Ministry of Environment and Food of Denmark
Environmental Protection Agency

Advancing the application of alternative test methods in chemical risk assessment by incorporating metabolism into *in vitro* assays (In-Vita)

Pesticide Research
no. 189

September 2020

Publisher: The Danish Environmental Protection Agency

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ISBN: 978-87-7038-228-1

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Preface

The project “Advancing the application of alternative test methods in chemical risk assessment by incorporating metabolism into *in vitro* assays” (InVita) (MST 667-00190) was conducted at the National Food Institute at DTU in 2016-2018.

The overall aim of the project was to address the general uncertainty and lack of knowledge concerning the metabolic capacities of cell-based assays for endocrine disrupting effects, as well as assess general methods for incorporating metabolism into various *in vitro* assays. This report includes a description of the project and presents the results obtained from the work performed in the project.

Summary

Background

Recent years of research have meant that a larger number of chemicals have been identified as potential endocrine disrupters (EDCs), among them several pesticides. It is therefore likely that legislation and prospective legislative proposals may require pesticides and other chemicals to be tested for their hormone disruptive properties prior to approval.

There are a growing number of *in vitro* test guidelines to investigate hormone disruptive mechanisms. However, a significant limitation of many *in vitro* assays is their limited metabolism of test substances.

In vitro data can be used for prioritization of chemicals for further testing, and for performing tests that are more targeted for specific endpoints, as well as to move towards an ultimate replacement of *in vivo* tests with *in vitro.* However*,* currently the application and use of *in vitro* tests are hampered by insufficient knowledge on chemical metabolism, bioactivation, and bioavailability. In addition, it is important to understand the metabolic capacity of a given *in vitro* system so that the results can be better understood, and used scientifically as well as regulatory.

Objectives

In the InVita project we wanted to address the general lack of knowledge concerning the intrinsic metabolic capacity of the different cell lines used in the various *in vitro* assays for endocrine disruption, as well as evaluate methods for incorporating metabolism in different currently used *in vitro* assays.

Specifically we have evaluated the endogenous metabolic potential of the CHO and H295R cell lines, the cells used in two *in vitro* OEDC test guidelines for endocrine disruption, namely the TG458 for testing effect on androgen receptor (AR) activity and TG456, the H295R Steroidogenesis Assay, for testing effects on steroid hormone production.

Results and Conclusions

To evaluate the endogenous metabolic potential of the CHO and H295R cells we performed gene expression analyses on the two cell lines, following enzyme induction, to determine whether Phase I and Phase II metabolic genes were expressed. Following exposure to different enzyme inducers, in order to increase potential metabolic activity, we only saw an increased expression of genes for a few Phase I enzymes (CYP1A1 and CYP19A1) in the H295R cells, thus indicating a very limited metabolic potential of the H295R cells. We found no significant induction on any of the major Phase I or Phase II enzymes in the CHO cells, and therefor conclude that the CHO cells does not have any significant endogenous metabolic capacity.

In order to add a metabolic module to the AR and H295R *in vitro* assays we used pre-incubation of the five test compounds with rat liver S9 as an add-on to the cell-based *in vitro* assays. Overall, the application of rat S9 looks to be a promising method for the incorporation of metabolism in *in vitro* assays for endocrine disruption.

All in all, this project has contributed to the work currently going on internationally aiming at improving the predictivity and application of *in vitro* assays for endocrine disruption. Our conclusion that the application of S9 liver fractions is a promising method for incorporating metabolism in *in vitro* assays is supported by others in recently published studies, and we believe that with further optimizations and eventually validated protocols the inclusion of a metabolising system in *in vitro* testing will strengthen the use of *in vitro* tests in the risk assessment of chemicals.

1. Introduction

Future legislation will require that pesticides, as well as other chemicals, are tested for their endocrine disrupting capabilities.
Both *in vivo* and *in vitro* tests are presently being proposed for the testing of endocrine disrupting activity of different compounds. *In vitro* models are valuable tools for testing the endocrine disrupting abilities of compounds, but the animal tests are currently necessary because it is not possible to model the complex responses of a whole body to endocrine disrupters (EDCs), including biotransformation.

EDCs, including many pesticides, act mainly by interfering with natural hormones, for instance by binding to various hormone and/or nuclear receptors, and several *in vitro* assays exist for testing the ability of a compound to interfere with the activity of various hormone receptors, as well as with the synthesis of hormones. In addition suppression of prostaglandin (PGD2) synthesis has been suggested as another possible mode of action for EDCs, although it is currently unclear whether compounds that have a PGD2-mediated mode of action will induce adverse effects[1].
At present the application of *in vitro* models is hampered by insufficient knowledge on chemical metabolism, bio-activation, and bioavailability.
A step towards increasing the predictivity of *in vitro* assays is improved knowledge about the metabolic capacity of the various cell lines, as well as validated methods for incorporating metabolism into various *in vitro* assay protocols. Although some cell-based assays are expected to have some inherent capacity to metabolise xenobiotics, actual knowledge on the metabolic capacity of the individual cell lines is largely lacking. Thus, there is a great need to investigate this further in order to both interpret results and define whether or not a supplementary metabolising system should be added to the assay.
While internationally a growing body of *in vitro* test guidelines exists addressing endocrine disrupting mechanisms and modes of action, there are still few – if any - standardized methods to incorporate metabolic and toxicokinetic parameters into these tests.

According to an OECD report from 2008[2] suitable candidates of *in vitro* assays that could benefit from the incorporation of metabolic systems include the estrogen and androgen receptor-mediated transcriptional activation assays, as well as the H295R assay for steroidogenesis. For these assays OECD test guidelines exists, and thus, these tests are in common use in research laboratories throughout the world[3–6], and potential improvement of their predictivity by the incorporation of a metabolic system would be of interest for both academic researchers and the industry.

1.1 Metabolism of xenobiotics

The liver is usually the primary site of metabolism, although extra-hepatic tissues may also play a significant role. The metabolism of xenobiotics can be divided into two steps: Phase I - modification, and Phase II – conjugation[7,8] (Fig.1).

Phase I metabolism encompasses the biochemical reactions that introduce reactive and polar groups into xenobiotic compounds by oxidation, reduction, or hydrolytic reactions. One of the most common Phase I modifications is hydroxylation catalyzed by the many isoforms of the cytochrome P450 (CYP) family of enzymes[9].

Phase II metabolism often involves the further conjugation of the metabolite with polar molecules, such as sulphate, amino acids, glutathione or glucuronic acid, facilitated by various transferases, generating metabolites that are more soluble and thus easily eliminated[7,8].The elimination is the final step and is sometimes referred to as Phase III (Fig. 1).

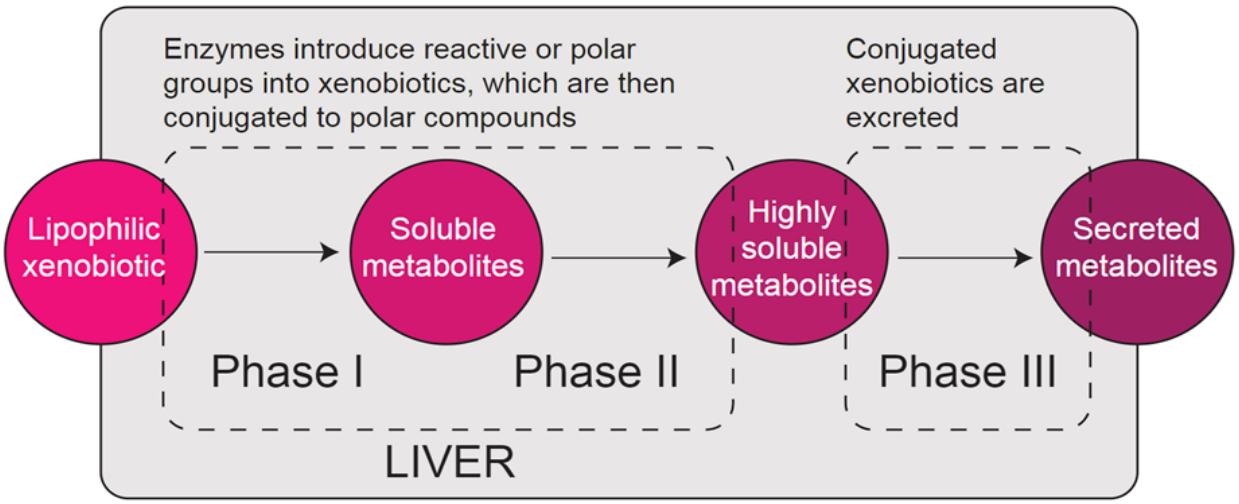

FIGURE 1: Metabolism of xenobiotics
Xenobiotic metabolism in the liver is divided into two steps: Phase I that includes reactions that introduce reactive and p
groups into
xenobiotic compounds by oxidation, reduction, or hydrolytic reactions. In subsequent Phase II reactions, the xenobiotic
tabolites are

Different metabolising systems are available for including or adding metabolism *in vitro*. They include both subcellular (e.g., enzyme homogenates, S9, cytosol, or microsomes) and cellular components (e.g., the residual metabolism of metabolically competent cells, such as cultured hepatocyte cell lines).

2. Aim

In this project the focus has been to address the general uncertainty and lack of knowledge concerning the metabolic capacities of cell-based assays for endocrine disrupting effects. The overall aim was to

- Evaluate the endogenous metabolic capacity of two *in vitro* assays: i.e. hAR and H295R assays, by evaluating the expression of various metabolic enzymes
- Evaluate methods for incorporating metabolism in currently used *in vitro* assays
- Examine the effects of incorporating a metabolising system based on rat liver enzymes (S9) in the hAR and H295R assays for 5 pesticides on selected endpoints
- Compare the metabolism of 5 pesticides using two different metabolising systems: rat liver S9 fractions and the human HepaRG® cell line

3. Methods

3.1 *In vitro* assays for testing endocrine disrupting endpoints

A key objective in this project was to evaluate the endogenous metabolic capacity of two commonly used *in vitro* assays for studying endocrine effects, namely the AR reporter gene assay (AR-Eco) and the H295R steroidogenesis assay.

In the AR-Eco assay, effect on AR activity is measured using Chinese hamster ovary (CHO) cells that have been stably transfected with the human AR. The stably transfected CHO-K1 cells have three inserted constructs: a human AR expression construct, a firefly luciferase reporter construct followed by a minimal heat shock protein promoter, and a Renilla luciferase reporter construct under the SV40 promoter. The Renilla luciferase reporter is constitutively and non-inducible expressed to distinguish pure antagonism from a decrease in luciferase activity due to cytotoxicity. Binding of a ligand to the AR will increase (agonist) or decrease (antagonist) the cellular expression of the luciferase reporter gene, resulting in a change in luminescence, which can then be quantitatively measured. The potential antagonistic activity was detected by measuring firefly luminescence using a LUMIstar® Galaxy luminometer. After measurement of the firefly luminescence, cytotoxicity was evaluated by measuring Renilla luminescence.

In the H295R assay, effects on steroid hormone production are studied using the H295R human adrenal cortico-carcinoma cell line. The H295R cell line is a steroid hormone producing cell line which expresses all enzymes in the steroid synthesis pathway. Thus in principle the assay can be used to assess effects on all steroid hormones, but in the OECD guideline (TG 456) it is only testosterone (T) and estradiol (E) that is included. In this project we evaluated the effects on testosterone, androstenedione and estradiol production. The levels of the hormones were measured in cell supernatant using the HPLC-MS/MS.

The rationale for selecting these assays included *a)* the high relevance for these endpoints when studying EDCs, *b)* these tests have both been adopted as OECD test guidelines and are extensively used, and *c)* our knowledge from previous studies and the literature showing that these targets are promiscuous and are affected by many pesticides or chemicals in general [10].

3.2 Characterization of the metabolic capacity of cell lines

To characterize the metabolic capacity of the CHO and H295R cell lines expression levels of key Phase I and II enzymes were evaluated, by performing gene expression analyses before and after enzyme induction to increase potential metabolic activity.

The cells of each of the two cell lines were exposed to two different concentrations of three induction compounds: Phenobarbital (a prototypical CYP2B inducer), 3-Methylcholanthrene (a prototypical CYP1A enzyme inducer), and Rifampicin (prototypical CYP3A and GST enzyme inducer) known to induce the expression of various CYP450 enzymes. The induction compounds and concentrations were selected based on literature on recommended protocols for assessing enzyme induction. The three compounds chosen are known to induce members of

the CYP-families (i.e., CYP1, CYP2, and CYP3) which in humans are responsible for the metabolism of the majority of drugs and other xenobiotics[11].
Following enzyme induction, gene expression analysis was performed on the samples from the enzyme induction experiments, looking at the expression level of a panel of Phase I and II enzymes, in order to evaluate the metabolic potential of the cells. In *Appendix I* is shown the list of the genes included in the initial gene array.
After the initial gene array, further RT-qPCR validation was performed on a selection of the differentially expressed genes.

3.3 Tested pesticides

In the project the following five pesticides were included: Prochloraz, Vinclozolin, Fludioxonil, Procymidone and Imazalil.
The rationale for the selection of these pesticides was based on their reported endocrine disrupting properties including androgen receptor binding, activation or inhibition of receptor activity, and/or effect on hormone production[10,12].

3.4 Methods for incorporating metabolism of test compounds

Another objective in this project was to evaluate the effects of incorporating a metabolising system based on liver enzymes (S9) in the two assays. Therefore, the selected pesticides were tested using a standard protocol for the respective assays, as well as with the incorporation of a metabolising step, where the pesticides were pre-incubated with rat liver S9 mix prior to standard testing in the assays.
The commercially available S9 fraction applied in the experiments was prepared from the livers of male Sprague Dawley rats that had been treated with a mixture of β-naphthoflavone and the peroxisome proliferator, phenobarbital, proven to causes induction of certain liver microsomal CYP enzymes. In addition a commercially available NADHP regenerating system was added to the S9 incubations to support reactions catalysed by cytochrome P450, flavin-containing monooxygenases and certain other enzymes.
For the S9 incubations a 100X (10mM) stock of each of the five pesticides was prepared in acetonitrile. The incubation reactions were performed in sterile glass test tube containing a final concentration of: 84 mM Phosphate buffer, 2.0 mg/mL rat S9, 100µM test compound, and 1 mM NADPH-RS (NADPH Regenerating System from Xeno Tech).
The glass test tubes were placed into a 37°C shaking water bath, and the samples were incubated for 60 min.
After incubation the reaction was stopped by adding 5x sample volume of cold methanol. The samples were centrifuged at 16,000g, 4°C, for 5 min. and the supernatants were transferred to clearly labeled HPLC vials and store at -20°C until they were analysed by HPLC.
As Phase II enzymes mainly results in decreased activity of the compounds, the primary focus in the current study was on Phase I metabolism and therefore the NADPH-RS (consisting of NADP(H), glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was the only exogenous cofactors added.

3.5 Comparison of two metabolising systems: liver S9 and the human HepaRG™ cell line

In relation to the conditional commitment for the final acceptance of the project it was requested that we included a study using the human cryopreserved HepaRG™ cell line. Thus as part of the project we wished to compare the metabolism of the selected pesticides using two different metabolising systems: liver S9 fractions and the human HepaRG™ cell line.
The human HepaRG cell line is originally derived from a liver tumor of a single female patient suffering from chronic hepatitis C infection and hepatocarcinoma. The HepaRG cells can undergo differentiation leading to hepatocyte-like cells, exhibiting a phenotype close to that of human hepatocytes [13]. Studies have shown that terminally differentiated HepaRG cells express high functional levels of most of the major xenobiotic metabolising cytochrome P450 enzymes[14].
Therefore, terminally differentiated HepaRG cells were applied to metabolise the five pesticides, and the metabolism was evaluated by measuring the remaining levels of parent pesticides (% recovery of parent compound) following incubation of the parent compounds with the HepaRG cells according to the protocol provided by the supplier of the HepaRG™ cells. These data were then compared with data obtained from the chemical analysis (HPLC MS-MS) of the pesticide samples from the S9-incubation experiments.

3.6 HPLC-MS/MS analysis

The chemical analysis was performed by LC-MS/MS. In brief the LC-MS/MS analysis were conducted on an 1100 series LC coupled to a MSD ion trap SL mass spectrometer using positive and negative electrospray ionisation depending on the analysed compounds. Analytes were semi quantified using external standard calibration. Semi quantification was based on the total sum of the dominant ions for each analyte.
In the current study analysis to get quantitative information on the metabolic fate (quantification of single metabolites) of the selected test compounds was not performed. The focus was to get qualitative data, to see if the test compounds could be metabolized by the different *in vitro* metabolising systems tested and to get information on the metabolic capability of the cells in the selected *in vitro* assays.

4. Results

4.1 The endogenous metabolic capacity of the CHO and H295R cell line

To characterize the metabolic capacity of the CHO and H295R cell lines, the cells used in the AR and H295R assay, respectively, gene expression analysis were performed on cells following enzyme induction with different compounds to increase metabolic activity.

No enzymes (neither Phase I nor II) were significantly induced in the CHO cells following enzyme induction with Phenobarbital (PB), Rifampicin (RIF) or 3-Methylcholanthrene (3-MC).
In the H295R cells CYP1A1 and CYP19A1 were significantly up-regulated (Fig. 2) following induction with 3-MC, a known inducer of specifically CYP1A1/2. None of the measured enzymes were found to be significantly affected following induction with PB or RIF.
Several Phase II enzymes were also found to be expressed, but none of the major Phase II enzyme groups like: the UDP-glucuronosyltransferases (UGTs), Glutathione S-transfrases (GSTs) or sulfotransferases (STs) were found to be significantly up-regulated.
Thus, overall the results from the H295R cells show that the cells could potentially have some Phase I metabolic capacity through the activity of CYP1A1and CYP19A1, although the overall metabolic activity looks to be very limited.

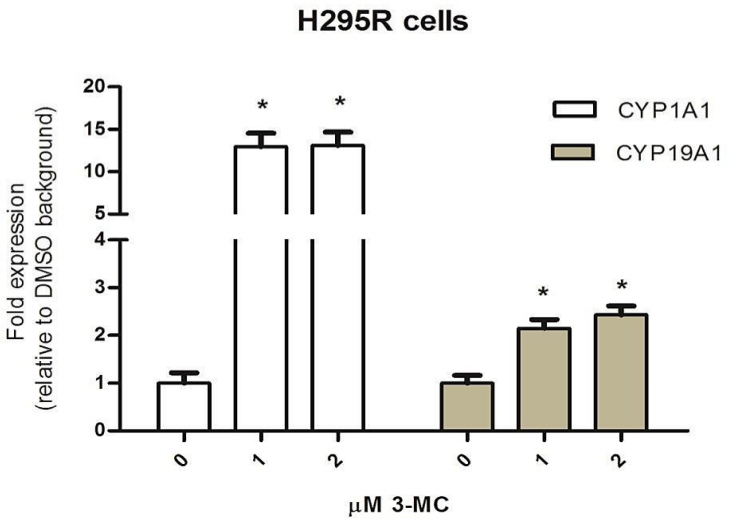

FIGURE. 2: Significantly expressed Phase I enzymes in the H295R cell line
Following enzyme induction with 3- Methylcholanthrene (3-MC), CYP1A1 and CYP19A1 were significantly up-regulated in the H295R cells. Data represent Mean ± SEM. *p>0.05 relative to non-induced DMSO controls

4.2 Metabolic activity of rat S9 fractions and human HepaRG™ cells

To measure the effect of the S9 fractions and the HepaRG cells on the metabolism of the selected pesticides, chemical analysis, in the form of high performance liquid chromatography—high resolution mass spectrometry (HPLC-HR/MS), was performed on samples of the pesticides after incubation with rat S9 or following incubation of the pesticides with the HepaRG hepatocyte-like cells.
In Table 1 and Table 2 is shown the recovery of the parent compounds (%) after incubation with rat S9 or the HepaRG cells, respectively.

TABLE 1. Metabolism of test compounds by use of rat S9-fraction

Parent compound	Recovery of parent compound (%)
Prochloraz	48
Imazalil	70
Fludioxonil	39
Vinclozolin	40
Procymidone	64

TABLE 2. Metabolism of test compounds by use of human HepaRG cells

Parent compound	Recovery of parent compound (%)
Prochloraz	98
Imazalil	91
Fludioxonil	75
Vinclozolin	91
Procymidone	97

The HPLC LC–MS analysis of the S9 extracts for the pesticides showed a decrease in the recovery of the parent compounds, suggesting that part of the parent compound had been metabolised by the S9, although to different degrees (Table 1). The compound showing the largest degree of biotransformation was fludioxonil with a recovery of the parent compound from the S9 extracts of 39% followed by vinclozolin with a recovery of 40% (Table 1).
When using the human HepaRG cells only very little metabolism had occurred. Overall, the recovery of the parent compounds following incubation with the HepaRG cells was between 91-98% (Table 2), except for fludioxonil, which as with the S9 was the compound showing the largest degree of metabolic transformation with a recovery of the parent compound of 75% (Table 2).

4.3 Effect of test compounds without and with S9 in the AR antagonist assay

The five pesticides were tested for AR antagonistic effects alone and following incubation with S9. As expected all five parent compounds showed AR antagonistic effects with varying potency (Fig. 3).

With the addition of S9 the AR antagonistic effects decrease dramatically (Fig.3), which is also reflected by the increase in the IC_{50} values for all five compounds, except procymidone (Table 3), suggesting that it is the parent compounds and not the metabolites that are the most potent AR antagonists. For procymidone a slight shift of the concentration-response curve to the left was seen following incubation with S9 (Fig. 3), accompanied by a small decrease in the IC_{50} value (Table 3). This could indicate the present of a more potent metabolite; however the shift in the concentration-response curve was only seen at concentrations below 0.2 µM. Thus the observed effect could also be due to the parent compound still being present to some degree (above 60% recovery of parent compound), as procymidone when tested alone is very potent and show significant AR antagonistic effect already at 0.05 µM (Fig. 3).

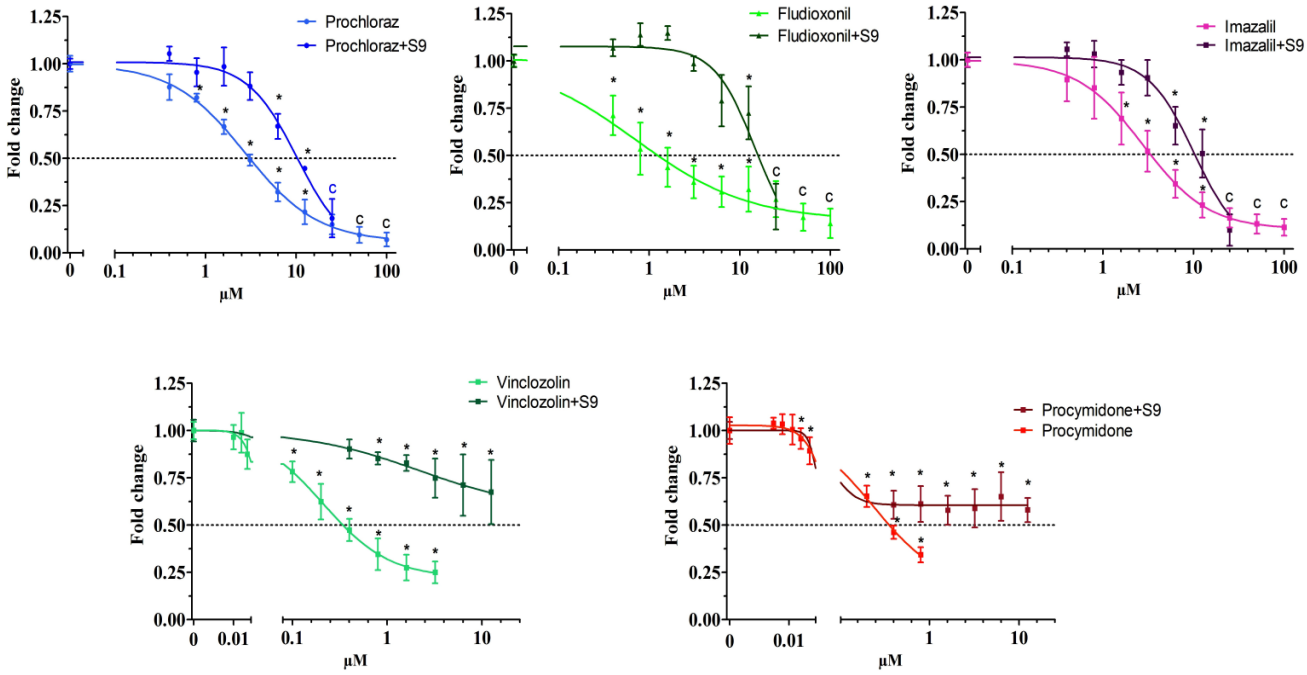

FIGURE. 3 AR antagonistic effects of test compounds with and without S9
The concentration-response curves shows the effect of the pesticides Prochloraz, Fludioxonil, Imazalil, Vinclozolin and Procymidone in the AR antagonist assay alone and following incubation with rat S9. Data represents Mean ± SD. * indicates statistically significant from the background control. C: cytotoxicity.

TABLE 3: The IC_{50} from the AR antagonist assay with and without S9

Parent Compound	IC_{50} -/+ S9
Prochloraz	2.7 µM / 10.2 µM
Fludioxonil	0.7 µM / 14.8 µM
Imazalil	2.8 µM / 10.2 µM
Vinclozolin	0.2 µM / 21.3 µM
Procymidone	0.2 µM/ 0.1 µM

4.4 Effect of test compounds alone and with S9 in the H295R steroidogenesis assay

As in the AR assay the five pesticides were tested alone and following incubation with S9 in order to evaluate their effects on steroid hormone production in the H295R steroidogenesis assay. Several pesticides have been found to affect steroid hormone production *in vitro* including several conazole fungicides like prochloraz [15], which is also included in the OECD test guideline as one of the quality control compounds[16]. However, very little, if anything, is known regarding the potential endocrine disrupting effect of the metabolites of many of these pesticides. In figure 4 is shown the effects on the levels of a selection of steroid hormones (testosterone, androstenedione and estradiol) for the five pesticides alone and following incubation with S9.

For prochloraz no difference in the effects between the compound tested alone or with S9 was seen (Fig. 4, top). For imazalil the overall effects (a decrease) on the hormone levels was the same with and without S9. For fludioxonil the significant decrease in testosterone and androstenedione seen for the parent compound was less pronounced following incubation with S9. In contrast the significant increase in estradiol seen without S9 was even more pronounced with S9 (Fig. 4). This more pronounced increase in estradiol following incubation with S9 was also seen for Procymidone and vinclozolin (Fig. 4.) However, the significant decrease in testosterone and androstenedione seen for fludioxonil alone was not seen for fludioxonil together with S9 (Fig. 4).

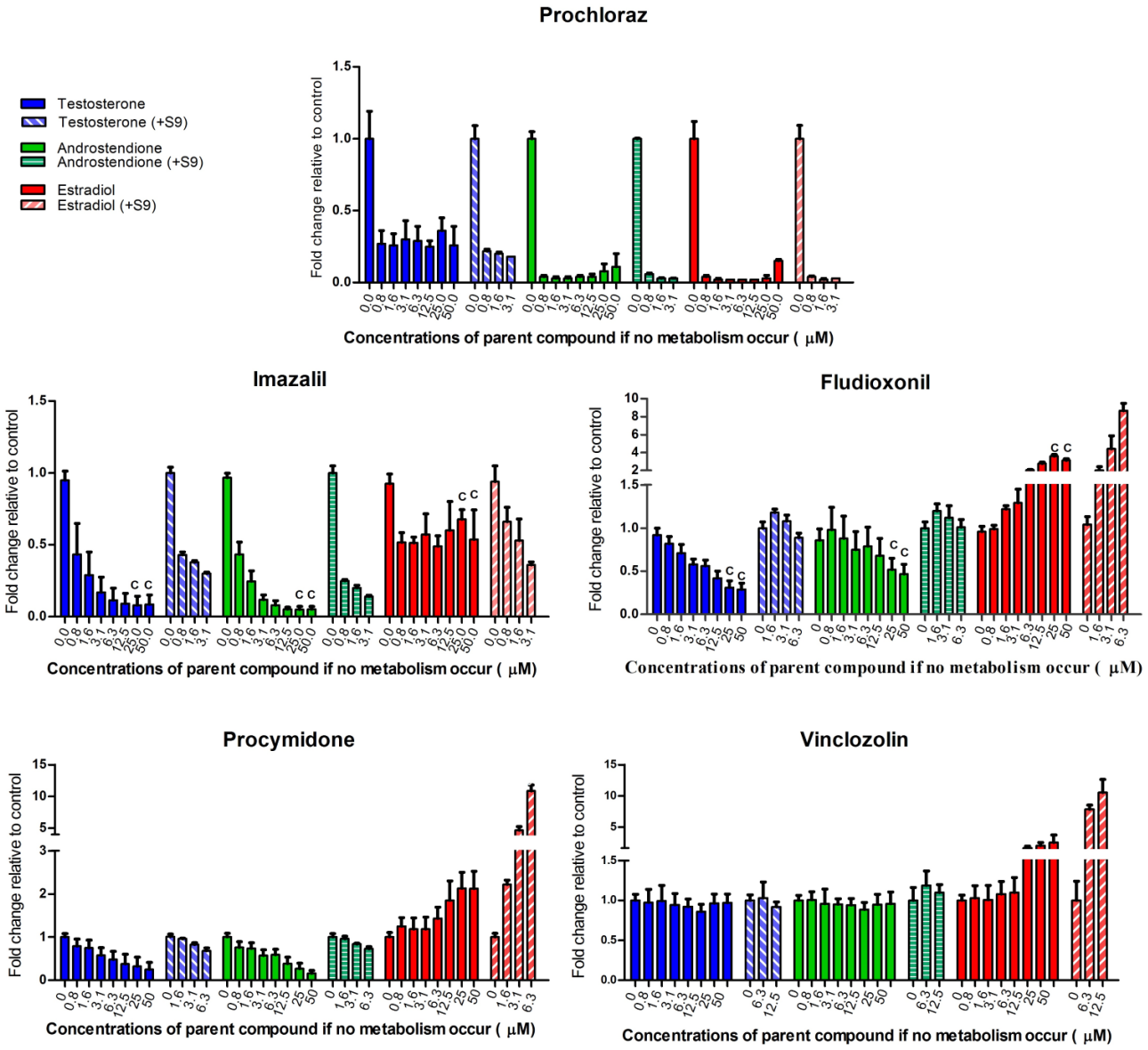

FIGURE. 4 Effects on steroid hormone production in the H295R steroidogenesis assay
The graphs show the effects of the five pesticides alone or following incubation with S9. The bars show the effect on the production of testosterone (blue), androstendione (green) and estradiol (red). Data represents Mean ± SD. C: cytotoxicity

Overall, for three out of the five pesticides a more marked increase in estradiol production was observed following incubation with S9. This could suggest that some of the metabolites of these compounds are able to affect estradiol production more potently than the parent compounds. This has to our knowledge not previously been reported, and it would be interesting to explore if these differences between the effects with and without S9 can be seen for other steroid hormones than estradiol.

5. Discussion

One of the most frequently cited limitations of *in vitro* assays concerns the qualitative and quantitative deficiencies in the metabolism of test chemicals, in comparison with *in vivo* metabolism. However, currently there is little information on the intrinsic metabolising potential of the cell systems traditionally used to test for endocrine disrupting effect. Thus, one of the objectives in this project (InVita) was to evaluate the endogenous metabolic capacity of the CHO and H295R cell lines. These cells are used in two commonly applied OECD test guidelines for endocrine disruption, namely TG458 for testing effect on AR activity and TG456, the H295R Steroidogenesis Assay, for testing effects on steroid hormone production.

The results of our studies showed that following enzyme induction to increase potential metabolic activity in the two cell lines, none of the major Phase I or II enzymes were significantly induced in the CHO cell line and only a few Phase I CYP enzymes were significantly induced in the H295R cells. In the H295R cells a significant increased expression of CYP1A1 and CYP19A1 was seen following induction with 3-MC. The most marked effect was that on CYP1A1 expression which was increased by around 12-fold compared to non-induced cells. However, the role or effect of CYP1A1 and/or other metabolic enzymes in the metabolism of xenobiotics in the H295R cells will of cause depend on how the individual compounds are metabolised, and the metabolic reactions will also depend on if the needed co-factors for the enzymatic reactions are present.

Of the different CYP enzymes, CYP3A4 is the most abundant in the human liver and play a key role in the metabolism of both steroids and xenobiotics, metabolising more than 50% of all currently prescribed drugs (Lin and Lu 2001). In the initial gene array analysis CYP3A4 was found to be expressed in the H295R cells following induction with Rifampicin a known inducer of CYP3A4. However, in the subsequent RT-PCR verification analysis no significant induction of CYP3A4 was found.

So, based on our results, both the CHO and the H295R cell assays could benefit from the incorporation of exogenous metabolism in order to improve the cell assays metabolic capabilities.

To date, there is little experience with incorporating metabolic and toxicokinetic aspects into *in vitro* tests for endocrine disruption, although work is currently ongoing concerning the incorporation of metabolism in various assays for testing EDCs.

This is a situation in sharp contrast to other areas of toxicity testing such as the genotoxicity area where *in vitro* tests are routinely conducted with and without metabolic capacity, like S9 liver fractions.

When using S9 liver fractions the S9 may be co-incubated with the cells and the chemical or the chemical or test compound may be pre-incubated with S9 and the extract applied to the assay. In general co-incubation is a time-saving approach, but it is recognised that there could be difficulties when adding exogenous metabolising fractions to a cell system, e.g. protein binding, as some chemicals can bind extensively to proteins in the enzyme fractions which can affect chemical availability[17] or the addition of S9 to the target cells can cause cytotoxicity.

In this project we pre-incubated the five tested pesticides with S9 and tested the metabolic extracts in the two *in vitro* assays. After incubation of the test compound with S9, the enzyme activity of the fraction is inactivated by the addition of methanol (following standard protocol), which is subsequently evaporated, and the metabolic extract is reconstituted in the respective assay media. By using this approach there is of cause the potential risk of losing relevant metabolites during the extraction and evaporation. In the case of the H295R steroidogenesis assay this approach, where the enzyme activity of the S9 fractions is removed before the extracts are applied to the assay, has a benefit. It is known that endogenous steroids are extensively metabolised by Phase I and II enzymes in both the liver and their target tissues[18]. If the S9 fraction were added directly to the H295R cells in the assay, the enzyme activity in the fraction could have a negative effect on the results of the assay, as it is the levels of the steroid hormones that is the endpoint, and the test compounds ability to either increase or decrease the production of the steroid hormones that is evaluated in the assay.

Also in relation to the AR antagonist assay, by inactivating the enzymes before adding the extracts to the assay we circumvent the risk of the agonist, which is used when running an antagonist assay, is metabolised or affected by the S9. If you add the S9 directly to antagonist assay, you should make sure that the S9 is not able to metabolise or otherwise inactivate your agonist and consequently diminish its agonistic activity.

The focus in this project was mostly on method evaluation and development and not the potential differences between the effects of the pesticides alone and with S9. However, we did get some interesting results, particularly in the H295R assay where we for three of the five pesticides saw a more marked increase in estradiol production for the metabolic extracts than for the parent compounds. This is to our knowledge new results that will be interesting to exploring further. In the AR assay we generally saw a decrease in the AR antagonistic effects with the addition of S9. It was somewhat unexpected that an increase in the AR antagonistic response was not seen for vinclozolin following incubation with S9. Several studies have reported that not only vinclozolin, but also the two main metabolites referred to as M1 and M2 exhibit AR antagonistic activity *in vitro.* Metabolite M2 has been reported to be the most potent antagonist followed by vinclozolin itself and the metabolite M1 [19,20]. It is known that vinclozolin is unstable in methanolic, ethanolic, as well as in aqueous solutions. In aqueous media vinclozolin is in a reversible equilibrium state with the metabolite 1 (M1).

In a study by Bursztyka et al., 2008 it was reported that after 2 hours incubation of vinclozolin in the cell culture medium, vinclozolin is almost totally hydrolyzed into about 20% M2 while around 80% is converted into M1[21].

In a study by Szeto et al. 1989, on the hydrolysis of vinclozolin in aqueous buffers the authors demonstrated that the rate of hydrolysis was proportional to pH and also increases with temperature[22]. So, even though the conversion of vinclozolin to M1 is known to be reversible, the formation of vinclozolin from M1 by re-cyclization only occurs at a significant extent at acidic pH (Szeto et al. 19891). Taking into consideration the conditions at which most cell assays are undertaken, it is likely that only very small amounts of M1, if any, is converted back to vinclozolin.

Taken this information into consideration it can be hypothesized that when we test vinclozolin alone (as parent compound) without S9 in our cell assays, the majority of vinclozolin is likely present in the form of it major metabolites M1 and M2.

An explanation for why the AR antagonistic effect in the AR assay, meaning the concentration-response curves are not more alike with and without S9, could be that without S9 there might be more of the more potent M2 present compared to what is formed following incubation with S9. Additionally, it could also be that with the addition of rat S9 and the co-factors used, vinclozolin is metabolised in a greater extent to less active metabolites than M1 and M2. In rats it has been demonstrated that M1 and M2 are not the end products of the biotransformation of vinclozolin. In rats vinclozolin and M2 are quickly biotransformed by dihydroxylation of the vinyl group and by further conjugation to glucuronic acid, and the activity of these metabolites toward the AR remains to be established[21].

As an additional objective in the InVita project we compared the metabolism of the selected pesticides using two different metabolising systems: liver S9 fractions and the human HepaRG™ cells.
When fully, differentiated HepaRG cells are seeded at high density they contain about 50% of hepatocyte-like cells shortly after plating and later during culturing[23]. According to the suppliers of the HepaRG cell line, terminally differentiated HepaRG cells exhibit many characteristics of primary human hepatocytes including morphology and expression of key metabolic enzymes, specifically CYP2C9 but also CYP3A4, CYP1A2, CYP2E1, and CYP2B6, the levels being dependent on the duration of confluency and culturing.
The results of our metabolism experiment using the terminally differentiated HepaRG cells showed that only very little metabolism had occurred, and in our hands using the rat S9 is a more effective *in vitro* metabolising system compared to the HepaRG cells.

Since terminally differentiated HepaRG cells do not divide, they cannot be propagated in culture.
Because the experiments with the HepaRG cells were not initially included in the project plan, as previously mentioned, we did not have in-depth knowledge concerning these cells, their specific culturing and related recruitments before the actual planning and execution of the experiments. Specifically the fact that the cells cannot be propagated in culture was unexpected and resulted in an unforeseen extra economical expense for the project as more cells needed to be bought than originally planned. A single metabolism experiment with all five test compounds costs close to 20.000 DKK.
Part of the aim of the InVita project was to evaluate and find a method that can be used as a kind of a standard method and that have a broad applicability across several *in vitro* assays. Because of the high cost for conducting these experiments we don't see the application of the HepaRG cells as a method appropriate for a broad and general use for performing *in vitro* metabolism. Based on this we also decided not to repeat the experiment with the HepaRG cells.
We conducted the experiment in full accordance with the protocol and recommendations of the supplier. However, the very low metabolism we found with the HepaRG cells was unexpected, and we can of cause not rule out that we might have gotten better results if we had repeated the experiments.
Our results from the S9 experiments showed a recovery of the parent compounds following incubation with S9 of 39-70 %. At present there are several publications where they have used S9 as a metabolising system in connection with *in vitro* testing of various chemicals. However, we have not been able to find any publications where they show how much of the parent compound is left following treatment with S9. Thus, currently we cannot say anything about if our

results are within the range of what can be expected for biotransformation using rat S9. The level of biotransformation will of cause also be very compound specific, as the enzymes (Phase I and II) involved in the primary metabolism of a compound can differ from compound to compound, as well as the overall stability of the parent compound itself under different conditions.

Finally, an option in order to take in to consideration the potential lack of metabolism in cell-based assays for endocrine disruption, and thus the possible endocrine disruption effects of the metabolites, could be to test pure (synthesized) metabolites directly in the assays.

This could be conceders in the cases where the various metabolites of a compound is known and thus can be synthesized or bought of a high purity. However, if information on the metabolic products is lacking this approach cannot be used. Also if the metabolites are not commercially available getting them synthesized by requests can in some cases be quite expressive. An advantage with using a so called an-on metabolic system like S9 is that the metabolism facilitated by the S9 if likely to be representative of the *in vivo* metabolism, meaning that the combination of several metabolites simultaneously and the ratios between the various metabolic products in the extracts could be more representative of the *in vivo* situation.

6. Conclusions

The major objectives of the InVita project were *a)* to assess the endogenous metabolic potential of the CHO and H295R cell line, the cells used in two OECD test guidelines for endocrine disruption, and *b)* to evaluate methods for incorporating a metabolising system in *in vitro* assays for endocrine disruption.

Based on the results obtained in this project we conclude the following:

- The H295R cell line have limited metabolic potential, although based on our results the cells could have a some Phase I metabolic capacity through the activity of CYP1A1 and CYP19A1.
- The CHO cells do not have any significant endogenous metabolic capacity.
- The application of rat S9 fractions looks to be a promising method for the incorporation of metabolism in *in vitro* assays for endocrine disruption.
- Overall, for the five pesticides tested in the project the addition of S9 lead to a decrease in the AR antagonistic effects.
- For three (fludioxonil, procymidone, and vinclozolin) out of the five pesticides a more marked increase in estradiol production was observed following incubation with S9.

7. References

1 S. Kugathas, K. Audouze, S. Ermler, F. Orton, E. Rosivatz, M. Scholze and A. Kortenkamp, *Env. Heal. Perspect*, 2016, **124**, 452–459.

2 OECD, *Detailed Review Paper On The Use Of Metabolising Systems For In vitro Testing Of Endocrine Disruptors.*, Organisation for Economic Co-operation and Development, OECD, 2008.

3 OECD, *Test No. 456: H295R Steroidogenesis Assay, OECD Guidelines for the Testing of Chemicals, Section 4,* , OECD Publishing, 2011.

4 OECD, *Guidance Document on Standardised Test Guidelines For Evaluating Chemicals For Endocrine Disruption*, 2012.

5 OECD, *Test No. 457: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists*, OECD Publishing, 2012.

6 O. (2012), *Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists, OECD Guidelines for the Testing of Chemicals, Section 4,* , OECD Publishing, 2012.

7 R. D. Combes, in *Progress in Drug Metabolism* , ed. G. G. Gibson, Taylor and Francis Limited, London, 1992, vol. 13, pp. 295–321.

8 S. Coecke, H. Ahr, B. J. Blaauboer, S. Bremer, S. Casati, J. Castell, R. Combes, R. Corvi, C. L. Crespi, M. L. Cunningham, G. Elaut, B. Eletti, A. Freidig, A. Gennari, J. F. Ghersi-Egea, A. Guillouzo, T. Hartung, P. Hoet, M. Ingelman-Sundberg, S. Munn, W. Janssens, B. Ladstetter, D. Leahy, A. Long, A. Meneguz, M. Monshouwer, S. Morath, F. Nagelkerke, O. Pelkonen, J. Ponti, P. Prieto, L. Richert, E. Sabbioni, B. Schaack, W. Steiling, E. Testai, J. A. Vericat and A. Worth, *Altern Lab Anim*, 2006, **34**, 49–84.

9 D. Werck-Reichhart and R. Feyereisen, *Genome Biol*, 2000, **1**, reviews3003.1.

10 W. Mnif, A. I. H. Hassine, A. Bouaziz, A. Bartegi, O. Thomas and B. Roig, *Int. J. Environ. Res. Public Health*, 2011, **8**, 2265–2303.

11 B. S. P. Thomas H. Rushmore and B. S. P. A.-N. Tony Kong, *Curr. Drug Metab.*, 2002, **3**, 481–490.

12 F. Orton, I. Lutz, W. Kloas and E. J. Routledge, *Env. Sci Technol*, 2009, **43**, 2144–2150.

13 M.-J. Marion, O. Hantz and D. Durantel, in *Hepatocytes: Methods and Protocols*, ed. P. Maurel, Humana Press, Totowa, NJ, 2010, pp. 261–272.

14 T. B. Andersson, K. P. Kanebratt and J. G. Kenna, *Expert Opin. Drug Metab. Toxicol.*, 2012, **8**, 909–920.

15 M. B. Kjærstad, C. Taxvig, C. Nellemann, A. M. Vinggaard and H. R. Andersen, *Reprod. Toxicol.*, 2010, **30**, 573–582.

16 M. Hecker, H. Hollert, R. Cooper, A. Vinggaard, Y. Akahori, M. Murphy, C. Nellemann, E. Higley, J. Newsted, J. Laskey, A. Buckalew, S. Grund, S. Maletz, J. Giesy and G. Timm, *Environ. Sci. Pollut. Res.*, 2011, **18**, 503–515.

17 M. B. Heringa, R. H. M. M. Schreurs, F. Busser, P. T. van der Saag, B. van der Burg and J. L. M. Hermens, *Environ. Sci. Technol.*, 2004, **38**, 6263–70.

18 T. Steimer, *Steroid hormone metabolism*, Geneva Foundation for Medical Research, 2003.

19 J. M. Molina-Molina, A. Hillenweck, I. Jouanin, D. Zalko, J. P. Cravedi, M. F. Fernández, A. Pillon, J. C. Nicolas, N. Olea and P. Balaguer, *Toxicol. Appl.*

Pharmacol., 2006, **216**, 44–54.

20 C. Wong, W. R. Kelce, M. Sar and E. M. Wilson, *J. Biol. Chem.*, 1995, **270**, 19998–20003.

21 J. Bursztyka, L. Debrauwer, E. Perdu, I. Jouanin, J.-P. Jaeg and J.-P. Cravedi, *J. Agric. Food Chem.*, 2008, **56**, 4832–4839.

22 S. Y. Szeto, N. E. Burlinson, J. E. Rahe and P. C. Oloffs, *Kinetics of hydrolysis of the dicarboximide fungicide vinclozolin*, 1989, vol. 37.

23 M. Szabo, Z. Veres, Z. Baranyai, F. Jakab and K. Jemnitz, *PLoS One*, 2013, **8**, e59432.

Appendix I

List of genes included in the "Human Drug Metabolism RT² Profiler PCR Array":

Drug Transporters

Metallothioneins: MT2A, MT3.

P-Glycoprotein Family Members: ABCB1, ABCC1, GPI.

Phase I Metabolizing Enzymes

CYP11B2, CYP17A1, CYP19A1, CYP1A1, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP3A4, CYP3A5.

Phase II Metabolizing Enzymes

Carboxylesterases: CES1, CES2, CES3.

Decarboxylases: GAD1, GAD2.

Dehydrogenases: ADH1B, ADH1C, ADH4, ADH5, ADH6, ALAD, ALDH1A1, HSD17B1, HSD17B2, HSD17B3.

Glutathione Peroxidases: GPX1, GPX2, GPX3, GPX4, GPX5, GSTA3, GSTA4, GSTM2, GSTM3, GSTM5, GSTP1, GSTT1, GSTZ1, LPO, MPO.

Lipoxygenases: ALOX12, ALOX15, ALOX5, APOE.

Hydrolases: ASNA1, EPHX1, FAAH, FBP1.

Kinases: HK2, PKLR, PKM.

Oxidoreductases: AOC1, BLVRA, BLVRB, CYB5R3, GPX1, GPX2, GSR, MTHFR, NOS3, NQO1, SRD5A1, SRD5A2.

Paraoxonases: PON1, PON2, PON3.

Glutathione S-Transferases: GSTA3, GSTA4, GSTM2, GSTM3, GSTM5, GSTP1, GSTT1, MGST1, MGST2, MGST3.

Other Phase II Metabolizing Enzymes: CHST1, NAT1, NAT2, COMT.

Other Drug Metabolism Genes

AHR, ARNT, GCKR, SNN.

Advancing the application of alternative test methods in chemical risk assessment by incorporating metabolism into in vitro assays (InVita)

A significant limitation of many in vitro assays is their limited metabolism of test substances.

The aim of this project was to address the general lack of knowledge concerning the intrinsic metabolic capacity of the different cell lines used in the various in vitro assays for endocrine disruption, as well as evaluate methods for incorporating metabolism in different currently used in vitro assays.

Specifically we have evaluated the endogenous metabolic potential of the CHO-K1 and H295R cell lines, the cells used in two in vitro OEDC test guidelines (TG458 and TG456) for endocrine disruption.

Following enzyme induction, in order to increase potential metabolic activity, we only saw an increased expression of genes for a few Phase I enzymes in the H295R cells. No significant induction of any of the major Phase I or Phase II enzymes were seen in the CHO-K1 cells.

In order to add a metabolic module to the AR and H295R in vitro assays, pre-incubation of test compounds with rat liver S9 was used as an add-on to the cell-based in vitro assays.

Our conclusion that the application of S9 liver fractions is a promising method for incorporating metabolism in in vitro assays is supported by others in recently published studies, and we believe that with further optimizations and eventually validated protocols the inclusion of a metabolising system in in vitro testing will strengthen the use of in vitro tests in the risk assessment of chemicals.

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