3.2 Chemically Activated Luciferase Gene Expression (CALUX)

The chemically activated luciferase gene expression (CALUX) in vitro cell bioassay is a bioanalytical method to measure biological toxic equivalency (bio-TEQ) of certain chemicals, like dioxins, dioxin-like PCB's, PAHs or (anti)estrogens and (anti)androgen compounds depending on the cell line used. These modified cell lines can have response to PAHs with the induction of luciferase in a dose, time, species and AhR dependent receptor. Compared to traditional HRGC/MS method, CALUX is an economical, practical and quick method in analyzing dioxin and PAHs compounds. Moreover, this bioassay has also shown its value as a screening tool for dioxin and dioxin like compound in the environment. The AhR-response firefly luciferase reporter gene can
stably transfec t and give the response of tested compounds (Han, Nagy, & Denison, 2004). The schematic diagram of CALUX is shown in figure 1.

Figure 1. Schematic Mechanism of CALUX.
This figure is based on the figure from Pieterse (B. Pieterse, 2013)
2. Objectives

The aim for this master thesis is to test pure PAH compounds: BaP, anthracene and 7-methylbenzo-a-pyrene, as well as a n-Paraffin mix by using the H1L7.5c1 CALUX bioassay.

2.1 Benzo(a)pyrene (BaP)

Benzo[a]pyrene is the byproduct during incomplete combustion or organic material pyrolysis and present in air, water, sediments and soils, generally at trace levels except near the sources. BaP can be found in several foods and in some pharmaceutical commodities relying on coal tar which is used on the skin. In urban daily life, tobacco and cooking smoke contains relatively high concentrations of BaP (IARC, 2010). BaP was used as a reference in calculation for PAHs for many years and as a priority PAH for bioassay (M.M. Mumtaz, 1996) (EPA, 2012). However, BaP is much more sensitive than other PAHs as it can be easily influenced by different exposure time related to its rapid metabolization. (B. Pieterse, 2013) So during the experiment exposure time for BaP dosing on mouse hepatoma cells should be well considered and strictly followed due to its biochemical effects from both interactions with metabolic activation and detoxification.

2.2 Anthracene

Anthracene is a colorless solid PAH of formula $C_{14}H_{10}$, consisting of three fused rings, derived from coal tar. Although anthracene is on EPA-16 list, detailed information of toxicity is quite limited.
2.3 7-methylbenzo-a-pyrene (7M-BaP)

7-methylbenzo-a-pyrene is a mono-methylated BaP. Some studies showed that this methylated BaP, (7M-BaP), was metabolized to metabolic 7-methylbenzo-a-pyrene 9,10-dihydrodiol (major) ,7-methylbenzo-a-pyrene 7,8- and 4,5-dihydrodiol and moreover, its metabolites can covalently bind to DNA in rat liver microsomes. Aside from the differences in metabolites, the metabolic pathways of 7M-BaP appear to be qualitatively similar with that reported for BaP. The methyl group on 7M-BaP suppresses but does not restrain the microsomal enzymes from reacting with the parent PAH to form the 7,8-epoxide first and, finally, the trans-7,8-diol (Takeshi Kinoshita, 1982) (T.K.Wong, 1981). Additionally, recent study showed 7M-BaP was more potent than BaP (Monika M. Lam, 2018)

2.3 n-Paraffin Mix

The last analyzed compound, however, is not a ePAH. It is the n-Paraffin Mix that contains eleven non-polycyclic hydrocarbon components (see detailed information on table 2.) In general, liquid paraffin is a complex organic mixture of highly processed saturated hydrocarbons, derived from petroleum industry through various refining steps and subsequently purified by acid treatment and/or catalytic hydro-treatment. Liquid paraffin was widely used as lubricants for machines and cosmetics, and ointments for pharmaceuticals. It is also allowed to be food additives in Japan (T. Shoda, 1997). Previous research tested its toxicity and proved that liquid paraffin can cause granulomas, an inflammation found in many diseases, in rat liver cells and lack carcinogenicity given in the diet to Fisher 344 rats. (Baldwin MK, 1992) Moreover, some research shows that chlorinated paraffin has adequately high carcinogenic potential to dose rat liver, especially for short chlorinated paraffin of chain length C10 to C12 (John R. Bucher, 1987). And recent study showed that the concentration of chlorinated paraffin (C9 to C31) in dust has significant positive correlation with dioxin-
like activity (Wong F, 2017). As such, it was deemed interesting to have a non-PAH compounds or mixture that could be tested on the CALUX bioassay.

Table 3. n-Paraffin mix compounds (sigma-aldrich, product number 44585-U, 2018)

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>WEIGHT PERCENTAGE (WT. %)</th>
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<tr>
<td>PENTANE</td>
<td>9.38</td>
</tr>
<tr>
<td>HEXANE</td>
<td>9.51</td>
</tr>
<tr>
<td>HEPTANE</td>
<td>9.78</td>
</tr>
<tr>
<td>OCTANE</td>
<td>9.54</td>
</tr>
<tr>
<td>NONANE</td>
<td>9.04</td>
</tr>
<tr>
<td>DECANE</td>
<td>9.24</td>
</tr>
<tr>
<td>UNDECANE</td>
<td>9.31</td>
</tr>
<tr>
<td>DODECANE</td>
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<tr>
<td>TRIDECANE</td>
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<tr>
<td>TETRADECANE</td>
<td>8.79</td>
</tr>
<tr>
<td>PENTADECANE</td>
<td>7.08</td>
</tr>
</tbody>
</table>
3. Material and method

The experiment was done with the following steps. First of all, prepare the standard solution of each tested compound. Then, defrozen H1L7.5c1 cells and cultivate them on tissue culture plates. Thirdly, seed cultivated cells in 96-well plates and incubate them further. Afterwards, use previously prepared standard solution to dose cells and incubate dosed cells for a certain exposure time. Finally, detect toxic effects of tested compounds on cells by using Glomax plate reader and further analyze these effects by Excel and Solver.

3.1 BaP standard preparation

For each CALUX analysis, standard curves of BaP were needed. A known amount of pure BaP (Sigma Aldrich, purity ≥ 96% (HPLC)) was dissolved in DMSO to make a series of concentrations of BaP standard solutions (Table 4). The highest concentration of BaP standards (H1) was 7mM which was made by adding 0.25mg pure BaP powder (BaP molar mass 252.31 g/mol) into 1.5mL DMSO (Thermo-Fisher, Catalog number: 85190, purity >99.5%). H2 was made by taking out 0.1ml H1 solution and adding DMSO whose volume was decided by a known dilution factor. Same diluting procedure was followed for making H3 to H10 standards. The standard concentrations were chosen to finally yield a full sigmoidal dose-response curve.
<table>
<thead>
<tr>
<th>STANDARDS</th>
<th>CONCENTRATION</th>
<th>DILUTION FACTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>6.5E-3</td>
<td>Na.</td>
</tr>
<tr>
<td>H2</td>
<td>6.5E-4</td>
<td>10</td>
</tr>
<tr>
<td>H3</td>
<td>6.5E-6</td>
<td>100</td>
</tr>
<tr>
<td>H4</td>
<td>6.5E-7</td>
<td>10</td>
</tr>
<tr>
<td>H5</td>
<td>3.2E-7</td>
<td>2</td>
</tr>
<tr>
<td>H6</td>
<td>1.6E-7</td>
<td>2</td>
</tr>
<tr>
<td>H7</td>
<td>8.7E-8</td>
<td>2</td>
</tr>
<tr>
<td>H8</td>
<td>8.2E-9</td>
<td>10</td>
</tr>
<tr>
<td>H9</td>
<td>8.2E-10</td>
<td>10</td>
</tr>
<tr>
<td>H10</td>
<td>8.2E-11</td>
<td>10</td>
</tr>
</tbody>
</table>

3.2 Pure Compound preparation

Pure anthracene and 7-methylbenzo[a]pyrene are powders (both obtained from Sigma-Aldrich) with known molar mass (178.03 g/mol and 242.32 g/mol, respectively). Their ten standard solutions were made with similar procedures as used for BaP standards. For n-Paraffin mix, it is a liquid mixture with unknown molar mass. But what we know is its density (0.80 g/cm$^3$). As a result, the unit of each standard of n-Paraffin mix is the total mass of paraffin in one vial. The highest mass of paraffin mix in one vials (P1) was done by adding 0.1ml paraffin mix into 0.9ml DMSO which made 0.0080 g in 1ml solutions. For P2 to P10, diluting the solution step by step with the dilution factor of 5. All concentrations of these three compounds were shown on table 4.
Table 5. tested compounds concentration

<table>
<thead>
<tr>
<th>Anthracene</th>
<th>concentration (mol/L)</th>
<th>dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.013</td>
<td>N.A.</td>
</tr>
<tr>
<td>A2</td>
<td>0.0033</td>
<td>4</td>
</tr>
<tr>
<td>A3</td>
<td>0.00081</td>
<td>4</td>
</tr>
<tr>
<td>A4</td>
<td>0.00020</td>
<td>4</td>
</tr>
<tr>
<td>A5</td>
<td>5.1E-05</td>
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</tr>
<tr>
<td>A6</td>
<td>1.3E-05</td>
<td>4</td>
</tr>
<tr>
<td>A7</td>
<td>3.2E-06</td>
<td>4</td>
</tr>
<tr>
<td>A8</td>
<td>8.0E-07</td>
<td>4</td>
</tr>
<tr>
<td>A9</td>
<td>2.0E-07</td>
<td>4</td>
</tr>
<tr>
<td>A10</td>
<td>5.0E-08</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7M-BaP</th>
<th>concentration(mol/L)</th>
<th>dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.00016</td>
<td>N.A.</td>
</tr>
<tr>
<td>M2</td>
<td>4.0E-05</td>
<td>4</td>
</tr>
<tr>
<td>M3</td>
<td>2.4E-06</td>
<td>4</td>
</tr>
<tr>
<td>M4</td>
<td>6.1E-07</td>
<td>4</td>
</tr>
<tr>
<td>M5</td>
<td>1.5E-07</td>
<td>4</td>
</tr>
<tr>
<td>M6</td>
<td>3.8E-08</td>
<td>4</td>
</tr>
<tr>
<td>M7</td>
<td>9.5E-09</td>
<td>4</td>
</tr>
<tr>
<td>M8</td>
<td>2.4E-09</td>
<td>4</td>
</tr>
<tr>
<td>M9</td>
<td>2.4E-10</td>
<td>10</td>
</tr>
<tr>
<td>M10</td>
<td>2.4E-11</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-Paraffin mix</th>
<th>mass in one standard (g/L)</th>
<th>dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>8.0E-05</td>
<td>N.A.</td>
</tr>
<tr>
<td>P2</td>
<td>1.6E-05</td>
<td>5</td>
</tr>
<tr>
<td>P3</td>
<td>3.2E-06</td>
<td>5</td>
</tr>
<tr>
<td>P4</td>
<td>6.4E-07</td>
<td>5</td>
</tr>
</tbody>
</table>
TCDD standards used were previously made in-house and the concentrations of TCDD standards are shown in table 6.

<table>
<thead>
<tr>
<th>TCDD standard</th>
<th>concentration</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD 3</td>
<td>1.2E-06</td>
<td>N.A.</td>
</tr>
<tr>
<td>TCDD 7</td>
<td>7.7E-08</td>
<td>16</td>
</tr>
<tr>
<td>TCDD 10</td>
<td>9.7E-09</td>
<td>8</td>
</tr>
<tr>
<td>TCDD 12</td>
<td>2.4E-09</td>
<td>4</td>
</tr>
<tr>
<td>TCDD 13</td>
<td>1.2E-09</td>
<td>2</td>
</tr>
<tr>
<td>TCDD 14</td>
<td>6.0E-10</td>
<td>2</td>
</tr>
<tr>
<td>TCDD 15</td>
<td>3.0E-10</td>
<td>2</td>
</tr>
<tr>
<td>TCDD 17</td>
<td>7.5E-11</td>
<td>4</td>
</tr>
<tr>
<td>TCDD 20</td>
<td>9.4E-12</td>
<td>8</td>
</tr>
<tr>
<td>TCDD 25</td>
<td>3.0E-13</td>
<td>32.5</td>
</tr>
</tbody>
</table>

3.3 Storage of H1L7.5c1 cells
H1L7,5c1 cells are kept in liquid nitrogen for long term storage. Thawed cells are transferred into the biological lab and grow in a Ø100 mm tissue culture (TC) plates (Greiner Bio One, Belgium) containing supplemented growth medium, 10 ml 90% (v/v) alpha-minimal essential medium (α-MEM from Gibco, Life Science Technologies Belgium) supplemented with 10% (v/v) foetal bovine serum (FBS) (SA approved from Gibco, Life Science Technologies) in a CO2 incubator (Forma Steri-Cult, Thermo Scientific) operated at 37.0°C, 80% RH (Relative Humidity) and 5.0% CO2. All operating steps relating to biological cells and cell culture are implemented in a Class II biological safety cabinet (MSC 1.8 from Thermo Scientific). All equipment used during the manipulations such as tips, plates, pipette etc. was sterilized with 75% ethanol before getting into the cabinet.

3.4 Cultivation

When cells confluency (the percentage of cells covering the plate surface) reached around 70% to 80% on a tissue culture plate, as observed under a microscope, the cells on TC plates should be harvested. TC plates were carefully transported into the bio- hood, and the old medium was removed with a sterile Pasteur glass pipette and a two-stage vacuum flask assembly connected to a membrane pump. Then the cell layer is rinsed twice with 5 ml sterile PBS buffer 1X. The rinse was removed identical to old medium. Afterwards, 2 ml of 0.05% trypsin-EDTA solution (containing phenol red as pH indicator) was added into each plate to separate cells from the plate. To have cells detach better, the plates were placed inside the incubator for 4 min. As these plates were taken out of the incubator, cells were well homogenized by pipetting continuously up and down the cell suspension with a pipette (1000 µl volume) and then all cells suspensions were transferred into one plate. Afterwards, the cell suspension was transferred to new tissue culture plates (about 4 to 8 plates depending on the needs for future experiments and/or continuation of the cell line) previously containing supplemented growth medium.

A few drops of cell suspension mixture (total volume is approximate 250 µl) are placed in to each freshly prepared culture plate. The medium in new plates can support cell
growth for future experiments. These plates were incubated for 72 hours at 37.0°C and with 5.0% CO₂.

3.5 Seeding and dosing of cells

After 72h incubation, the confluence of cells for each plate should be around 80%-90%. Then some drops of cell suspension liquid were transferred to two or three TC plates containing fresh media for next time usage. Then, the rest of the cell suspension was transferred to a conical centrifuge tube (50 mL, GBO) with fresh alpha-MEM (approximately double the volume of expected cell solution, 15 ml) and centrifuged at 400 rpm 8 min. After centrifugation, the 50mL vials were transferred to the biological hood and the supernatant was removed using a Pasteur pipette (vacuum assembly) in such a way as to not damage the cell pellet. Afterwards, alpha medium was added into the tube at three times (each time add approximate 10ml) for the cells mixing well with alpha medium by repeating up and down pipetting of the cell suspension with pipette.

After mixing, 300 µl cell suspension was taken twice and transferred to 2 Eppendorf cups (1.5 ml). One tube is additionally added 300 µl lysis buffer A solution and 300 µl stabilizing buffer B. This tube was used to count both living and dead cells to obtain a total cell count, the other is used to monitor the dead number of cells/ml. The number of cells can be counted by implementing NucleoCounter (Chemometec). Finally, cell suspension density was diluted to 300,000 cells/ml gained by adding fresh medium. After dilution, the cells suspension ought to be mixed completely by repeating up and down pipetting.

Then the diluted cells solution was poured into a 60ml tray container to let a 12-channel multi-pipettor (Thermo, 30-300 µl) seed the solution into plates. 100 µl cell suspension was seeded into the sterile 96-well plates (Perkin Elmer, USA) obtain a final amount of 30,000 cells per well. The 96-well plates were finally incubated for 24 hours at 37.0°C and 5.0% CO₂. Two types of experiments were carried out: testing of pure compounds with TCDD as a reference compound and those with BaP as a reference.
compound. The 96-well plates for TCDD measurements were placed in the incubator for 24h followed by dosing, those intended for BaP were left in the incubator for 48 hours prior to dosing (see below).

After 24 hours (for TCDD and tested compounds) or 48 hours (for BaP and tested compounds) for settling and growing well in 96-well plates, a monolayer of cells was formed. The plates were taken out from the incubator to the bio-hood and the media in plates were removed by quickly reverse dumping 96-well plates. Then, the 96-well plates were dosed with diluted TCDD/BaP standard solutions and compounds, following incubation for strict 24 hours (for TCDD) or 2.5 hours (for BaP) under the same conditions.

i) Dosing step for the plates with BaP as a reference:

Standard concentrations of BaP and tested compound solutions (see table 4) and DMSO blank solvent were prepared in 2 ml vials as previously discussed (see section 3.1). All standards were arranged from H10 to H1, H1 referring to the highest standard (6.5×10⁻³ mol/L) and H10 (8.2×10⁻¹¹mol/L) referring to the lowest standard. All concentrations and DMSO solvent blanks needed to be mixed with a-MEM prior to dosing. The ratio of original compound to the mixture are 1:100(v/v). So each tube was filled with 396 µl alpha medium and 4 µl corresponding concentration BaP solution was added. Then the mixtures were well mixed one after another with a Vortex in a touch mode (7-8 stalls). The plates were finally dosed with these mixed solutions according to the dosing scheme in figure 2. The three border sides of the plate were added into 100 µl medium with 200 µl pipette as medium blank, as shown in Fig.2. Each concentration was tested in three replicates.
Figure 2. Layout of a 96-well plate

- **BaP or TCDD standard**
- **DMSO blank**
- **Tested Sample**
- **Basket without circle**
- **Media blank**
- **Green basket**
- Detected by Luminometer (see further)

ii) Dosing step for the plates with TCDD as a reference:

The TCDD standard concentration was shown in Table 1. And all plates with TCDD as a reference had 24h exposure time. The dosing process was same as BaP dosing step.
3.6 Analysis

i) Luminescence measurements

For TCDD reference plate, the exposure time was 24h. For BaP reference plate, the exposure time was 2.5h (with a prior seeding time of 48h). After following the strict exposure time, the plates were taken out of the incubator and then were analyzed by Luminometer (Glomax 96-well plate luminometer from Promega, USA).

When the 96-well plates were exposed for certain time, the plates were taken out from the incubator and rinsed again. Then, the cells in 96-well plates were checked under the microscope to visually evaluate possible toxicity. After that, a white backing tape was placed on the bottom of the plate to prevent light scattering and reduce well-to-well crosstalk for further luminometer analysis. 50 µL of cell lysis reagent (homemade, recipe refers to (Frank R. Wettey, 2006)) was transformed to each well of 66-well by a continuous back pietting injection mode. And the whole plate was shaken for 900 seconds by a digital shaker. Finally, the plate was placed in the luminometer (Glomax 96-well plate luminometer from Promega, USA); then luciferin reagent (50 µL, homemade) was automatically injected into 68 chosen wells (see Figure 2.) and the light output, in relative light units (RLUs), was measured after a lag time of 6 s and an integration time of 3 s. RLU's were then converted into response percentages relative to the highest RLU value (100%) by further calculation.

ii) Calculation

The produced light output was measured with a luminometer (Glomax 96-well plate luminometer from Promega, USA) and both the result data of referenced TCDD/BaP standard curve and sample curve followed a 4-parameter Hill equation (Vandemarken T., 2016). A 4-parameter Hill equation was fitted to the data points (RLUs of the
TCDD/BaP standard solutions or samples solutions as a function of the concentrations, in amount per well) using a weighted least squares regression (M. Elskens, 2011).

\[
y_i = y_0 + \frac{m \cdot x_i^h}{k^h + x_i^h} + \varepsilon_i
\]  
(Equation 3)

Eqn. (3):  Four parameter Hill equation.

- \(y_i\): the measured RLU of observation I;
- \(y_0\): the background RLU for the full dose–response curve;
- \(m\): the maximum plateau of RLU for the full dose–response curve;
- \(x_i\): the amount of TCDD/BaP standard or samples compounds in the each measured well;
- \(k\): the amount of TCDD/BaP standard or sample compounds at 50% of the maximum of the dose-response curve (=EC\(_{50}\)),
- \(h\): Hill-coefficient that defines the shape of the curve
- \(\varepsilon_i\): residual term.

When the data points are plotted on a logarithmic scale, a typical sigmoid curve appears on Figure 3.
By using Solver software (excel add-in), the four parameters can be calculated. And $EC_{20}$ and $EC_{80}$ were also carried out by this software. To determine potency of tested compound, the relative potencies (REP values) was calculated (see equation 2).

iii) Uncertainty calculation

Uncertainties on EC and background values are calculated from the results of the nonlinear regressions as described in (Elskens et al., 2011) and are expressed as standard errors. Moreover, different results may be compared by their relative standard deviation (RSD%), a dimensionless measure of uncertainty.
4. Results and discussion

4.1 BaP characterization

4.1.1 BaP exposure time optimization

As previously mentioned, PAHs exert their carcinogenic effects by metabolizing to reactive intermediates, as catalyzed by cytochrome P450 (CYP) enzymes and epoxide hydrolases. This process is called bio-activation. The bio-activation for BaP is a complex and requires that BaP was firstly transformed to metabolic (+) and (-) BaP-7,8-epoxide by CYP enzymes. This epoxide rapidly transforms to (+) and (-) BaP-7,8-diol stereo-isomers by epoxide hydrolase. Subsequently, the two kinds of BaP-diols again undergo the activation by CYP enzyme to become ultimate carcinogenic metabolites, (+)- and (-)- BaP-7,8-diol-9,10-epoxides, which are both highly reactive towards DNA. Moreover, B[a]P-4,5-epoxide also generated by CYP enzymes from BaP was thought to be another carcinogenic metabolite. However, this B[a]P-4,5-epoxide was readily hydrolyzed to an inactive BaP-4,5-diol metabolite which was concluded not to play a major role in carcinogenesis of rat cells (Tsutomu Shimada, 2003). One thing should be noticed is that the rate of metabolism of BaP is relatively rapid (highest activation of metabolites to target gene at 3-4h) in murine hepatoma cells, Hepa1c1c7. (Michaelson, 2011). Comparison of the response to BaP after an exposure time of 6 h versus 24 h exhibits a significant decrease in fold induction for cell lines. (B. Pieterse, 2013) As a result, the exposure time of BaP needs to be optimized and deviates from the conventional 24h which offer good fold induction for TCDD. B. Pieterse used H4IIE-pDREtataLuc cell lines for BaP bioassay and found 4h was the best exposure time for this cell lines (B. Pieterse, 2013). Vrabie chose 6h as exposure time for H4IIE.luc cell lines. But the other exposure times he used to compare are all too long (24h, 48h, 72h) which made BaP fully metabolized in cell lines (Cozmina M.
To optimize exposure time of BaP for H1L7.5c1 cells, BaP standards were tested under 6 different exposure times. The sigmoid dose-response curves are shown in Figure 4.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>EC50 (pg / well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>85</td>
</tr>
<tr>
<td>3.5</td>
<td>150</td>
</tr>
<tr>
<td>4.5</td>
<td>240</td>
</tr>
<tr>
<td>5</td>
<td>620</td>
</tr>
<tr>
<td>24</td>
<td>1400</td>
</tr>
</tbody>
</table>

Figure 4. Dose−response curves for the exposure of the CALUX cell line with BaP at various exposure times.

From fig.4, it is obvious that the dose-response curve was significantly shifted under different exposure time and became more typical sigmoidal shape under relatively shorter exposure time. The $EC_{50}$ value under different exposure times can be seen on the left top side on fig.4. It showed that $EC_{50}$ value was increased when prolonging the exposure time, which means the potency of BaP itself was decreased more and more. In total, this figure indicated that metabolism of BaP was taking place in a time-dependent manner. Based on these results, it was opted to keep the exposure time short in order to have full dose data on BaP, allowing more accurate determination of $EC_{50}$ values and, subsequently, the ability to provide relative potency values for other pure PAHs.
4.1.2 The influence of dosing direction

As previously mentioned, the chemicals was normally dosed following the order: H10, H9, H8, … H2, H1, which is from low concentration to high concentration, while it was interesting to figure out if the results were influenced by reverse dosing, which was from H1 to H10. The experiment was done by 4 times repetitions on 4 different plates. Choosing the normal dosing as a reference and reverse dosing as a “test sample” on one plate. The results are shown in table 7.

<table>
<thead>
<tr>
<th>NORMAL DOSING</th>
<th>REVERSE DOSING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>EC(_{20})</td>
<td>4</td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>4</td>
</tr>
<tr>
<td>EC(_{80})</td>
<td>4</td>
</tr>
</tbody>
</table>

From this table we can see that the variation between these two methods is not statistically significant. The results of p-values for student t-tests on \(EC_{20}\), \(EC_{50}\), \(EC_{80}\) are 0.90, 0.60, and 0.99, respectively.
4.1.3 BaP characterization.

From the previous sections we were able to deduce the best procedure regarding exposure time and how the 96-well plates should be analyzed (starting with the high or low standard solutions). The next step is to generate multiple dose response curves to characterize the BaP activity by using the 4-parameter Hill function. To this end, we need to determine the concentrations of BaP standards that will provide a full dose-response curve (see Figure 5.) gives an overview of the BaP standard curves obtained during this study for an exposure time of 2.5 hours.

![Graph showing BaP dose-response sigmoid curves.](image)

**Figure 5.** An overview of whole BaP dose-response sigmoid curves (n=24, exposure time=2.5h)

Overall, all BaP curves exhibit a sigmoid type when plotted on a log-dose scale. However, some curves display high background values ($y_0$) and the upper plateau is not clearly reached by all curves. In addition, the slopes between curves are rather variable with steeper values and others more attenuated. These all need further explanation.
4.1.4 TCDD standard curves

Since BaP and TCDD are both reference ligands for other PAHs under different exposure time. This section will focus on the dose-response curve comparison between these two compounds. TCDD was chosen as the reference compound for dioxin assessment with the CALUX bioassay since 1990s. TCDD was not metabolized in mouse cell lines under long exposure time. For example, an exposure time of 24h yield a good induction response. (Isabelle Windal, 2005) Comparing with BaP curves, the TCDD dose-response curves (see figure 6) are less scattered reflecting that TCDD (24h) is more stable or persistent than BaP. The background values ($y_0$) were always lower than 5% (data shown on figure 6) and the upper plateau were close to 100 for the 14 assays, while the latter for BaP curves ranged from 80 to 100.

Figure 6. An overview of TCDD dose-response sigmoid curves
(n=14, exposure time=24h)
4.1.5  Background value and parameter discussion for BaP

i)  Background value

Media blank, DMSO blank and Background ($y_0$) values for BaP and TCDD are shown in Figure 7.

Figure 7. DMSO blank, media blank and $y_0$ RLU values in a) BaP standards under 2.5h exposure time and b) TCDD standards under 24h exposure time. Error bars indicate standard error of the means over triplicate measurements.
RLUS values of these three blanks (DMSO, media, $y_0$) are different between TCDD and BaP standards. The background values ($y_0$) for all 24h exposure time (TCDD reference) were lower than 5%. This is an acceptable level for CALUX assay. But $y_0$ values for 2.5h (BaP reference) are not always acceptable. The $y_0$ on Apr. 25 is higher than 30%. This may be caused by contamination during the experiment and will not further be considered in the following paragraph. Moreover, $y_0$ values from last few days are also relatively high which vary from 8% to 13%. Possibly, the cells have been used for too many sub-culturing. Even though cells have been stably transfected, loss of reporter genes may occur and affect assay responsiveness (Khurana, 2014) Media blank values for both exposure time are lower than 5%. This means the influence from media is quite low and indicates DMSO or manipulations associated here with could be responsible for elevated levels.

DMSO values under 24h exposure time (TCDD reference) are always lower than 4.5% which indicates that the measurement process is under control. (S D Seidel, 2000) DMSO blank values for BaP standards suggest abnormality in the measurement process. We found that DMSO values below 10% were acceptable at the beginning of this study (Figure 7a), but after April 18, they increased significantly. DMSO used in the whole study was always issued from the same stock. Moreover, some other studies showed similar situation without further explanations. (Yue Sun, 2014) While it can be cautiously said that some high result are linked to operator mistakes, a general trend is also observed with higher DMSO induction levels as cells are more subcultured. Further investigation is needed.
ii) $m$ discussion

$m$ value is the maximum plateau of RLU for a typical dose–response curve. For usual curves, the maximum plateau indicates a stable response regardless of the (increased) concentration of tested compound. In the case of TCDD, a good example of obtaining such a plateau can be shown. This is clearly not the case for BaP (see Figure 8). After a growing phase, the BaP curve seems to reach a plateau, but sometimes can increase slightly further when assaying a higher concentration of BaP. The large variation in $m$ values can probably be attributed to the metabolism of BaP by the cell lines used. For TCDD, there is no metabolization and the upper plateau is linked to the AhR saturation. In the case of BaP, it is known that metabolism happened and one can expect a complex interplay between BaP concentration, cell density, physiological cell status and exposure time, which all may impact the dose-response curves. In our experiments, these variables are controlled as much as possible, but it cannot be denied that there is greater variability in the cellular responses when using BaP.

For other studies, BaP curves are not shown this phenomenon under longer exposure time (6h) (Cozmina M. Vrabie, 2009) or are not reached relatively high dosing concentration to see reach the higher plateau (B. Pieterse, 2013).
Figure 8. Maximum plateau comparison between a) TCDD 24h exposure time and b) BaP 2.5h exposure time
4.1.6 $EC_{50}$ values of BaP.

i) $EC_{50}$ values under 2.5 hours exposure time

Since BaP is used as a positive reference congener (agonist) for other PAHs and PACs, quantifying the $EC$ values of BaP is important for PAHs bioassay, which can further calculate the BEQ values to know other PAHs and PACs potency. (Delistraty D., 1997) (B. Pieterse, 2013) All $EC_{50}$ values for BaP under 2.5h exposure time is shown below.

![Graph showing BaP $EC_{50}$ values](image)

Figure 9. BaP $EC_{50}$ value (pg/well) distribution for all 24 tests under 2.5h exposure time. The data was arranged according to test date

The average value±standard deviation of all $EC_{50}$ of BaP is $46\pm30$ pg/well. Except the results from one day (three dots in left top in figure 9), BaP $EC_{50}$ values show an increasing trend with subculturing (while previously DMSO blank values showed a small increase).
ii) EC values under 24 hours exposure time

BaP was treated as a sample while TCDD was chosen as a reference. The tests were repeated for five times. The curves are below (see figure 10) where BaP curves are all posted and one TCDD curve was posted.

![Figure 10. TCDD/BaP curves under 24h exposure time](image)

It is not hard to see that the response of BaP standards after 24h exposure time deviate from a typical sigmoid shape. The reason for this is due to BaP metabolic activity. An EC values comparison for all BaP curves under different exposure time and TCDD under 24h are on table 8.
Table 8. TCDD, BaP EC values under different exposure times. n, test times, EC value unit is pg per well

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exposure time</th>
<th>n</th>
<th>EC&lt;sub&gt;20&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>24h</td>
<td>14</td>
<td>0.11±0.021</td>
<td>0.25±0.064</td>
<td>0.43±0.11</td>
</tr>
<tr>
<td>BaP</td>
<td>24h</td>
<td>5</td>
<td>290±120</td>
<td>2100±1300</td>
<td>16000±14000</td>
</tr>
<tr>
<td>BaP</td>
<td>2.5h</td>
<td>24</td>
<td>7.4±5.0</td>
<td>46±30</td>
<td>320±360</td>
</tr>
</tbody>
</table>

When comparing the EC<sub>50</sub> values of BaP for exposures time 2.5 and 24h, we can calculate that the loss in potency is about 50-fold. Indeed, after 24h exposure, the potency of BaP amounts to 2100±1300pg/well.
4.2 Pure compound characterization

4.2.1 7-Methylbenzo[a]pyrene

7-Methylbenzo[a]pyrene (7M-BaP) was tested under 2.5h and 24h exposure time using BaP and TCDD as a reference compound. The dose-response curves and EC values are shown in Figure 11.

![BaP/7M-BaP curves under 2.5h exposure time](image1)

**a) BaP/7M-BaP curves under 2.5h exposure time**

![TCDD/7M-BaP curves under 24h exposure time](image2)

**b) TCDD/7M-BaP curves under 24h exposure time**
Figure 11. 7M-BaP under 2.5h (a) and 24h (b) exposure time using BaP and TCDD as reference (unit pg/well)

Table 9. EC values of 7M-BaP under 2 exposure time

<table>
<thead>
<tr>
<th>exposure time</th>
<th>n</th>
<th>EC&lt;sub&gt;20&lt;/sub&gt;</th>
<th>RSD%</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>RSD%</th>
<th>EC&lt;sub&gt;80&lt;/sub&gt;</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td></td>
<td>pg/well</td>
<td></td>
<td>pg/well</td>
<td></td>
<td>pg/well</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>4</td>
<td>2.8±1.4</td>
<td>52</td>
<td>24±7.0</td>
<td>30</td>
<td>210±60</td>
<td>28</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>200000±</td>
<td>150</td>
<td>1900000±</td>
<td>120</td>
<td>23000000±</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300000</td>
<td>230000</td>
<td>1900000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tested compound: 7M-BaP; Mw= 266.3g/mol; n tested times

7M-BaP dosing response curves under 2.5h exposure time are similar to BaP dosing response curve. The EC values for 7M-BaP under 2.5h exposure is quite close and about 2 times lower than BaP EC values which means 7M-BaP is even more potent than BaP. However, Lam (Monika M. Lam, 2018) found that 7-Methylbenzo[a]pyrene is more potent than BaP based on comparison of REP values after 24h exposure time by the H4IIE-luc bioassay. The potency of 7M-BaP under 24h exposure time observed in this paper is approximately 1000 times lower than BaP under 24h exposure time. The reasons for this seemingly paradoxical result are not clear for now. Further research is needed.
4.2.2 Anthracene

CALUX bioassay of anthracene was done under 2.5h and 24h exposure time respectively using BaP and TCDD as a reference compound. Its sigmoid curves and EC values are shown on below.

![BaP/Anthracene 2.5h exposure time](image1)

![TCDD/Anthracene 24h exposure time](image2)

Figure 12. Anthracene under 2.5h (a) and 24h (b) exposure time using BaP and TCDD as reference (unit pg/well)
<table>
<thead>
<tr>
<th>E.T.</th>
<th>n</th>
<th>EC\textsubscript{20} pg/well</th>
<th>RSD%</th>
<th>EC\textsubscript{50} pg/well</th>
<th>RSD%</th>
<th>EC\textsubscript{80} pg/well</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>3</td>
<td>1120± 230</td>
<td>21</td>
<td>5900.0±</td>
<td>26</td>
<td>31000±</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1513.3</td>
<td></td>
<td>11000</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>280000±</td>
<td>64</td>
<td>2000000±</td>
<td>59</td>
<td>15000000±</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1780000</td>
<td></td>
<td>8100000</td>
<td></td>
</tr>
</tbody>
</table>

Tested compound: Anthracene; Mw= 178.2 g/mol; E.T. exposure time; n tested times;

Anthracene under 2.5h exposure time still provides a clear sigmoid dose-response curve. And the location of anthracene curves is on the right side of BaP curves which means Anthracene is less potent than BaP. Nevertheless, as other PAHs shows, it is hard to see that anthracene can give a sigmoid curve under 24h exposure. EC values for anthracene under 2.5h exposure is hundred times higher than BaP EC values which implies anthracene is 100 times less potent than BaP.
4.2.3 n-Paraffin mix

n-Paraffin mix is a liquid mixture of eleven non-polycyclic hydrocarbon components which only roughly know each compound weight percentage. So only consider mass unit of n-Paraffin mix. The dose-response curves and EC values are shown below.

Figure 13. n-Paraffin mix under 2.5h and 24h exposure time using BaP and TCDD as reference (unit pg/well)
From figure 13, it is hard to find that n-Paraffin mixture can have any induction for cell lines under 2.5h or 24 h exposure time. As the result, the corresponding EC values for paraffin are meaningless. Therefore, Paraffin mixture cannot have some positive response on CALUX bioassay. This might because of the lack of polycyclic formation of paraffin. Previous study also has proved there is a lack of carcinogenicity on liquid paraffin. (T.Shoda, 1997)
4.3 REP value

REP value for every tested compound under 2 exposure time was listed on table 11. During the calculation, each compound and reference EC values are their average of all tests (see Equation 2).

Table 11. REP value for all tested compound

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>REP\textsubscript{Bap}(2.5h)</th>
<th>REP\textsubscript{Tcdd}(24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC\textsubscript{20}</td>
<td>EC\textsubscript{50}</td>
</tr>
<tr>
<td>BaP</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7M-BaP</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>n-Paraffin mix</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A. not applicable. BaP, benzo[a]pyrene. 7M-BaP, 7-Methylbenzo[a]pyrene. REP values are calculated by the average of

For 2.5h exposure time, it is interesting for the result of REP value. Just like the results from the curves, 7M-BaP is more potent than the reference compound, BaP. But under 24h exposure time, 7M-BaP acts less potent which suggests it has been metabolized completely in cells. Anthracene showed one percent potency comparing with BaP at 2.5 exposure time. It is relatively high REP value comparing to previous studies. (EPA, 2012) (Miroslav Machala, Jan Vondrác, 2001). Still it goes almost fully metabolized after 24 exposure time. For n paraffin-mix, there is no induction under both exposure time. So the REP values for paraffin is no existent. But since the research on
liquid paraffin by CALUX bioassay is limited, it is interesting to test if paraffin mixing with PAHs can have some positive effect on PAHs for further study.
5. Conclusion

The response and potency of BaP, anthracene, 7-methylbenzo-[a]-pyrene and liquid paraffin mix (C5 to C15) towards the AhR was tested by using CALUX bioassay at two exposure time (2.5h and 24h). Strong induction and clear dose-response curves were obtained for BaP, anthracene and 7-methylbenzo-a-pyrene under 2.5h exposure time. The n-Paraffin mix did not give any response whatever the exposure time considered. Afterwards, the results of REP calculation showed that 7-methylbenzo-[a]-pyrene and anthracene were, respectively, twice and 0.01 times potent comparing with BaP under 2.5h exposure time.

Some studied showed alkylation of PAHs had been reported to enhance or induce the genotoxic activity of PAHs depending on both size and location of the substituents. For future works, it is interesting to test different mono-methylated and double-methylated BaP by CALUX. Then compare their potencies with BaP to see what kind of influence of substituents’ size and location. Moreover, more research is needed for detecting if longer carbon number liquid paraffin mix (C15 to C31) can give any response by CALUX.
6. Reference


EPA. (2012). *Consolidated List of Chemicals Subject to the Emergency Planning and Community Right- To-Know Act (EPCRA), Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) and Section 112(r) of the Clean Air Act*. Office of Emergency Management.


Kersten Van Langenhovea, K. C. (2011, 3 29). The CALUX bio-assay: Analytical comparison between mouse hepatoma cell lines with a low (H1L6.1c3) and high (H1L7.5c1) number of dioxin response elements. *Talanta*, pp. 2039–2046.


Sigma-aldrich. (2018). *n-Paraffins Mix (analytical standard)*. Retrieved from sigma-aldrich:


