Research Article

Incorporation of a Metabolizing System in Biodetection Assays for Endocrine Active Substances

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Summary

The use of *in vitro* assays for the biodetection of endocrine active substances (EAS) aims to reduce and replace *in vivo* studies required for regulatory assessment. However, this approach often fails to take into account the role of biotransformation on the activity of the test substances. A method introducing an S9 metabolic system into the CALUX-reporter gene assays for estrogen receptor α- and anti-androgen receptor-mediated activities was developed. Methoxychlor, which exhibits increased estrogenic and anti-androgenic activities after biotransformation, was used to establish the method in ERα and anti-AR CALUX. For the anti-androgenic assay, stanozolol was used as a competing agonist not metabolized by S9. The method was first applied in both agonist and antagonist modes to methoxychlor and bisphenol A, as positive and negative controls, respectively. Then, benzo(a)pyrene and flutamide were also tested for their bioactivation potential. Co-treatment with S9 successfully increased the ERα agonist and AR antagonist potency of methoxychlor; no change was observed for bisphenol A. Incubation with S9 also enhanced the anti-androgenic activity of flutamide. Interestingly, the metabolism of benzo(a)pyrene by S9 resulted in a greatly increased estrogen receptor-mediated transcriptional activation, while the shift in EC_{50} was only minor. It is likely that both enzyme kinetics and metabolite stability affect the composition of the final metabolite mixture and thus influence the observed effects. Together these results demonstrate the relevance of including biotransformation in *in vitro* bioassays for the detection of EAS.

Keywords: endocrine active substances, biodetection, metabolism, S9 fraction, CALUX® assay

1 Introduction

Biodetection is gaining importance, especially for samples for which little toxicological data is available (Krewski et al., 2010). In this context, cell culture-based assays are recognized to play an increasingly important role in the detection of endocrine active substances (EAS). These test systems provide insight into the intrinsic biological properties of both pure chemicals and previously uncharacterized mixtures. These test systems may reduce or even eliminate *in vivo* testing required for safety assessments.

Among the *in vitro* assays for the detection of endocrine active substances (EAS), the CALUX assay (Chemically Activated LUciferase gene eXpression) allows the detection of substances (i.e., ligands) with the potential to interact with various nuclear receptors and affect the subsequent transcriptional response (Sonneveld et al., 2005). Such assessments generally fail to take into account the role of metabolism. Indeed, the majority of cell-based assays use cells that are not metabolically competent and do not include a metabolizing step.

Some attempts to assess the role of metabolism in endocrine bioassays have been made previously. Most of these included

Abbreviations

NF/PB, β-naphtoflavone/phenobarbital; AR, androgen receptor; BaP, benzo(a)pyrene; BPA, bisphenol A; CALUX, chemically activated luciferase gene expression; CYP, cytochrome P450; DHT, dihydrotestosterone; DMSO, dimethylsulfoxide; E2, 17β estradiol; EAS, endocrine active substances; EC_{50}, half-maximal effective concentration; EDTA, ethylenediaminetetraacetic acid; ERα, estrogen receptor α; HPTE, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane; IC_{50}, half-maximal inhibitory concentration; MC, methoxychlor; RLU, relative light unit; S9, hepatic 9 000 x G supernatant; STZ, stanozolol

Received November 2, 2016;
Accepted December 19, 2016;
Epub December 22, 2016;
doi:10.14573/altex.1611021

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additional steps consisting of the pre-incubation of the compound of interest with rat liver S9 or microsomal fractions, followed by extraction of the final mixture which was then tested *in vitro* (Kang et al., 2014; Legler et al., 2002; van Lipzig et al., 2005; Yoshihara et al., 2001, 2004). However, this method is labor-intensive and the risk of losing relevant metabolites during extraction and evaporation is non-negligible.

Metabolism is classically divided into phase I (hydroxylation, dealkylation, oxidation or reduction) and phase II (conjugation). Many of these enzymatic reactions are oxidative and catalyzed by the mixed function oxidase activities of the cytochrome P450 (CYP) family of enzymes (Reinen and Vermeulen, 2015). However, the reactions are not necessarily detoxifying; a pro-toxicant can also be converted into one or more toxicants by these reactions. Indeed, phase I metabolism may generate metabolite(s) with increased, decreased or similar activity to that of the parent compound. Moreover, the first-generation metabolite(s) may also be substrate(s) of metabolic enzymes, further altering their activities. A number of reports indicate that bioactivation is a critical step in the *in vivo* activities of a variety of well-characterized EAS including methoxychlor (MC), dichlorodiphenyltrichloroethane (DDT), tamoxifen and vinclozolin (Jacobs et al., 2013; Kelce et al., 1997; Reinen and Vermeulen, 2015; Shelby et al., 1996).

Without a bioactivation step, current *in vitro* testing may underpredict human hazard by failing to identify the full potential of a substance for endocrine activity. Results from these screening tests can fail to eliminate candidates with less favorable profiles early in development (precluding safety by design). Such failures may also prevent the identification of hazard potential in cases where it is easier, faster and/or cheaper to manage this potential than clarify whether the hazard is genuine with further testing (thwarting proactive management).

To address the potential role of metabolism in the endocrine activity of EAS, liver S9 and cofactors for the cytochrome P450 enzyme family were incorporated into the CALUX bioassay as an exogenous activation system. Although this method may eventually be broadly applicable to screening of a wide-variety of nuclear receptor-mediated mechanisms, the feasibility and relevance were investigated for specific assays of estrogen receptor α agonism and androgen receptor antagonism for the purposes of this study. Metabolic activation is relevant to these two common endocrine activities (Jacobs et al., 2008).

The organochlorine pesticide MC was considered ideal for initial testing. Metabolites of this formerly used pesticide, are known to induce adverse reproductive and developmental effects via nuclear receptor-mediated transcriptional changes. MC is reported to have weak binding affinity for the estrogen receptor *in vitro*, but has pronounced estrogenic activity in the rat uterotrophic assay (Aoyama and Chapin, 2014) owing to CYP-mediated bio-activation. The pathways involved in the metabolism of MC have been well-characterized (Hu and Kupfer, 2002). One of the identified metabolites, di-hydroxylated HPTE, is believed to be responsible for the increased estrogenic but also anti-androgenic activity *in vivo* (Gaido et al., 2000; Maness et al., 1998). MC was therefore studied in the ERα and anti-AR CALUX, in the presence or absence of different sources of S9.

The newly established method was then applied to benzo(a)pyrene (BaP) and flutamide. BaP and other polycyclic aromatic hydrocarbons are widespread environmental contaminants formed during incomplete combustion or pyrolysis of organic material with estrogenic and class 1 carcinogenic properties (IARC, 2012). Flutamide is a synthetic androgen receptor antagonist used in the treatment of prostate cancer. These two chemicals were considered to be good test candidates, since they are metabolized into forms that are more ERα- and AR active than the parent compounds (Fertuck et al., 2001; Shet et al., 1997; Tevell et al., 2006; van Lipzig et al., 2005).

Bisphenol A (BPA) was also studied for its estrogenic and anti-androgenic properties. BPA is a monomer of polycarbonate and is used in the manufacture of epoxys and plastics, including food contact materials. BPA is mainly metabolized by phase II enzymes to biologically inactive forms (EFSA Panel on Food Contact Materials, 2015; Teeguarden et al., 2015). Therefore, it was tested as a negative control of the activities of Phase I metabolites.

The commercially available pure active metabolites of methoxychlor and flutamide (HPTE and 2-hydroxyflutamide, respectively) were also tested in parallel to both compare the difference observed between the pure metabolite and the metabolite mixture generated by the S9, as well as to investigate whether the known active metabolite is itself a substrate for further metabolism.

2 Materials and methods

2.1 Materials

All test compounds were purchased from Sigma-Aldrich (Buchs, Switzerland). For further information, please refer to Table S1 (doi:10.14573/altex.1611021s). All test chemicals were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Buchs, Switzerland) at a fixed stock concentration between 25-100 mM, depending on the desired final concentration. These stock solutions were then diluted in DMSO to standard concentrations 200-times the final treatment concentration for each well. A vehicle control of DMSO was included in each concentration range. The DMSO stock solutions were diluted 1:200 into assay medium immediately prior to treatment.

S9 fractions were purchased commercially from Moltox (Aroclor 1254-induced rat S9 and Human S9 from 5 donor livers; Giessen, Germany) and Xenometrix (rat S9 induced with a mixture of β-naphtoflavone and phenobarbital (βNF/PB); Allschwil, Switzerland). S9 stock solutions contained 0.1 mg/ml S9, 3 units/ml glucose-6-phosphate dehydrogenase, 2 mM β-NADPH, 30 mM glucose-6-phosphate and 50 mM $MgCl_2$*$6H_2O$ (Sigma, Buchs, Switzerland) in assay medium.

2.2 CALUX cell culture

CALUX® cell lines, consisting of human U2OS osteosarcoma cells co-transfected with constructs of either a human androgen (AR) or estrogen receptor (ERα) and a luciferase reporter gene under the direct transcriptional control of repeated copies of the respective hormonal response element (Sonneveld et al., 2005),

were licensed from BioDetection Systems (Amsterdam, Netherlands). CALUX cells were cultured in 75 cm^2 cell culture flasks (VWR, Dietikon, Switzerland) at 37°C, 5% CO_2 and 100% humidity. The cells were routinely grown in cell culture medium containing DMEM/F12 (Dulbecco's Modified Eagle's Medium and Ham's F12) supplemented with non-essential amino acids (1%), fetal bovine serum (7.5%) (Life Technologies, Zug, Switzerland), 10 units/ml penicillin and 10 µg/ml streptomycin (Sigma-Aldrich, Buchs, Switzerland) with G418 (0.2 mg/ml) (Roche, Mannheim, Germany) as a selection antibiotic. Maintenance cultures were sub-cultured using trypsin (Sigma-Aldrich, Buchs, Switzerland) twice per week and re-suspended 1:3 to 1:8 in growth medium.

For seeding, cells were resuspended in assay medium instead of growth medium at subculture. The assay medium was identical to DMEM/F12 growth medium; except that the hormonally-active phenol red pH indicator, G418, and fetal bovine serum were removed and replaced with 5% charcoal stripped serum (Life Technologies, Zug, Switzerland) to ensure the culture medium was free of background hormonal activity.

2.3 CALUX reporter gene assay

CALUX cells were seeded into 96-well tissue culture treated polystyrene plates (VWR, Dietikon, Switzerland) at a density of 10,000 cells per well. Test plates were then cultured for 24 h at 37°C, 5% CO_2 and 100% humidity after which the medium was replaced with 200 µl assay medium containing test substance. 20 µl S9 stock solution was added to S9 treated wells for 24 h before luciferase activity determination. Each concentration was tested in triplicate. Hence the cells were exposed to a constant concentration of 0.5% DMSO and 0.01 mg/ml S9. Prior experience in our lab has indicated that this concentration of DMSO is neither cytotoxic to the cells nor does it interfere with the CALUX assay (data not shown).

Following exposure, the cells were observed under microscopy following exposure to check for any signs of test-substance induced cytotoxicity. The medium was removed and the membranes of the cells were disrupted by adding 30 µl of lysis buffer (BioDetection System, Amsterdam, Netherlands) (1% Triton X-100, 10% glycerol, 25 mM Tris buffer pH 7.8, 2 mM 1,4-dithiothreitol (DTT) and 2 mM 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA)) to each well and shaking the plate for 5 min at room temperature, releasing the luciferase. Luminescence was measured well-by-well in a Centro XS3 LB960 microplate luminometer (Berthold Technologies, Zug, Switzerland), 4 sec after the automatic injection of 100 µl of glow-mix (20 mM tricine buffer pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2$.5, 10 µM EDTA, 1.5 mM DTT, 539 µM D-luciferin and 5.49 mM ATP) (BioDetection System, Amsterdam, Netherlands). The light produced was recorded in relative light units (RLUs).

Agonist activity testing

In the agonistic version of the assay, substances were tested for their potential to induce hormone receptor-mediated transcription of the luciferase reporter gene, which was quantified luminometrically. Dihydrotestosterone (DHT) ($1x10^{-12}$ - $1x10^{-07}$ M) and 17β-estradiol (E2) ($1x10^{-13}$ - $1x10^{-09}$ M) were used as positive controls for the AR and ERα CALUX, respectively.

To determine the hormone receptor-mediated induction of the luciferase gene in the agonistic version of the assay, the mean luminescence background (RLUs) from cells exposed to the 0.5% DMSO vehicle alone or to vehicle + S9 was subtracted from all wells. The resulting background-subtracted data was then normalized such that saturating levels of the positive control were set to 100% ($1x10^{-09}$ M E2 and $1x10^{-07}$ M DHT, respectively for the ERα and AR CALUX). The concentrations were then log transformed using the software GraphPad Prism, version 6.04 (La Jolla, CA) and non-linear sigmoidal regression was performed using the command "log (agonist) vs response – variable slope (four parameters)" in the same software, which calculated the EC_{50} and maximum saturation level for each test compound.

Antagonist activity testing

In the antagonistic version of the assay, substances were tested for their potential to inhibit hormone receptor-mediated transcription of the luciferase reporter gene by a fixed quantity of agonist. Androgen receptor agonist concentrations were chosen based on experimentally derived EC_{50} in agonist mode: 0.3 nM for DHT and 1.5 nM stanozolol (STZ). The potential of each test substance to antagonize this agonist concentration was then quantified in the anti-AR CALUX relative to the reference compound flutamide ($1x10^{-09}$ - $3x10^{-05}$ M).

As in the agonist activity testing, the mean luminescence background (RLUs) from cells exposed to the 0.5% DMSO vehicle alone or to vehicle + S9 was subtracted from all wells. To determine the relative decrease of the hormone receptor-mediated induction of the luciferase gene, the measured luminescence in all plate-wells was normalized to the mean of the agonist-only positive control wells (0.3 nM DHT), which was set to 100% luciferase induction. All further data processing was performed as described in the agonist version of the assay except the command used for the sigmoidal regression was "log (inhibitor) vs. response – variable slope (4 parameters)".

3 Results

3.1 Method set up in ERα and Anti-AR CALUX

U2OS CALUX cells, transfected with ERα and a reporter gene, were exposed to MC in the presence of rat liver S9 induced with Aroclor 1254 or βNF/PB (Tab. 1). In both cases, co-treatment with induced rat S9 successfully increased ERα agonist potency as compared to MC alone. EC_{50} values in the presence of the induced rat S9 were about 40 times below that of MC alone. Similar experiments were performed with human S9; however, the activity of MC after incubation with S9 was similar to MC alone.

The reproducibility and robustness of this method was demonstrated by inter-laboratory testing in 3 different laboratories. Varying concentrations of MC were tested for estrogenic activity in the presence and absence of 0.01 mg/ml Arochlor 1254 or βNF/PB-induced S9. Each lab followed the ERα CALUX assay protocol with only minor inter-laboratory differences. The re-

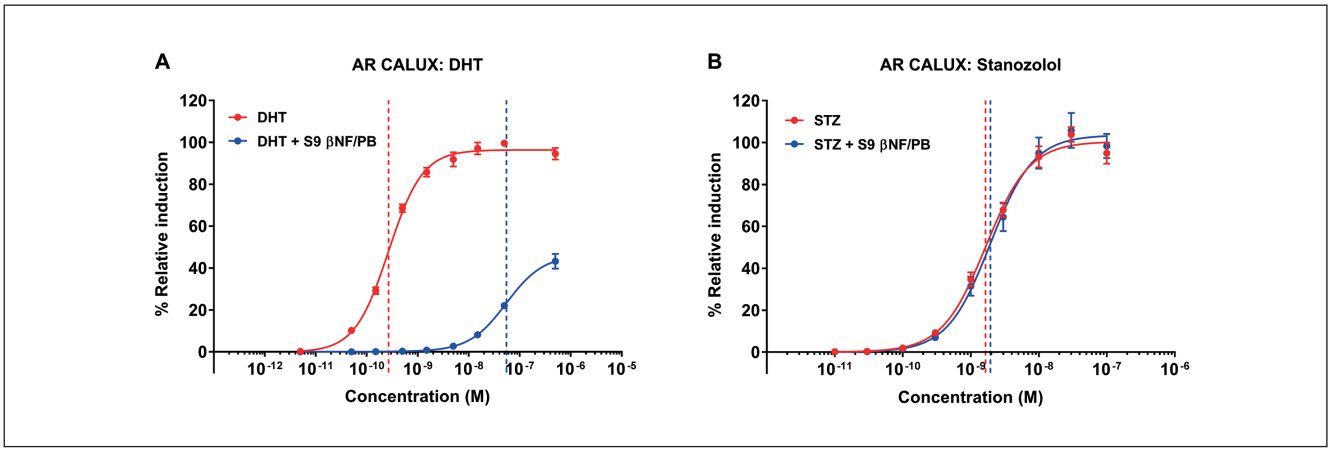

Fig. 1: Androgenic reporter activity of dihydrotestosterone (DHT, panel A) and stanozolol (STZ, panel B) in AR CALUX cells incubated in the presence or absence of βNF/PB-induced S9

This graph combines the results of 3 biologically-independent experiments made up of 3 technical replicates within each experiment. Results are expressed as a percentage of the maximal luciferase induction. Each point signifies the mean of means and the error bars represent the standard error of that mean of means (SEM).

Dashed line: EC_{50} of DHT and STZ (in red: without incubation with S9, in blue: after incubation with S9)

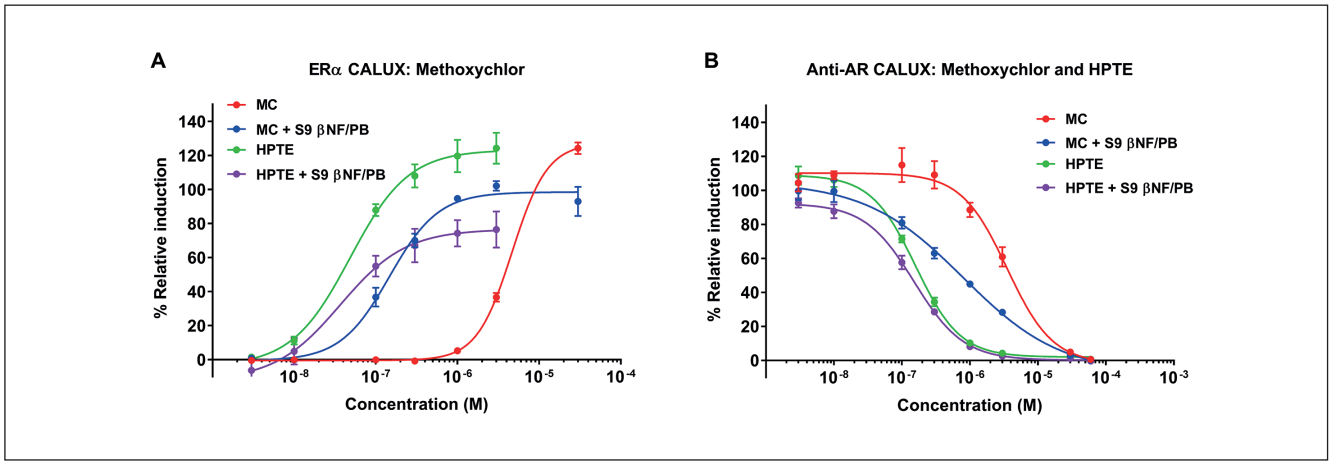

Fig. 2: Estrogenic (Panel A) and anti-androgenic (Panel B) activities of methoxychlor and its metabolite HPTE in the presence or absence of βNF/PB-induced S9

This graph combines the results of 3 biologically-independent experiments made up of 3 technical replicates within each experiment. Results are expressed as a percentage of the maximal luciferase induction. Each point signifies the mean of means and the error bars represent the standard error of that mean of means (SEM).

Tab. 1: Influence of different S9 treatments on the estrogenic activity of methoxychlor in ERα CALUX

	EC_{50}	95% confidence interval	Maximum induction**
E2	8.3 pM	7.4 – 9.2 pM	99.6% ± 0.6
MC - S9	4.6 µM	3.7 – 6.4 µM	115.3% ± 5.5
MC + human S9	3.03 µM	2.5 – 4.3 µM	66.1% ± 1.7
MC + S9 Aroclor 1254	0.11 µM	0.04 – 0.21 µM	89.9% ± 13.6
MC + S9 βNF/PB	0.15 µM	0.11 – 0.19 µM	102.1% ± 2.8

** Maximum induction ± standard error of the mean of means

Tab. 2: Inter-laboratory differences of the EC_{50} of methoxychlor in ERα CALUX
Each lab normalizes the measured raw RLU values slightly differently. Lab 3 always sets the 100% relative induction to the luciferase activity produced by 0.5 nM E2. Labs 1 and 2 establish 100% relative induction as the triplicate inducing the maximum relative light units (RLUs) on the plate. This value is similar to, but not exactly the same as, the RLU produced by 0.5 nM E2; thus the normalized data are comparable, but not exactly equivalent.

	Without S9			With S9 βNF/PB			With S9 Aroclor		
	EC_{50}	CI 95%*	Maximum induction	EC_{50}	CI 95%*	Maximum induction	EC_{50}	CI 95%*	Maximum induction
Lab 1	9.4 µM	7.6 – 11.6 µM	113%	0.25 µM	0.19 – 0.34 µM	136%	0.24 µM	0.17 – 0.29 µM	153%
Lab 2	4.8 µM	4.4 – 5.2 µM	154%	0.24 µM	0.19 – 0.30 µM	139%	0.25 µM	0.19 – 0.34 µM	157%
Lab 3	2.28 µM	2.1 – 2.5 µM	124%	0.18 µM	0.06 – 0.53 µM	93%	0.18 µM	0.11 – 0.29 µM	90%

*CI: confidence interval

Tab. 3: Influence of βNF/PB-induced S9 on the estrogenic or anti-androgenic activity of chemicals in the CALUX assays

	ERα CALUX					
	Without S9			With S9 βNF/PB		
	EC_{50} (PC10)	CI 95%*	Maximum induction**	EC_{50} (PC10)	CI 95%*	Maximum induction**
Methoxychlor	4.6 µM	3.7 – 6.4 µM	115.3% ± 5.5	0.15 µM	0.11 – 0.19 µM	102.1% ± 2.8
HPTE	0.05 µM	0.02 – 0.08 µM	124.2% ± 9.06	0.04 µM	Not calculated – 0.1 µM	76.4% ±10.6
Benzo(a)pyrene	7.4 µM (4.9 µM)	4.0 – 15 µM (2.7 – 6.8 µM)	30.2% ± 4.6	3.0 µM (1.0 µM)	2.1 – 3.9 µM (not calculated – 1.7 µM)	112.8% ± 15.5
Bisphenol A	1.6 µM	1.1 – 3.4 µM	155.6% ± 4.1	1.2 µM	0.7 – 2.7 µM	108.7% ± 4.9
	AR CALUX					
	Without S9			**With S9 βNF/PB**		
	EC_{50}	CI 95%*	Maximum induction **	EC_{50}	CI 95%*	Maximum induction **
Dihydrotestosterone	0.29 nM	0.23 – 0.31 nM	99.6% ± 0.4	54.6 nM	43.3 nM – 82.4 nM	43.2% ± 3.6
Stanozolol	1.65 nM	1.36 – 2.02 nM	103.9% ± 3.5	1.94 nM	1.47 – 2.58 nM	105.8% ± 8.3
	Anti-AR CALUX (agonist: STZ)					
	Without S9			**With S9 βNF/PB**		
	EC_{50}	CI 95%*		EC_{50}	CI 95%*	
Flutamide	0.59 µM	0.51 – 0.67 µM		0.053 µM	0.021 – 0.081 µM	
2 OH-flutamide	0.029 µM	0.027– 0.03 µM		0.024 µM	0.022 – 0.026 µM	
Methoxychlor	3.5 µM	2.6 – 5.8 µM		0.78 µM	0.54 – 1.18 µM	
HPTE	0.16 µM	0.14 – 0.18 µM		0.15 µM	0.13 – 0.18 µM	

*CI: confidence interval

** Maximum induction ± standard error of the mean of means

sults were both qualitatively and quantitatively similar between the laboratories (Tab. 2).

MC is also known to antagonize the androgen receptor (AR). The final protocol used for the ERα CALUX (i.e., 0.01 mg/ml βNF/PB induced rat S9 for 24 h exposure) was also incorporated into the anti-AR CALUX. In this version of the assay, cells are exposed to the test substance as well as an agonist ligand to see if the test sample is able to inhibit luciferase transcription. For an accurate interpretation of these results, it is critical that the activity of the agonist remains constant. Therefore, when incorporating the S9, it is important to ensure that the potency of the agonist is not affected by metabolic transformation (no shift in EC_{50}). Hence, preliminary tests were performed in agonist mode to test the susceptibility of DHT to metabolism by the βNF/PB-induced S9. As shown in Figure 1A, DHT potency was considerably diminished as a result of metabolism (Tab. 3). Consequently, another AR agonist is needed to study the role of S9 in the anti-AR CALUX.

The anabolic steroid STZ was tested with and without S9 for its agonistic potential in the AR CALUX assay (Fig. 1B). STZ is a potent AR agonist, with an EC_{50} only one log higher than DHT. This potency was unaffected (no shift of the EC_{50}) by co-incubation with S9 βNF/PB (Tab. 3). Therefore, STZ is a suitable agonist which can be used instead of DHT in tests of the anti-androgenic properties of a substance. The effects of MC biotransformation become apparent when using STZ as the AR agonist. Indeed, co-treatment with S9 βNF/PB generated metabolites with more potent AR antagonistic activities as compared to the MC parent (5-fold lower IC_{50}) (Fig. 2B).

Exposure to MC and HPTE in the presence or absence of S9 all resulted in estrogenic and anti-androgenic activities (Fig. 2A,B). HPTE was found to be more potent than its parent compound in both assays. Interestingly, co-incubation of MC with S9 resulted in a response similar to HPTE alone (EC_{50} in ERα CALUX and IC_{50} in anti-AR CALUX of MC with S9 4-times more than EC_{50} and IC_{50} of HPTE), suggesting that much MC was metabolized to HPTE by P450 cytochromes in the S9 (Tab. 3).

3.2 Application to other model chemicals

ERα CALUX tests of BaP alone demonstrated that it binds the ERα with weak potency and affinity (Fig. 3). Interestingly, the ERα-mediated transcriptional response changed dramatically after BaP metabolism (a 4-fold increase in luciferase induction). The potency was somewhat altered (a 5-fold decrease in PC10, the concentration that induces 10% of the maximum response, and a 2.5-fold decrease in EC_{50}), but this minor shift could be attributed to this increase in efficacy (Tab. 3).

As expected, the addition of S9 also increased the anti-androgenic potency of flutamide. Co-treatment with S9 βNF/PB generated metabolites with more potent AR antagonistic activities (Fig. 4) than the flutamide parent compound (11-fold lower IC_{50}) and the potency of pure 2-hydroxyflutamide metabolite tested approximately 2-times higher still. No difference in 2-hydroxyflutamide activity was observed with the addition of S9 (Tab. 3).

BPA was used as a negative control for S9 bioactivation in both the ERα and anti-AR CALUX assays. BPA is an ERα ag-

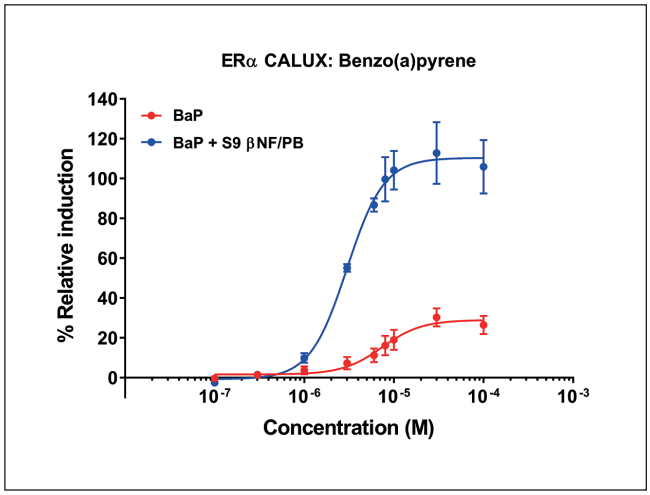

Fig. 3: Influence of the addition of βNF/PB-induced S9 on the potency and efficacy of the estrogenic activity of benzo(a) pyrene

This graph combines the results of 3 biologically-independent experiments made up of 3 technical replicates within each experiment. Results are expressed as a percentage of the maximal luciferase induction. Each point signifies the mean of means and the error bars represent the standard error of that mean of means (SEM).

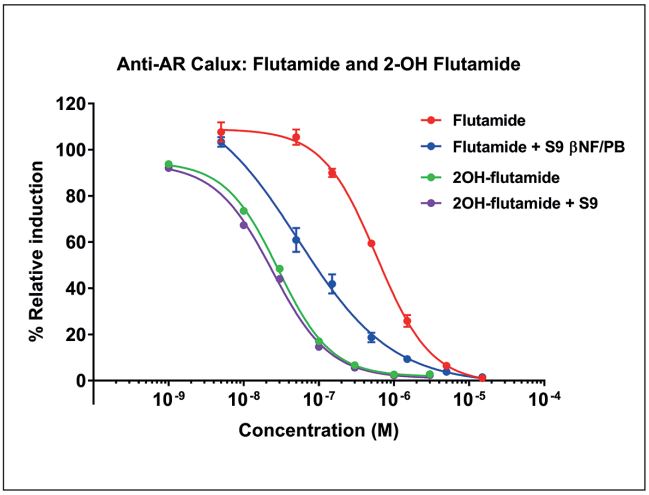

Fig. 4: Antagonistic activity of flutamide and its 2-OH metabolite on the androgen receptor in the presence or absence of βNF/PB-induced S9

This graph combines the results of 3 biologically-independent experiments made up of 3 technical replicates within each experiment. Results are expressed as a percentage of the maximal luciferase induction. Each point signifies the mean of means and the error bars represent the standard error of that mean of means (SEM).

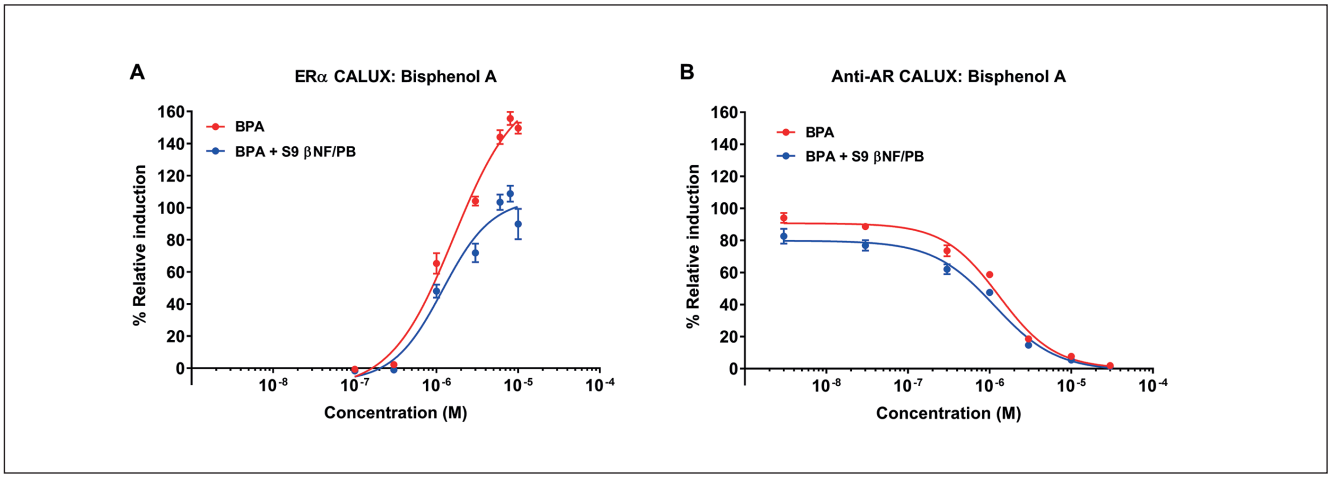

Fig. 5: Estrogenic (panel A) and anti-androgenic (panel B) activity of Bisphenol A in the presence or absence of βNF/PB-induced S9.

This graph combines the results of 3 biologically-independent experiments made up of 3 technical replicates within each experiment. Results are expressed as a percentage of the maximal luciferase induction. Each point signifies the mean of means and the error bars represent the standard error of that mean of means (SEM).

onist and AR antagonist (Fig. 5A,B and Tab. 3). As expected, both potency and efficacy of BPA remained the same after exposure to S9 βNF/PB.

4 Discussion

This study presents a methodology to supplement the CALUX assay for the detection of EAS with a metabolizing system that does not require any additional sample preparation steps. Liver S9 is a robust source of CYPs enabling the bioactivation of EAS. Its incorporation into this bioassay was valuable for the biodetection of substances which require phase I enzymatic biotransformation to express endocrine activity. Moreover, these results suggest that neglecting the role of metabolism in assays for EAS potentially results in the underestimation of the capability of a substance for endocrine activity.

A protocol using a low concentration of rat S9 (Aroclor 1254 or βNF/PB induced S9) during 24 h incubation was found to be suitable for the formation of active metabolites with a higher potency as demonstrated with MC. The magnitude of the shift in MC potency towards ERα was much greater after co-treatment with rat liver S9 than with human S9, suggesting that the CYP activity of the human S9 fraction was too low to significantly change the potency of MC. These results were confirmed by an inter-laboratory experiment. Although the donors were exposed to various chemicals throughout their lives, the different CYPs present in human S9 are not highly expressed when compared with S9 harvested from rats treated with either Aroclor 1254 or βNF/PB to induce hepatocellular CYP production (Parkinson et al., 2013). Since the interest in biodetection is in screening the greatest potential for metabolic activation of the endocrine response of unknowns, human S9 was considered to be inappropriate to identify metabolic activation of potential EAS. Based on the observations using MC, it appears that induced S9 from rat species is more suitable to screen whether endocrine activity of EAS is influenced by phase I metabolism.

Although no differences in MC bioactivation were observed between S9 fractions induced with different agents, they do differ somewhat in their CYP content. Aroclor 1254, which consists of a mixture of polychlorobiphenyls (PCBs), induces rat CYPs 1A1/2, 2B1/2 (roughly corresponding to 2B6 in humans) and 2C6 (2C8/9 in humans) (Ku et al., 2007). These CYPs are also induced by the combination of β-naphtoflavone and phenobarbital; however, phenobarbital is also a moderate inducer of 3A1/2 in rats (3A4/5 in humans) (Parkinson et al., 2013), a significant difference as compared to Arochlor 1254 induction. Even though experiments with MC did not show any difference in potency between the inducing agents, βNF/PB-induced S9 was preferred to Aroclor, since it contained the broadest possible induction spectrum of cytochromes P450. However, as not all CYPs are induced by βNF/PB, it is still theoretically possible that the metabolic activation of EAS, whose bioactivation requires specific CYPs not represented by this S9, may not be detected in this assay.

In the case of MC, the CYPs involved in its metabolism are well-identified. MC undergoes different metabolic steps, involving various CYPs: CYP1A2, 2A6, 2C8, 2C9, 2C19, and 2D6 exhibit mainly O demethylation, leading to the formation of the di-hydroxylated metabolite HPTE. CYP3A4, 3A5, and rat 2B1 catalyze primarily ortho-hydroxylation, leading to the final product tri-hydroxylated MC (Hu and Kupfer, 2002).

Bioactivation of MC also led to increased anti-androgenic activity in the anti-AR CALUX. In our experiments, the IC_{50} of MC after S9 treatment was one order of magnitude lower than for MC without S9. Gaido and colleagues identified the mono-

and di-hydroxylated (HPTE) metabolites of MC as agonists and antagonists of the ERα and AR, respectively, in HepG2 cells, with HPTE being the most potent (Gaido et al., 2000). The combined binding affinities of MC after S9 treatment did not appear to differ greatly from that of pure HPTE, suggesting that either HPTE was a principle component of the metabolite mixture, or that the other metabolites present had similar anti-androgenic and estrogenic activities, or both. This hypothesis would need to be confirmed by analytical studies.

As well as the S9 source (human/rodent species, inducing agent), another parameter to consider when incorporating S9 into the antagonist version of the CALUX assay is the choice of agonist. For proper interpretation of the data, it is important that the EC_{50} of the agonist should not be affected by the S9. In our own experiments, it appears that CYPs present in the S9 were able to inactivate DHT and consequently diminish its agonistic activity. Another AR agonist, STZ, was found to be a suitable replacement for DHT in the anti-AR CALUX assay, permitting the detection of the augmented anti-androgenic effects of MC or flutamide following metabolism. Thus, the observed shift in the anti-androgenic potency of MC with S9 co-treatment (detailed above) was observed using STZ as an agonist. Tevell and colleagues (2006) studied the metabolism of flutamide and identified extensive hydroxylation. Of these metabolites, the 2-hydroxy-metabolite has been reported to be among the most potent, certainly more so than its flutamide parent, as confirmed in our assay. Hence, it is likely that hydroxylation increases the anti-androgenic properties of a chemical, which could explain the increased antagonistic activity of flutamide after S9 biotransformation.

Bioactivation resulting from the addition of a metabolizing system is not always reflected by a change in relative potency. This appears to be the case with BaP. One explanation is that BaP is only a partial agonist of ERα. Partial agonists act by binding to the ligand binding domain of the receptor in a similar manner to that of full-agonists, however these agonists do not fully activate the receptor to promote gene transcription. *In vitro* studies demonstrated that hydroxylated metabolites of BaP were more effective in terms of estrogen mediated gene-expression than the parent compound BaP (Fertuck et al., 2001; Meerman et al., 2003; van Lipzig et al., 2005). In our experiment, the maximum response to BaP at ERα is only maximally 12% of that of the maximum response to E2. However, co-incubation of BaP and S9 resulted in a dramatic increase in the efficacy of the response to approximately 100% luciferase induction as compared to E2. It seems possible that the binding of the metabolites to the receptor have a greater capacity to change ERα conformation and activate it, resulting in much more estrogen response element-mediated DNA transcription, and therefore luciferase activity. These results suggest a transition from a partial to a full agonistic response at ERα as a result of bioactivation by S9.

Another hypothesis for explaining these results could be linked to the solubility of BaP and its metabolites. BaP is reported to be poorly water soluble (May et al., 1983). It seems likely that at the highest concentration tested in our assay, the limit of solubility for free BaP may have already been reached, resulting in a maximum response of only 12% induction. Phase I metabolism typically enhances solubility and the free-fraction of these metabolites is generally larger. It is possible that the maximum response of the metabolites is no longer limited by solubility and is therefore in the same range as for E2. Thus, changes in solubility as a result of metabolism may also explain the amplified estrogenic response of BaP after S9 addition.

Regardless of the exact reason, in the particular case of BaP, biotransformation was able to influence the efficacy of the transcriptional response, increasing luciferase synthesis, without dramatically changing the potency of the ligand, which is usually related to the strength of receptor-ligand binding. The exact mechanism underlying this alteration is still not clear and requires further investigation.

The addition of a phase I metabolizing system did not always generate metabolites which altered the activity of the parent compound, as demonstrated with BPA, used as a negative control in this study, and the 2-hydroxyflutamide anti-androgenic metabolite. Following oral absorption, BPA is reported to be rapidly metabolized (99%) to the biologically inactive BPA-glucuronide by polymorphic UDP-glucuronyltransferases (UGTs) in the gut wall and the liver (first-pass effect) before systemic circulation and urinary excretion (EFSA Panel on Food Contact Materials, 2015; Teeguarden et al., 2015). Similarly, 2-hydroxyflutamide is metabolized via phase II glucuronidation and thus is not a noteworthy substrate for the assay's phase I metabolic system (Tevell et al., 2006). While hepatic S9 does contain the enzymes needed for phase II glucuronidation, this metabolic system currently lacks the cofactors needed for UGT activity. Hence, S9 modification of BPA and 2-hydroxyflutamide activity was not expected to occur; this was reflected in both the ERα and anti-AR CALUX results.

It is also important to bear in mind that the major metabolites within a complex mixture may not necessarily be the ones responsible for the observed activity. Therefore, the major metabolites may not be represented as the major activities. Conversely, if the metabolites with greater activities than the parent compound are only present in the mixture in small quantities, then their greater activities may be "diluted" in the overall activity of the mixture, which may not substantially differ from the parent compound. Co-treatments of MC and flutamide with S9 resulted in a response similar to HPTE (98% and 81%, respectively for estrogenic and antiandrogenic activities) and 2-hydroxyflutamide (96% equivalent response). Since tests with the pure metabolites demonstrated that this activity was unlikely to be substantially altered by S9 addition, it seems logical that these substances could be principal components within the mixture of metabolites, but the veracity of this should be confirmed.

Enzyme kinetics is an important parameter to consider in the interpretation of these data. Within the exposure time of 24 h, many enzymatic reactions may occur, resulting in the generation of various metabolites with different properties at the nuclear receptor. While HPTE and 2-hydroxyflutamide seem, at least superficially, to be terminal and stable metabolites, other active metabolites of BPA, flutamide or MC may also exist in small quantities. This may be either because they are the final

products of minor pathways, or because these metabolites may be used as a substrate for other phase I reactions, resulting in their degradation. Thus, production of active metabolites may be transient due to enzyme kinetics. The importance of these "minor metabolites" should not be ignored when integrating an exogenous metabolizing system into endocrine bioassays. Moreover, this implies a need to exercise caution against over-interpreting the *in vitro* data to draw conclusions about the metabolites involved, especially without analytical identification of the specific metabolite species created.

Due to toxicokinetics, this *in vitro* model may not accurately reflect the *in vivo* situation during human exposure. The role of phase II conjugation reactions is not addressed by this method, nor was the S9 chosen to specifically model human metabolism. However, for the purposes of biodetection, the interest is in knowing if toxic metabolites may be created; it is important to promote the phase I bioactivation of EAS into metabolites with greater potential for endocrine activities. In such cases, the CALUX test system with S9 would be somewhat overly sensitive. This is desirable for biodetection, but does not necessarily produce data directly relevant for human risk assessment. Therefore, caution should be exercised when extrapolating these data to human-relevant situations.

The addition of a metabolic step will provide a better estimation of the potential hazard of EAS, providing stronger, reliable, more complete information to serve risk management. In this biodetection context, decision-making occurs based on the limited data set available and it is critical that these data do not underpredict human hazard. Hence, integrating metabolism in biodetection assays reinforces the decision-making and/or prioritization processes for chemicals of potential concern. In this context, the method provides a useful tool for rapidly assessing the potential role of metabolism in altering receptor agonism and antagonism by pure chemicals and mixtures. However, receptor-mediated modes of action are only some of many EAS activities that can be altered by metabolism. The addition of an exogenous metabolic system without sample preparation steps to remove the metabolizing system may also have wider applications, including assays for other endocrine disruption modes of action and other high-throughput toxicological screening methods.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgment

This work was supported in part by the Biotechnology-Based Ecologically Balanced Sustainable Industrial Consortium.

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