

Development and Application of a Reference Database for Androgen Receptor Activity

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Introduction

- As many as 30,000 commercial chemicals in the environment require testing to collect data on their potential androgen bioactivity, with several hundred new chemicals being added to this total each year (EPA 2011). This process will cost millions of dollars and take decades to complete using current validated methods.
- Alternative approaches for chemical testing used in the ToxCast and Tox21 programs (Kavlock et al. 2012, Tice et al. 2013) use high-throughput screening (HTS) assays and computational toxicology methods. These approaches, which are accepted by the U.S. Environmental Protection Agency (EPA) for testing for estrogen receptor bioactivity (Browne et al. 2015), could also be used to identify potential androgen-active chemicals rapidly and cost-effectively.
- Evaluation of these HTS approaches and further development of alternative test methods and testing strategies require high-quality reference data for androgenic and anti-androgenic activity.
- Using a comprehensive list of putative androgen-active or inactive chemicals from international validation studies, we performed a literature search to compile high-quality in vitro androgen receptor (AR) binding and transactivation (TA) assay data. No ToxCast or Tox21 assay data were included in the literature search.
- We then identified the chemicals with reliable and reproducible in vitro results, and binned chemicals into potency categories. This yielded a list of reference chemicals that can be used for validation of Tox21 and ToxCast results and other novel AR assays.
- We used this list to evaluate a computational model of AR pathway activity based on 11 Tox21 and ToxCast assays.

Literature Search

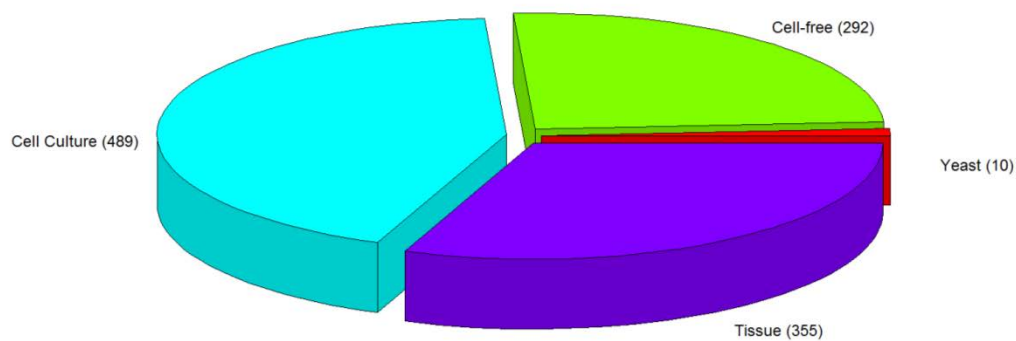
- We selected 158 chemicals identified in the following assay validation efforts as having potential AR agonist or antagonist activity (or lack of activity) :
 - Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003)
 - European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM, ongoing)
 - Organization for Economic Cooperation and Development (OECD 2010)
 - EPA Endocrine Disruptor Screening Program (EPA 2011)

- We conducted semi-automated literature searches for in vitro androgen activity data on these chemicals using PubMatrix (<http://pubmatrix.grc.nia.nih.gov/>) and Scopus (<http://www.scopus.com/>). Search keywords included “androgen”, “androgenic”, “anti-androgen”, and related terms.
- Data from in vitro AR binding and TA assays were extracted from identified references and compiled into a single database. (The database is expected to be made available to the public later in 2016. Availability will be announced via the NICEATM News email list.)
- Using a standardized ontology, the following information was recorded for each chemical-study combination:
 - PubMed Identifier, author, year
 - Chemical tested, Chemical Abstracts Service Registry Number
 - Table or figure where results were reported
 - Hit, response, response notes
 - Half-maximal activity concentration (AC50 or IC50), standard error measurement, units
 - Assay type (tissue or cell culture), tissue of origin (for cell culture), species of origin
 - Receptor information, species source
 - Reference androgen or anti-androgen
 - Number of concentrations tested, highest concentration tested, units, incubation time
 - Binding assays only: binding affinity, dissociation constant, relative binding affinity (RBA)
 - TA assays only: agonist or antagonist mode, whether cytotoxicity was evaluated, extent of cytotoxicity observed (i.e. at IC50)
 - TA assays only: reporter type, reporter construct, whether construct was native, transient, or stable

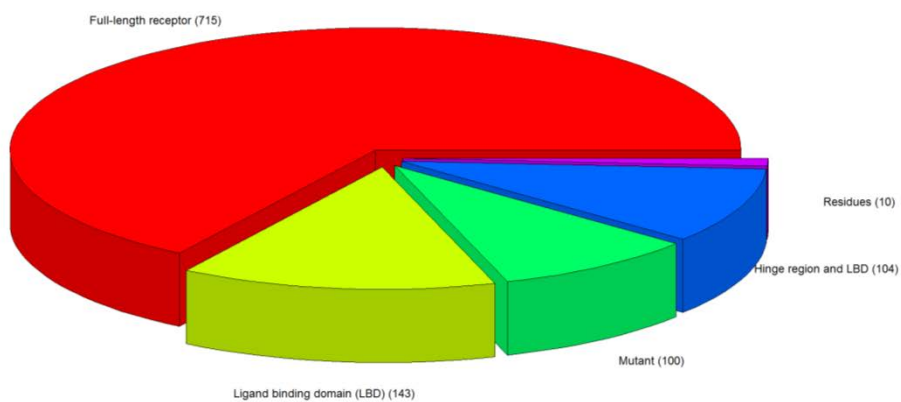
Literature Results: AR Binding

- AR binding data were compiled for 103 chemicals (1167 experiments from 159 papers).
- Commonly used assay platforms included cell culture, tissue preparations, and cell-free systems (**Figure 1a**).
- The majority of assays used full-length receptors (**Figure 1b**).
- Most assays used human (39%) or rat (33%) receptors, but receptors from a total of 26 species were represented among all assays in the database.
- The four most commonly used reference androgens were: methyltrienolone (R1881; 475 assays, 41%), 5 α -dihydrotestosterone (DHT; 400 assays, 34%), testosterone (203 assays, 17%), mibolerone (84 assays, 7%).

Figure 1 AR Binding: (a) Assay Types and (b) Receptor Types



(a)



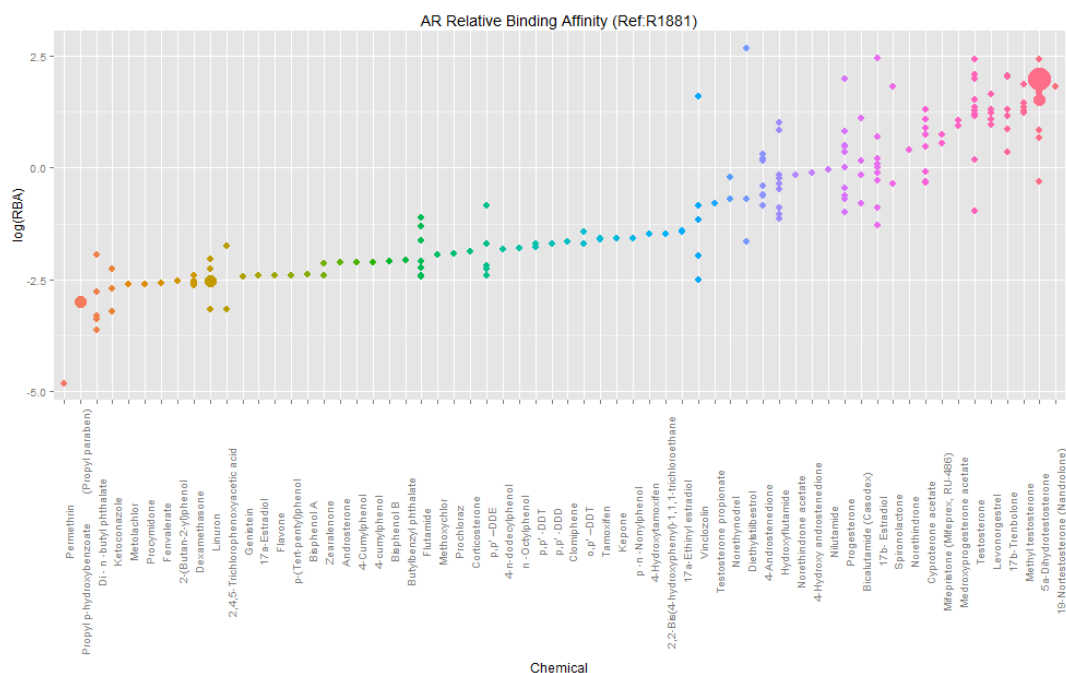
(b)

Abbreviation: AR = androgen receptor. The number of experiments conducted using each assay type is shown in parentheses.

Analysis of Experimental Results for AR Binding Assays

- Further analyses were conducted on data from assays using the full-length receptor and the ligand binding domain. Positive and negative AR binding assay results were reported for 957 of these experiments on 95 chemicals.
 - Multiple positive binding results with no negative results were reported for 38 chemicals.
 - Atrazine, cycloheximide, and 2,4-dinitrophenol had multiple negative binding results and no positive results.
 - There were 14 chemicals with only one positive result (and no negatives), and six chemicals with only one negative result (and no positives).
 - The remaining 34 chemicals had both positive and negative binding results reported, although there was usually a clear majority of positive or negative results for each chemical.
- Results for binding affinity were reported in many different formats, the most common being RBA or log RBA relative to a positive control: R1881 (240 results), DHT (168 results), testosterone (97 results), mibolerone (30 results).
- As an example, results for log RBA on 61 chemicals relative to the most common positive control compound, R1881, are shown in **Figure 2**.

Figure 2 AR Relative Binding Affinities (Reference: R1881)



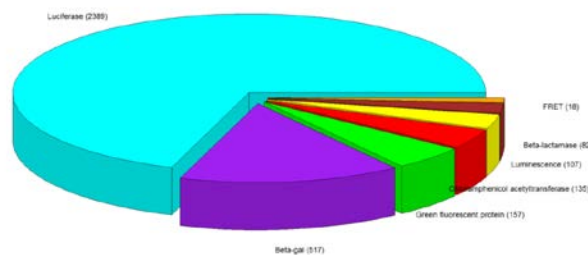
Abbreviations: AR = androgen receptor; R1881 = methyltrienolone; RBA = relative binding affinity.

Chemicals are listed along the x-axis; y-axis represents the log₁₀ (RBA). The size of the dot increases with the number of observations.

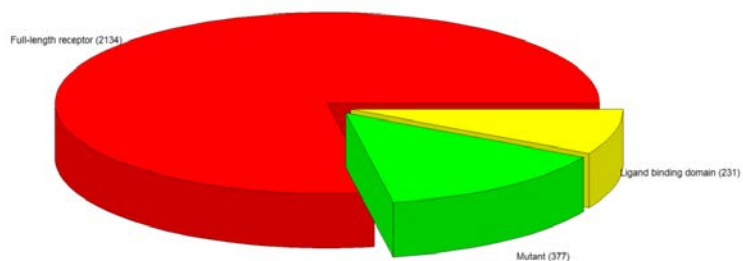
Literature Results: AR Transactivation

- AR transactivation data were compiled for 133 chemicals (3444 experiments from 284 papers).
- While six different reporter types were used in the experiments, the majority of experiments used assays with a luciferase reporter (**Figure 3a**).
- Use of the full-length receptors was most common (**Figure 3b**).
- Many assays used a transiently transfected AR (46%) or stably integrated AR (39%), followed by native receptor expression (14%).
- Most TA assays used the human AR (93%), but receptors from a total of 14 species were represented among all assays in the database.

Figure 3 AR Transactivation: (a) Assay Types and (b) Receptor Types



(a)



(b)

Abbreviation: AR = androgen receptor. The number of experiments conducted using each assay type is shown in parentheses.

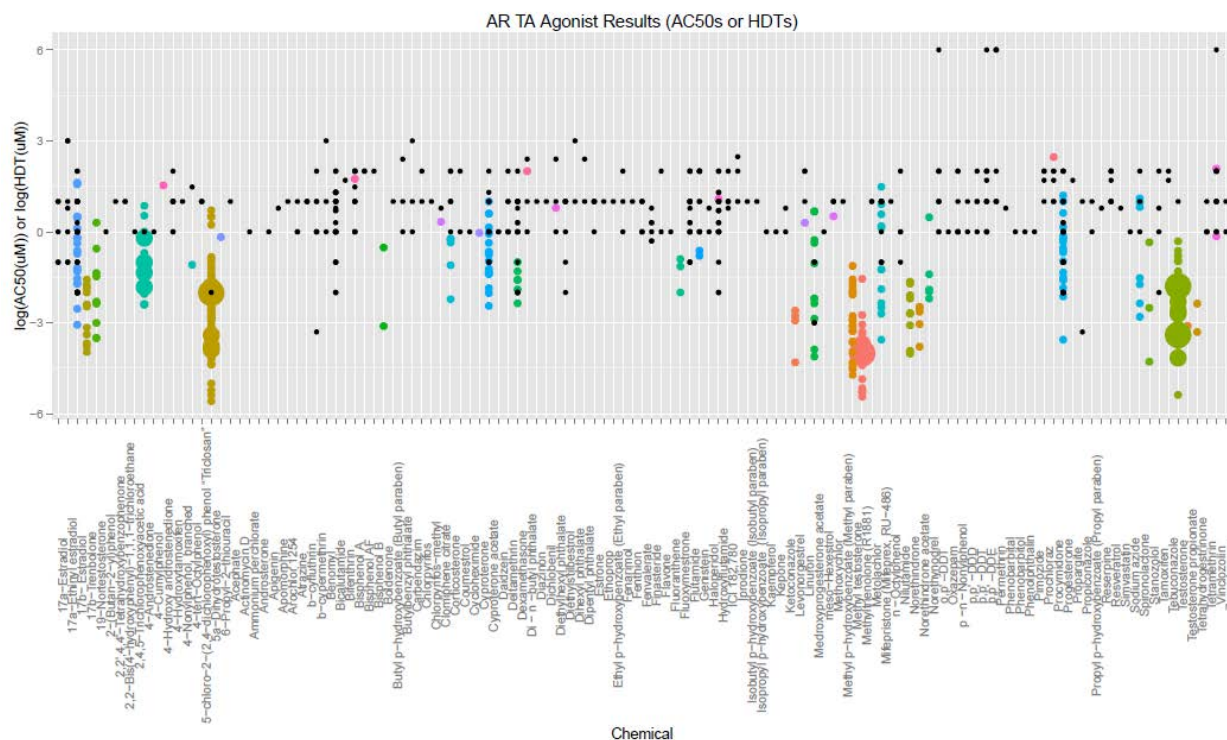
Analysis of Experimental Results for AR Transactivation Assays

- Further analyses were conducted on data from assays using the full length receptor and the ligand binding domain. Positive and negative AR TA assay results were reported for 2393 experiments on 133 chemicals. Results were subdivided into modes measuring agonist activity (1447 experiments, 60%) and antagonist activity (946 experiments, 40%).
 - There were 13 chemicals with multiple positive agonist results (increase in TA) and no antagonist results.
 - All of these chemicals also had negative results reported (no agonist or antagonist activity), but for most of these chemicals the number of positive agonist results far outnumbered the number of negative results.
 - Negative outcomes tended to occur in specific cell or receptor types and/or at very low concentrations.
 - There were 32 chemicals with multiple positive antagonist results (decrease in TA) and no agonist results.
 - All of these chemicals also had negative results that tended to occur in specific cell types and/or at low concentrations.
 - There were 17 chemicals with multiple negative results and no positive (agonist or antagonist) results.
 - There were 15 chemicals with only one TA result in any category.
 - The remaining 56 chemicals had a mix of positive (agonist and/or antagonist) and negative results. However, for most chemicals there was a clear majority of either agonist or antagonist results.

Transactivation Agonist Potency

- Positive results for TA agonist activity were reported in many different formats and with many different units, the most common being lowest effect level (LEL, 415 results, 49%) and half-maximal activity concentration (AC50, 406 results, 48%).
- All TA agonist results were converted into log μ M units where possible, and the respective agonist potencies based on AC50s for each chemical (colored dots) were compared to negative results in terms of highest dose tested (HDT, black dots), as shown in **Figure 4**.

Figure 4 Comparing AR TA Agonist Results



Abbreviations: AC50 = half-maximal activity concentration; AR = androgen receptor; HDT = highest dose tested; TA = transactivation.

Chemicals are listed along the x-axes and the log transformed doses along the y-axis. The colored dots represent positive results in log10 (AC50), and the black dots represent negative results in log10 (HDT). The size of the dot increases with the number of observations.

Transactivation Antagonist Potency

- We evaluated AR TA antagonist potency using only data from experiments that concurrently measured cytotoxicity (520 experiments [55%] representing 105 chemicals).
- Positive results for antagonist activity were reported in many different formats and with many different units, the most common being half-maximal inhibition activity concentration (IC₅₀, 224 results, 64%) and LEL (114 results, 33%).
- All TA antagonist results were converted to -log μ M units where possible, and the respective antagonist potencies based on IC₅₀ (colored dots) were compared to the negative results in terms of HDT (black dots), as shown in **Figure 5**.

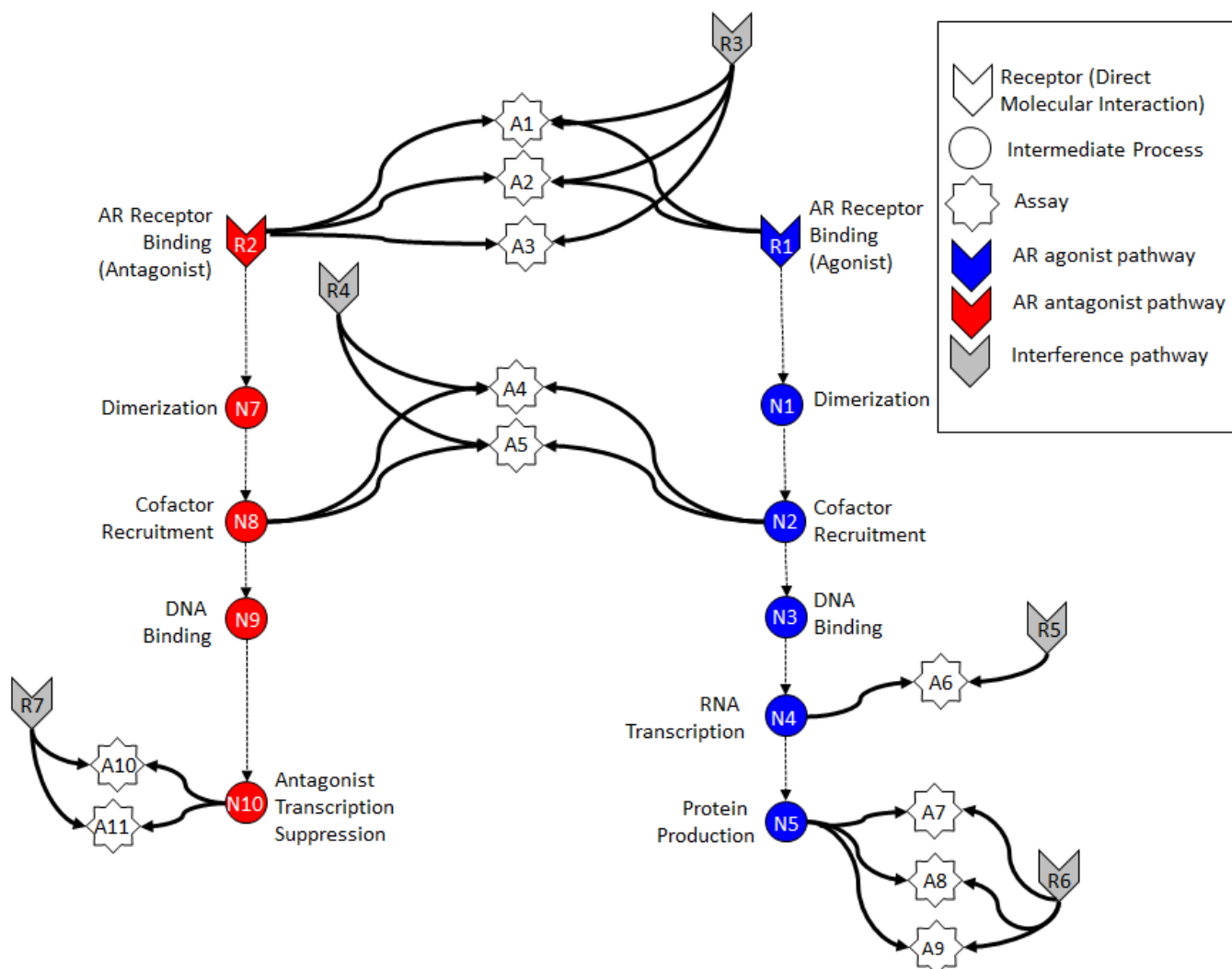
- To establish reference chemical lists, we first identified all quantitative TA assay data from the literature search that could be converted to μM units. Using these data, we then calculated mean, standard deviation, 95% confidence interval, and number of observations for each chemical. We used the results to define agonist and antagonist reference chemical lists and potency categories according to the following criteria.
- Agonist:
 - **Positives:** at least three experiments, of which at least 70% yielded positive results
 - Strong: mean AC50 less than or equal to $0.1\ \mu\text{M}$
 - Moderate: mean AC50 greater than $0.1\ \mu\text{M}$ and less than or equal to $1\ \mu\text{M}$
 - Weak: mean AC50 greater than $1\ \mu\text{M}$

- **Negatives:** at least three experiments yielding negative results, and no experiments yielding positive results
- Antagonist:
 - **Positives:** at least three experiments, of which at least 70% yielded positive results that were not due to cytotoxicity
 - Strong: mean IC50 less than or equal to 0.5µM
 - Moderate: mean IC50 greater than 0.5µM and less than or equal to 5µM
 - Weak: mean IC50 greater than 5µM and less than or equal to 25µM
 - Very Weak: mean IC50 greater than 25µM
 - **Negatives:** at least two experiments yielding negative results, and no experiments yielding positive results
- Chemicals with upper 95% confidence intervals that spanned potency categories were given combined category designations such as “Strong/Moderate” or “Moderate/Weak.”
- This evaluation produced 29 agonist reference chemicals and 28 antagonist reference chemicals covering a wide range of potencies.

AR Pathway Model

- A computational model for AR pathway activity (**Figure 6**) was built using 11 Tox21 and ToxCast assays (**Table 1**) that map to key events in the biological pathway (Kleinstreuer et al., manuscript in preparation).
- An additional run of the Tox21 antagonist luciferase assay in the MDAKB2 cell line (**Figure 6, A11**) with a higher concentration of the synthetic ligand R-1881 was run as a confirmation assay to verify chemical activity specific to the AR pathway. A shift in potency in this experiment indicates that a chemical was likely a true AR antagonist, whereas a lack of a shift indicates that the data should be flagged for potential non-specific activity.

Figure 6 AR Pathway Model Based on Tox21/ToxCast Assays



Abbreviations: AR = androgen receptor.

Colored arrow nodes (R1/R2) represent “receptors” with which a chemical can directly interact (true agonism/antagonism, respectively). Colored circles represent intermediate biological processes that are not directly observable. White stars represent the assays that measure activity at the biological nodes. Arrows represent transfer of information. Grey arrow nodes represent biological interference pathways (R3-R7) or technology-specific interference.

Table 1 Tox21/ToxCast Assays Used in AR Pathway Model

ID	Assay Name	Source	Gene	Species	Type
1	NVS human AR	Novascreen	AR	Homo sapiens	Receptor Binding
2	NVS chimpanzee AR	Novascreen	AR	P. troglodytes	Receptor Binding
3	NVS rat AR	Novascreen	AR	Rattus norvegicus	Receptor Binding
4	OT_AR_ARSRC1_0480	Odyssey Thera	AR;SRC	Homo sapiens	Cofactor Recruitment
5	OT_AR_ARSRC1_0960	Odyssey Thera	AR;SRC	Homo sapiens	Cofactor Recruitment
6	ATG_AR_TRANS	Attagene	AR	Homo sapiens	RNA Reporter Gene
7	OT_AR_ARELUC_AG_1440	Odyssey Thera	AR;ARE	Homo sapiens	Reporter Gene
8	Tox21_AR_BLA_Agonist_ratio	NCGC	AR	Homo sapiens	Reporter Gene
9	Tox21_AR_LUC_MDAKB2_Agonist	NCGC	AR	Homo sapiens	Reporter Gene
10	Tox21_AR_BLA_Antagonist_ratio	NCGC	AR	Homo sapiens	Reporter Gene
11a	Tox21_AR_LUC_MDAKB2_Antagonist	NCGC	AR	Homo sapiens	Reporter Gene
11b*	Tox21_AR_LUC_MDAKB2_Antagonist-c	NCGC	AR	Homo sapiens	Reporter Gene

Abbreviations: AR = androgen receptor; ARE = androgen response element; NCGC = National Center for Advancing Translational Sciences (NCATS) Chemical Genomics Center; SRC = c-Src tyrosine kinase.

*Confirmation assay data (different concentration of R-1881)

AR Pathway Model Performance Against Reference Chemicals

- We used the AR pathway model to predict activities of the reference chemicals that were independently identified in the literature review. The results of these predictions are shown in **Figure 7a** (29 agonist reference chemicals) and **Figure 7b** (28 antagonist reference chemicals). An AR pathway model score greater than 0.05 (activity at concentrations less than 200µM) was considered positive, with higher model scores corresponding to stronger potency.
- The AR pathway model was 96.6% accurate at predicting the agonist reference chemicals.
 - 17a-Estradiol was the only false positive and there were no false negatives.
 - 17a-Estradiol was classified negative for agonism based on multiple literature results; however, the HDTs were 10 µM.
 - All 11 Tox21/ToxCast assays were activated by 17a-Estradiol (AC50/IC50 range 0.1 – 10 µM), resulting in a model prediction of both agonist and antagonist activity.
 - These results could be indicative of true selective AR modulation by this chemical, or heightened sensitivity of the HTS assays to strong steroid pharmaceuticals, especially at higher concentrations.
- The AR pathway model was 92.9% accurate at predicting the antagonist reference chemicals.

- Zearalenone and o,p'-DDT, both categorized in the literature review as weak antagonists, were false negatives, but hit both Tox21 antagonist assays so were predicted to produce assay interference through R7 (**Figure 6**). There were no false positives.
- When tested in the confirmation assay, o,p'-DDT exhibited a potency shift, confirming it as a true antagonist. Zearalenone also exhibited a potency shift in the expected direction, flagging it as potential true antagonist, although the shift was not statistically significant due to overlapping confidence intervals around the AC50s.

Figure 7 AR Pathway Model Results for Reference Chemicals

Figure 7a. Agonist Reference Chemicals

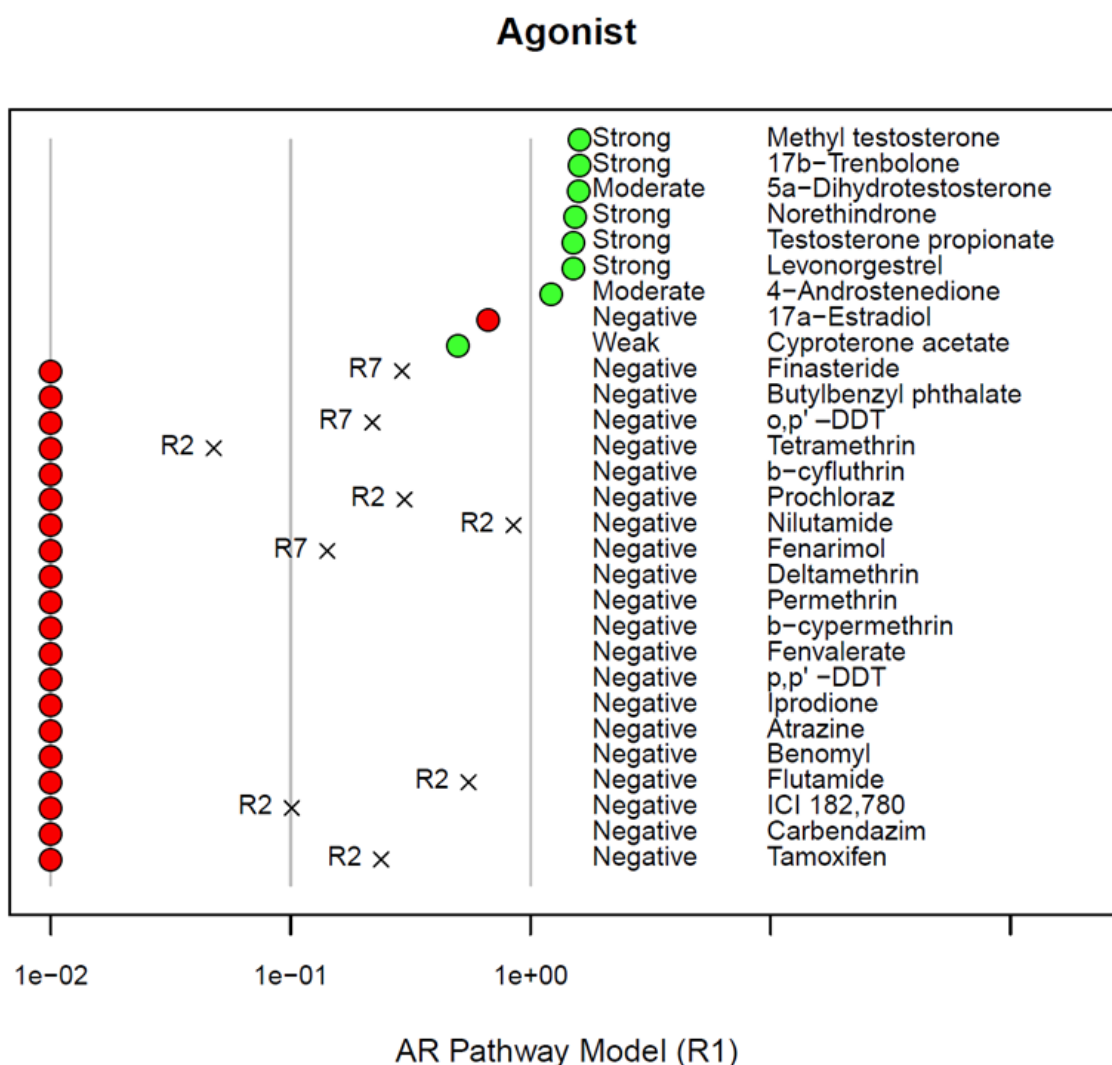
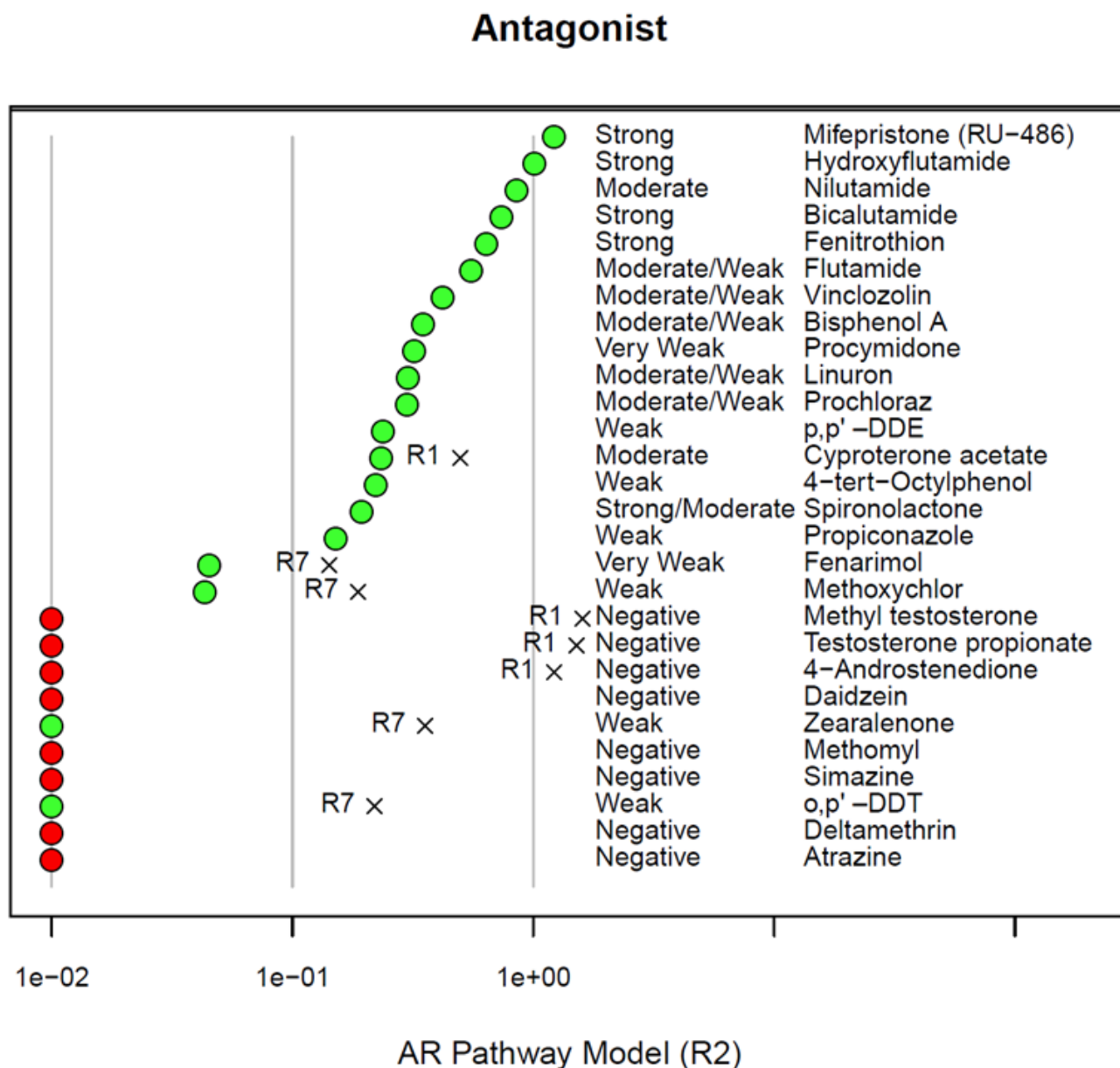


Figure 7b. Antagonist Reference Chemicals



Reference chemicals and associated potency categories (from the literature search) are listed along the y-axes and the AR pathway model score for (a) agonism, R1, or (b) antagonism, R2, along the x-axes. Green dots represent positive reference chemicals and red dots represent negative reference chemicals. Assay interference predictions (e.g. R7) are marked with an “x”. AR pathway model scores below 0.01 were truncated at 0.01 for plotting purposes. There was one false positive for agonism (17a-estradiol). Two initial false negatives for antagonism (zearalenone and o,p' -DDT) were confirmed as true positives by a confirmation assay.

Conclusions

- Our database includes a wide array of binding and TA data from many different sources for a range of potential AR agonist and antagonist reference chemicals.
- Additional data collection efforts are ongoing. These are occurring in parallel with a data curation effort led by the EPA Office of Science Coordination and Policy compiling in vivo androgen and anti-androgen data from the EDSP Tier 1 Hershberger assay (EPA 2009).
- The proposed reference chemical lists and associated potency categories will be made available to the public and submitted to OECD via the Validation Management Group–Non-Animal to facilitate international harmonization of test method evaluations.
- The AR pathway model based on Tox21/ToxCast assays was validated against an independently curated set of AR reference chemicals and shown to be over 90% accurate for both agonism and antagonism.
- The Tox21 confirmation assay data assisted in identifying chemicals that exhibited a shift in potency indicative of a true AR antagonist response, where both chemicals initially identified by the AR pathway model as false negatives were flagged as true positives.

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