Grade's final project

COMPARING ASSAYS FOR THE MEASUREMENT OF AHR LIGAND ACTIVITY

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1. SUMMARY

In this thesis (TFG) the results of the comparison of three assays for the measurement of AhR ligand activity are exposed. This study was part of a collaborative project aiming at the characterization of the AhR signaling activities of known naturally occurring compounds to explore the potential of using non-toxic compounds to treat inflammatory diseases via oral administration.

The first goal of this project was to find an assay able to measure AhR-activity, so the comparison of different assays has been done in order to find the most convenient one according to the efficiency, sensitivity and precision. Moreover, other elements with operational nature such as price, toxicity of components or ease of use has been considered. From the use of compounds known from the literature to be AhR ligands, three assays have been tested: (1) P450-Glo™ CYP1A2 Induction/Inhibition assay, (2) quantitative Polymerase Chain Reaction (qPCR) and (3) DR. CALUX® Bioassay. Moreover, a different experiment using the last assay was performed for the study *in vivo* of the transport of the compounds tested.

The results of the TFG suggested the DR. CALUX® Bioassay as the most promising assay to be used for the screening of samples as AhR-ligands because it is quicker, easier to handle and less expensive than qPCR and more reproducible than the CYP1A2 Induction/Inhibition assay. Moreover, the use of this assay allowed having a first idea of which compounds are uptaken by the epithelial barrier and in with direction the transport happens.

2. INTRODUCTION

2.1. Definition

Aryl hydrocarbon receptor (AhR) is a ligand-activated cytosolic transcription factor initially known for its role in the regulation of dioxin toxicity, as it recognizes numerous small xenobiotics and natural molecules and regulate some metabolizing enzymes that detoxify them. Recent findings has brought AhR into mainstream research on mucosal immunology and intestinal health research as it provides a molecular pathway by which endogenous and environmental signals can influence the immune response. Thus, AhR is seen as a possible target for therapeutic intervention in immune-mediated disorders [1]. Moreover, there is recent data from animal models that has demonstrated the possibility of targeting the AhR to treat inflammatory and autoimmune diseases [2, 3].

One of these studies had focused on the role of natural plant derived dietary compounds found in the conventional rodent diet as agonists of AhR. The replacement of this conventional mouse diet containing plant AhR agonists with a synthetic highly refined diet devoid of plant products have an impact on the intestinal homeostasis[4] (Figure 1).

The picture in Figure 1A shows how a balanced diet maintains intestinal mucosal homeostasis by many mechanisms. The most important is the role of intraepithelial lymphocytes (IELs) as they support epithelial cell growth and have a role in immune surveillance. IELs are involved in stimulating epithelial cell turnover in the small intestine, thereby maintaining the intestinal villi and providing protection against mechanical or microorganism-induced damage. Moreover, IELs are essential mediators of host-microorganism homeostasis, as they can directly lyse target cells through the expression of granzymes and perforin and secrete antimicrobial factors [5].

Decrease in the number of IELs when a diet is low in AhR ligands alters intestinal homeostasis (Figure 1B). Thus, decrease or absence in the number of IELs reduces the ability of the intestine to repair tissue damage. Therefore, the epithelial barrier is compromised and immunity is reduced, increasing the risk of bacterial dissemination into the lamina propria, leaving to microbial infection and tissue damage so finally resulting in an overt inflammatory response.

The addition of synthetic ligands for AhR into the synthetic diet restored normal numbers of these IELs, highlighting the importance of dietary components rich in AhR ligands, especially early in life for the maintenance of immune populations within the gut mucosa.

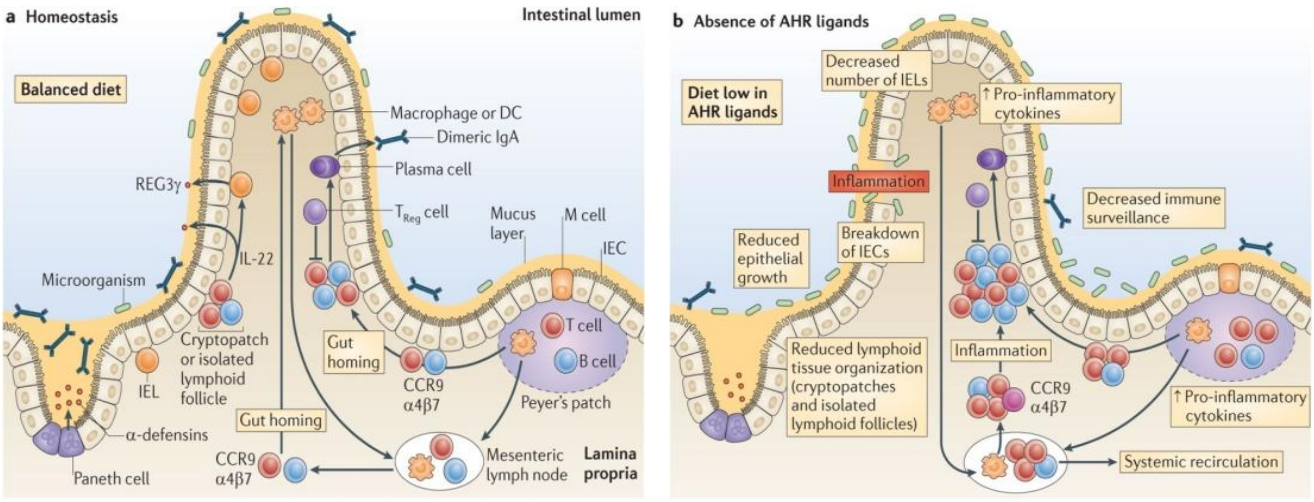

Figure 1. *Role of AhR in maintainance of the intestinal homeostasis*

2.2. AhR signaling pathway

AhR is a ligand-activated member of the Per-Arnt-Sim family of basic helix–loop–helix transcription factors. Normally, AhR forms cytoplasmic complexes with various proteins, such as heat shock protein 90, AhR-interacting protein, chaperones and p23. Binding of AhR to ligands, such as TCDD, induces translocation of the Ahr complex into the nucleus, where ligand-bound AhR complex dissociates from chaperone proteins after dimerization with AhR nuclear translocator (Arnt).

In the nucleus, AhR–Arnt heterodimers bind xenobiotic-responsive elements (DREs/XREs) in the promoters of responsive genes, thereby inducing AhR-dependent gene expression, including those encoding members of the cytochrome P450 (CYP) family [6] (Figure 2).

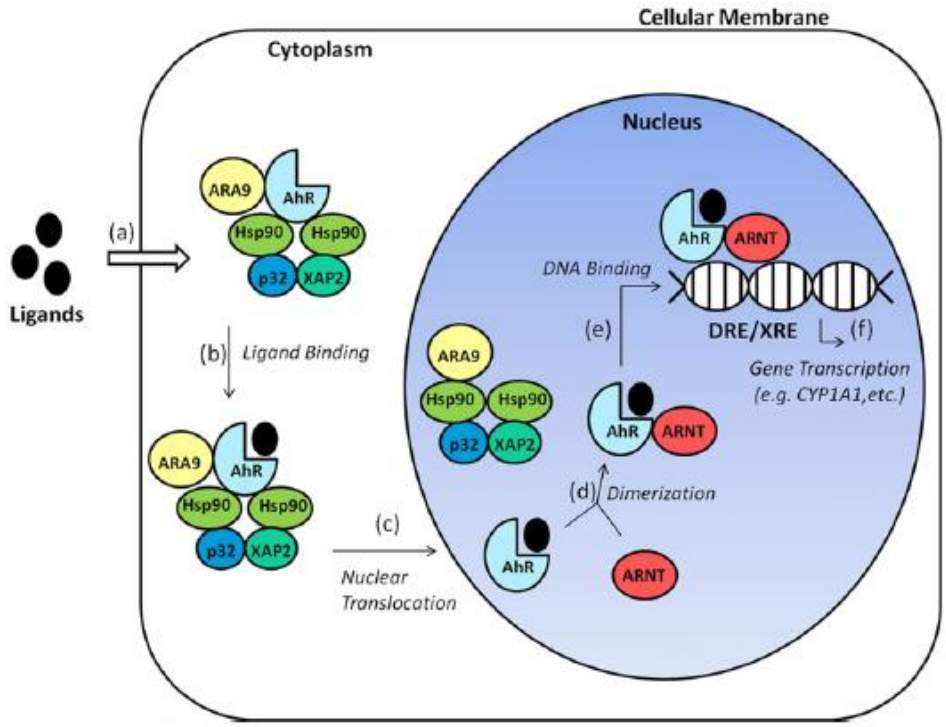

Figure 2. *AhR signaling pathway*. (a) Ligand passes through plasma membrane into the cellular cytoplasm. (b) Ligand binds to cytosolic AhR complex. (c) The ligand-bound AhR complex is translocated into the nucleus. (d) The ligand-bound AhR complex dissociates from chaperone proteins after dimerization with ARNT. (e) Ligand-bound AhR/ARNT complex binds to DREs/XREs, (f) which leads to transcriptional activation of target genes.

2.3. AhR ligands

The diet, particularly vegetables, fruits and teas [7,8], is an important source of AhR ligands, mostly flavonoids and other phytochemicals such as quercetin, curcumin, resveratrol, and tryptophan metabolites as DIM (3-3 diindolylmethane) and L-kynurenine. These natural compounds have been shown to be less toxic but still able to elicit responses through the AhR pathway [9].

Quercetin is one of the most abundant polyphenolic compounds in the human diet [10] and can be found in numerous vegetables, fruits, seeds and nuts, as well as in tea and red wine [11] and it has been shown to elicit anti-inflammatory properties [12, 13, 14, 15, 16].

Curcumin is a lipophilic polyphenol found in the rhizome of the plant Curcuma longa [17], used in the traditional Indian medicine to treat a number of ailments like anorexia, inflammation, wound healing, arthritis, and sinusitis [18] and it has been shown to exert antioxidant, antiproliferative, antiangiogenic, apoptotic, and anticancer properties, supporting its use in traditional Indian medicine and to protect many tissue types, such as brain, heart, liver, lungs, kidneys and skin, from oxidative agents [19].

Resveratrol is a naturally occurring nonflavonoid polyphenol that can be found in a variety of dietary sources, including grape seeds, peanuts, and mulberries [20]. Plants produce resveratrol as a stress response against invading fungus, which highlights the natural antifungal properties of this compound [21]. It is an important component of the root of Polygonum cuspidatum, also known as ko-jo-kon, which is used in Eastern medicine to treat diseases of the blood vessels, heart, and liver [21-28]. Resveratrol has also been suggested to have possible therapeutic applications in numerous areas due to its cardioprotective [28, 29], anticancer [30], antioxidant [31], cholesterol-lowering [32] and antiaging effects [33].

Resveratrol is also able to downregulate various proinflammatory cytokines and the number of Th17 cells, supporting the potential usefulness in inflammatory conditions [9].

DIM is an indole compound, a by-product of I3C (indole-3-carbinol) after the acid hydrolysis during digestion within the gut [34]. Recent studies have indicated that this compound may play a critical role in modulating immune responses, particularly with regards to inflammation and inflammatory diseases [35, 36].

Finally, L-Kynurenine is a metabolite of the amino acid L-tryptophan, synthesized by the enzyme tryptophan dioxygenase, which is made primarily but not exclusively in the liver, and indoleamine 2,3-dioxygenase, which is made in many tissues in response to immune activation. L-Kynurenine and its further breakdown products carry out diverse biological functions, including dilating blood vessels during inflammation and regulating the immune response [37].

In this study previous known AhR-ligand compounds were used to optimize different assays for the screening of potential AhR-ligands.

2.4. AhR Assays

In order to investigate the effect of different natural occurring compounds on the activation of the AhR pathway, three previous steps have to be done. First, the comparison of different assays able to measure AhR- activity is needed to find the most convenient one according to the efficiency, sensitivity and precision. Moreover, other elements with operational nature such as price, toxicity of components or ease of use should also be considered. Second, the optimization of the chosen assay to characterize AhR ligands has to be done and finally, by using the optimized assay, a third step will allow the screening of samples as possible ligands.

In this study the first step was developed and three different assays were used to check which one meets most of the conditions listed above.

P450-Glo™ CYP1A2 Induction/Inhibition assay is based on the power of CYP enzymes to catalyze a reaction that converts a luminogenic substrate (proluciferin) to luciferin. Luciferin can be detected with the Luciferin Detection reagent [38]. With this assay the amount of CYP1A2 produced in cells as a consequence of AhR activation can be quantified as it is proportional to the light output of the luciferase reaction (Figure 3).

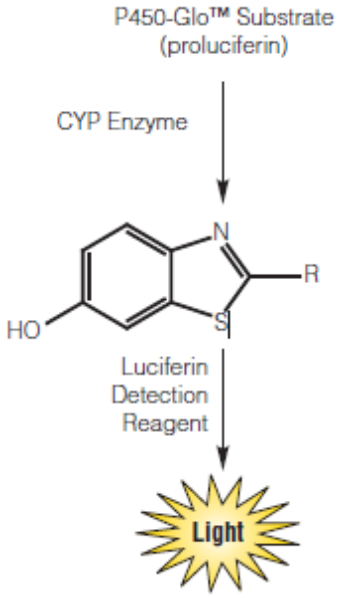

Figure 3. *Luciferase reaction.*

A quantitative polymerase chain reaction (qPCR), also called real-time polymerase chain reaction, is a molecular biology technique used to amplify and simultaneously quantify a targeted DNA molecule, in this case CYP1A1 gene. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing. Relative quantification is the most used and it calculates the ratio between a housekeeping gene, a gene which is constitutive expressed (in this case GAPDH), and the gene of interest (CYP1A1). Thus, by dividing the signal of the gene of interest by the signal of the normalized gene it is possible to compare the results without knowing their absolute level of expression.

QPCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorophore. The qPCR process generally consists of a series of temperature changes that are repeated 25 – 40 times, which normally consist of a first step, at around 95 °C, which allows the separation of the nucleic acid double chain and a second one, at a temperature of around 50-60 °C, that allows the binding of the primers with the DNA template. In addition, some thermal cyclers add another short temperature

phase lasting only a few seconds to each cycle, called melting curve, in order to reduce the noise caused by the presence of primer dimers when a non-specific dye is used.

The qPCR technique used in this study uses a non-specific fluorochrome (SYBR-Green), a fluorescent dye that intercalate with any double-stranded DNA. Thus, while exiting the sample using blue light (λmax = 488 nm) it emits green light (λmax = 522 nm), which can be detected. This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down. However, it is only possible to amplify one product in each reaction.

The Dioxin Receptor Chemical-Activated LUciferase gene eXpression assay (DR. CALUX® Bioassay) was developed for the detection of dioxins in samples and is based on the principle that dioxins can activate AhR pathway for the translation of detoxifying enzymes [39, 40, 41].

This assay is a receptor based reporter gene assay which utilizes Rat H4IIE hepatoma cells, stably transfected with an AhR-controlled luciferase reporter gene construct, so in response to compounds that stimulate AhR pathway, particularly the binding of AhR-ligand complex to DRE element, this cell-line will synthesize luciferase in a dose-dependent way [42], which can subsequently be quantified by an enzymatic light producing reaction (Figure 4).

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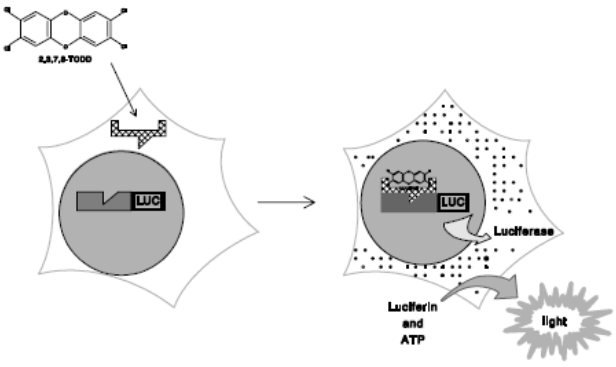

Figure 4. *Schematic representation of the mechanism of action of the DR-CALUX®.*

In all assays TCDD has been used as a positive control as it gives strong signals, but the screening of different compounds may allow the detection of a less toxic substitute for TCDD.

As the aim of this study was the comparison of different assays to check which one is more useful in an overall for the detection of AhR-activity, assays based on the measurement of different components in the AhR pathway were chosen. P450-Glo™ CYP1A2 Induction/Inhibition assay detects the activity of CYP1A2 gene, qPCR the expression of CYP1A1 gene and DR. CALUX® Bioassay the activity of the transcription factors which activates the cellular detoxifying system. The first two assays focus on different genes transcribed after the binding of the AhR complex to the transcription factor and the third one on the binding itself.

2.5. Intestinal uptake of Ahr-ligand compounds

The first step for the study of transport, secretion and absorption of the ligands by the intestine is to simulate the gut epithelial barrier. This is possible by using a transwell system, which produces a cell culture environment that closely resembles the in-vivo state and enables growth of specialized cell types, in particular polarized intestinal epithelial cells (Figure 5).

By stimulating the apical or the basolateral compartments with different compounds, transwell system allows the study of the uptake of those compounds in the gut, from the luminal to the serosal side or the other way around.

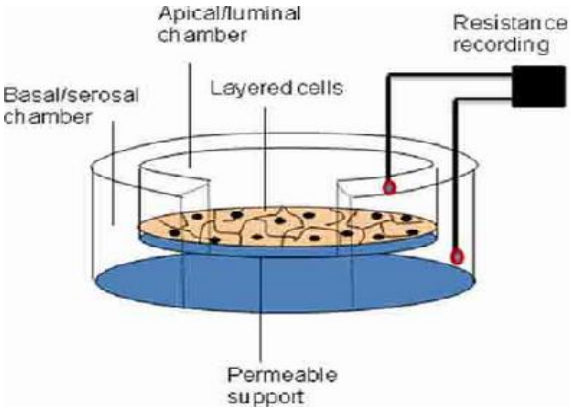

Figure 5. *Transwell system*.

3. OBJECTIVES

3.1. General objective

This work is part of a collaborative project aiming at the characterization of the AhR signaling activities of known naturally occurring compounds to explore the potential of using non-toxic compounds to treat inflammatory diseases via oral administration.

3.2. Specific objectives

1) Comparison of the currently available assays for measurement of AhR –ligand activity:

- CYP1A2 Assay
- qPCR
- DR-CALUX Bioassay

2) Study of the transport, secretion and ligand absorption using polarized CaCo2 monolayers grown in transwell filter inserts, in order to understand the mechanisms by which compounds reach the lumen.

3) Look for a non-toxic substitute of TCDD as a positive control for all the assays tested.

4. MATERIALS AND METHODS

For the selection of an assay for the screening of samples as AhR ligands, compounds known from literature that act as AhR-ligands and the right concentrations of them were used to compare different parameters of each assay. Before the performance of each assay, HepG2 cells for the CYP1A2 Induction/Inhibition assay and QPCR and CALUX cells for DR. CALUX Bioassay were stimulated with the compounds for 24 hours in order to let them go into the cells and bind to the AhR, so activate the AhR pathway. HepG2 were selected for the stimulation because even though they are not intestinal cells it is known from literature that they are the most used in these studies.

Once the assay that met more requirements was chosen, an in vitro study of the transport of the compounds was performed. Caco-2 cells, an intestinal cell line, was used to try to mimic the real behavior of the intestine, as they can be grown in a transwell system to reproduce the gut epithelial layer. In order to know if the compounds go either through or in between the cells in the monolayer (from the apical or luminal side to the serosal or basolateral side or vice versa) once the cells were stimulated either in the apical or basolateral compartment for 24 hours the supernatants of both sides were tested with the DR. CALUX Bioassay. As it is known that this compounds are AhR-ligands, if they are present in the supernatants this assay will detect them. Thus, this assay permits to know in which compartment there is compound and quantify it.

4.1. Cell lines

HepG2 (DSMZ-ACC180) cells, derived from a well-differentiated hepatocellular carcinoma from human liver tissue, were obtained from DSMZ, Germany. Cells were cultured at 37ºC in 5% CO_2 atmosphere in RPMI medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% penicillin/streptomycin (P/S) (Sigma-Aldrich).

The Caco-2 cell line, derived from heterogeneous human epithelial colorectal adenocarcinoma cells, was obtained from ATCC (LGC Standards GmbH, Germany) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated FBS and 1% P/S.

The CALUX cell line, which is derived from Rat H4IIE hepatoma cells and stably transfected with an AhR-controlled luciferase reporter gene construct, (pGudLuc1.1) was obtained from BioDetection Systems (Amsterdam, The Netherlands) and cultured in α-MEM medium (Gibco) supplemented with 10% heat-inactivated FBS and 0.6% P/S.

4.2. Culture conditions

HepG2 cells were seeded at a concentration of $3 \cdot 10^5$ cells/ mL in 48 and 96-well plates and incubated to allow cell adherence. After 24 hours the cells were stimulated with different compounds and incubated overnight before the analysis.

Caco-2 cells were seeded on filters at a concentration of $2 \cdot 10^4$ cells/ mL in 48 well plates and incubated for 2 weeks to allow monolayer and tight junction formation. Medium was refreshed every other day. Stimulation with different compounds was done 24 hours before the analysis.

4.3. AhR-ligand compounds

TCDD (SUPELCO) and DIM (3-3 diindolylmethane) were dissolved in DMSO, β-Naphthoflavone in $CHCl_3$, α-Naphthoflavone in CH_3OH, L-Kynuririne in 0.5M HCl, Quercetin in 1M NaOH and Resveratrol and Curcumin in EtOH (all Sigma-Aldrich, Germany) before their use.

4.4. CYP1A2 Assay

HepG2 cells[43] were stimulated with the following compounds; 10nM TCDD, 6nM DIM, 100µM curcumin, 1mM L-kynurinine, 100µM β-naphtophlavone, 100µM quercetin and 100µM resveratrol. After 24 hours

incubation cells were washed twice with PBS before adding 50 µL luciferin/well. After 45 minutes incubation, 50 µL detection reagent was added in each well (P450-GloTM CYP1A2 Induction/Inhibition assay, Promega, Madison, WI, USA). Plates were shaken briefly prior to measurement. Luminescence was measured after 10 minutes in a Spectramax M5 (Molecular devices, CA, USA).

4.5. RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated using RNeasy kit (Quiagen, Venlo, the Netherlands), with a DNase digestion step according to the manufacturer's protocol. One µg was reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol.

For qPCR 5µL cDNA (1:20 diluted from cDNA synthesis mixture) was used, together with 300nM forward and reverse primers (qPCR Primers for Human CYP1A1: PPH01271F from SABiosciences and the forward primer: "TGCACCACCAACTGCTTAGC" and reverse primer: "GGCATGGACTGTGGTCATGAG" for GAPDH gene), 6.25µL 2X Rotor-Gene SYBR Green PCR kit (Qiagen), and demineralized water up to a total volume of 12.5 µL. QPCR was performed (5 min 95ºC, then 40 cycles of 5 sec at 95ºC, 10 sec at 60ºC, and a final melt step ramp from 60ºC till 95ºC rising 1ºC each step) on a Rotorgene 6000 real-time cycler (Qiagen).

The raw data was analyzed using the Rotor-gene 6000 Series Software 1.7. Changes in transcript levels were calculated relative to the housekeeping gene according to the following equation:

$$\text{Ratio} = \frac{\text{Ereference}^{\text{Ct reference}}}{\text{Etarget}^{\text{Ct target}}}$$

Where E is the amplification efficiency and Ct is the number of PCR-cycles needed for the signal to exceed a predetermined threshold value.

Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was incorporated in all qPCR experiments as internal reference gene. Reactions lacking reverse transcriptase (-RT) or template were included as controls in all experiments and no amplification above background levels was observed for these controls. Non-template controls were included for each gene in each run and no amplification above background levels was observed. The melting temperature and profile of each melting curve was checked to ensure specificity of the amplification product.

4.6. CALUX Bioassay

CALUX cells were grown confluent in a 96 well white clear bottom plate and exposed overnight in triplicate to TCDD as standard(0 nM, 0.1 nM, 1 nM, 5 nM and 10 nM TCDD) and non-standard stimulatory compounds (100 µM Curcumin, 100 µM β-naphtophlavone, 100 µM Quercetin) for the first study and with the Caco-2 supernatants (after Caco-2 monolayer stimulation with 10 µM TCDD, 100 µM β-naphtophlavone, 100 µM Quercetin and 6 nM DIM) for the second study. Each well contained 100 µL medium with standards and samples diluted and dissolved in the medium. Following 24 hours exposure, cells were washed twice with 250 µl pre-warmed PBS and lysed in 20 µL lysis reagent (Luciferase cell culture lysis 5X reagent, Promega, Madison, WI, USA). After 10 minutes 100 µL assay buffer (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA, 33.3 mM DTT, 261 µM Coenzyme A, 470 µM Luciferin, 530 µM ATP, pH 7.8) was added and luminescence measured in a Spectramax M5. Plates were shaken briefly prior to measurement.

4.7. Electrical resistance measurements in monolayer cell culture

CaCo-2 cells were cultured on transwell insert filters for 2 weeks in order to let cells monolayers reach a density of $2.6 \cdot 10^5$ cells/cm^2 and trans-epithelial resistance (TER) was measured using chopsticks.

4.8. Statistical analysis

Results are presented as the mean ±standard deviation (SD) of triplicates. All data were analyzed by a t-test. A value of $p < 0.05$ was considered as statistically significant. All statistical analyses were performed using Graphpad Prism software.

5. RESULTS AND DISCUSSION

CYP1A2 assay

The CYP1A2 Induction/Inhibition assay was the first assay tested for the screening of known AhR ligands for its simplicity in the procedure. It was performed after 24 and 48 hours stimulation of HepG2 cells with different compounds (DIM, curcumin, L-kynurenine, β-naphtophlavone, quercetin, resveratrol and TCDD as a positive control and medium as a negative control).

The differences between the positive (TCDD) and negative (medium) control values, despite they are significant, were too small (around 40 RLU) to detect subtle changes, as it is known that in the assays based on luminescence measurement the differences between controls are in the order of 1000 RLU. Furthermore, the variation between duplicates and different assays performed was too big to trust the results. Luminescence was measured after 24h and 48h stimulation to see what the best point to measure luminescence signal was but no significant differences could be observed between the times (Figure 6).

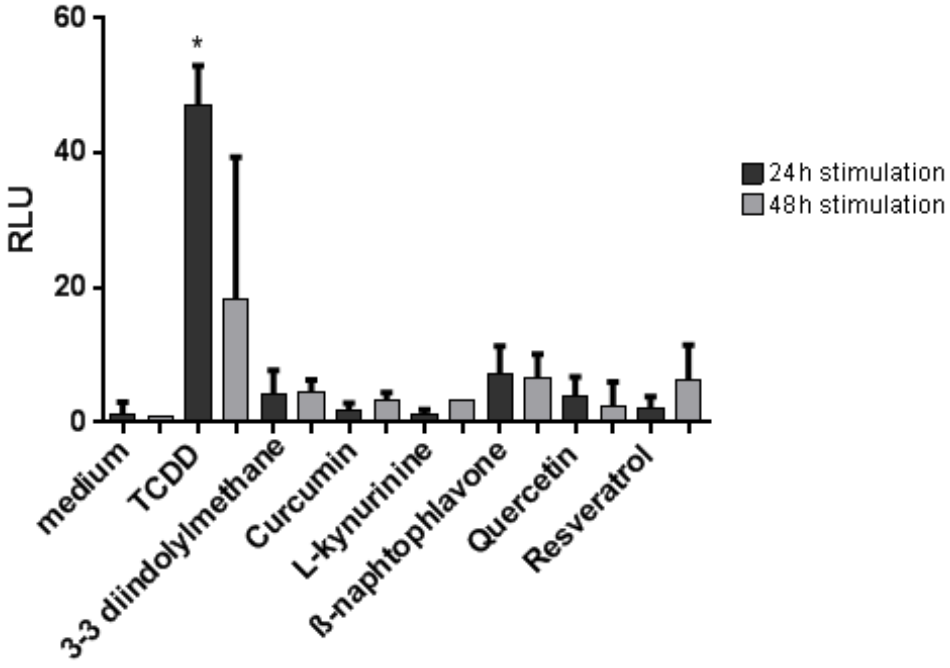

Figure 6. *CYP1A2 assay results.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium.

qPCR

Quantitative PCR with mRNA isolated from stimulated HepG2 cells was performed as the CYP1A2 assay was not sensible enough to detect differences between induced cultures and the negative control.

All compounds tested (β-naphthoflavone, quercetin, resveratrol, DIM and L-kynurenine) induced CYP1A1 expression, which was not due to the solvents that the compounds were dissolved in but because of the compounds themselves. This results were expected as the product that has the power to bind to AhR ligand is the compound and the solvent is only used to dilute the compound in order to achieve the appropriate concentration (Figure 7). As it is known from literature that α-naphthoflavone has an inhibitory effect on the AhR signaling pathway [44], cells were stimulated either with TCDD and TCDD mixed with α-naphthoflavone (TCDD+) to confirm the effect of α-naphthoflavone inhibition on the CYP1A1 expression. As observed in the graph, the expression of TCDD when α-naphthoflavone was added (TCDD+) is significantly lower than without (TCDD) which is thus in agreement with our expectations.

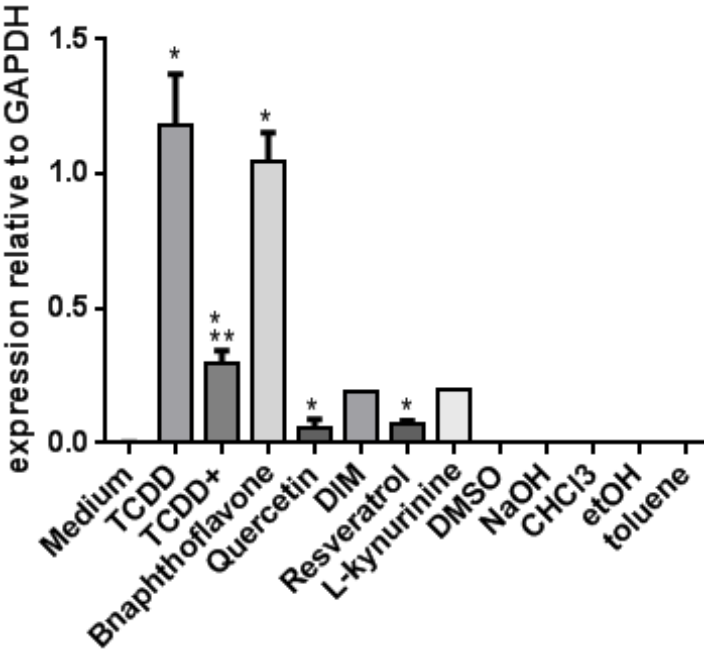

Figure 7. *qPCR results.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium, **p < 0.01 compared to TCDD.

DR-CALUX™ Bioassay

DR-CALUX Bioassay was performed to evaluate its potential as a replacement technique for qPCR, as it is less expensive, quicker and easier to handle.

The clear effect of β-naphtophlavone (β-Nap) on the binding of AhR-complex to DRE element is also appreciated, as its signal is around 35 times higher than the control. Results for quercetin are also statistically significant although the signal is just 1.24 times higher compared to the control.

Moreover, as this assay gave the best results compared to CYP1A2 Induction/Inhibition assay or QPCR, a comparison between freshly prepared compounds and compounds prepared some days before the assay was made in order to test the stability of the compounds. Figure 8 shows a progressive decline between day 0 and day 7, but the signal is significant in day 0 and day 5, suggesting that the compounds can be used 5 days after their preparation and they will still gave a strong signal.

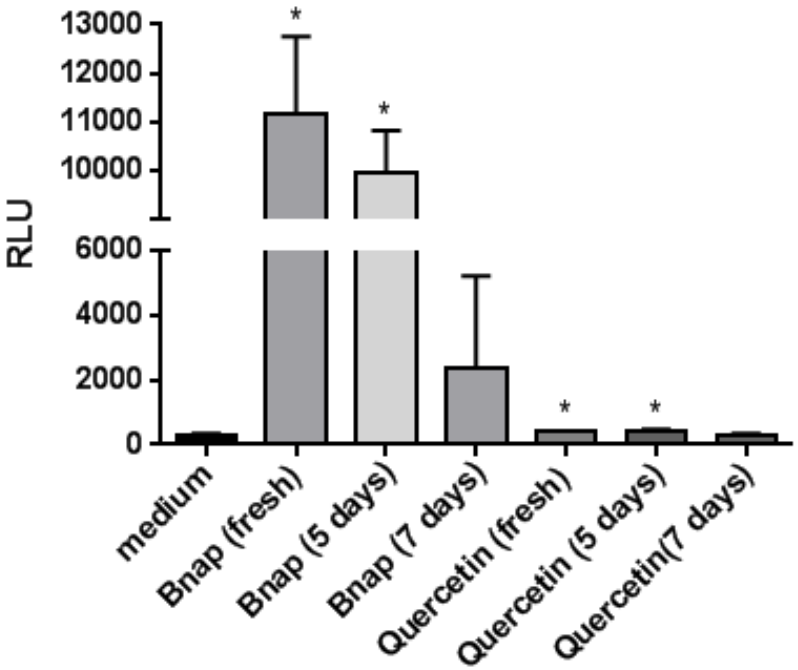

Figure 8. *DR. CALUX Bioassay results.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium.

Comparison between the CYP1A2 assay (Figure 6) and the DR.CALUX Bioassay (Figure 8) signals suggest that the second one is more reliable. Although the two methods measure different phenomena we are interested in finding the system with the higher sensibility and precision to evaluate the effect of Ahr ligands on cell cultures. So, Dr. CALUX showed better results in terms of both parameters, as the RLU is comparable with the values obtained in other experiments done with the same assay and not a big deviation in the duplicates is appreciated.

Intestinal uptake of AhR-ligand compounds

Electrical resistance measurements in polarized Caco-2 monolayers grown in transwell filter inserts where done in order to test the concentration effect of the compounds on epithelial permeability, as higher doses of those compounds can kill the cells and brake the monolayer. Thus, it is important to check the TER, as it permits to know if the compounds damage the cell monolayer, leaving the samples unusable, and adjust the concentration tested.

After culturing Caco-2 cells on filters for 2 weeks to allow monolayer and tight junction formation and cell polarization, TER was measured before and after the stimulation of the apical (A) or basolateral (B) compartments, for 24 hours with different compounds. Table 1 shows that none of the Ahr ligands increased significantly the permeability of the membrane as the TER values were similar to that of the negative control. This means that none of the ligands, at the tested concentrations, damaged the monolayer. These results were as expected since the concentration of the compounds used was taken from the literature, where non of the concentrations showed epithelial barrier damage.

	TER (Ω)	
	Before stimulation	24h stimulation
Medium (A)	1,70	5,51
Quercetin (A)	1,46	5,45
β-Naphthoflavone (A)	1,32	5,14
DIM (A)	1,38	4,91
TCDD (A)	1,32	4,20
TCDD (B)	1,33	5,16

Table 1. *TER measurements*.

Once it was known that the monolayers were not damaged, so they could be used in further experiments, we wanted to know whether the cells are able to take compounds from the basolateral side (which represents the lamina propria, or the serosal side) and/or if they can do it from the apical side (luminal side). Therefore, cells were stimulated apically (A) or basolaterally (B) with TCDD (used as positive control) and the apical supernatants were tested with the CALUX assay. Figure 9 shows that the signal from the cells stimulated basolaterally is the same as that of the control medium, confirming that the compounds are not able to be transported from the lumen. The apical stimulation is much stronger, as it doubles the control value, suggesting that Caco-2 cells use the compounds, but the basolateral compartment of the apical stimulated sample (A) should be tested to know if transport from the lumen exists

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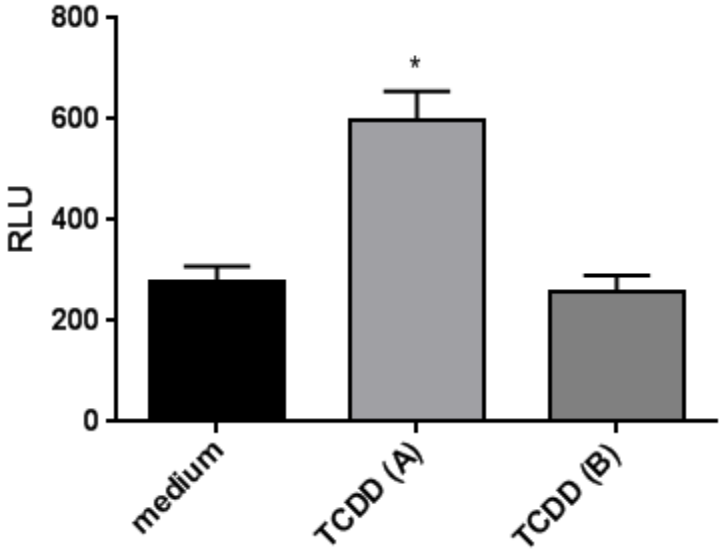

Figure 9. *CALUX Bioassay results from apical supernatants of polarized Caco-2 cells after apical (A) and basolateral (B) stimulation with TCDD.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium.

Thus, to know if the compounds pass through the cell layer, so from the apical compartment to the basolateral compartment, cells were stimulated apically with different compounds and the apical (A) and basolateral (B) supernatants were tested with the CALUX assay. Figure 10 shows more signal for all compounds in the apical supernatants compared to the basolateral ones, suggesting that the compounds tested pass through the cell layer, as signal in the supernatant of both compartments is seen. With DIM, signal is higher in the basolateral side than in the apical one, and this could be due to a faster uptake of this component than the others or as a result of a more active transport.

For now, whether the compounds are taken up by the Caco-2 cells and secreted (so transcellular transport from apical to basolateral) or the compounds go paracelullar cannot be confirmed. Furthermore, other assays are needed to learn whether the compounds are converted by the cells.

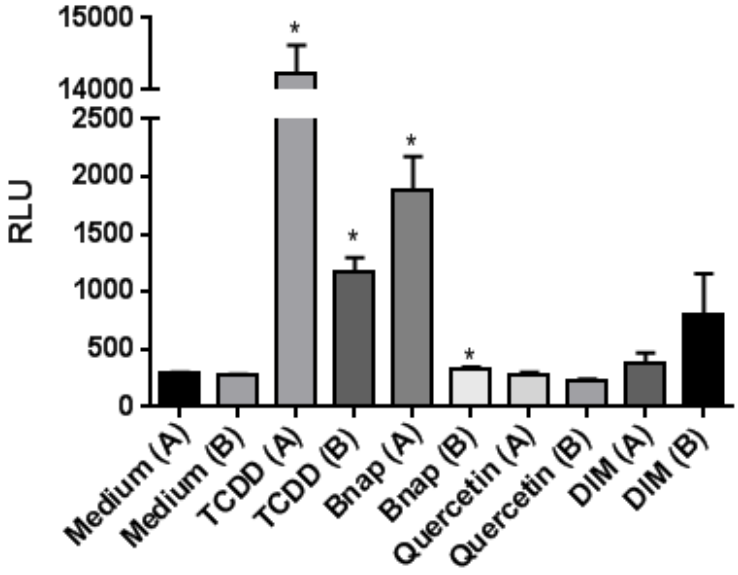

Figure 10. *CALUX Bioassay results from apical (A) and basolateral (B) supernatants of polarized Caco-2 cells after apical stimulation with different compounds.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium.

Finally, once verified that cells takes the compounds from the apical side and not from the basolateral one, cells were stimulated apically with different compounds and the apical supernatants were tested with the CALUX assay. The basolateral supernatants were not tested as the aim of this last experiment was to check which compounds significantly signals as AhR ligands. In Figure 11 it can be seen that β-naphthoflavone and DIM induce the AhR pathway.

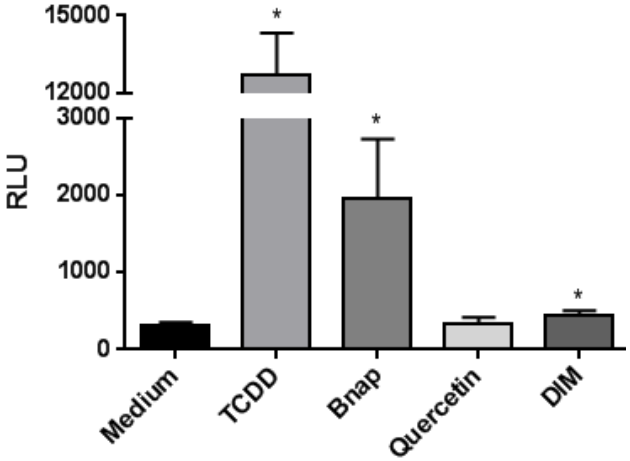

Figure 11. *CALUX Bioassay results from apical supernatants of polarized Caco-2 cells after apical stimulation with different compounds.* Values are expressed as the relative light units (RLUs) and represent the mean ± SD of triplicate determinations. *p < 0.05 compared to medium.

6. CONCLUSIONS

The results described in this report indicate that DR.CALUX Bioassay seems to be the most promising assay to be used in further studies because it is quicker, easier to handle and less expensive than qPCR and more reproducible than the CYP1A2 Induction/Inhibition assay. Moreover, DR.CALUX Bioassay detects the amount of AhR complex bound to the DRE/XRE transcription element so it seems to be more specific for the measurement of AhR activity, as it is focus on one step of the AhR-pathway. On the contrary, the other two assays tested measures the activity of CYP genes, which can also be transcribed by other pathways.

The inclusion of this bioassay will also allow rapid screening and detection of both, known and new AhR agonists. Although the induction seen was not the one expected based on literature, the signals obtained with β-naphthoflavone were high enough to think that this compound can potentially replace TCDD as a positive control, avoiding in that way the use of toxic compounds.

Finally, it is important to test the half-life of compounds as it was seen that they can be used at least 5 days after their preparation, because this will enable to use less amount of compounds as they will not have to be prepared fresh every day and save money as a counterpart.

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