Chapter 3

Improved androgen specificity of AR-EcoScreen by CRISPR based glucocorticoid receptor knockout

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Abstract

The AR-EcoScreen is a widely used reporter assay for the detection of androgens and anti-androgens. Endogenous expression of glucocorticoid receptors and their affinity for the androgen responsive element that drives reporter expression, however, makes the reporter cells sensitive to interference by glucocorticoids and less specific for (anti-)androgens. To create a glucocorticoid insensitive derivative of the AR-EcoScreen, CRISPR/Cas9 genome editing was used to develop glucocorticoid receptor knockout mutants by targeting various sites in the glucocorticoid gene. Two mutant cell lines were further characterized and validated against the unmodified AR-EcoScreen with a set of 19 environmentally relevant chemicals and a series of environmental passive sampler extracts with (anti-)androgenic activity. Sequencing of the targeted sites revealed premature stop codons following frame-shift mutations, leading to an absence of functional glucocorticoid receptor expression. The introduced mutations rendered cell lines insensitive to glucocorticoid activation and caused no significant difference in the responsiveness towards (anti-)androgens, compared to the unmodified AR-EcoScreen cells, allowing the selective, GR-independent, determination of (anti-)androgenicity in environmental passive sampler extracts. The increase in selectivity for (anti-)androgens improves reliability of the AR-EcoScreen and will provide higher accuracy in determining (anti-)androgenic potential when applied in toxicity screening and environmental monitoring of both single compounds and mixtures.

Introduction

Androgenicity is an important endocrine disruption endpoint in toxicological and environmental screening. Brominated flame retardants, pesticides, pharmaceuticals, food packaging constituents and various industrial chemicals have previously been identified as (anti-)androgens. Such (anti-)androgens are present in the environment as (persistent) pollutants, mainly due to the release of particles or leachate from consumer products, runoff water from agricultural activity or urban or industrial wastewater. Exposure can result from contact with house dust, consuming contaminated food or drink or via air. Xenobiotic (anti-)androgens can potentially disrupt signaling of endogenous androgens in both humans and wild-life. The ability to selectively detect (anti-)androgenic potential is essential for
accurate characterization of the endocrine disruptive potency of individual compounds and complex mixtures as found in environmental samples.

The AR-EcoScreen is a cell-based reporter assay for the detection of (anti-)androgenic activity. The assay, developed in 2004\(^4\), uses Chinese hamster ovary (CHO) cells, stably transfected with a human androgen receptor (hAR) and a 6x androgen response element (ARE) (5\(^\prime\)-AGTACGnnnTGTTCT-3\(^\prime\)) from the C3 gene\(^{14}\) regulating expression of a luciferase reporter gene. Luciferase is expressed in a dose-dependent manner and its resulting activity can be measured quantitatively by a luminometer after a 24 hour exposure period. In contrast to a number of other cell-based AR reporter assays, the CHO cells used in the AR-EcoScreen lack metabolizing capacity for steroid hormones that might otherwise eliminate androgenic potency of tested compounds\(^4\). Recently, an OECD guideline has been established for application of the AR-EcoScreen in the testing of chemicals (Test No. 458, 2016)\(^{15}\).

The AR luciferase reporter system shows high sensitivity towards androgens. However, luciferase can also be induced by glucocorticoid receptors (GR) activated by glucocorticoids (GCC) like cortisol or the synthetic GCC dexamethasone\(^{16}\). The GR, endogenously expressed in CHO cells from the NR3C1 gene\(^{17}\), shares homology with the AR and cross-talk between the two nuclear hormone receptors is known\(^{18}\). The DNA binding domain (DBD) is well conserved between the AR and GR\(^{19,20}\). As a result the DNA binding sites to which the activated receptors bind share similarities. Consequently, the GR has affinity for the ARE which causes a non-AR-specific reporter activation by GCCs, which may lead to misclassification of compounds as being androgenic when luciferase is actually induced through activated GRs in the AR-EcoScreen. Likewise, the androgenic potency of environmental samples can be overestimated if GCCs are present. By preventing the expression of functional GR proteins, through introduction of mutations on the GR gene in the CHO genome, responsiveness to GCCs can be reduced to undetectable levels. Various genome editing methods have been developed for introducing mutations with the most recent being the CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9 editing\(^{21}\).

The CRISPR/Cas9 technique is a novel, scarless genome editing technique that can introduce mutations at a specific location without leaving remnants of the editing machinery. CRISPR genome editing was adapted from a prokaryotic immune system
which defends against viruses by inducing double stranded breaks (DSBs) at specific sites on viral DNA. The principle behind this immune system is that a single-guide RNA (sgRNA), which is complementary to the viral DNA sequence, associates with Cas9 nuclease. The sgRNA is expressed from viral DNA sequences incorporated into the prokaryote’s own genome following previous encounters with the virus. The sgRNA subsequently directs the Cas9 nuclease towards its complementary site on the viral DNA where Cas9 nuclease activity will create a DSB. Binding of Cas9 to DNA requires the presence of an NGG protospacer adjacent motif (PAM) sequence, which is located three nucleotides downstream of the position where the DSB will occur.

In mammalian cells, this system can be used to induce DSBs at very specific sites in order to induce mutations or allow insertion of DNA. The CRISPR/Cas9 machinery is expressed from plasmid DNA after transient transfection. Ligation of a target sequence into the plasmid guides the CRISPR/Cas9 complex to the specific location of the operator’s choice to introduce a dsDNA break. This break can be repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR). HDR is used to knock-in genes or DNA sequences with 3’- and 5’-end sequences homologous to sequences found at either side of the break. NHEJ, however, is an error-prone DNA repair mechanism which can introduce random mutations during imprecise repair of the break site. Deletion or insertion mutations in exons can cause disruptive frame-shifts that lead to non-functional proteins or truncated/extended proteins with altered activity. In-frame mutations, like single nucleotide polymorphisms (SNPs), can lead to changes in the protein sequence and alter activity in a more subtle manner.

To develop a mutant AR-EcoScreen cell line with functional knockout of the GR (i.e. lacking GCC responsiveness), the CRISPR/Cas9 genome editing technique was used to introduce either SNPs or knockout mutations in the NR3C1 (GR) gene. Five SNPs in the NR3C1 gene related to a human or in vitro GCC resistant phenotype were selected at residues that shared homology between human and Chinese hamster. The mutations, which were targeted at different functional domains, were analyzed by sequencing the targeted site. Mutant clones were screened for GCC responsiveness and selected mutant cell lines were validated against the unmodified AR-EcoScreen cells by comparing their response to (1) a selection of (anti-)androgens and glucocorticoid dexamethasone described in literature, (2) a mixture of anti-androgens, and (3) a collection of extracts from passive samplers deployed at river water and...
wastewater treatment plant (WWTP) effluent water. The responses were analyzed for significant differences between the cell lines.

The obtained functional GR knockout in the well-established AR-EcoScreen provides more selective determination of (anti-)androgenic potency of compounds and (environmental) mixtures while maintaining the sensitivity for (anti-)androgenic compounds of the original AR-EcoScreen. When applied in toxicological screening, compounds can be accurately classified for their androgenic potential, while the concurrent presence of GCCs in (environmental) mixtures will not lead to an over- or underestimation of the androgenic potency.

**Materials and methods**

**Materials**

DMEM/F12 with glutamax medium, DMEM/F12, phenol-free, L-glutamine medium and fetal bovine serum were obtained from Gibco (Eggenstein, Germany). Penicillin/streptomycin, hygromycin, zeocin, ampicillin, ATP, co-enzyme A and DNA oligonucleotides were obtained from Sigma (Zwijndrecht, The Netherlands). Luciferin was obtained from Promega (Fitchburg, WI, USA). All restriction enzymes and Proteinase-K were obtained from New England Biolabs (Ipswich, MA, USA). All plasmid isolations were performed using a Plasmid DNA Miniprep kit (Invitrogen, Carlsbad, CA) and all PCR-clean ups were performed with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Lipofectamine 2000 was obtained from Life technologies (Waltham, MA, USA). (Anti-)androgens used in validation of the cell lines were obtained from various suppliers (see Supporting Information Table S1) and were dissolved in DMSO (Acros, Geel, Belgium). The pSpCas9(BB)-2A-GFP (PX458) plasmid was a kind gift from Dr. Feng Zhang (Addgene plasmid # 48138).

**CRISPR/Cas9 plasmid construction**

Five different CRISPR/Cas9 plasmids were constructed by introducing sgRNA coding sequences to the pSpCas9(BB)-2A-GFP (PX458) plasmid. Target sequences were designed against the Chinese hamster genome (RefSeq Assembly ID GCF_000223135.1). The dsDNA coding the sgRNA was formed by annealing single stranded DNA sequences (see Supporting Information Table S2) at equimolar
concentrations in annealing buffer (10 mM TRIS (pH 7.5-8.0), 50 mM NaCl and 1 mM EDTA). The reaction was performed in a PCR Thermocycler with a linear temperature gradient from 95°C to 4°C over a period of 2.5 hours. The resulting fragment with overhanging ends was ligated to the BbsI restriction product of the PX458 plasmid in a 3:1 ratio. Clones of successfully transformed E. coli (DH5α) cells (Invitrogen, Carlsbad, CA, USA) carrying the targeting sequence containing PX458 plasmid were selected by ampicillin resistance and the correct insert was validated by PCR (see Supporting Information Table S2).

Transfection and cell isolation
AR-EcoScreen cells were maintained as described by Satoh et al.14. Eighteen hours prior to transfection, 70-90% confluent cells were trypsinized, diluted in DMEM/F12 culture medium (with 10% fetal bovine serum and 1% penicillin/streptomycin) to 200,000 cells/ml and seeded in 200 µL aliquots on 24-well plates. In the morning, 1 µg of the CRISPR/Cas9 plasmid with target sequence insert was mixed with 4 µl Lipofectamine-2000 and incubated from 20 to a maximum of 30 minutes to form plasmid-carrying liposomes. The mixture was added to the seeded AR-EcoScreen cells which were incubated at 37°C and 5% CO2. After 6 hours, the medium was changed with 500 µL fresh medium. After 24 hours, cells were trypsinized and transferred to polypropylene round bottom tubes and applied to fluorescent-activated cell sorting (FACS) for sorting of successfully transfected cells (based on expressed GFP). Transfected cells were then collected under semi-sterile conditions, in separate wells on a 96-well plates, filled with culture medium. Individual mutant cells were left to grow to form a culture while medium was refreshed every other day. After 10 days, the cells from each individual mutant culture were trypsinized, seeded and maintained on 48-well plates for further testing.

Confirmation of mutation by sequencing
Cells were prepared for DNA isolation by centrifugation of trypsinized and resuspended cells for 5 minutes at 3000 rpm. Pelleted cells were washed in 50 mM Tris HCL (pH 8.8), taken up in 100 µL 5 mM Tris HCL (pH 8.8), boiled for 10 minutes and rapidly cooled on ice. The lysed cell material was incubated for 1 hour at 37°C with 1 mg/ml Proteinase-K to digest DNA associated proteins. The reaction was boiled for 10 minutes to inactivate Proteinase-K, cooled on ice and the resulting lysate was used in PCR amplification. Sequences from the mutation sites were amplified by PCR in 20 µL
reactions (1 mM MgCl₂, T_a 55°C) introducing a M13 tag for amplification during sequencing (see Supporting Information Table S2) in the process and correct fragment size (~300 base pair (bp)) was checked by gel electrophoresis. Amplified fragments were purified by gel filtration over Sephadex G-50 medium (GE Healthcare Life Sciences, Piscataway, NJ, USA). Sephadex resin was applied to a multiscreen 0.45 µm low protein binding filter plate (Millipore, Billerica, MA, USA). The column material was conditioned with 300 µL MQ water for 2 hours, centrifuged for 5 minutes at 910 g and washed two times with 150 µL water. The PCR reaction products were brought to a volume of 32 µL and applied to the columns. The purified reaction products were collected by centrifuge and sent for sequencing (Baseclear, Leiden, The Netherlands). Resulting sequence data of mutants and wild-type were compared to the Chinese hamster genome retrieved from the NCBI Genbank database (taxonomy ID: 10029 RefSeq Assembly ID GCF_000223135.1).

Sample preparation
Adsorption-based Speedisk (SD), containing a styrene divinylbenzene sorbent with affinity for polar compounds, and partitioning-based silicone rubber (SR) passive samplers, with affinity for hydrophobic compounds, were deployed for a six week period between August and October 2014 in the river Meuse at Eijsden, in the Rhine-Meuse estuary at Hollands Diep (Bovensluis), and in the effluent stream of two wastewater treatment plants (WWTP) in the Netherlands. Briefly, sample preparation consisted of dichloromethane extraction of SD and hexane extraction of SR samplers. SD and SR extracts were solvent-exchanged from dichloromethane and hexane, respectively, into DMSO in final concentrations of 14 SD/mL or 100 g SR/mL, respectively.

Reporter assay protocol
Cells were seeded (at 200,000 cells/ml) in 34 µL cell suspension aliquots per well in 384-well plates. Plates were incubated for 24 hours at 37°C and 5% CO₂. In each experiment (n=1), seeded cells were exposed at t=0 in triplicate to compounds or extracts dissolved in 34 µL phenol-free DMEM/F12 L-glutamine medium at a final DMSO concentration of 1 µL/mL. In antagonism experiments, cells were additionally exposed to compounds or extracts in the presence of 200 pM DHT. Cytotoxicity was measured in the exposed cells by adding resazurin dissolved in PBS at t=22 h leading to a final concentration of 21 nM and measuring fluorescence (λ_ex=530 nm; λ_em=590 nm).
Conversion rate of resazurin into resorufin in cells exposed to test substances was compared to the conversion rate in cells exposed to vehicle (DMSO). At t=24h, medium was aspirated and cells were lysed for 10 minutes on an orbital shaker at 700 rpm with 17 µL lysis mix (25 mM TRIS (pH 7.8), 2 mM DDT, 2 mM 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA), 10% Glycerol and 1% Triton-X100). Luminescence was measured on a Varioskan luminometer (Thermo Fisher Scientific, Waltham, MA, USA) after injection of 34 µL of glow-mix (2 mM Trycin, 1.07 mM C4H2Mg5O14, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 270 mM Co-enzyme A, 470 mM Luciferin, and 530 mM ATP) for one second followed by quenching of the reaction with 34 µL 0.1 M NaOH. GCC insensitive cells that retained androgen responsiveness were selected by exposing transfected cells seeded on 384-well plates to either 100 nM dexamethasone or 200 pM DHT and measuring the luciferase response. Selected GR knockout mutant and AR-EcoScreen cell lines were then exposed to serial dilutions of (anti-) androgens and glucocorticoid dexamethasone (see Supporting Information Table S1), an equipotent mixture consisting of six anti-androgens (i.e. BDE-19, estriol, PCB-125, PCB-168, permethrin and vinclozolin at their respective PC50 concentrations, at which luciferase induction corresponds to 50% of the induction by reference AR-antagonist flutamide, calculated from previously collected (historical) data in the unmodified AR-EcoScreen (see Supporting Information Table S3)), and passive sampler extracts to compare responsiveness to androgens.

Data and statistical analysis
Data was analyzed in Prism 5.04 (Graphpad Software Inc, San Diego, CA). For each data set, D’Agostino-Pearson test was used to test data normality, and Levene’s test to test homogeneity of variance (significance P < 0.05). Dose response curves were fitted with the Hill-equation \[Y=A+(B-A)/(1+(x/C)^D)\], where A and B denote minimal and maximal response respectively, C is the EC50 or IC50, D is the hillslope and x represents the tested concentration. Significant differences (P < 0.05) between the responses of the GR knockout mutants and unmodified AR-EcoScreen cell line was determined by performing an F-test on fitted curves based on the EC50/IC50 and hillslope parameters. Additionally, response towards a mixture of anti-androgens was analyzed after converting exposure concentrations to toxic units (TU). TUs were calculated as the ratio of the concentration of an individual compound or mixture at
which the response equals that of the EC50 response of AR-antagonist flutamide (PC50) versus the actual exposure concentration. The total TU of the mixture, as present at the highest exposure concentration, was expressed as the sum TU of the individual compounds. A serial dilution curve of the mixture was converted to TU and the responses between the cell lines were compared with the F-test.

**Results**

**AR-EcoScreen mutants exhibited insensitivity towards glucocorticoid dexamethasone**

Five target sequences in the NR3C1 gene on the Chinese hamster 773 AA X1 isoform GR (NCBI accession XM_016975585.1; XP_016831074.1) were selected for NHEJ-based editing by CRISPR/Cas9 (Table 1). Cleavage was directed at nucleotides homologous to SNP positions on the human 777AA GR alpha (NCBI accession NM_000176.2; NP_000167.1) (which shares 90% homology with the Chinese hamster X1 isoform GR) or polymorphisms generated *in vitro* associated with GCC-resistance.

Three homologous amino acid residues were located within the ligand binding domain (LBD), one in the DNA binding domain (DBD) and one in the N-terminal domain (NTD). Selection of target sites was limited by the required presence of a PAM sequence (NGG) three bp upstream of the position where a DSB was intended to occur. sgRNA sequences were aligned with NCBI BLAST against the full Chinese hamster (taxonomy ID: 10029 RefSeq Assembly ID GCF_000223135.1) genome and indicated no off-target binding site. Initial testing of clones focused on determining their responsiveness to androgens and GCC dexamethasone (Fig. 1). Responsiveness towards the androgen DHT in the mutant cells was equal or greater compared to the response in the unmodified AR-EcoScreen cells (panel A). Induced mutations at four of the five sites resulted in total loss of GCC responsiveness (Panel B) while mutant 3 showed no loss of GCC responsiveness (data not shown). Two mutants with the highest reporter induction (M1 and M2) were selected for further evaluation of (anti-)androgen and glucocorticoid responsiveness.
Table 1. Polymorphisms linked to GCC resistance in humans or human cell models and the resulting amino acid (AA) change in human

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AA change</th>
<th>nt change</th>
<th>Effect</th>
<th>Domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>N363S</td>
<td>1220 A&gt;G</td>
<td>Altered transactivation</td>
<td>NTD</td>
<td>24</td>
</tr>
<tr>
<td>M2</td>
<td>C421Y</td>
<td>1394 G&gt;A</td>
<td>No transactivation</td>
<td>DBD</td>
<td>25</td>
</tr>
<tr>
<td>M3</td>
<td>G567A</td>
<td>1832 G&gt;C</td>
<td>No ligand binding</td>
<td>LBD</td>
<td>26</td>
</tr>
<tr>
<td>M4</td>
<td>N766N</td>
<td>2430 T&gt;C</td>
<td>Untested</td>
<td>LBD</td>
<td>27</td>
</tr>
<tr>
<td>M5</td>
<td>Q710x</td>
<td>2260 C&gt;T</td>
<td>67-bp truncation of mRNA</td>
<td>LBD</td>
<td>28</td>
</tr>
</tbody>
</table>

Nucleotides (nt) in the Chinese hamster GR mRNA sequence corresponding with the targeted polymorphisms in human GR mRNA sequence linked to the AA residue altered by the polymorphism could be targeted by CRISPR/Cas9 modification due to proximity of PAM sequence used to direct endonuclease activity of Cas9. Described polymorphisms affected protein function at the ligand binding domain (LBD), N-terminal domain (NTD) or DNA binding domain (DBD).

Figure 1. Response of the unmodified AR-EcoScreen and mutant cells to androgen DHT (panel A) and glucocorticoid dexamethasone (panel B). The response to DHT was not significantly different (F-test) between AR-EcoScreen and mutant cells (n=1). While AR-EcoScreen cells responded to dexamethasone, mutant cells M1, M2, M4, and M5 did not.

Sequencing of mutation sites in two mutant strains

Sequence data of 200-bp target sequences was collected for mutant 1 and mutant 2. Although successful generation of a GCC insensitive phenotype was achieved, the mutants appeared not to possess the intended SNP (Fig. 2). Furthermore no base-pair substitutions or in-frame insertion or excision of codons were observed that could lead to alternate SNPs that influence functionality of the receptor. The target sites of mutant 1 and mutant 2 contained a deletion and insertion, respectively, which caused a frame-shift mutation (Fig. 2), leading to a premature stop codon in both mutants (in green).
**Mutant 1 (NTD Nucleotide position 1220)**  
AR-EcoScreen: CCTGTTGGTTCTGAAAAACTGGAATAAGG  
Mutant: CCTGTTGGTT......AACTGGAATAAGG

**Mutant 2 (DBD nucleotide position 1394)**  
AR-EcoScreen: CCCAAACTCT.GCCTGGTGCTCTGATGA  
Mutant: CCCAAACTCTTGCCCTGGTGCTCTGATGA

Figure 2. Sequences at the targeted NR3C1 gene in AR-EcoScreen and mutant 1 and 2 with the modified sequence in red and the intended nucleotide to be changed (see Table I) in yellow. In mutant 1 a five base-pair deletion and in mutant 2 a one base-pair insertion occurred leading to premature stop codons (in green). The PAM sequence (NGG), three bp upstream of the target, used to coordinate endonuclease activity is underlined. The only PAM site available in mutant 1, in contrast to the PAM site of mutant 2, was located two bp upstream of the intended position.

**Validation of responsiveness to (anti-)androgens and glucocorticoid dexamethasone**

To investigate alteration in the (anti-)androgen reporter system response as a result from GR knockout, the response to a set of (anti-)androgens and glucocorticoid dexamethasone (see supplementary table S1) and a mixture of anti-androgens was compared between the unmodified AR-EcoScreen and the two GR knockout mutants. The set consisted of the reference compounds DHT and flutamide as agonist and antagonist respectively, 13 antagonists, 3 partial agonists and GCC dexamethasone. All 19 compounds produced dose-response curves which could be compared between the cell lines (Fig. 3). Cytotoxicity was only observed with bisphenol A and p,p’-DDE at concentrations above 30 µM (data not shown). Responses of the unmodified AR-EcoScreen and mutant cell lines to 18 of the 19 compounds were not significantly different (F-test) and dose-response curves from all three cell lines could be fitted with the same parameters (Table 2 and Fig. 3). Dose-response curves of the GCC dexamethasone, however, differed between cell lines. The response of dexamethasone (in the presence of 200 pM DHT) in the unmodified AR-EcoScreen was agonistic at low concentrations and antagonistic at concentrations above, approximately, 300 nM while the response from the two GR knockout mutants was exclusively antagonistic. Although significantly different from the response of the unmodified AR-EcoScreen, the responses from the two GR knockout mutants did not differ significantly (p=0.8366).
Figure 3. Dose-response curves of 19 compounds measured with AR-EcoScreen, mutant 1 and mutant 2. Measurements were performed either at n=4 (BDE-19, DHT, estriol, flutamide, PCB-125, PCB-168, permethrin and vinclozolin) with errors bars representing the SEM or n=1 (other compounds). (Continued on next page).
Figure 3. (continued).
The responses towards 18 out of 19 AR-(ant)agonists were not significantly different (F-test) in the unmodified AR-EcoScreen and two mutant cell lines and responses of all three cell lines could be fitted with the same (shared) parameters. Measurements were performed either at n=4 (BDE-19, DHT, estriol, flutamide, PCB-125, PCB-168, permethrin and vinclozolin), with results expressed as the IC50 or EC50 ± standard error, or n=1 (other compounds) with results expressed as IC50 or EC50. a Values determined based on mutant 1 and mutant 2 responses.

Dose-response curves of the unmodified AR-EcoScreen and two GR knockout mutants exposed to an equitopent mixture (based on historical PC50 values measured in the unmodified AR-EcoScreen) (see Supporting Information Table S3) consisting of six AR-antagonists (i.e. BDE-19, estriol, PCB-125, PCB-168, permethrin and vinclozolin) were analyzed by F-test to determine comparability between the cell lines when analyzing mixtures (Fig. 4). No significant difference was found between unmodified AR-EcoScreen and two mutant cell lines (F-test P-value 0.9603). Calculated toxic units at the IC50 concentration of flutamide were 0.98 (95% CI: 0.83-1.13 TU), 1.01 (95% CI: 0.88-1.15 TU) and 0.91 (95% CI: 0.87-0.95 TU) in the AR-EcoScreen, mutant 1 and mutant 2 cells respectively. Out of the two selected mutants, AR-antagonistic response of mutant 1 corresponded best with the unmodified AR-

### Table 2. EC50 and IC50 values of the responses in the unmodified AR-EcoScreen and two GR knockout mutant cell lines towards 19 AR-agonists and antagonists

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity</th>
<th>AR-EcoScreen</th>
<th>IC50/EC50 (M) p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-DHT</td>
<td>Agonist</td>
<td>2.51±0.14 X 10^-10</td>
<td>1.07±0.07 X 10^-7</td>
</tr>
<tr>
<td>BDE-19</td>
<td>Antagonist</td>
<td>1.31±0.21 X 10^-7</td>
<td>9.24±1.24 X 10^-8</td>
</tr>
<tr>
<td>BDE-100</td>
<td>Antagonist</td>
<td>2.82±0.41 X 10^-10</td>
<td>8.10±0.12 X 10^-7</td>
</tr>
<tr>
<td>Benzoantrone</td>
<td>Partial agonist</td>
<td>1.97±0.11 X 10^-6</td>
<td>3.14±0.12 X 10^-6</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>Antagonist</td>
<td>1.72±0.10 X 10^-6</td>
<td>2.89±0.10 X 10^-6</td>
</tr>
<tr>
<td>Cyprotilin</td>
<td>Partial agonist</td>
<td>1.40±0.10 X 10^-6</td>
<td>8.10±0.12 X 10^-7</td>
</tr>
<tr>
<td>DB(a,h)A</td>
<td>Partial agonist</td>
<td>1.31±0.11 X 10^-6</td>
<td>7.71±0.10 X 10^-7</td>
</tr>
<tr>
<td>Estriol (E3)</td>
<td>Antagonist</td>
<td>1.50±0.15 X 10^-6</td>
<td>1.63±0.08 X 10^-6</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Antagonist</td>
<td>1.02±0.27 X 10^-6</td>
<td>1.05±0.10 X 10^-7</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Antagonist</td>
<td>1.53±0.10 X 10^-6</td>
<td>1.33±0.10 X 10^-9</td>
</tr>
<tr>
<td>PCB-19</td>
<td>Antagonist</td>
<td>3.14±0.10 X 10^-6</td>
<td>2.89±0.10 X 10^-6</td>
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<tr>
<td>PCB-122</td>
<td>Antagonist</td>
<td>9.07±0.15 X 10^-7</td>
<td>7.09±0.10 X 10^-7</td>
</tr>
<tr>
<td>PCB-125</td>
<td>Antagonist</td>
<td>4.13±1.22 X 10^-7</td>
<td>6.80±1.10 X 10^-7</td>
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<tr>
<td>PCB-128</td>
<td>Antagonist</td>
<td>8.10±0.10 X 10^-7</td>
<td>7.93±0.10 X 10^-7</td>
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<tr>
<td>PCB-168</td>
<td>Antagonist</td>
<td>3.74±1.20 X 10^-7</td>
<td>4.72±1.10 X 10^-7</td>
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<tr>
<td>Permethrin</td>
<td>Antagonist</td>
<td>8.02±1.07 X 10^-6</td>
<td>8.60±0.10 X 10^-6</td>
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<td>p,p’-DDE</td>
<td>Antagonist</td>
<td>1.17±0.10 X 10^-6</td>
<td>1.16±0.10 X 10^-6</td>
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<tr>
<td>Progestosterone</td>
<td>Partial antagonist</td>
<td>3.62±0.10 X 10^-8</td>
<td>3.17±0.10 X 10^-8</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>Antagonist</td>
<td>1.07±0.12 X 10^-7</td>
<td>1.16±0.07 X 10^-7</td>
</tr>
</tbody>
</table>

The responses towards 18 out of 19 AR-(ant)agonists were not significantly different (F-test) in the unmodified AR-EcoScreen and two mutant cell lines and responses of all three cell lines could be fitted with the same (shared) parameters. Measurements were performed either at n=4 (BDE-19, DHT, estriol, flutamide, PCB-125, PCB-168, permethrin and vinclozolin), with results expressed as the IC50 or EC50 ± standard error, or n=1 (other compounds) with results expressed as IC50 or EC50. a Values determined based on mutant 1 and mutant 2 responses.
EcoScreen (p=0.6866). For this reason, mutant 1 was further selected to test passive sampler extracts.

**Antagonistic mixture**

![Graph showing dose-response curves for AR-EcoScreen, Mutant 1, and Mutant 2](image)

**Figure 4.** Dose-response curves of the unmodified AR-EcoScreen and two GR knockout mutant cells in response to a mixture of six anti-androgenic compounds (i.e. BDE-19, estriol, PCB-125, PCB-168, permethrin and vinclozolin) in the presence of 200 pM DHT (n=3). No significant difference was observed when dose-response curves of all three cell lines combined (p=0.0646) or only the unmodified AR-EcoScreen and mutant 1 (p=0.6866) were compared by F-test based on the EC50 and Hill-slope parameters.

**Application to environmental passive sampler extracts**

The functionality of the GR knockout was demonstrated by comparing the responses in the unmodified AR-EcoScreen and GR knockout mutant 1 to extracts from 8 passive samplers that had been deployed for six weeks in river water or WWTP effluent (Fig. 5). While increased luciferase activity (normalized against reference androgen DHT) was observed for all samples in all three cell lines, the GR knockout mutant reported lower activity in a number of samples. Antagonism, which was observed in some SR extracts, was stronger in the GR knockout mutant. Both the weaker agonistic and stronger antagonistic responses in the mutant cell lines indicate an overestimation of AR-agonism and an underestimation of AR-antagonism when extracts are analyzed in the unmodified AR-EcoScreen.
Figure 5. Agonistic response (compared to the maximal DHT induction) (panel A) and antagonistic response (compared to the maximal flutamide induction) (panel B) of the unmodified AR-EcoScreen cells and GR knockout mutant 1 (n=1) to Speedisk and silicone rubber passive sampler extracts at specific dilutions (indicated in brackets in figure legend) to avoid cytotoxicity (data not shown). A lowered agonistic response in mutant cells compared to the AR-EcoScreen cells indicates the presence of GCCs which activate luciferase transcription through the GR. A lowered anti-androgenic response (measured in the presence of 200 pM DHT) in mutant cells compared to AR-EcoScreen cells indicates a masking effect of anti-androgenicity by the presence of GCCs.
Discussion

Mutations in NR3C1 gene eliminate glucocorticoid agonism

Through induction of mutations in the NR3C1 (GR) gene of AR-EcoScreen (CHO) cell line, we developed an AR-EcoScreen reporter cell line lacking GCC responsiveness. As a result of homology between the AR DBD and GR DBD, endogenously expressed GR in CHO cells activated by GCCs can induce expression of luciferase under ARE control due to low affinity for the C3 ARE sequence (5'-AGTACGtgaTGTTCT-3')\textsuperscript{19,20}. We demonstrated that CRISPR/Cas9 genome editing induced mutations at four out of five targeted sites within functional domains, resulting in a GCC insensitive reporter system. As a result, the luciferase reporter now specifically reports (ant)agonism on the AR. This allowed to measure the effect of GCCs specifically on the AR. Exposure of mutants to dexamethasone in the presence of 200 pM DHT revealed AR-antagonistic activity (IC\textsubscript{50} 13 nM) (Table 2 and Fig. 3). This confirms the AR-antagonistic potency of dexamethasone (IC\textsubscript{50} 190±100 µM) earlier observed in a ligand binding assay\textsuperscript{29}.

Sequencing of the target regions revealed frame-shift mutations rather than SNPs in GCC insensitive mutants (Fig. 2). SNPs were also not observed in any of the other sequenced clones (data not shown). This may be explained by the random nature of insertions and deletions during NHEJ which can involve changes of multiple base pairs. This makes the chance of a single base pair being replaced relatively small. In addition, minor changes in the target sequence (e.g. single base-pair substitutions) may allow binding of the sgRNA (at reduced affinity) and lead to subsequent induction of DSBs and mutations until the target site is unrecognizable to the sgRNA. In the validated mutant cell lines (mutant 1 and mutant 2), frame-shift mutations resulted in the generation of premature stop codons (Fig. 2). As a consequence, the observed GCC resistant phenotype may be the result of either the nonsense-mediated decay of the transcribed (partial) mRNA\textsuperscript{30} or the lack of functionality of the translated but truncated peptide. Truncated variants of hormone receptors may retain (partial) activity or mediate the activity of the full-length receptors as was observed for the estrogen receptor\textsuperscript{31} and an AR variant missing the LBD\textsuperscript{32}. A truncated human GR (22-525), lacking the LBD, showed constitutive localization to the nucleus and induction of target gene expression\textsuperscript{33}. GR mutations targeted at N363S (NTD) and C421Y (DBD), in
mutant 1 and mutant 2 respectively, leave the AF1 domain (77-262)\textsuperscript{34}, which was identified as the mediator of constitutive activity in LBD truncated AR\textsuperscript{35,36}, intact. Loss of the DBD in both mutant 1 and 2, however, eliminates potential of DNA binding and subsequent induction of luciferase in the AR-EcoScreen altogether. No constitutive activity was observed in the current study in any of the tested mutant clones (data not shown).

**Validation of GR knockout mutants with (anti-)androgens**

Conserved homology between the AR and GR allows dimerization and interaction with common coactivator proteins\textsuperscript{18,37}. The formation of AR/GR heterodimers, however, has been linked to inhibition of either receptor marked by a reduction in transcriptional activity of their respective target genes\textsuperscript{38}. Furthermore, cytosolic, but mainly plasma membrane bound GRs can exert non-genomic effects through modulation of signal transduction cascades in target tissue\textsuperscript{39}. As a consequence of the interaction between AR and GR and the involvement of the GR in cellular processes, knockout of the GR could disrupt cell homeostasis or AR function and negatively impact responsiveness to (anti-)androgens. Therefore, AR-EcoScreen and mutant cell lines were compared based on their response to 19 (anti-)androgenic compounds described in literature and to a mixture of six anti-androgens. Described androgenic potency could be reproduced and no significant difference in responsiveness towards 18 of 19 compounds (Table 2 and Fig. 3) or the mixture (Fig. 4) was observed between the three cell lines. Responses were significantly different for GCC dexamethasone (Table 2 and Fig. 3). The response to dexamethasone, while partially agonistic in the unmodified AR-EcoScreen, was exclusively antagonistic in the GR knockout mutants revealing anti-androgenic activity by dexamethasone which had not earlier been described. Similarly, (anti-)androgenicity of other environmentally relevant GR activators may be revealed using the GR knockout mutants. In a study by Schriks et al.\textsuperscript{40}, GCCs and their derivatives were identified as the main contributors to glucocorticogenic activity in wastewater samples. The identified GCCs provide interesting candidates for further investigation in the GR knockout mutants and, belonging to the same chemical class, may exhibit anti-androgenic activity similar to dexamethasone. GR agonism was also observed for the AR-antagonist bisphenol A by Sargis et al.\textsuperscript{41} and for the partial AR-agonist progesterone by Attardi et al.\textsuperscript{42}, however, EC50s in the current study were not lower in the unmodified AR-EcoScreen compared to the mutant cell lines (Table 2 and
Fig. 3). Progesterone concentrations similar to those used in the current study (3-3000 nM) induced 30-50% of the maximum response of dexamethasone. The partial AR-agonist cyprodinil was also reported as a weak GR agonist inducing GRE-mediated transcriptional activity 1.2- to 1.3-fold (compared to 12- to 17-fold induction by dexamethasone)43. The response in the current study, however, did not indicate significant differences between the unmodified AR-EcoScreen and mutant cell lines (Table 2 and Fig. 3). This suggests that weaker GCCs lack the potential to influence the response in the unmodified AR-EcoScreen. Dexamethasone concentrations >1 nM, however, induced luciferase expression (Fig. 1). Consequently, samples with a dexamethasone equivalent concentration of >1 nM can influence the response and lead to over- or underestimation of androgenic or anti-androgenic potency respectively, in the unmodified AR-EcoScreen.

Mutants 1 and 2 were further validated for mixture toxicity assessment by testing for concentration additivity (in toxic units) of a mixture of six anti-androgens (Fig. 4). Similar as for the single compounds, no significant difference was observed between the unmodified AR-EcoScreen and mutant cells. Best concentration additivity was observed for the unmodified AR-EcoScreen and GR knockout mutant 1, for which highest correspondence (p=0.6866) was observed in dose-response curves (Fig. 4) and the confidence intervals of the estimated IC50 values included the expected IC50 value (i.e. 1 TU).

**Application to environmental samples**

The AR-EcoScreen and GR knockout mutant 1 were applied to environmental samples. Androgenic and (anti-)androgenic activity were detected by both cell lines (Fig. 5). In general, lower agonistic activity was reported by the mutant cell line. This loss in signal suggests the presence of GCCs in the sample, which induced additional luciferase induction in the unmodified AR-EcoScreen. The 50% lower responses in GR knockout mutants exposed to WWTP effluent extracts compared to the unmodified AR-EcoScreen (Fig. 5) suggest highest concentrations of GCCs in these samples. When testing anti-androgenicity, the GR knockout mutant reported stronger AR-antagonism compared to the unmodified AR-EcoScreen. This corresponds with a loss of GR agonism, which masks part of the observed anti-androgenic activity. The anti-androgenicity was, however, only observed in SR extracts suggesting either a role for
less polar compounds in AR-antagonism and/or for more polar compounds in AR-agonism as found in SD extracts.

GCCs are found in surface water, as well as in higher concentrations in wastewater, in the presence of androgens subsequently, levels of androgenicity measured in environmental samples in previous studies using the AR-EcoScreen may have been influenced by the presence of GCCs. Despite the presence of GCCs in the tested samples, the GR knockout AR-EcoScreen was able to specifically report AR (ant)agonism in environmental mixtures. Cross-talk between nuclear hormone receptors like AR and GR, however, occurs in vivo. While this is a toxicologically relevant interaction, the availability of glucocorticoid specific reporter cell lines make reporter cell lines better suited for specific and sensitive determination of their intended endpoint. More complex models are required to investigate such mechanisms of toxicity.

Most of the known AR disrupting environmental pollutants are anti-androgens, which can be explained by the ability of compounds to affect receptor function through sites other than the LBD on the C-terminus. Inhibitors were identified that acted on the AR by binding the N-terminal AF1 domain and disrupting interaction with coactivator proteins. In our samples, however, AR-agonism was more common in the passive sampler extracts than AR-antagonism (Fig. 5). Few environmental pollutants have been identified to which the observed AR-agonistic activity could possibly be attributed. Examples of AR-agonistic environmental pollutants include benzoanthrone, dibenzo(a,h)anthracene and cyprodinil, which were all confirmed as partial agonists in the present study (Table 2 and Fig. 3). Alternatively, the AR-agonistic potency of the passive sampler extracts might be due to the presence of natural and/or synthetic steroid hormones and their metabolites. Further investigation will be needed to elucidate the identity of these and other unknown (anti-)androgens.

**Conclusion**

Introduction of a knockout mutation in the NC3C1 (GR) gene of the AR-EcoScreen (Chinese hamster) genome with the CRISPR/Cas9 genome editing technique prevented the induction of luciferase expression by GCCs. This knockout of a functional GR did not affect the sensitivity of the reporter system to (anti-)androgens as determined with single compounds and a mixture of anti-androgens. Application of
the obtained mutant reporter cell line on passive sampler extracts revealed that, in
case of the WWTP effluent extracts, up to 50% of the observed activity in the
unmodified AR-EcoScreen was the result of GR activation. Consequently, AR-
EcoScreen mutants reported lower AR-agonism and stronger AR-antagonism. The
improved selectivity to (anti-)androgens and insensitivity towards disruption by GCCs
make the developed mutant cell lines a suitable derivative of the AR-EcoScreen for
selective testing of both single compounds and mixtures for (anti-)androgenic potential
in risk assessment and environmental screening.

Acknowledgements

The developed mutant 1 cell line will be deposited for distribution at the Japanese
Collection of Research Bioresources (JCRB) Cell Bank (http://cellbank.nibiohn.go.jp/english/) under the name “AR-EcoScreen GR KO M1”.
This research was funded by the Dutch Technology Foundation (STW), project number
12396. The authors acknowledge Dr. Feng Zhang (Massachusetts Institute of
Technology, Cambridge, MA, USA) for providing the pSpCas9(BB)-2A-GFP plasmid,
Marjo den Broeder (Brunel University, London, United Kingdom) for assistance with
experimental design and Tom O’Toole (Vrije Universiteit Medical Center, Amsterdam,
The Netherlands) for assistance with FACS. Passive samplers were obtained within
the CEFIC-LRI ECO-23 funded TIPTOP project.

References

Andersson, P. L.; Legler, J.; Brouwer, A. In vitro profiling of the endocrine-
157–173.

Estrogen and Androgen Receptor Activities in 200 Pesticides by In Vitro

(3) Runnalls, T. J.; Margiotta-Casaluci, L.; Kugathas, S.; Sumpter, J. P.
Pharmaceuticals in the Aquatic Environment: Steroids and Anti-Steroids as
1318–1338.

effects of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether
(BFDGE) and their derivatives using cells stably transfected with human


(16) Satoh, K.; Nonaka, R.; Ohyama, K.; Nagai, F. Androgenic and antiandrogenic
Chapter 3 AR-EcoScreen GR-KO cell line


(20) Schoenmakers, E.; Verrijdt, G.; Peeters, B.; Verhoeven, G.; Rombauts, W.; Claessens, F. Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. J. Biol. Chem. 2000, 275 (16).


(28) Hala, M.; Hartmann, B. L.; Böck, G.; Geley, S.; Kofler, R. Glucocorticoid-receptor-gene defects and resistance to glucocorticoid-induced apoptosis in


(53) Khalaf, H.; Larsson, A.; Berg, H.; McCrindle, R.; Arsenaught, G.; Olsson, P.-E. Diastereomers of the brominated flame retardant 1,2-dibromo-4-(1,2

## Chapter 3 - Supporting information

**Table S1.** List of 19 tested compounds to compare the responsiveness to (anti-)androgens and GCC dexamethasone between the AR-EcoScreen and mutant cell lines.

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Supplier</th>
<th>CAS No.</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-androgenic chemicals</strong></td>
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<td></td>
</tr>
<tr>
<td>BDE-19</td>
<td>Neosyn</td>
<td>147217-73-0</td>
<td>&gt;98</td>
</tr>
<tr>
<td>BDE-100</td>
<td>Neosyn</td>
<td>189084-64-8</td>
<td>&gt;99</td>
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<tr>
<td>PCB-19</td>
<td>Neosyn</td>
<td>38444-73-4</td>
<td>&gt;98</td>
</tr>
<tr>
<td>PCB-122</td>
<td>Neosyn</td>
<td>76842-07-4</td>
<td>&gt;98</td>
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<td>PCB-125</td>
<td>Neosyn</td>
<td>74472-39-2</td>
<td>&gt;98</td>
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<tr>
<td>PCB-128</td>
<td>Neosyn</td>
<td>38380-07-3</td>
<td>&gt;98</td>
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<td>PCB-168</td>
<td>Neosyn</td>
<td>59291-65-5</td>
<td>&gt;98</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>Fluka</td>
<td>80-05-7</td>
<td>&gt;97</td>
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<td>Estriol (E3)</td>
<td>Sigma</td>
<td>50-27-1</td>
<td>&gt;98</td>
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<tr>
<td>Flutamide</td>
<td>Sigma</td>
<td>13311-84-7</td>
<td>&gt;98</td>
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<tr>
<td>p,p’-DDE</td>
<td>Sigma</td>
<td>72-55-9</td>
<td>&gt;99</td>
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<td>Permethrin</td>
<td>R&amp;H</td>
<td>52645-53-1</td>
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<td>Vinclozolin</td>
<td>R&amp;H</td>
<td>50471-44-8</td>
<td>&gt;99</td>
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<td><strong>Androgenic chemicals - (partial) agonists</strong></td>
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<td>5α-dihydrotestosterone (DHT)</td>
<td>R&amp;H</td>
<td>521-18-6</td>
<td>&gt;99</td>
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<td>Benzoanthrone</td>
<td>Supelco</td>
<td>82-05-3</td>
<td>&gt;98</td>
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<td>Cyprodinil</td>
<td>Dr. Ehrenstorfer</td>
<td>121552-61-2</td>
<td>99</td>
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<tr>
<td>Dibenzo(a,h)anthracene</td>
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<td>53-70-3</td>
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<td>Progesterone</td>
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<td><strong>Glucocorticoids</strong></td>
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<tr>
<td>Dexamethasone</td>
<td>Sigma</td>
<td>50-02-2</td>
<td>&gt;98</td>
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**Table S2.** Sequences of target sequence oligos for ligation into the PX458 plasmid and the primer sequences for the amplification of mutation sites of mutant clones for sequencing.

<table>
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<tr>
<th>Mutation</th>
<th>Target sequence oligos</th>
<th>Sequencing primers</th>
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<tbody>
<tr>
<td>dN363S</td>
<td>F CACCGTTCCCTGTTGTTCTGAAAAC</td>
<td>GTTTTCCCAAGTGCCAGACATGGCGTGAGTACCTGG</td>
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<tr>
<td></td>
<td>R CAAGGACAACAAAGACTTTTTGCAA</td>
<td>TGTACAACTATCCGAAGCACATC</td>
</tr>
<tr>
<td>dC421Y</td>
<td>F CACCGACCACCTCCCAACCTCTGCCC</td>
<td>GTTTTCCCAAGTGCCAGACGGCCTGGAATGAGACCAGA</td>
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<tr>
<td></td>
<td>R CTGGTGGAGGGTTTGGAGGCGAAA</td>
<td>GCCCTGGTGAGCTTGCGGCTGGTA</td>
</tr>
<tr>
<td>dG567A</td>
<td>F CACCGCCACACTCAACATGTTAGGT</td>
<td>GTTTTCCCAAGTGCCAGACACCTGTTATCAGCTGCTGA</td>
</tr>
<tr>
<td></td>
<td>R CGGTGTGAGGTGTGACTAAATCCAAA</td>
<td>GCCGGTGCTGGTGATGGGTAA</td>
</tr>
<tr>
<td>dN766N</td>
<td>F CACCGCAGATACCAAAATATTAAAAA</td>
<td>GTTTTCCCCAGTGCCAGACCTTCCTTCGAGGTTGAG</td>
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<tr>
<td></td>
<td>R CTGGTGGAGGGTTTGGAGGCGAAA</td>
<td>GCCCTGGTGAGCTTGCGGCTGGTA</td>
</tr>
<tr>
<td>dQ710X</td>
<td>F CACCGAAGGAAACTCCAGTCAGAAC</td>
<td>GTTTTCCCCAGTGCCAGACAGGAAGGTTGAGGAGGCTGG</td>
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<tr>
<td></td>
<td>R CTTCCCTTGGAGGTCTGTCTGGCAAA</td>
<td>ACTGAAGGAGGGGGAATGACTT</td>
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Annealing phosphorylated ssDNA target sequence oligos yields dsDNA with overhang compatible with BbsI restriction sites. Sequencing primers contain an M13 tag (in bold) on the forward primer used to initiate amplification during sequencing.

**Table S3.** PC50 values, inducing a response corresponding to 50% of the maximum luciferase activity induced by flutamide, from six anti-androgens measured in the AR-EcoScreen, mutant 1 and mutant 2 presented as mean ± SEM (N=3) and combined average ± SEM and PC50 values calculated from historical pilot data measured in the AR-EcoScreen

<table>
<thead>
<tr>
<th>Compound</th>
<th>AR-EcoScreen</th>
<th>Mutant 1</th>
<th>Mutant 2</th>
<th>Combined average</th>
<th>AR-EcoScreen (historical)</th>
</tr>
</thead>
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<tr>
<td>BDE-19</td>
<td>2.00 ± 0.21 X 10^-7</td>
<td>1.75 ± 0.21 X 10^-7</td>
<td>2.10 ± 0.42 X 10^-7</td>
<td>1.95 ± 0.16 X 10^-7</td>
<td>1.47 X 10^-7</td>
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<td>Estriol</td>
<td>2.09 ± 0.15 X 10^-6</td>
<td>1.95 ± 0.08 X 10^-8</td>
<td>2.10 ± 0.31 X 10^-6</td>
<td>2.05 ± 0.11 X 10^-6</td>
<td>2.05 X 10^-6</td>
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<td>PCB-125</td>
<td>8.53 ± 0.72 X 10^-7</td>
<td>8.25 ± 0.86 X 10^-7</td>
<td>7.63 ± 0.99 X 10^-7</td>
<td>8.14 ± 0.45 X 10^-7</td>
<td>8.25 X 10^-7</td>
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<td>PCB-168</td>
<td>6.83 ± 0.48 X 10^-7</td>
<td>5.98 ± 0.32 X 10^-7</td>
<td>5.36 ± 0.74 X 10^-7</td>
<td>6.06 ± 0.35 X 10^-7</td>
<td>4.26 X 10^-7</td>
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<tr>
<td>Permethrin</td>
<td>1.65 ± 0.15 X 10^-5</td>
<td>1.58 ± 0.16 X 10^-5</td>
<td>1.74 ± 0.50 X 10^-5</td>
<td>1.66 ± 0.16 X 10^-5</td>
<td>3.18 X 10^-5</td>
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<td>Vinclozolin</td>
<td>1.61 ± 0.03 X 10^-7</td>
<td>1.46 ± 0.07 X 10^-7</td>
<td>1.72 ± 0.33 X 10^-7</td>
<td>1.60 ± 0.11 X 10^-7</td>
<td>9.20 X 10^-8</td>
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