Is there an added value to the incorporation of a metabolizing system in biodetection assays for endocrine active substances?

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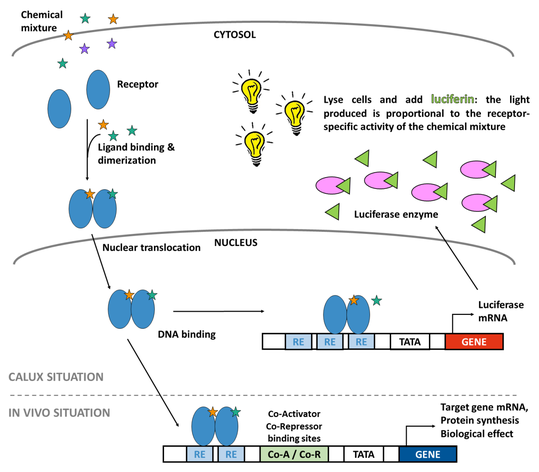
INTRODUCTION:

The use of *in vitro* assays is important for the detection of endocrine active substances (EAS). However, such assessments often fail to take into account the role of biotransformation on the activity of tested substances. To address the potential role of metabolism in the endocrine activity of EAS, a method has been developed incorporating an S9 metabolic system into the CALUX-reporter gene assays (Chemically Activated LUciferase gene eXpression) for estrogen receptor α- and androgen receptor-mediated activities.



Simplified scheme of the CALUX bioassay

METHODOLOGY:

In the agonist version of the assay, cells are exposed to varying concentrations of receptor agonist for 24h in the presence or absence of 0.01 mg/ml Aroclor 1254 or βNF/PB-induced rat liver S9 fraction containing cofactors for the cytochromes P450 (phase I enzymes). These cells dose-dependently express luciferase whose activity is quantified luminometrically. Results are expressed as a percentage of the maximal luciferase activity obtained with the reference agonist standard. In antagonist mode, cells are similarly treated with a suspected antagonist, except that this treatment occurs in the presence of a fixed concentration of agonist standard at a concentration near its EC50.

RESULTS

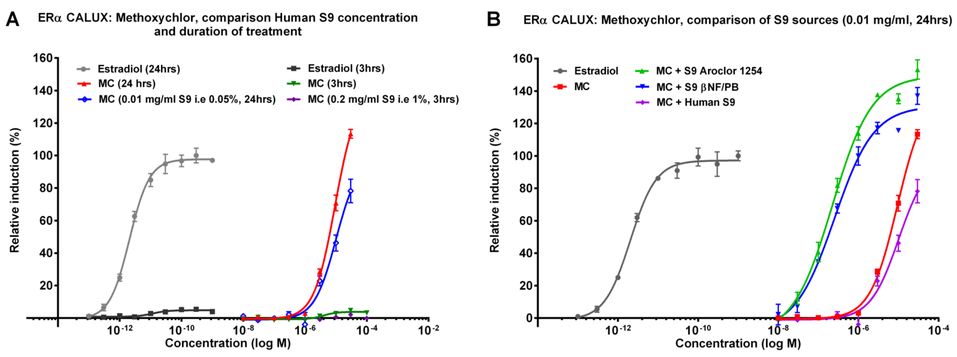


Figure 1. Influence of different treatments of S9 on the estrogenic activity of methoxychlor (MC) in the ERα CALUX assay. As observed with both MC and estradiol, a 3 h exposure time was not long enough to induce an estrogenic response, regardless of S9 treatment (Panel A). Over a 24 h exposure time, a slightly lower concentration of human S9 was unable to alter estrogenic activity (higher concentrations of S9 are cytotoxic at 24 h). In contrast, Aroclor 1254- and βNF/PB-induced rat liver S9 substantially enhanced the estrogenic activity of methoxychlor (Panel B).

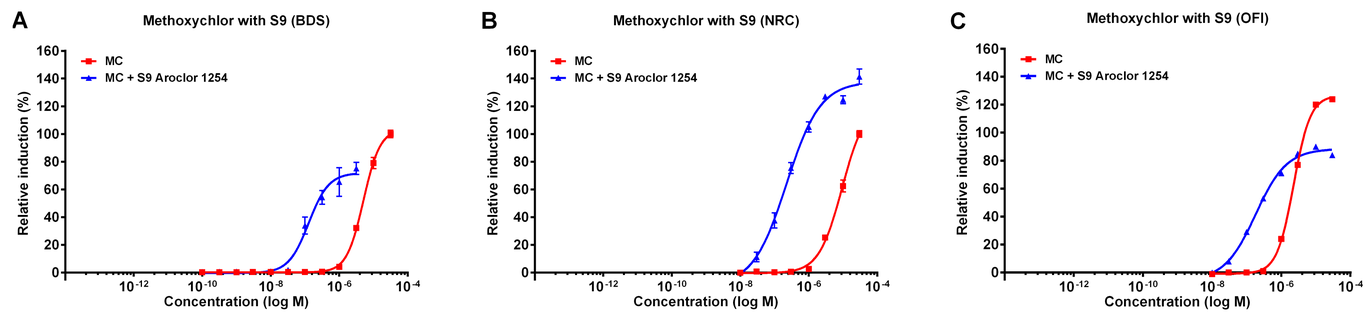


Figure 2. Inter-laboratory differences. Varying concentrations of methoxychlor were tested by 3 different laboratories for estrogenic activity in the presence and absence of 0.01 mg/ml Arochlor 1254-induced S9. Each lab followed the same ERα CALUX assay protocol, but used their own reagents and equipment. Results were similar between the labs, demonstrating the reproducibility of the metabolic method. EC50s from BDS experiment (Panel A): MC = 5.08×10^{-6}, MC+S9 = 1.28×10^{-7}; EC50s from Nestlé experiment (Panel B): MC = 9.38×10^{-6}, MC+S9 = 2.42×10^{-7}. EC50s from OFI experiment (Panel C): MC = 2.28×10^{-6}, MC+S9 = 2.13×10^{-7}

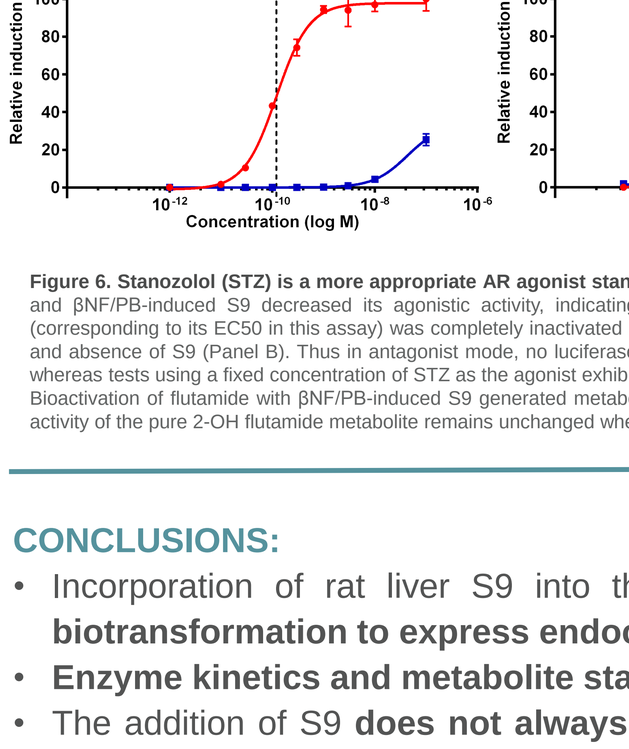


Figure 3. The estrogen receptor agonist activity of methoxychlor is enhanced by biotransformation. Exposure to MC-alone resulted in a dose-dependent ERα agonist activity, the potency of which increased 38-fold when co-incubated with βNF/PB-induced S9. A pure sample of a phase I metabolite of MC, HPTE, was also a more potent ERα agonist than methoxychlor.

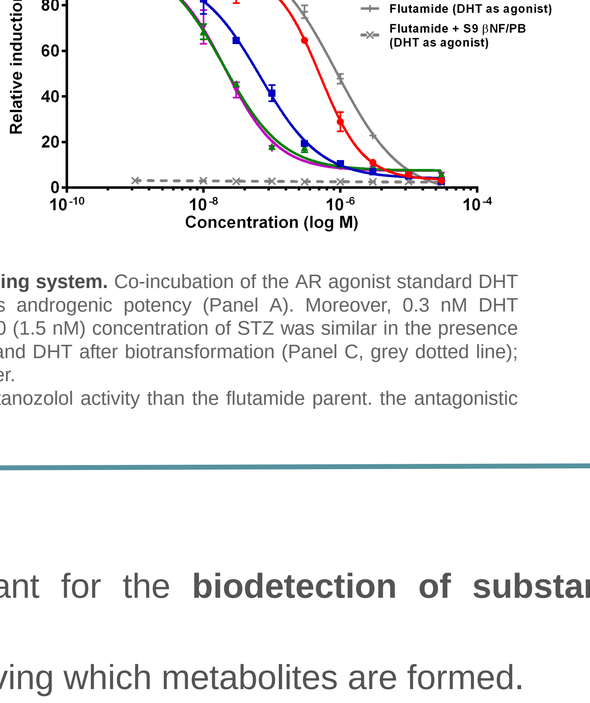


Figure 4. Potency versus efficacy: biotransformation of benzo(a)pyrene markedly alters the efficacy, much more than the potency of the response in the ERα CALUX assay.

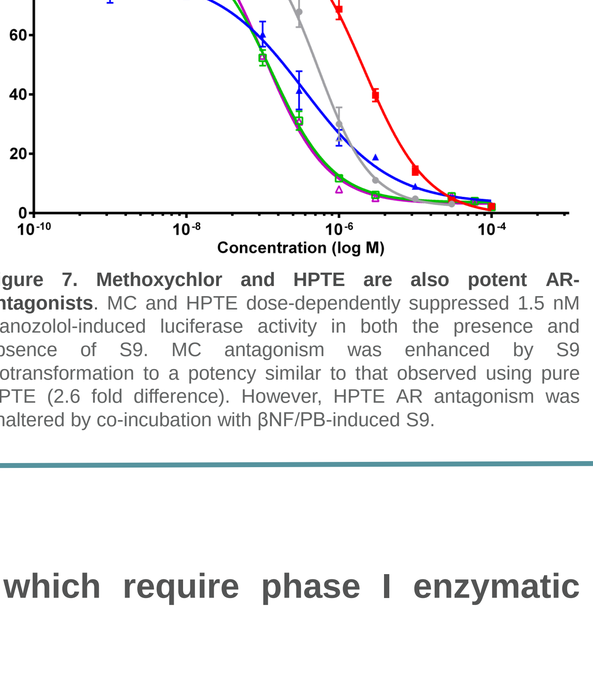


Figure 5. Addition of an S9 fraction does not always provide a full picture of metabolic potential. The estrogenic activity of Bisphenol A was unaltered by co-incubation with βNF/PB-induced S9.

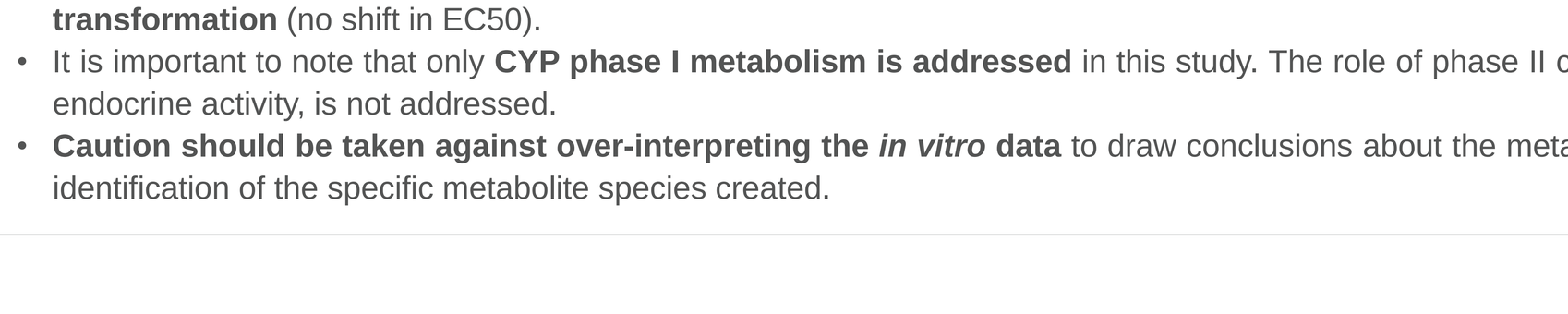


Figure 6. Stanozolol (STZ) is a more appropriate AR agonist standard than DHT for assays employing an S9 metabolizing system. Co-incubation of the AR agonist standard DHT and βNF/PB-induced S9 decreased its agonistic activity, indicating DHT was readily metabolized to a mixture with less androgenic potency (Panel A). Moreover, 0.3 nM DHT (corresponding to its EC50 in this assay) was completely inactivated by the S9. In contrast, the androgenic activity of the EC50 (1.5 nM) concentration of STZ was similar in the presence and absence of S9 (Panel B). Thus in antagonist mode, no luciferase activity can be observed in cells exposed to flutamide and DHT after biotransformation (Panel C, grey dotted line); whereas tests using a fixed concentration of STZ as the agonist exhibited activity in a flutamide-concentration dependent manner.
Bioactivation of flutamide with βNF/PB-induced S9 generated metabolites with a 7-fold more potent suppression of 1.5 nM stanozolol activity than the flutamide parent. the antagonistic activity of the pure 2-OH flutamide metabolite remains unchanged when biotransformed (Panel C).

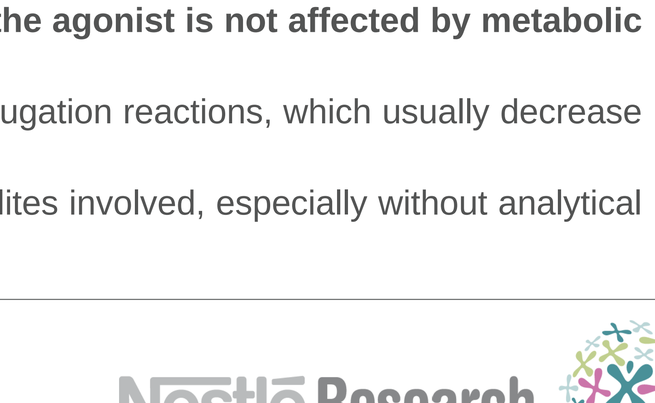


Figure 7. Methoxychlor and HPTE are also potent AR-antagonists. MC and HPTE dose-dependently suppressed 1.5 nM stanozolol-induced luciferase activity in both the presence and absence of S9. MC antagonism was enhanced by S9 biotransformation to a potency similar to that observed using pure HPTE (2.6 fold difference). However, HPTE AR antagonism was unaltered by co-incubation with βNF/PB-induced S9.

CONCLUSIONS:

- Incorporation of rat liver S9 into the CALUX bioassay is important for the **biodetection of substances which require phase I enzymatic biotransformation to express endocrine activity**.
- **Enzyme kinetics and metabolite stability** are likely major factors driving which metabolites are formed.
- The addition of S9 **does not always result in changes in relative potency. Efficacy may also be altered**, as demonstrated with B(a)P. This effect should not be dismissed although the exact mechanism is still unclear.
- When incorporating S9 into the antagonist version of the assay, it is important to **ensure that the potency of the agonist is not affected by metabolic transformation** (no shift in EC50).
- It is important to note that only **CYP phase I metabolism is addressed** in this study. The role of phase II conjugation reactions, which usually decrease endocrine activity, is not addressed.
- **Caution should be taken against over-interpreting the *in vitro* data** to draw conclusions about the metabolites involved, especially without analytical identification of the specific metabolite species created.