Retrofitting an Estrogen Receptor Transactivation Assay with Metabolic Competence Using Alginate Immobilization of Metabolic Enzymes (AIME)

Danica E. DeGroot[1], Russell S. Thomas[1], Paul Carmichael[2], Mi-Young Lee[2], and Chad Deisenroth[1]

[1]National Center for Computational Toxicology, U.S. EPA, Research Triangle Park, NC, USA
[2]Safety and Environmental Assurance Centre, Unilever, Colworth Science Park, Sharnbrook, Bedford MK44 1LQ, United Kingdom

Danica E. DeGroot | degroot.danica@epa.gov | 919-541-2482

www.epa.gov

Objective

To develop a platform to retrofit existing high-throughput screening assays with metabolic competence.

Introduction

The VM7Luc4E2 estrogen receptor (ER) transactivation assay is an OECD approved method (TG 457) for the detection of ER agonists and antagonists, and is also part of the Tox21 high-throughput screening (HTS) portfolio. Despite international acceptance as a screening assay, immortalized cell lines such as VM7Luc4E2 do not express a full complement of metabolizing enzymes. This has led to calls for improved methods for the incorporation of metabolic competence into *in vitro* assays, particularly those used in the detection of endocrine active chemicals. The Alginate Immobilization of Metabolic Enzymes (AIME) platform is an HTS-compatible solution that retrofits existing assays with metabolic competence by attaching alginate-hepatic S9 microspheres to solid supports extending from microplate lids. Herein we demonstrate the successful retrofit of the VM7Luc4E2 ER transactivation assay with metabolic competence using the AIME platform and methoxychlor as a proof-of-concept reference compound.

Impact – This work supports US EPA efforts to enhance evaluation of potential chemical hazards by incorporating xenobiotic metabolism into existing high-throughput assays.

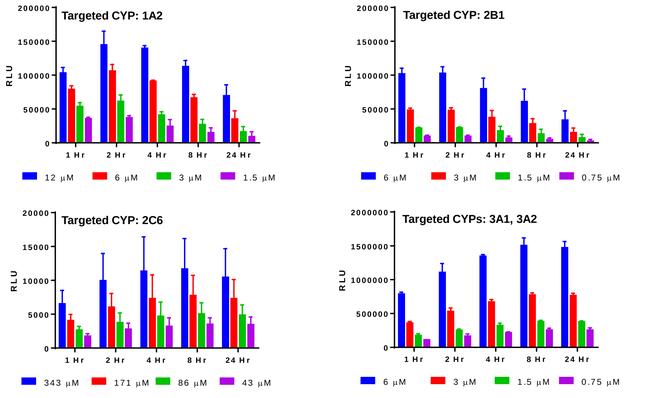


Figure 1: Encapsulated induced rat hepatic S9 maintains significant cytochrome P450 activity over time. The targeted CYP for each assay is indicated in the top left of each panel and the concentrations of pro-luciferin substrates are listed below each panel. Values represent the mean ± SEM of two experiments. *RLU: Relative Luciferase Units

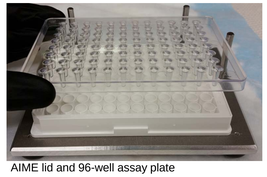
Materials & Methods

Chemicals – 17β-estradiol (E2) and methoxychlor (MXC) were purchased from Sigma-Aldrich and stock solutions prepared in DMSO.

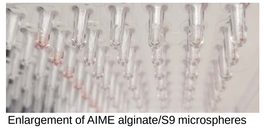
Alginate Immobilization of Metabolic Enzymes (AIME): Phenobarbital/β-naphthoflavone- or Aroclor 1254-induced rat hepatic S9 (Molecular Toxicology, Inc) was encapsulated in alginate microspheres using a modification of a cell encapsulation protocol by Lee et al. (1). The microspheres were made using 10% S9 (20 mg/mL) in an alginate solution.

Cytochrome P450 (CYP) Enzyme Time Course – CYP enzyme activity was quantified using P450-Glo assays (Promega) using the following modifications: assays were performed in phenol red free DMEM/1% charcoal-dextran treated FBS with an NADPH regeneration system (NRS) in order to replicate conditions in the VM7Luc4E2 ER transactivation assay. Pro-luciferin substrates (Luciferin-1A2, 2B6, H and IPA) were added at time zero and P450 activity determined at the indicated time points.

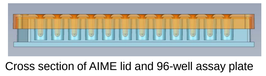
VM7Luc4E2 Estrogen Receptor Transaction Assay with AIME: Human breast carcinoma cells containing a stably integrated ER-responsive luciferase reporter gene have been previously described (2). Estrogen-stripped cells were plated at 75,000 cells/well in white, 96-well microplates and allowed to attach overnight. Post-attachment, AIME lids were prepared and immediately added to microplates containing MXC in an 8-point concentration range in estrogen stripped medium with an NRS. Incubation with encapsulated S9 proceeded for 2 hours after which the AIME lid was removed and the conditioned medium (containing metabolites) was substituted for the growth medium on the plated cells. The VM7Luc4E2 cells were incubated for 24 hours at 37°C after which luciferase activity was determined.



AIME lid and 96-well assay plate



Enlargement of AIME alginate/S9 microspheres



Cross section of AIME lid and 96-well assay plate

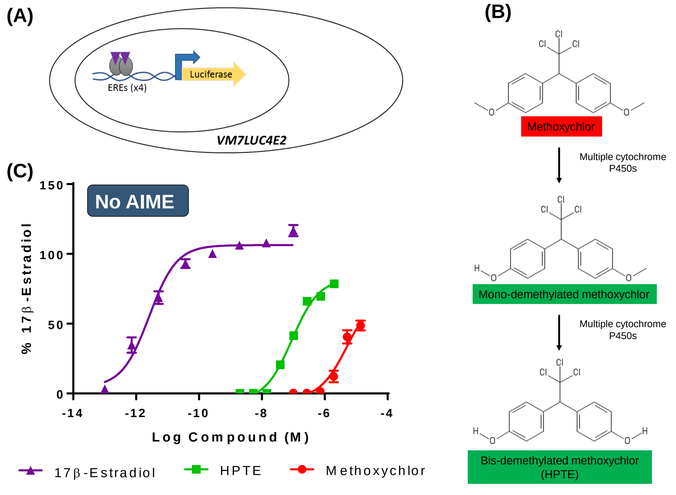
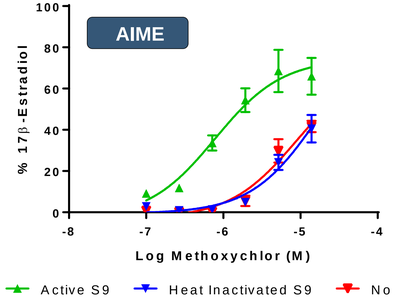


Figure 2: In the VM7Luc4E2 ER transactivation assay, methoxychlor is a weak ER agonist (A) Schematic diagram of the VM7Luc4E2 ER TA; (B) Simplified metabolic scheme of methoxychlor with metabolites indicated in green; (C) Activity of E2 and MXC in the VM7Luc4E2 ER TA. Values represent the mean ± SEM of three experiments in 384-well assay format.



96-Well	EC50 (µM)	AUC
Active S9	0.78	88.20
Heat Inactivated S9	16.89	22.82
No AIME	8.44	24.91
384-Well	**EC50 (µM)**	**AUC**
Active S9	1.20	92.87
Heat Inactivated S9	8.40	37.10
No AIME	5.07	33.86

Figure 3: Retrofitting the VM7Luc4E2 ER transactivation assay with the AIME platform shifts the EC50 value of methoxychlor (Graph, left) ER activity of MXC with and without metabolic activation in a 96-well AIME format. Values represent the mean ± SD of a single experiment. (Table, right) Comparison of MXC EC50 values with and without metabolic activation in 96- and 384-well AIME formats.

Results & Conclusions

- We have successfully produced functional AIME microspheres on lids compatible with 96- and 384-well microplates.
- AIME microspheres encapsulating phenobarbital/β-naphthoflavone-induced rat hepatic S9 demonstrate a variety of CYP activities relevant to xenobiotic metabolism. This activity is detectable over several hours in estrogen-stripped cell culture medium indicating the ability of the AIME platform to couple with the VM7Luc4E2 estrogen receptor transactivation assay.
- At two hours, most CYP activity examined using the P450-Glo assays had reached a maximal or near maximal level. Thus, a 2-hour incubation with the AIME lid was selected for use the VM7Luc4E2 ER assay.
- Retrofitting the VM7Luc4E2 ER transactivation assay with the AIME platform and a conditioned medium transfer (CMT) approach successfully produced the expected increase in MXC agonist activity.

Future Directions

- Identification and quantitation of reference metabolites using LC-MS/MS to validate the metabolic capacity of the AIME platform as well as the ratio of parent/metabolite(s) produced.
- Continued functional validation of the AIME platform using a training set of chemicals known to be bioactivated by various CYP enzymes to ER-active compounds.
- Deployment of the AIME platform to additional cell-free and cell-based assays with expanded sets of chemicals.

References

1. Lee et al. (2013) Sens. Actuators, B 177: 78-85
2. Rogers and Denison (2000) In Vitro Mol. Toxicol. 13(1): 67-82

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