Integrating In Silico And In Vitro Approaches to Understand Cross-Species Predictions of Chemical Susceptibility for Aromatase

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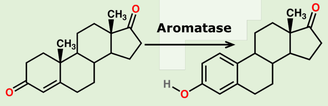
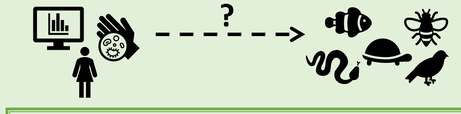
Background

- Endocrine active chemicals are of concern for chemical hazard evaluation and several new approach methodologies (NAMs), defined as animal alternatives, have been developed to rapidly screen chemicals for biological activities leading to endocrine disruption
- Aromatase (CYP19A1), for example, catalyzes the biosynthesis of estrogens from androgens and can be inhibited by environmental chemicals



- However, many NAMs (e.g., U.S. EPA ToxCast Program) that focus on aromatase currently rely on mammalian-based test systems, resulting in uncertainty surrounding the applicability of these approaches to non-mammalian targets
- The U.S. EPA's Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool identifies whether known critical amino acids involved in catalytic function are conserved across species and makes predictions of susceptibility based on amino acid molecular weight and side chain classification



This study employs in silico predictions, in vitro confirmation assays, and molecular modeling approaches to investigate cross-species predictions of chemical susceptibility for aromatase inhibition

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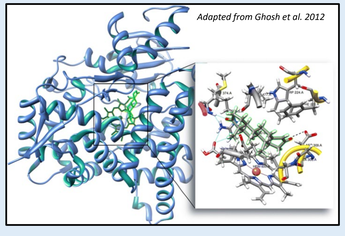
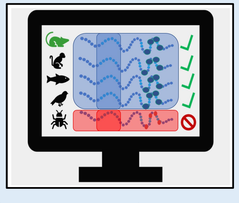
Methods 1: Identification of Critical Amino Acids

- Aromatase inhibitors can block aromatase action via competition mechanism inside aromatase binding site
  - **Steroidal inhibitors (Androstenedione):** Irreversible binding, requires recycling of the aromatase enzyme
  - **Nonsteroidal Inhibitors (Letrozole)**: Reversable, non-covalent binding
- Literature search was conducted to identify the amino acid residues important in both types of aromatase-ligand binding



Critical Amino Acids			
Androstenedione (ASD)		Letrozole	
Asp309	Hydrogen bond	Asp309	van der Waals
Met374	Hydrogen bond	Met374	Hydrogen bond
Arg115	Weak H-bond	Arg115	van der Waals
Pro308	van der Waals	Pro308	van der Waals
Thr310	van der Waals	Thr310	van der Waals
Phe134	van der Waals	Phe134	van der Waals
		Ser478	Hydrogen bond
Val370	van der Waals	Val370	van der Waals
I133	van der Waals		
Phe221	van der Waals	Phe221	van der Waals
Trp224	van der Waals	Trp224	van der Waals

Methods 2: SeqAPASS Analysis



- The SeqAPASS tool identifies whether known critical amino acids involved in catalytic function are conserved across species and makes predictions of susceptibility based on amino acid molecular weight and side chain classification
- Five critical amino acid residues were chosen for further SeqAPASS analysis to predict conservation across vertebrate species
- This SeqAPASS analysis revealed the presence of amino acid substitutions among bony fishes (Actinopteri), with only the channel catfish predicted to not be similarly susceptible

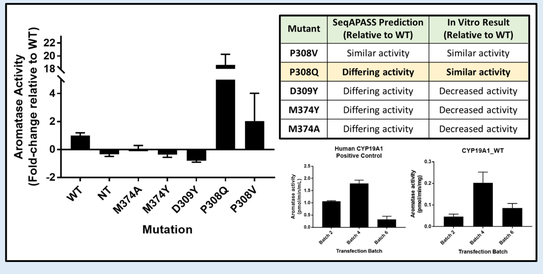
Taxon	Number of Species	Number Y	Number N	Majority Similar?	hCYP19A1 Pos. 115	Pos. 308	Pos. 309	Pos. 310	Pos. 374
Mammalia	108	108	0	Y	R	P	D	T	M
Crocodylia	2	2	0	Y	R	P	D	T	M
Testudines	6	6	0	Y	R	P	D	T	M
Aves	74	74	0	Y	R	P	D	T	M
Actinopteri	123	122	1	Y	R	P, V	D, E	T	M, P, Y
Lepidosauria	7	7	0	Y	R	P	D	T	M
Amphibia	7	7	0	Y	R	P	D	T	M
Chondrichthyes	4	4	0	Y	R	P	D	T	M
Ceratodontimorpha	1	1	0	Y	R	P	D	T	M

Taxon	Species Name	Similar?	hCYP19A1 Pos. 115	Pos. 308	Pos. 309	Pos. 310	Pos. 374
Mammalia	Human	Y	R	P	D	T	M
Actinopteri	Channel catfish	N	R	P	D	T	Y

Methods 3: In Vitro Site-Directed Mutagenesis

- To further investigate these predictions and understand how SeqAPASS comparisons relate to in vitro aromatase assays, five mutations were selected, across three positions for in vitro site-directed mutagenesis (SDM) studies
- Selected mutations spanned a range of molecular weight and side chain classification, two mutants (P308V and M374Y) were chosen based on their occurrence in fish species
- In vitro SDM of the wildtype (WT) human Cyp19A1 gene sequence was used to create enzyme variants representing selected aromatase mutants and mutant enzyme activity was measured relative to wild type (WT)

Mutant	Mutation Rational
P308V	**Size:** Nonpolar residue substituted by similar sized nonpolar residue
P308Q	**Size & Hydrophobicity:** Nonpolar residue, substituted with larger polar residue
D309Y	**Size & Charge:** Negatively charged residue substituted with large uncharged residue
M374Y	**Size:** Nonpolar residue substituted with larger nonpolar residue
M374A	**Size:** Large, nonpolar residue substituted with smaller nonpolar residue



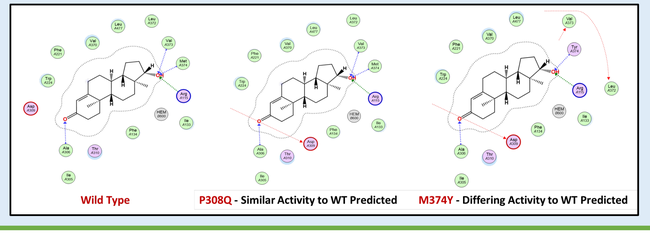
Mutant	SeqAPASS Prediction (Relative to WT)	In Vitro Result (Relative to WT)
P308V	Similar activity	Similar activity
P308Q	**Differing activity**	**Similar activity**
D309Y	Differing activity	Decreased activity
M374Y	Differing activity	Decreased activity
M374A	Differing activity	Decreased activity

- While in vitro results were largely consistent with SeqAPASS predictions, mutant P308Q demonstrated similar aromatase activity to WT, rather than differing activity as predicted

Methods 4: Molecular Modeling

- To further investigate these predictions, mutations were modeled using the Molecular Modeling Environment (MOE) using aromatase complexed with testosterone (RCSB Protein Data Bank, 5JKW)
- Overall, modeling results were consistent with in vitro SDM results, predicting similar ligand interaction with P308Q compared to WT

Mutant	Modeling Result (Relative to WT)
P308V	Similar activity
P308Q	**Similar activity**
D309Y	Differing activity
M374Y	Differing activity
M374A	Differing activity



Summary & Future Directions

- SeqAPASS analysis of amino acid residues critical for aromatase enzymatic activity reveal that aromatase, and potential chemical susceptibility, is conserved across vertebrate species, but not among invertebrate species
- These predictions are consistent with the current evolutionary hypothesis that suggests the aromatase gene arose at the origin of vertebrates
- Subsequent in vitro SDM of wildtype human aromatase and select enzyme variants revealed that the majority of predictions aligned with SDM results, with only mutant P308Q demonstrating similar aromatase activity relative to WT, rather than decreased activity as predicted
- Further assessment through molecular modeling revealed that the P308Q mutant is not predicted to demonstrate altered mutant-ligand interactions

Mutant	Mutation Rational	SeqAPASS Prediction (Relative to WT)	In Vitro Result (Relative to WT)	Modeling Result (Relative to WT)
P308V	**Size:** Nonpolar residue substituted by similar sized nonpolar residue	Similar activity	Similar activity	Similar activity
P308Q	**Size & Hydrophobicity:** Nonpolar residue, substituted with larger polar residue	**Differing activity**	**Similar activity**	**Similar activity**
D309Y	**Size & Charge:** Negatively charged residue substituted with large uncharged residue	Differing activity	Differing activity	Differing activity
M374Y	**Size:** Nonpolar residue substituted with larger nonpolar residue	Differing activity	Differing activity	Differing activity
M374A	**Size:** Large, nonpolar residue substituted with smaller nonpolar residue	Differing activity	Differing activity	Differing activity

- Overall, these results suggest that predictions that can incorporate both sequence-based and structural-based evaluations of protein conservation provide the greatest potential for predicting the likelihood for chemical susceptibility across species
- New capabilities are being developed within the SeqAPASS tool to integrate structural assessments into cross-species predictions and are on track to be released in 2023

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