Identifying Reference Chemicals for Androgen Receptor Activity

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Introduction

- As many as 30,000 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 such as those developed in the U.S. Environmental Protection Agency (EPA) ToxCast chemical research program (Dix et al. 2007; Judson et al. 2010) and the Tox21 U.S. federal partnership (Tice et al. 2013) use high-throughput screening (HTS) assays and computational toxicology technologies. These approaches can identify potential androgen-active chemicals rapidly and cost-effectively.
- Evaluation of these HTS approaches and further development of alternative test methods and testing strategies will require high-quality reference data for androgenic and anti-androgenic activity (Wilson et al. 2008).
- We compiled a list of putative androgen-active or inactive chemicals for which Tox21 or ToxCast data were available, performed a literature search on these chemicals to identify in vitro androgen receptor binding and transactivation (TA) assay data, and analyzed the results to facilitate creating a list of reference chemicals and associated potencies that can be used for future validation of Tox21/ToxCast results and other novel AR assays.

Chemical Selection

- We selected 158 chemicals that were identified as having potential androgen receptor (AR) agonist or antagonist activity (or inactivity) in past or ongoing assay validation efforts by:
  - Interagency Coordinating Committee for Validation of Alternative Methods (ICCVAM 2003)
  - European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)
  - Organization for Economic Cooperation and Development (OECD 2010)
  - U.S. EPA Endocrine Disruptor Screening Program (EDSP, EPA 2011)
- Of the 158 chemicals, 127 are included in the ToxCast/E1K library. AR agonism or antagonism data on these chemicals is available from nine ToxCast assays that measure biological events in the AR pathway. These assays have been mapped to a computational model for AR agonism/antagonism and used to train predictive models (see **Zang et al. poster** in this session).

Literature Search

- 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/) with keywords such as “androgen”, “androgenic”, “anti-androgen”, etc.
- Data from in vitro binding and TA assays in the published scientific literature were extracted from identified references and compiled into a single database, which will be publicly available on the National Toxicology Program (NTP) website (http://ntp.niehs.nih.gov/go/40658).
- The following pieces of information were recorded for each chemical/study combination using a standardized ontology:
  - PubMed Identifier, author, year
  - Chemical tested, Chemical Abstracts Service Registry Number (CASRN), table/figure where results are found
  - Hit, response, response notes
  - Half-maximal activity concentration (AC50, EC50, or IC50), standard error measurement (SEM), units
  - Assay type (tissue or cell culture), tissue of origin, species of origin
  - Species receptor source, receptor information
  - Reference androgen/anti-androgen
  - Number of doses, highest dose tested, units, incubation time
  - Binding assays only: binding affinity, dissociation constant, relative binding affinity (RBA)
  - TA assays only: agonist/antagonist mode, cytotoxicity evaluated, cytotoxicity level observed
  - TA assays only: reporter type, reporter construct, construct was native/transient/stable

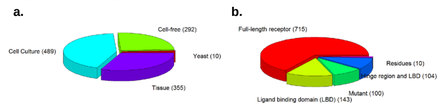
Results

- We analyzed the assay data collected for reproducibility and specificity of results across assay systems and receptor types.
- Analyses were conducted only on data from assays using the full length receptor and the ligand binding domain (957 binding assay experiments on 95 chemicals and 2393 TA assay experiments on 133 chemicals).
- For analysis of chemical-specific results, TA assays were subdivided into modes measuring agonist activity (1447 assays, 60%) and antagonist activity (946 assays, 40%).

Binding Assays

- AR binding data was found for 103 chemicals (1167 experiments from 159 papers).
- The predominant binding assay platform was cell culture (43%), followed by tissue (31%), cell-free (25%), and yeast (1%) (**Figure 1a**).
- Use of full-length receptors (FLR) was most common (66%), followed by receptor ligand-binding domain (LBD) (14%), mutant receptors (9%), hinge region and LBD (10%), and residues 668–919 (1%) (**Figure 1b**).
- Most assays used receptors from human (443 assays, 39%) or rat (372 assays, 33%), 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%), 5α-dihydrotestosterone (DHT; 400 assays, 34%), testosterone (203 assays, 17%), mibolerone (84 assays, 7%).

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

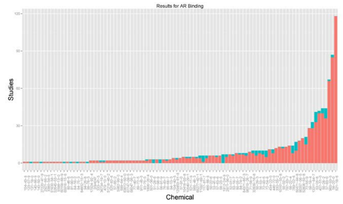


Abbreviation: AR = androgen receptor. Numbers of assays shown in parentheses.

Positive and Negative AR Binding

- Positive and negative AR binding assay results (on FLR and LBD) for 95 chemicals are shown in **Figure 2**.
  - 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 6 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.

Figure 2. AR Binding Assay Results



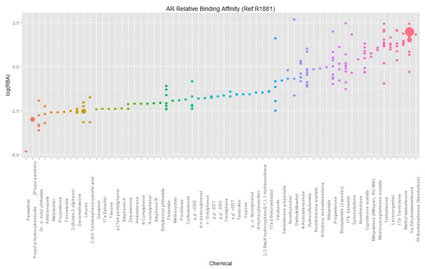
Abbreviation: AR = androgen receptor.

Chemicals are listed along the x-axis; height of bars represents number of positive (red) or negative (blue) assay results for each chemical.

Binding Affinity

- Results for binding affinity were reported in many different formats, the most common being RBA or log RBA with respect to a positive control:
  - R1881 (240 results), DHT (168 results), testosterone (97 results), mibolerone (30 results).
- Results for log RBA on 61 chemicals with respect to the most common positive control compound, R1881, are shown in **Figure 3**.

Figure 3. AR Relative Binding Affinities (Reference: R1881)



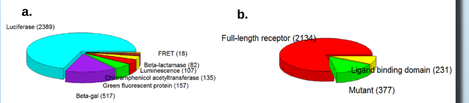
Abbreviations: AR = androgen receptor; R1881 = methyltrienolone; RBA = relative binding affinity.

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

Transactivation Assays

- AR transactivation data was found for 133 chemicals (3444 experiments from 284 papers).
- The reporter types used in TA assays were luciferase (69%), beta-gal (15%), green fluorescent protein (5%), chloramphenicol acetyltransferase (4%), luminescence (3%), beta-lactamase (2%), and FRET (1%) (**Figure 4a**)
- When reported, use of the FLR was most common (77%), followed by mutant receptors (14%), and the LBD (8%) (**Figure 4b**)
- Most assays used a transiently transfected AR (1529 assays, 46%), followed by a stably integrated AR (1309, 39%), and native receptor expression (476, 14%).
- Most TA assays used the human AR (3049 assays, 93%), but receptors from a total of 14 species were represented among all assays in the database.

Figure 4. AR Transactivation: (a) Assay Types and (b) Receptor Types

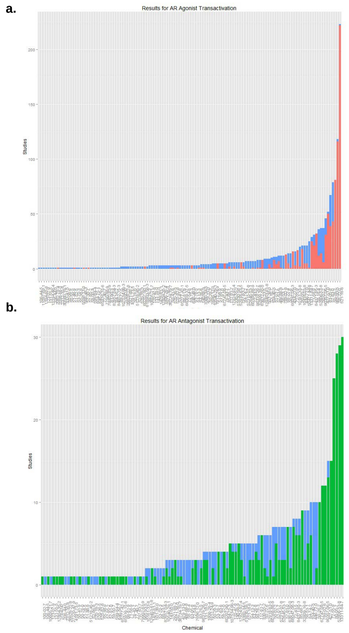


Abbreviation: AR = androgen receptor. Numbers of assays shown in parentheses.

Transactivation Results

- Positive and negative AR TA results for 133 chemicals are shown for agonist **(Figure 5a)** and antagonist **(Figure 5b)** modes.
  - There were 13 chemicals with multiple agonist results (increase in TA) and no antagonist results. Most of these 13 chemicals also had negative results reported, but in most cases the number of agonist reports far outnumbered the number of negative reports. Negative outcomes tended to occur in specific cell types or at very low concentrations.
  - There were 32 chemicals with multiple antagonist results (decrease in TA) and no agonist results. All of these also had negative results that tended to occur in specific cell types 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, though in most of the mixed cases there was a clear majority of either agonist or antagonist results.

Figure 5. AR TA Assay Results for (a) Agonist and (b) Antagonist Mode



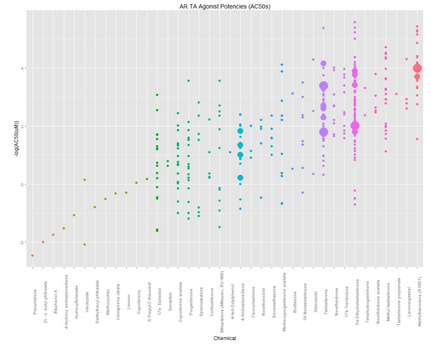
Abbreviations: AR = androgen receptor; TA = transactivation.

Chemicals are listed along the x-axis (differs by graph); height of bars represents the number of positive (agonist [red] or antagonist [green]) and negative (blue) assay results.

Transactivation Agonist Potency

- Positive results for 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%).
- Results were converted to -log micromolar (-log μM) units, and the respective agonist potencies based on AC50 are shown in **Figure 6**.

Figure 6. AR TA Agonist Potencies by AC50

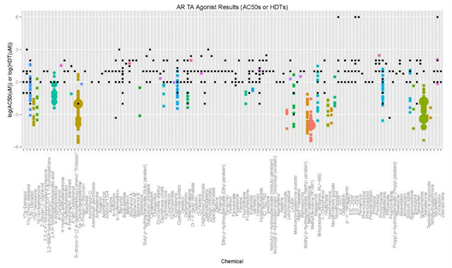


Abbreviations: AC50 = half-maximal activity concentration; AR = androgen receptor; TA = transactivation.

Chemicals are listed along the x-axes and the –log(AC50) along the y-axes. The size of the dot increases with the number of observations.

- To compare all TA agonist results where it was possible to convert into log μM units, negative results were plotted in terms of highest dose tested (HDT) and compared to the AC50 values for each chemical, shown in **Figure 7**.

Figure 7. Comparing AR TA Agonist Positive and Negative Doses



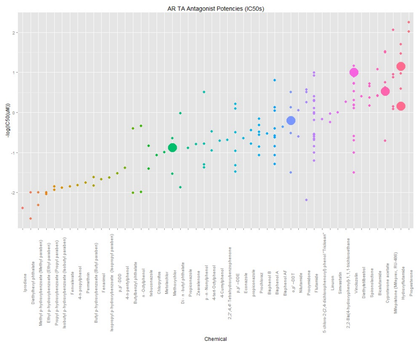
Abbreviations: AC50 = half-maximal activity concentration; AR = androgen receptor; HDT = highest dose tested; TA = transactivation.

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

Transactivation Antagonist Potency

- Analyses for the AR TA antagonist data were further limited to those assays that concurrently measured cytotoxicity (520 assays, 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%).
- Results were converted to -log μM units, and the respective agonist potencies based on IC50 are shown in **Figure 8**.

Figure 8. AR TA Antagonist Potencies by IC50

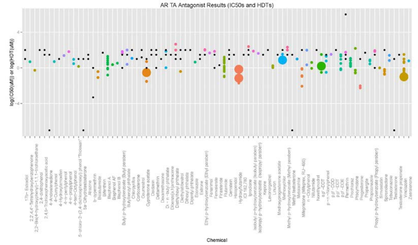


Abbreviations: AR = androgen receptor; IC50 = half-maximal inhibitory concentration; TA = transactivation.

Chemicals are listed along the x-axes and the -log(LEL) or –log(IC50) along the y-axes. The size of the dot increases with the number of observations.

- To compare all TA antagonist results where it was possible to convert into log μM units, negative results were plotted in terms of highest dose tested (HDT) and compared to the IC50 values for each chemical, shown in **Figure 9**.

Figure 9. Comparing AR TA Antagonist Positive and Negative Doses



Abbreviations: AR = androgen receptor; HDT = highest dose tested; IC50 = half-maximal inhibitory concentration; 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 log(IC50), and the black dots represent negative results in log(HDT). The size of the dot increases with the number of observations.

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Conclusions

- The AR in vitro literature review database provides a wide array of binding and transactivation data from many different sources for a range of potential AR agonist and antagonist reference chemicals.
- The data collection effort is ongoing and is occurring in parallel with an EPA/OSCP-led data curation effort focusing on the in vivo Hershberger study.
- After finalization of the results, the in vitro database will be publicly available on the NTP website (http://ntp.niehs.nih.gov/go/40658).
- The ICCVAM AR Reference Chemical Working Group is evaluating these analyses and integrating the results to propose lists of in vitro and in vivo reference chemicals for AR agonism and antagonism that may be used for assay characterization and validation.
- The proposed reference chemical list and associated potency categories will be made available to the public and submitted to OECD via the Validation Management Group–Non-Animal to facilitate the international harmonization of test method evaluations.