Evaluation of two high through-put (HTP) androgen receptor based assays: Utility of data for prioritization for further testing versus prediction of adverse effects.

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ABSTRACT

The androgen signaling pathway plays a critical role in sexual differentiation during development in mammals and is one of the better understood pathways in human development. Thus it was chosen as a model pathway to evaluate the potential of HTP in vitro assays as risk assessment tools. This study examined the interaction of chemicals with the androgen receptor (AR) using *in vitro* cell-based transcriptional activation assays. The chemicals identified as positive agonists or antagonists were then tested in competitive binding assays to confirm receptor interaction. An initial set of about sixty well-characterized compounds with varying affinities for the AR were tested. The in vitro results from these known compounds were compared to available data from in vivo Hershberger assays to evaluate the predictive capacity of the in vitro assays when compared to in vivo results. About fifty unknown chemicals were also tested in vitro and evaluated using the criteria developed. Results for chemicals with known activity in vitro and in vivo indicate that most with ED50s lower than 10-6 M were drugs or natural steroids. The pesticides and toxic substances known to have in vivo effects via the AR fell in the 10-4 to 10-6 M range. In the 10-4 to 10-6 M ED50 range, there was no correlation between in vitro activity and the in vivo potency. An examination of the chemicals in this range indicates that the limitations of the in vitro assays (failure to account for metabolic inactivation, activation, and half-life of a compound.) result in a high rate of "false positives" precluding their use for accurate prediction of in vivo effects. However, since there were no "false negatives" (in vitro versus in vivo) these in vitro receptor assays can be used to prioritize chemicals for additional in vitro or short-term in vivo screening for compounds that act via the AR signaling pathway in an HTP mode. Disclaimer: This abstract does not necessarily reflect EPA policy.

OVERVIEW

Purpose: This extramural contract was designed to test the use of an *in vitro* pre-screening strategy as a method to prioritize chemicals for testing. Compounds were first tested in AR transcriptional activation (TA) assays for both agonist and antagonist activity. Positives were then tested in AR binding assays to confirm interaction with the receptor.

Chemicals:

- Overall, about 125 chemicals were tested in three phases of work.
- Phase 1 - using 17 well-characterized compounds, the contractor established their proficiency with the assays.
- Phase 2 - Fifty compounds were tested. Most (46) were selected from the ICCVAM list of Reference Substances for the Validation of *In Vitro* AR Assays plus four additional compounds of interest. (Note: A total of 58 compounds from the ICCVAM list were tested in either Phases 1 or 2). Phase 1 and 2 results were compared to in vivo results from the literature and used to set criteria for evaluation of Phase 3 unknowns
- In Phase 3 - Fifty-seven unknown compounds were evaluated.

METHODS

Transcriptional Activation (TA) Assays:
Androgen receptor mediated TA - MDA-kb2. These cells have endogenous AR and stably express an androgen-responsive promoter (MMTV) linked to a luciferase reporter gene.

Competitive Binding:
AR Binding - Androgen Receptor-FP protocol (Androgen Receptor Competitor Assay (Invitrogen/Panvera).

Cytotoxicity:
Cell viability in all cell based assays was monitored by propidium iodide (PI) uptake. Additional cytotoxicity assay (ATP assay) was performed for some Phase 3 compounds.

Solubility:
Limit of solubility was determined by a light scattering procedure using Nephelometry (Nepheloskan Ascent by Labsystems).

Luciferase interference:
The two highest concentrations of each compound in Phase 3 were also tested for their ability to directly interfere with the luciferase enzyme itself (i.e. a non- receptor-mediated effect).

Data Analysis
Curve fits and EC50 analyses were conducted using GraphPad Prism software. TA assay results were fit using a non-linear (sigmoidal) fit (variable slope) model with bottom fold induction constrained to 1(1=vehicle control value) and competitive binding was fit using a one-site competition model with bottom and top constrained to 0 and 100% binding, respectively.

LIST OF CHEMICALS TESTED

Compound #/ID	COMPOUND NAME	CAS #	LogEC50 agonism	LogEC50 antagonism	LogEC50 binding	
DHT	Dihydrotestosterone, 5a-androstan-17b-ol-3-one	521-18-6	-9.824		-7.844	
TREN	Trenbolone (17β-Hydroxyestra-4,9,11-trien-3-one)	10161-33-8	-9.808		-7.670	1
R1881	Metribolone (Synonyms:Methyltrienolone, 17ß-Hydroxy-17a-methylestra-4,9,11-trien-3-one	965-63-5	-9.631		-7.873	
35	Methyl testosterone (17α-Methyltestosterone)	58-18-4	-9.231		-7.771	
47	Testosterone	58-22-0	-9.081		-8.149	1
27	Fluoxymestrone	76-43-7	-8.484		-7.672	
34	Medroxyprogesterone acetate (6α-Methyl-17α-hydroxyprogesterone acetate)	71-58-9	-8.089		-7.929	1
36	Mifepristone	84371-65-3	-7.942	-6.388	-7.701	2
39	Norethynodrel	68-23-5	-7.817		-6.976	
17	Dexamethasone	50-02-2	-7.814		-4.846	
AD	Delta-4-androstenedione (4-Androstene-3,17-dione)	63-05-8	-7.031	n/a	-6.113	
46	Spironolactone	52-01-7	-6.704	n/a	-6.873	2
10	Corticosterone	50-22-6	-6.144		-5.638	
14	Cyproterone acetate	427-51-0	-5.933	-6.072	-7.221	2
45	Progesterone	57-83-0	-5.419	-6.216	-6.906	2
2	Anastrazole	120511-73-1				
3	Atrazine (Aatrex)	1912-24-9				
4	Bicalutamide	90357-06-5		-5.948	-6.847	
5	Bisphenol A	80-05-7		-4.945	-5.349	
6	Bisphenol B	77-40-7		-5.033	-5.407	
7	2-sec butylphenol	89-72-5		-6.389	-4.540	
8	5-chloro-2-(2,4-dichlorophenoxul) phenol (Triclosan)	3380-34-5		-5.358	-5.875	3
9	Clomiphene citrate	50-41-9				
11	Coumestrol	479-13-0		-5.293	-5.327	1
12	4-Cumylphenol (phenol, 4-(1-methyl-1-phenylethyl))	599-64-4		-5.069	-5.020	1
13	Cycloheximide	66-81-9		-5.901		1
15	Daidzein	486-66-8				
16	o,p'-DDT	789-02-6		-4.923	-5.442	
18	Dibenzo[a,h]anthracene	53-70-3				
19	Diethylhexyl phthalate [Bis(2-ethylhexyl) phthalate]	117-81-7				
20	Diethylstilbestrol	56-53-1				
21	Dopamine	51-61-6 [62-31-7]				
22	17α-Estradiol	57-91-0		-6.159	-6.092	1
23	Estrone	53-16-7		-5.533	-5.778	
24	Fenarimol	60168-88-9				
25	Flavone	525-82-6		-4.782	-4.375	1,3
26	Fluoranthene	206-44-0		-5.091	-5.186	
28	Flutamide	13311-84-7		-5.633	-5.247	
29	Meso-Hexestrol (Hexestrol)	84-16-2		n/a	-5.557	1
30	Hydroxyflutamide	52806-53-8		-6.856	-6.765	
31	4-Hydroxytamoxifen	68047-06-3				
32	Kaempferol	520-18-3				
33	Kepone (Chlordecone)	143-50-0				
37	4-nonylphenol	84852-15-3		-5.106	-5.300	
38	Nilutamide	63612-50-0		-6.973	-6.093	1
40	Neburon	555-37-3			-4.608	
41	1-octadecanaminium,N,N,N-trimethyl-chloride (Trimethylstearylammonium Chloride)	112-03-8				
42	4-tert-Octylphenol	140-66-9		-5.170	-5.364	1
43	Oxazepam	604-75-1		n/a	-5.709	
44	Procymidone	32809-16-8		-5.703	-5.283	1
48	12-O-Tetradecanoyl-phorbol-13-acetate (Phorbol 12-myristate)	16561-29-8				
49	2,4,5-Trichloro-phenoxyacetic acid	93-76-5				
50	Trimethylborate	121-43-7		n/a	n/a	
51	Zearalenone	17924-92-4		-4.918	-4.680	1,3
DBP	Dibutyl phthalate	84-74-2			n/a	
DDE	p,p'-DDE (4,4'-DDE, p,p'-dichlorodiphenyldichlorethylene)	72-55-9		-5.713	-5.080	
FLUT	Flutamide	13311-84-7		-4.960		
LIN	Linuron	330-55-2		-5.599	-4.922	
PZ	Prochloraz	67747-09-5		-4.907	-4.675	1
VIN	Vinclozolin	50471-44-8		-4.675	-4.525	1

Phase 1
Phase 2
Phase 3

Notes:
1: Follow up work is needed
2: Chemical is either a partial agonist or a mixed agonist and antagonist
3: R^2 value is low for antagonism (#25,51) or binding (#8)

Compound #	COMPOUND NAME	CAS #
1	1,2-benzisothiazoline-3-one	2634-33-5
2	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4
3	4,5-dichloro-2-n-octyl-3(2H)-isothiazolone, (Kathon 930)	64359-81-5
6	6-chloro-n(1,1-dimethylethyl)-n`-ethyl-1,3,5-triazine-2,4-diamine	5915-41-3
7	2,4-imidazolidinedione,-1-(hydroxymethyl)-5,5-dimethyl	116-25-6
10	1-methoxy-4-tert-pentylcyclohexane	??
11	di-ethylphthalate (DEP)	84-66-2
12	di-n-hexylphthalate; (DIETHYL PHTHALATE)	84-75-3
13	ethyl-2-methylbenzoate	87-24-1
14	ethoxyquin	91-53-2
15	4-cyclohexylcyclohexanone	92-68-2
16	tetrahydrofurfuryl alcohol ; (THF)	97-99-4
17	4-tert-butylcyclohexanol	98-52-2
18	N-dodecyltrimethylammonium chloride ; (DTA)	112-00-5
19	methylsalicylate	119-36-8
20	propyl-3,4,5-trihydroxybenzoate; (propyl gallate); (PG)	121-79-9
21	sodium 2-ethylhexylsulfate	126-92-1
22	di-isopropylphthalate ; (IPP)	605-45-8
23	4-tert-butylaniline	769-92-6
24	ethyl-2,4,6-trimethylbenzoate	1754-55-8
25	tert-butylhydroquinone	1948-33-0
26	4-cyclohexylcyclohexanol ; (CXC)	2433-14-9
27	4-tert-amylcyclohexanol	5349-51-9
28	4-n-butylchlorobenzene	15499-27-1
29	2-tert-butyl-4-methoxyphenol (TMOP)	25013-16-5
30	4-n-amylaniline (4-Pentylaniline) ; (AAN)	33228-44-3
31	4-hexylaniline ; (HAL)	33228-45-4
32	4-propylcyclohexanone ; (PCH)	40649-36-3
33	3-methyl-4-chlorophenol; (4-CHLORO-3-METHYLPHENOL)	59-50-7
34	4,4'-(1-methylethylidene)bis[2,6-dibromophenol]	79-94-7
36	benzoic acid, 2-hydroxy-, 4-(1,1-dimethylethyl)phenyl ester; (TBS)	87-18-3
37	phenol, 2,4-bis(1,1-dimethylethyl)-	96-76-4
38	1,3-benzenediol, 4,4'-thiobis- ; (RES)	97-29-0
39	1-decanol, (DECYL ALCOHOL) ; (DL)	112-30-1
40	10-undecenal (Undecylenic aldehyde)	112-45-8
41	phenyl salicylate	118-55-8
42	benzyl salicylate ; (BES)	118-58-1
43	benzaldehyde, 3,4-dimethoxy-	120-14-9
44	phenol, 4-(phenylamino)-, (4-HYDROXYDIPHENYLAMINE)	122-37-2
45	2-naphthol	135-19-3
46	1,3-benzenedicarboxylic acid, diphenyl ester	744-45-6
47	octyl aldehyde; octyltriethoxysilane	2943-75-1
48	Phenol, 4,4'-(9H-fluoren-9-ylidene)bis-	3236-71-3
49	sulcofuron-natrium monohydrate	3567-25-7
50	2-naphthalenol, 6,6'-dithiobis-	6088-51-3
51	2-bromohexadecanoic acid	18263-25-7
52	ethanone, 2,2-dimethoxy-1,2-diphenyl-	24650-42-8
53	cis-3-hexenylsalicylate	65405-77-8
54	peroxide, bis(1-methyl-1-phenylethyl)	80-43-3
55	1,3,5-triazine, 2,4,6-tris(2-propenyloxy)-	101-37-1
56	cyclotrisilazane, 2,2,4,4,6,6-hexamethyl-	1009-93-4
57	benzeneethanol, .beta.-methyl-	1123-85-9
58	methanone, phenyl(2,3,4-trihydroxyphenyl)-	1143-72-2
59	9,12-octadecadienoic acid (z,z)-, methyl ester	112-63-0
60	1-tetradecanamine, n,n-dimethyl-	112-75-4
61	Octanal	124-13-0
62	phenol, 4,4'- 2,2,2-trifluoro-1-(trifluoromethyl)ethylidene bis-	1478-61-1

Note: Omitted numbers were compounds not run due to unavailability

Agonist example

Fluoxymestrone 76-43-7
Compound 27 AR Induction

Fluoxymestrone
AR Agonism
LogEC50 -8.484

Fluoxymestrone
AR binding
LogEC50 -7.672

Antagonist example

Bicalutamide 90357-06-5
Compound 4 AR Induction Antagonism
CeeTox Phase-2 MDA-kb2 assay
(from 11.27, 12.07)

Bicalutamide
AR Antagonism
LogEC50 -5.948

Bicalutamide
AR binding
LogEC50 -6.847

Phase 1 and 2 ARTA and AR binding
(no cycloheximide)
Pearson r = 0.87, R2 = 74%

in vitro ARTA vs in vivo Hershberger Assay results using oral doses mg/kg/d

In vitro ARTA vs in vivo Hershberger Assay results using oral doses mg/kg/d
Excluding steroids and drugs

in vitro ARTA vs in vivo Hershberger Assay results using oral doses mg/kg/d
No steroids or drugs

Number	PHASE 3 PRIORITIZATION	arta	AR binding	comments on cyto	priority	luc
15	4-cyclohexylcyclohexanone	-5.318	-4.207		yes	no
26	4-cyclohexylcyclohexanol (CXC)	-5.1	-4.9		yes	no
27	4-tert-amylcyclohexanol	-4.955	-4.206		yes	no
50	2-naphthalenol, 6,6'-dithiobis-	-4.758	-4.549		yes	no
33	3-methyl-4-chlorophenol; (4-CHLORO-3-METHYLPHENOL)	-4.418	-4.064		yes	no
42	benzyl salicylate ; (BES)	-4.278	-4.138		yes	no
62	phenol, 4,4'- 2,2,2-trifluoro-1-(trifluoromethyl)ethylidene bis-	-5.203	-5.899		yes	yes
44	phenol, 4-(phenylamino)-, (4-HYDROXYDIPHENYLAMINE)	-4.699	-4.541		yes	yes
29	2-tert-butyl-4-methoxyphenol (TMOP)	-4.91	-3.871	mod weak binding	maybe	no
54	peroxide, bis(1-methyl-1-phenylethyl)	-4.792	-3.842	mod weak binding	maybe	no
10	1-methoxy-4-tert-pentylcyclohexane	-4.201	-3.664	weak binding	maybe	no
34	4,4'-(1-methylethylidene)bis[2,6-dibromophenol]	-5.14	-5.547	cytotoxic ARTA cur	maybe	yes
14	ethoxyquin	-4.38	-3.739	weak binding	maybe	yes
53	cis-3-hexenylsalicylate	-4.322	-3.508	weak binding	maybe	yes
45	2-naphthol	-4.248	-3.939	weak binding	maybe	yes
18	N-dodecyltrimethylammonium chloride ; (DTA)	-5.563	-3.2	very cytoxic in ART	no	
37	phenol, 2,4-bis(1,1-dimethylethyl)-	-4.911	nd	very cytoxic in ART	no	
24	ethyl-2,4,6-trimethylbenzoate	-4.799	-3.314	weak binding variab	no	
1	1,2-benzisothiazoline-3-one	-4.75	-3.26	weak AR binding	no	
3	4,5-dichloro-2-n-octyl-3(2H)-isothiazolone, (Kathon 930)	-4.658	.	ARTA concurrent w	no	
2	5-Chloro-2-methyl-4-isothiazolin-3-one	-4.307	-2.551	no binding	no	
22	di-isopropylphthalate ; (IPP)	-4.217	-3.266	no binding	no	
20	propyl-3,4,5-trihydroxybenzoate; (propyl gallate); (PG)	-4.21	only fit with cytoxic dose incl		no	
51	2-bromohexadecanoic acid	-4.19	-4.94	ugly bindin -40% lik	no	
17	4-tert-butylcyclohexanol	-4.154	-3.369	weak binding	no	
49	sulcofuron-natrium monohydrate	-4.055	only fit with cytoxic dose incl		no	

CONCLUSIONS

- The techniques used in this assessment can all be performed in an efficient high (or semi-high) through-put system. In addition, no animal tissues were needed with which to conduct any of the assays.
- Having positive results of more that one in vitro assay adds confidence to the interpretation.
- Additional in vitro assay results (such as KI determination) may aid in further defining equivocal results.
- Results for chemicals from Phase 1 and 2 with known activity in vitro and in vivo indicate that most with ED50s lower than 10-6 M were drugs or natural steroids. The pesticides and toxic substances known to have in vivo effects via the AR fell in the 10-4 to 10-6 M range. Therefore, compounds with ED50's less than 10-4 M would have higher priority for further testing.
- An examination of the chemicals in this range indicates that the limitations of the in vitro assays (failure to account for metabolic inactivation, activation, and half-life of a compound) result in a high rate of "false positives" precluding their use for accurate prediction of in vivo effects.
- However, since there were no "false negatives" (in vitro versus in vivo) these in vitro receptor assays can be used to prioritize chemicals for additional in vitro or short-term in vivo screening for compounds that act via the AR signaling pathway in an HTP mode