Development of a 5α-reductase High-throughput Screening Assay for Androgen Steroidogenesis

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Abstract

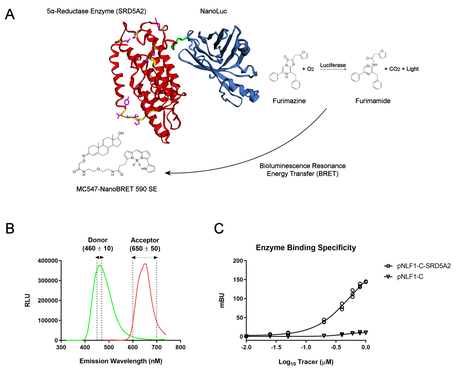
The US EPA employs high-throughput screening assays to identify environmental chemicals that may pose a risk to human health. Many assays are utilized by the Endocrine Disruptor Screening Program to evaluate effects on estrogen, androgen, and thyroid endocrine pathways. Altered androgen hormone biosynthesis contribute to endocrine disruption that may result in impaired reproductive and sexual development. Steroid 5α-reductase enzymes catalyze the conversion of testosterone into the more potent androgen 5α-dihydrotestosterone. Type 2 5α-reductase enzyme (SRD5A2) deficiency is associated with decreased virilization in males and presents an important mode-of-action when evaluating environmental chemical exposure. The objective of this study was to develop a high-throughput assay for screening inhibition of human SRD5A2. NanoBRET Target Engagement assay technology was used to evaluate modulation of testosterone binding to SRD5A2 in a 384-well cell-based format. Michaelis-Menten kinetics of the SRD5A2-NanoLuc fusion protein demonstrated normal conversion of testosterone to 5α-dihydrotestosterone (Km: 434 nM). Initial evaluation with 5α-reductase reference inhibitors dutasteride, finasteride, and epristeride revealed significant gain-of-signal. Screening of 1803 blinded ToxCast chemicals identified 91 chemicals with bioactivity hit counts. Additional filtering for assay-specific cytotoxicity and autofluorescence resulted in 24 chemicals with putative bioactivity toward SRD5A2. Overall, the NanoBRET assay technology demonstrated high precision (rCV: 5.2%), modest dynamic range (S/B: 1.41 FC), and marginal assay quality (rZ': 0.13). Finasteride was the most potent compound identified in the screen, suggesting sufficient sensitivity for identifying potent inhibitors of enzyme function.

Objective

NanoBRET Target Engagement assay technology uses bioluminescence resonance energy transfer (BRET) to directly measure interactions of drugs or chemicals with intracellular protein targets in living cells. The technology enables quantitative analysis of target engagement in both equilibrium and non-equilibrium conditions. Reporter complexes are formed when luciferase-tagged protein targets are bound to cell-permeable fluorescent tracer molecules. The technology has been applied to a wide variety of protein targets and is readily adapted to high-throughput, cell-based screening formats. The objective of this study was to adapt NanoBRET Target Engagement assay technology for high-throughput screening of SRD5A2 inhibition.

NanoBRET-SRD5A2 Target Engagement Assay Overview

Predicted protein tertiary structure of human 5α-reductase isozyme 2 (SRD5A2) fused to NanoLuc luciferase. The custom testosterone-fluorophore tracer (acceptor; MC547-NanoBRET 590 SE) binds to SRD5A2 at the ligand binding domain. When tracer is bound to the enzyme, catalysis of furimazine to furimamide by the NanoLuc moiety (donor) results in a bioluminescence resonance energy transfer (BRET) signal, indicating direct binding (A). Confirmation of the donor luminescence (460 ± 10 nM) and acceptor fluorescence (650 ± 50 nM) wavelength emission gates (B). Concentration-dependent enzyme binding specificity of tracer to the fusion protein (pNLF1-C-SRD5A2) (n=3) (C). RLU: Relative light units; mBU: milli BRET Units.



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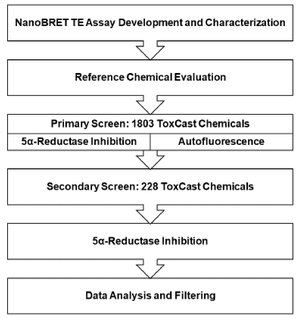
Study Workflow

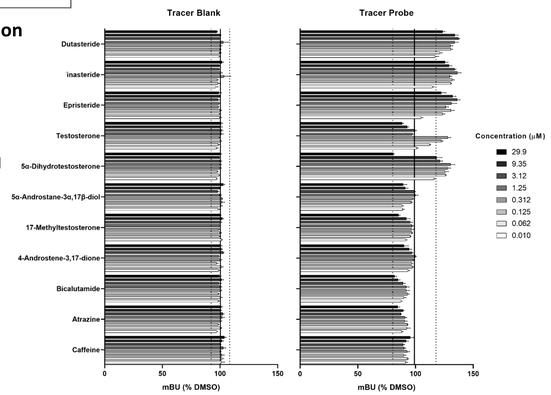


Workflow

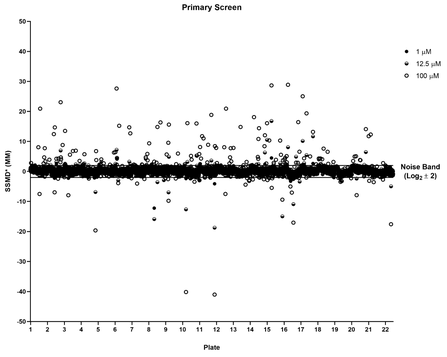
NanoBRET Target Engagement assay technology was adapted and characterized for inhibition of human 5α-reductase. Reference chemicals were used to evaluate the initial assay performance. Primary screening of 1803 ToxCast chemicals was conducted in limited concentration-response format in parallel with assay specific autofluorescence screening of the chemical library. 228 chemicals triaged from the primary screen were evaluated in a broader multiple concentration-response format for functional inhibition of 5α-reductase. Final data analysis of 91 chemical hits was performed to classify bioactivity and flag potential confounding effects of assay autofluorescence and cytotoxicity.

Reference Chemical Evaluation

A chemical training set consisting of validated 5α-reductase inhibitors, enzyme substrate and metabolites, substrate analogs, and negative control compounds were evaluated in concentration-response format in the absence (Tracer Blank) or presence (Tracer Probe) of tracer. mBU normalized data are plotted as a percentage of solvent control (% DMSO), representing the mean ± SD of four independent replicates. The solid vertical black line is the baseline % DMSO with dashed vertical lines indicating the noise band.



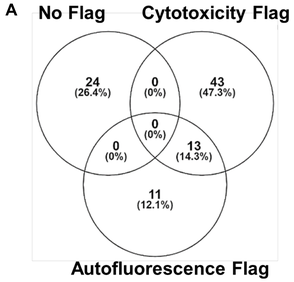
ToxCast Primary Screen

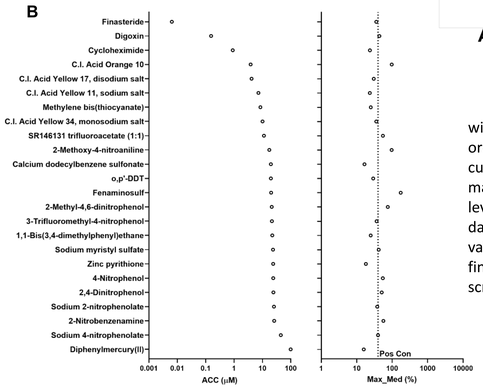


A blinded ToxCast chemical library of 1803 compounds was screened across three concentrations (1, 12.5, 100 μM) in one experimental replicate. Effect size across 22 assay plates was determined using robust strictly standardized mean difference method-of-moment (SSMD* (MM)). The horizontal black bars represent the Log2 ± 2 noise band.

ToxCast Secondary Screen

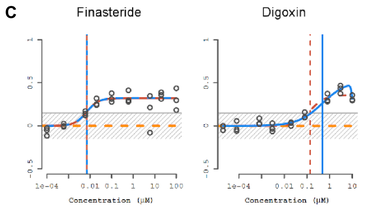
The 228 chemicals triaged from the primary screen were evaluated in a broader multiple concentration-response format for functional inhibition of 5α-reductase. The secondary screen identified 91 total chemicals with potential for inhibition of 5α-reductase. Chemicals were flagged and binned according to potential confounding factors including autofluorescence interference and cytotoxicity (A).





The 24 chemicals identified without any flags were rank-ordered by active concentration at cutoff (ACC) with corresponding maximum observed median effect level (Max_Med (%)). The vertical dashed line indicates the mean value for positive control (Pos Con) finasteride run on each plate in the screen (B).

Concentration-response plots for Finasteride and Digoxin. The gray hashed line indicates the assay noise band, the red vertical line indicates the AC50 value for the ToxCast Pipeline Hill model, the blue vertical line indicates the AC50 value for the ToxCast Pipeline gain-loss model. The winning model is shown with a solid line. (C).



Conclusions

- NanoBRET target engagement assay technology was successfully adapted to directly measure interactions of testosterone substrate with intracellular 5α-reductase enzymes in living cells.
- The NanoBRET-SRD5A2 assay demonstrated high precision, modest dynamic range, and marginal assay quality.
- Finasteride was the most potent compound identified in the blinded screen, suggesting sufficient sensitivity for identifying potent inhibitors of enzyme function.
- Few environmental chemicals were identified as potential inhibitors of 5α-reductase enzyme.

The views expressed do not reflect the views or policies of the U.S. EPA.