Alginate Immobilization of Metabolic Enzymes (AIME) Coupled to an Estrogen Receptor Transactivation Assay Detects Bioactivated and Bioinactivated Estrogen Receptor Agonists

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Objective

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

Introduction

Characterization of chemical hazard is incomplete without evaluating the bioactivity of compounds processed through metabolic transformation and many high throughput screening (HTS) assays used for toxicity assessment lack xenobiotic metabolism. The Alginate Immobilization of Metabolic Enzymes (AIME) platform is a HTS-compatible solution that retrofits existing *in vitro* assays with metabolic competence by attaching alginate-hepatic S9 microspheres to solid supports extending from custom microplate lids. We have previously demonstrated that the AIME platform can be coupled with the VM7Luc4E2 estrogen receptor (ER) transactivation assay (TA) using methoxychlor as a reference chemical for bioactivation to a more potent ER agonist. In this study, we describe a proof-of-principle chemical screen of 48 compounds using the AIME-VM7Luc4E2 ER TA, and show that the method can effectively detect bioactivated and bioinactivated ER compounds.

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

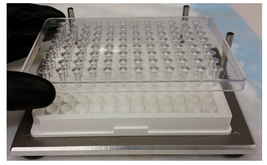
Materials & Methods

Test Chemicals & Controls: Test compounds were identified through ER QSAR analysis and literature review (1). The test sets were comprised of 34 compounds reported to have more ER activity after biotransformation (Metabolism Positive Test Set) and 14 compounds reported to be unaffected by xenobiotic metabolism (Metabolism Negative Test Set). Assay-specific controls were identified from OECD Test Guideline 455.

Alginate Immobilization of Metabolic Enzymes (AIME): Phenobarbital/β-naphthoflavone- induced rat hepatic S9 (Molecular Toxicology, Inc.) was encapsulated in alginate microspheres using a modification of a cell encapsulation protocol by Lee et al. (2). The microspheres were made using 10% S9 (20 mg/mL) in an alginate solution.

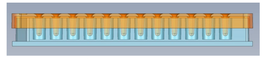
VM7Luc4E2 Estrogen Receptor Transaction Assay with AIME: Human breast carcinoma cells containing a stably integrated ER-responsive luciferase reporter gene have been previously described (3). AIME lids were prepared and immediately added to microplates containing test compounds in an 8-point concentration range (2 nM – 200 μM) in estrogen stripped medium containing an NADPH regeneration system. Metabolism proceeded for 2 hours, the AIME lid was removed and the conditioned medium (containing metabolites) was substituted for growth medium on estrogen-stripped VM7Luc4E2 cells plated in 96-well microplates. The cells were incubated for 24 hours with the conditioned medium, and cell viability (CellTiter Fluor; Promega) and ER-dependent luciferase activity determined. Assay controls were tested under (1) standard conditions; (2) with metabolic activation; and (3) without metabolic activation (Figure 1).

Curve Fitting and Data Analysis: Concentration-response data with and without metabolism from each assay endpoint were fit and analyzed using the ToxCast Data Analysis Pipeline (tcpl v.2.0.1). To assess model fitting uncertainty, an additional bootstrapping analysis of the data was completed using ToxBoot (v.0.1.2).

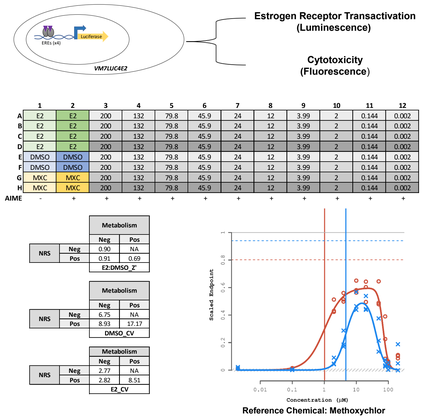


AIME lid and 96-well assay plate

Enlargement of AIME alginate/S9 microspheres



Cross section of AIME lid and 96-well assay plate



	1	2	3	4	5	6	7	8	9	10	11	12
A	E2	E2	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
B	E2	E2	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
C	E2	E2	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
D	E2	E2	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
E	DMSO	DMSO	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
F	DMSO	DMSO	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
G	MXC	MXC	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
H	MXC	MXC	200	132	79.8	45.9	24	12	3.99	2	0.144	0.002
AIME	-	+	+	+	+	+	+	+	+	+	+	+

Metabolism		Neg	Pos
NRS	Neg	0.90	NA
	Pos	0.91	0.69
E2:DMSO_Z'			

Metabolism		Neg	Pos
NRS	Neg	6.75	NA
	Pos	8.93	17.17
DMSO_CV			

Metabolism		Neg	Pos
NRS	Neg	2.77	NA
	Pos	2.82	8.51
E2_CV			

Figure 1: VM7Luc4E2 assay overview, plate diagram and coupled VM7Luc4E2-AIME assay performance. E2,17β-Estradiol; MXC, Methoxychlor; NRS, NADPH regeneration system.

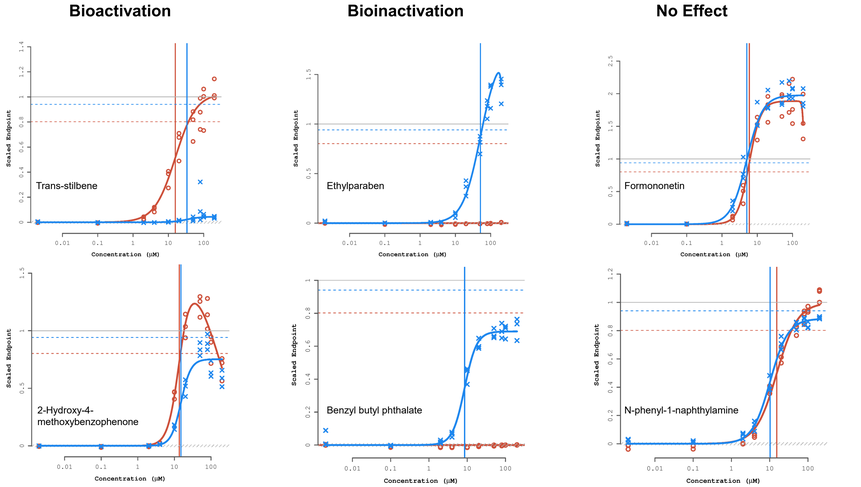


Figure 2: Representative graphs demonstrating bioactivation, bioinactivation, and no effect on ER transcriptional activity after the addition of xenobiotic metabolism. With xenobiotic metabolism (red), without xenobiotic metabolism (blue). The response to the E2 positive control under standard assay conditions (i.e. OECD TG 455) was set to 1 (grey line), and the data scaled accordingly. The red and blue dashed lines represent E2 positive control shifts under the ascribed assay conditions. Vertical red and blue lines represent the AC50 for each condition.

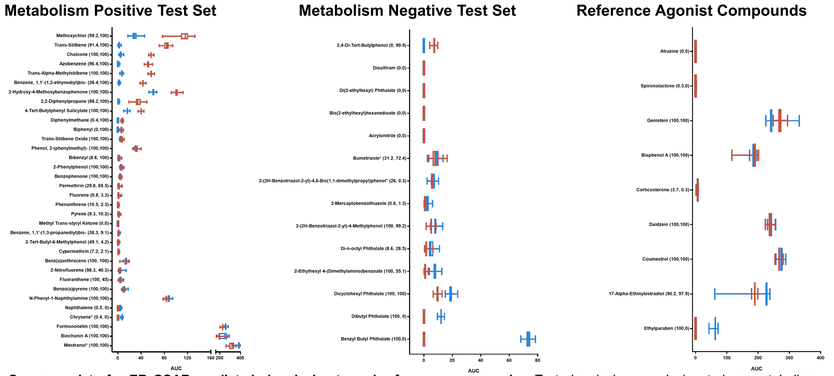


Figure 3: Summary data for ER QSAR predicted chemical sets and reference compounds. Test chemicals were designated as metabolism positive (compounds with more potent metabolites) or metabolism negative (compounds with no or low ER activity and no evidence of ER-active metabolites) based on Pinto et al. (1). Each data point displays a distribution of areas under the curve (AUC) generated from a bootstrapped resampling of 1000 curve fits. With xenobiotic metabolism (red), without xenobiotic metabolism (blue). Numbers following compound names indicate the hit count percentage of successful fits to either a Hill or Gain-Loss model in tcpl v.2.0.1 (no metabolism, with metabolism). Asterisks indicate compounds tested below a top concentration of 200 μM. Reference compounds from OECD TG 455.

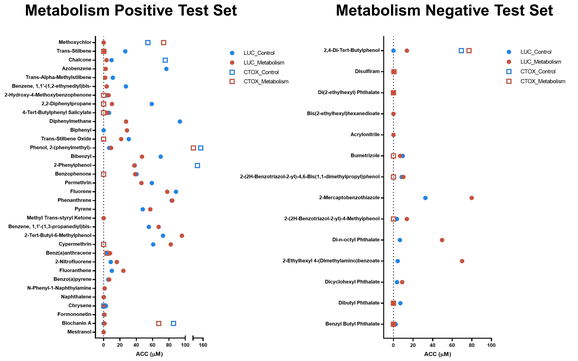


Figure 4: Activity concentration at cutoff (ACC) values for ER transactivation and cytotoxicity assays. With xenobiotic metabolism (red), without xenobiotic metabolism (blue). LUC: luciferase endpoint for ER transactivation (circles). CTOX: fluorescence endpoint for cytotoxicity (squares). If no cytotoxicity was observed, squares were omitted from the figure. Dashed line indicates the zero reference point.

Results & Conclusions

- We have successfully coupled the AIME platform to the VM7Luc4E2 estrogen receptor transactivation assay and demonstrated the utility of this method for identification of bioactivated and bioinactivated ER compounds.
- Two compounds, ethylparaben and trans-stilbene, were identified as key reference compounds for metabolism-dependent effects on ER transcriptional activity due to their robust bioinactivation and bioactivation, respectively.
- The cell viability data demonstrate a reduction of S9-induced cytotoxicity in cell-based assays coupled with the AIME platform. In addition, they provide context to potency and efficacy relationships of ER-active compounds for evaluating biological relevance.
- Preliminary findings demonstrate compounds (e.g., benzyl butyl phthalate) that tested ***positive*** in ToxCast ER assays, in fact, tested ***negative*** in both the *in vivo* rat uterotrophic studies and the metabolically competent VM7Luc4E2-AIME assay. By contrast, compounds such as trans-stilbene which are ***weakly*** estrogenic in ToxCast ER assays demonstrate ***increased efficacy and potency*** in the metabolically competent VM7Luc4E2-AIME assay which correlates with positive results in a murine uterotrophic study. Together this suggests that the AIME-driven incorporation of metabolism into the VM7Luc4E2 assay refines *in vitro* identification of ER active compounds and may provide greater relevance to biological systems.
- Future work will broaden the scope of AIME-coupled screening to refine the identification of chemical hazards that are a product of hepatic xenobiotic metabolism.

References

1. Pinto et al. (2016) Chem. Res. Toxicol. 29(9): 1410-27
2. Lee et al. (2013) Sens. Actuators, B 177: 78-85
3. Rogers and Denison (2000) In Vitro Mol. Toxicol. 13(1): 67-82

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