Development and Implementation of a High-Throughput High-Content Screening Assay to Identify Inhibitors of Androgen Receptor Nuclear Localization in Castration-Resistant Prostate Cancer Cells

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ABSTRACT

Patients with castration-resistant prostate cancer (CRPC) can be treated with abiraterone, a potent inhibitor of androgen synthesis, or enzalutamide, a second-generation androgen receptor

(AR) antagonist, both targeting AR signaling. However, most patients relapse after several months of therapy and a majority of patients with relapsed CRPC tumors express the AR target gene prostate-specific antigen (PSA), suggesting that AR signaling is reactivated and can be targeted again to inhibit the relapsed tumors. Novel small molecules capable of inhibiting AR function may lead to urgently needed therapies for patients resistant to abiraterone, enzalutamide, and/or other previously approved antiandrogen therapies. Here, we describe a high-throughput high-content screening (HCS) campaign to identify small-molecule inhibitors of AR nuclear localization in the C4-2 CRPC cell line stably transfected with GFP-AR-GFP (2GFP-AR). The implementation of this HCS assay to screen a National Institutes of Health library of 219,055 compounds led to the discovery of 3 small molecules capable of inhibiting AR nuclear localization and function in C4-2 cells, demonstrating the feasibility of using this cell-based phenotypic assay to identify small molecules targeting the subcellular localization of AR. Furthermore, the three hit compounds provide opportunities to develop novel AR drugs with potential for therapeutic intervention in CRPC patients who have relapsed after treatment with antiandrogens, such as abiraterone and/or enzalutamide.

INTRODUCTION

Castration-resistant prostate cancer (CRPC) is currently incurable, making prostate cancer the second most common cause of cancer death among men in the United States in 2012 with >28,000 deaths and >241,000 new cases diagnosed.¹ Multiple studies have shown that the androgen receptor (AR) is activated in prostate cancer through several mechanisms, including AR overexpression, mutation, hypersensitization, and/or intratumoral androgen synthesis in patients relapsed after androgen deprivation

therapy.²⁻⁸ Overexpression and knockdown studies have demonstrated that AR is a key molecular determinant and a validated therapeutic target for CRPC.^{9,10}

The importance of AR as a target in the majority of CRPC patients is emphasized by the mechanisms of the two drugs most recently approved by the federal drug administration for the treatment of CRPC, abiraterone, a potent inhibitor of testosterone synthesis,¹¹ and MDV3100 (Enzalutamide®), a novel AR antagonist.^{12,13} However, prostate cancers develop resistance to therapies, including the most recent second-generation antiandrogens.^{11,14-16} Also, some AR-positive prostate cancer cell models, such as 22Rv1, are insensitive to abiraterone and/or MDV3100.¹⁷⁻¹⁹ Therefore, there is a need for the development of more effective inhibitors of AR function to treat CRPC patients who have developed resistance to antiandrogens, including abiraterone and MDV3100.

As a member of the steroid receptor superfamily, AR is a ligand-dependent transcription factor that controls the expression of androgen-responsive genes.²⁰ Intracellular trafficking is an important mechanism in the regulation of many transcription factors, including AR. To transactivate its target genes, AR must translocate from the cytoplasm into the nucleus, and retention of AR in the cytoplasm is one mechanism to prevent its transactivation activity. Thus, a key regulatory step in the action of AR is its nuclear translocation. AR contains one nuclear localization signal (NL1) within the DNA-binding domain and hinge region, one ligand-induced nuclear localization signal (NL2) within the ligand-binding domain (LBD), and a nuclear export signal in the ligand-free LBD.²¹⁻²⁴ In addition, the N-terminal domain of AR contains amino acid sequences that can modulate subcellular localization.^{25,26} In androgen-sensitive cells, AR is localized to the cytoplasm in the absence of ligand.²⁷ On exposure to androgens, AR translocates to the nucleus where it binds to specific androgen response element DNA sequences to transactivate target genes. However, in CRPC cells, AR remains in the nucleus even in the absence of androgens and transactivates androgen-responsive genes, leading to uncontrolled growth of prostate tumors.^{6,28} Therefore, approaches that can reduce the level of nuclear AR may provide an effective therapy against CRPC.

To date, no high-throughput screens to identify small molecules capable of specifically and effectively reducing the nuclear localization of AR in CRPC cells have been published. In this study, we report the development and implementation of the first high-throughput high-content screening (HCS) assay to identify small molecules capable of reducing AR nuclear localization in CRPC cells.

MATERIALS AND METHODS

Reagents and Plasmid

Dimethyl sulfoxide (DMSO), 17-allylamino geldanamycin (17-AAG), formaldehyde and Lipofectamine™ were purchased from Sigma-Aldrich, St. Louis, MO. Hoechst 33342 was obtained from Invitrogen (Carlsbad, CA), phosphate-buffered saline (PBS) and RPMI-1640 medium from Corning Cellgro, fetal bovine serum (FBS) from Atlanta Biologicals (Flowery Branch, GA), L-glutamine from Gibco/Life Technology, and G418 from Gemini Bio-Products. The GFP-AR-GFP (2GFP-AR) expression vector was generated by adding another green fluorescent protein (GFP) cDNA at the C-terminus of the AR coding sequence of the GFP-AR expression vector, which is based on the expression vector pEGFP-C1 (Clontech).²⁴ The 2GFP-AR expression vector was verified by DNA sequencing.

Cell Culture and Stable Transfection

C4-2 cells were purchased from UroCor (Oklahoma City, OK).²⁹ Cells were maintained in the RPMI-1640 medium supplied with 10% FBS and 1% L-glutamine at 37°C with 5% CO₂. C4-2 cells were transfected with the 2GFP-AR expression vector using Lipofectamine according to the manufacturer's protocol (Invitrogen). The transfected cells were cultured in the presence of 800–1,000 µg/mL G418, individual C4-2 colonies expressing 2GFP-AR were selected, and the subcellular localization of 2GFP-AR was determined by fluorescence microscopy using a Nikon TE 2000U inverted microscope. The N3 C4-2-2GFP-AR clone was used for high-throughput screening.

Compound Library

A library of 219,055 compounds provided by the National Institutes of Health (NIH) was formatted at 10 mM concentration in DMSO and arrayed into 384-well microtiter master plates. The storage and use of the library in high-throughput screening were carried out as previously described.³⁰

Image Acquisition and Analysis

The images of 2GFP-AR in C4-2 cells cultured in 384-well plates were captured using the ArrayScan V^{TI} (AS-VTI) platform (Thermo Fisher Scientific, Waltham, MA) as described previously.^{31,32} Images of Hoechst-stained nuclei (Ch1) and 2GFP-AR (Ch2) in fixed cells were sequentially acquired on the AS-VTI using a 10X 0.3NA objective, and the 2 channel molecular translocation (MT) image analysis algorithm was used to quantify the expression of the 2GFP-AR biosensor in the digital images of the C4-2-2GFP-AR cells. Hoechst 33342 was used to stain the nuclei of the C4-2-2GFP-AR cells, and

the image analysis segmentation used the intensity over background, width and area parameters of this fluorescent signal in channel 1 (Ch1) to define a nuclear mask for each cell as described previously.³¹ The nuclear mask was contracted from the edge of the identified nucleus to reduce cytoplasmic contamination within the nuclear area, and the reduced mask was used to segment and quantify the amount of target channel, 2GFP-AR (channel 2, Ch2) fluorescence within the nucleus. The nuclear mask was also expanded to cover as much of the cytoplasmic region as possible within the cell boundary. Removal of the original nuclear region from this expanded mask creates a ring mask that covers the cytoplasmic region. The outputs of the MT image analysis algorithm are quantitative data such as the total and average fluorescent intensities of the Hoechst-stained objects (Ch1), the selected object or cell count (SCC) from Ch1, the total and average fluorescent intensities of the 2GFP-AR (Ch2) signals in the nucleus (circ) or cytoplasm (ring) regions as an overall average value, or on an individual cell basis. To quantify the changes in subcellular distribution between the nucleus and cytoplasm of 2GFP-AR, we used the MT image analysis algorithm, which calculates a mean average intensity difference by subtracting the average 2GFP-AR intensity in the ring (cytoplasm) region from the average 2GFP-AR intensity in the circ (nuclear) region of Ch2; mean circ–ring average intensity difference in channel 2 (MCRAID-Ch2). To quantify and compare the expression levels and subcellular localization of 2GFP-AR in C4-2-2GFP-AR cells in the presence or absence of small molecules, we analyzed images with the MT algorithm.

Automated 2GFP-AR Subcellular Localization HCS Assay Protocol

The 2GFP-AR translocation HCS assay protocol was very similar to the protocol developed for dexamethasone-induced glucocorticoid receptor nuclear translocation HCS assay (Table 1).³¹ N3 C4-2-2GFP-AR cells were seeded in 384-well plates at density of 3,000 cells/well in complete media and cultured overnight. The cells were then treated overnight with 0.2% DMSO vehicle, 0.2% DMSO in the presence of 20 μ M of the compounds in the library, or 0.2% DMSO plus 3.0 μ M 17-AAG. The treated cells were subsequently fixed using 3.7% formaldehyde containing 2 μ g/mL Hoechst 33342 in PBS at room temperature. Images of Hoechst-stained nuclei (Ch1) and 2GFP-AR (Ch2) expression in fixed cells were sequentially acquired on the AS-VTI using a 10 $\times$ 0.3NA objective and were analyzed using the MT image analysis algorithm. Data processing, visualization, statistical analysis, and curve fitting were conducted as described previously.³¹ Data processing for the 2GFP-AR nuclear localization screen was performed using

ActivityBase™ (IDBS, Guildford, Alameda, CA) and CytoMiner. Processed data and HCS multiparameter features were visualized using Spotfire™ DecisionSite™ (Somerville, MA) software. An ActivityBase primary HTS template was created that automatically calculated % inhibition together with plate control signal-to-background ratios (S:B) and Z'-factor coefficients.³³ For the 2GFP-AR nuclear localization screen, we utilized the MRAID-Ch2 values of the 0.2% DMSO maximum plate control wells ($n=32$) and the MCRAID-Ch2 values of the 3.0 μ M 17-AAG minimum plate control wells ($n=32$) to normalize the MCRAID-Ch2 values of the compound data and to represent 100% and 0% activation or translocation of 2GFP-AR to the nucleus, respectively. We also constructed an ActivityBase concentration–response template to calculate percent inhibition together with plate control S:B ratios and Z'-factor coefficients for quality control purposes.^{31,33,34} For the cytotoxic and fluorescent outlier analysis, we used the plate-based statistical scoring method z-score that is estimated from the 320 compound wells (no plate control wells) on an assay plate.³⁴ It is defined as $z\text{-score} = (X^i - \bar{X})/S_x$. Where X^i is the raw measurement on the i th compound, $\bar{X}$ and S_x are the mean and standard deviation of all the sample measurements on a plate. A deviation of -3 below the sample average on the plate is frequently utilized as a statistical threshold or cutoff for active compounds.³⁴

Western Blot Analysis

C4-2 cells were lysed in a modified radioimmune precipitation assay buffer containing 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and 1% protease inhibitor cocktail (Sigma-Aldrich).

Protein concentrations in cell lysates were measured using the bicinchoninic acid assay reagent. Lysates were subjected to SDS-PAGE, and Western blotting was carried out using antibodies against AR (N-20, sc-816) and prostate-specific antigen (PSA) (sc-7638) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). To determine the effect of SID 3712502 on AR and PSA protein levels, we treated C4-2 cells with various concentrations of SID 3712502 for 48 h and then harvested for cell lysate preparation. An antibody against GAPDH (sc-25778; Santa Cruz Biotechnology, Inc.) or β -actin (sc-47778; Santa Cruz Biotechnology, Inc.) was used as a control to demonstrate equal protein loading.

RESULTS

Generation and Characterization of 2GFP-AR Expressing Cell Lines in the C4-2 CRPC Cell Background

To establish a cell-based high-throughput screening assay to identify small molecules capable of reducing nuclear AR levels

Table 1. Androgen Receptor Nuclear Localization HCS Assay Protocol

Step	Parameter	Value	Description
1	Plate cells	60 μ L	3,000 N3 C4-2-2GFP-AR cells
2	Incubate cells overnight	16–24 h	Culture medium at 37°C, 5% CO ₂ , and 95% humidity
3	Library compounds/DMSO/DMSO +17-AAG to controls wells	20 μ L	Compounds –20 μ M final concentration in well +0.2% DMSO; controls –0.2% DMSO or 0.2% DMSO plus 3.0 μ M 17-AAG
4	Incubate assay plates overnight	16–24 h	At 37°C, 5% CO ₂ , and 95% humidity
5	Aspirate media and fix cells	50 μ L	3.7% formaldehyde containing 2 μ g/mL Hoechst 33342 in PBS without Ca ²⁺ and Mg ²⁺ , prewarmed to 37°C
6	Incubate plates	10–30 min	Ambient temperature
7	Aspirate fixative and wash 2x with PBS	50 μ L	Fixative was aspirated and plates were then washed twice with 50 μ L PBS without Ca ²⁺ and Mg ²⁺ , 50 μ L PBS in well
8	Seal plates	1x	Sealed with adhesive aluminum plate seals
9	Acquire images	10x, 0.3NA objective	Images of the Hoechst (Ch1) and AR-GFP (Ch2) were sequentially acquired on the ArrayScan V ^{TI} 10x using the XF100 excitation and emission filter set
10	Assay readout	MCRAID-Ch2	Images were analyzed using the MT image analysis algorithm using the mean circ (nucleus)–ring (cytoplasm) average intensity difference to quantify the AR-GFP localization

Step Notes

- 384-well black walled clear bottom plates, poly-L-lysine-coated plates Greiner Bio-one Cat No. 781091, Zoom liquid handler (Titertek, Huntsville, AL).
- RPMI-1640 medium supplied with 10% FBS, 1% L-glutamine, and 800–1,000 μ g/mL G418.
- Compounds added to wells in columns 3–22. Controls in columns 1, 2, 23, and 24. VPrep (Velocity 11, Menlo Park, CA) or an Evolution P3 (Perkin-Elmer, Waltham, MA) outfitted with a 384-well transfer head.
- Cells exposed to 20 μ M compound overnight.
- Aspiration of media and fixative addition automated on BioTek ELx405 (BioTek, Winooski, VT) plate washer.
- 10–30-min incubation at ambient temperature to fix cells and stain nuclei with Hoechst.
- Aspiration of fixative and PBS wash steps automated on BioTek ELx405 (BioTek) plate washer.
- Plates sealed with adhesive aluminum plate seals using the Abgene Seal-IT 100 plate sealer (Abgene, Rochester, NY).
- Plates loaded into the ArrayScan V^{TI} for scanning using a Twister II robotic plate handler (Thermo Fisher Scientific, Waltham, MA).
- MT bio-application (Thermo Fisher Scientific).

17-AAG, 17-allylamino geldanamycin; AR, androgen receptor; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GFP, green fluorescent protein; HCS, high-content screening; PBS, phosphate-buffered saline; MT, molecular translocation.

in prostate cancer cells, we transfected the C4-2 human prostate cancer cell line with the 2GFP-AR expression vector and isolated stably transfected clones. C4-2 was chosen because it is a well-characterized CRPC cell line derived from LNCaP cells.²⁹ We transfected the 2GFP-AR expression vector into C4-2 cells and then used G418 selection to isolate stably transfected clones for subsequent studies. The 2GFP-AR expression vector was used because GFP-AR did not provide a green fluorescence signal strong enough for the HCS assay (data not shown). The N3 clone of C4-2-2GFP-AR cells was selected for the development of AR nuclear localization HCS assay. In addition to endogenous expression of AR, the stably transfected cell line exhibited a slower migrating larger molecular weight immu-

noreactive band on Western blots probed with the anti-AR antibody (*Fig. 1A*). Endogenous AR and 2GFP-AR were reported previously to predominantly localize to the nuclei of C4-2 cells cultured in an androgen-free medium.³⁵ As expected, 2GFP-AR was predominantly localized to the nuclei of C4-2 cells cultured in androgen-free media, and exposure to DHT further enhanced that nuclear distribution (*Fig. 1B*). The androgen-independent nuclear localization of AR requires HSP90 function and can be effectively inhibited by the HSP90 inhibitor 17-AAG.³⁵ Exposure to 17-AAG for 16 h appeared to cause some reduction in the protein levels of both endogenous AR and 2GFP-AR in the N3 clone of C4-2-2GFP-AR cells (*Fig. 1A*). Both the androgen-independent

Fig. 1. Characterization of the N3 C4-2-2GFP-AR clone selected for assay development and screening. **(A)** Effects of 17-AAG on 2GFP-AR and endogenous AR protein levels in the stably transfected N3 clone of C4-2 cells. N3 C4-2-2GFP-AR cells were cultured in ligand-free conditions for 24 h before treatment with 200 nM 17-AAG for 5 or 16 h or 1 nM DHT for 16 h. Cell lysates from the treated cells were collected and separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-AR antibody. Beta-actin was probed to provide a protein loading control. **(B)** Effect of 17-AAG on the subcellular localization of 2GFP-AR in the N3 stably transfected C4-2 clone. N3 C4-2-2GFP-AR cells were cultured in ligand-free conditions for 24 h and then treated with 100 nM 17-AAG or vehicle in the presence or absence of 1 nM DHT overnight before fluorescent microscopy. **(C)** Inhibition of nuclear localization of 2GFP-AR in stably transfected C4-2 cells by 17-AAG. N3 C4-2-2GFP-AR cells cultured in ligand-free conditions were treated with either 500 nM 17-AAG or the DMSO vehicle control overnight before the visualization of 2GFP-AR subcellular localization by fluorescence microscopy. Nuclei of C4-2-2GFP-AR cells were visualized by Hoechst staining. 17-AAG, 17-allylamino geldanamycin; AR, androgen receptor; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein.

and, to a lesser extent, the DHT-induced, nuclear localization of 2GFP-AR in C4-2 cells were markedly reduced by exposure to 17-AAG (Fig. 1B, C). These observations indicate that the stability and subcellular localization of 2GFP-AR in the N3 clone of C4-2-2GFP-AR cells behave similarly to endogenous AR with respect to both androgen responsiveness and HSP90 inhibition by 17-AAG. These data further substantiated the selection of the N3 C4-2-2GFP-AR cell line for the development of the 2GFP-AR nuclear localization HCS assay and confirmed that 17-AAG was a suitable control compound that induced the desired cytoplasmic 2GFP-AR distribution phenotype in the C4-2 CRPC cell line.

Development and Optimization of the 2GFP-AR Nuclear Localization HCS Assay

To evaluate whether the 2GFP-AR nuclear localization assay would be compatible with HCS, we acquired 10× images of Hoechst-stained nuclei (Ch1) and 2GFP-AR (Ch2) on the AS-VTI automated HCS platform and used the MT image analysis algorithm to quantify the 2GFP-AR subcellular distribution phenotype in N3 C4-2-2GFP-AR cells cultured in the presence or absence of 312 nM 17-AAG (Figs. 2 and 3). Consistent with the images presented in Figure 1 that were acquired on a Nikon TE 2000U inverted fluorescent microscope, 2GFP-AR was predominantly localized to the nuclei of N3 C4-2-2GFP-AR cells cultured in the androgen-free medium, and the colocalization of the Hoechst and 2GFP-AR signals was indicated by the light blue nuclear staining of the color composite images acquired on the AS-VTI (Fig. 2A). However, in N3 C4-2-2GFP-AR cells cultured overnight in the presence of 312 nM 17-AAG, the 2GFP-AR was predominantly localized to the cytoplasm of the cell as indicated by the dark blue nuclear staining of the color composite images (Fig. 2A). The MT image analysis algorithm was used to quantify the relative subcellular distribution of 2GFP-AR between the nucleus and cytoplasm regions of N3 C4-2-2GFP-AR cells (Fig. 2B). The MT algorithm generates a mask of the nucleus for each cell by segmenting the Ch1 Hoechst images into background and stained objects that were classified as nuclei because they exhibited average intensities above a preset threshold and also had suitable width and area measurements (Fig. 2B). The MT algorithm then uses the nuclear masks for each cell to segment the corresponding Ch2 2GFP-AR images into nuclear “circ” and cytoplasm “ring” regions for each cell (Fig. 2B).

The MT image analysis algorithm extracts and outputs a large number of quantitative parameters such as the integrated and average fluorescent intensities of the Hoechst-stained objects (Ch1), the SCCs from Ch1, the total and average fluorescent intensities of the 2GFP-AR (Ch2) signals in the nucleus (circ) or cytoplasm (ring) regions as an overall well average value, or on an individual cell basis.³¹ The selected object counts from Ch1 correspond to the number of cells included in the analysis and provide a useful readout of compound-mediated cytotoxicity. For example, compared to low androgen medium control wells, there were ~30% fewer cells in the images of N3 C4-2-2GFP-AR cells that had been cultured overnight in the presence of 312 nM 17-AAG (Fig. 3A). In these same cell populations, cells that were exposed to 312 nM 17-AAG exhibited ~ a 75% reduction in the mean “circ” average intensity of 2GFP-AR in the nucleus compared to low androgen media controls (Fig. 3B), but only

Fig. 2. Image acquisition and analysis on the ArrayScan-VT[™] automated HCS platform. **(A)** Image Acquisition. Grayscale images of Hoechst-stained nuclei (Ch1) and 2GFP-AR expression (Ch2) in N3 C4-2-2GFP-AR cells that had been fixed in paraformaldehyde after being cultured overnight in low androgen conditions in the presence or absence of 312 nM 17-AAG were acquired sequentially on the AS-VT[™] using a 10×0.3NA objective. The corresponding color composite overlay images are also shown. The images presented are representative of similar images obtained in numerous independent experiments. **(B)** Nucleus and cytoplasm masks generated by the molecular translocation (MT) image analysis segmentation. The MT algorithm generates a mask of the nucleus (blue outline) for each cell after segmenting the Ch1 Hoechst images and classifying stained objects that exhibited average intensities above a preset threshold that also had suitable width and area measurements as nuclei. The nuclear mask was then contracted 1 pixel from the edge of the identified nucleus to create a nuclear “Circ” (yellow outline) mask that was used to quantify the total and average 2GFP-AR (Ch2) fluorescence within the nucleus. The nuclear mask was then expanded to cover as much of the cytoplasmic region as possible within the cell boundary and removal of the original nuclear region creates a cytoplasmic “Ring” mask (red donut) that was used to quantify the total and average 2GFP-AR (Ch2) fluorescence within the cytoplasm. HCS, high-content screening.

~ a 10% reduction in the mean “ring” average intensity of 2GFP-AR in the cytoplasm (Fig. 3C). The mean circ-ring average intensity difference (MCRAID) (Fig. 3D) and the mean circ:ring average intensity ratio (Fig. 3E) readouts both indicated that compared to low androgen medium controls, 17-AAG treatment significantly reduced the nuclear localization of 2GFP-AR. We selected the MCRAID readout as our primary indicator of 2GFP-AR nuclear localization.

To further optimize the 2GFP-AR nuclear localization assay, we evaluated several variables to determine if they would improve the performance of the 2GFP-AR nuclear localization assay (Fig. 4). Consistent with previous findings,³⁵ 17-AAG treatment of N3 C4-2-2GFP-AR cells significantly reduced the 2GFP-AR nuclear localization in cells cultured in medium prepared with both charcoal stripped FBS and normal FBS (Fig. 4A). Since the assay signal window between medium controls and 17-AAG-treated cells was slightly larger in the medium prepared with normal FBS, we elected to use normal FBS for all further assay development experiments. In an effort to reduce and/or control for cell loss in the automated HCS protocol, we tested the impact of different types of 384-well plates, including poly-D- and poly-L-lysine-coated plates from different suppliers (Fig. 4B). All of the manually and commercially coated poly-D- and poly-L-lysine plates worked well in the automated 2GFP-AR nuclear localization screening assay, with only the CellBIND plates, which do not have peptide-coated surfaces, exhibiting increased variability and therefore a lower assay signal window (Fig. 4B). We selected commercially available poly-L-lysine plates for all further assay development experiments. Next, we evaluated the effects of the 384-well N3 C4-2-2GFP-AR cell seeding density in the range from 1.25×10^3 to 10×10^3 cells per well on assay performance (Fig. 4C). Although all of the seeding densities produced very comparable results,

Fig. 3. Quantitative data extracted from the digital images of the Hoechst-stained nuclei and 2GFP-AR expression (Ch2) in N3 C4-2-2GFP-AR cells by the MT image analysis algorithm. 10× images of Hoechst-stained nuclei (Ch1) and 2GFP-AR expression (Ch2) in N3 C4-2-2GFP-AR cells that had been fixed in paraformaldehyde and stained with Hoechst after being cultured overnight in low androgen conditions in the presence or absence of 312 nM 17-AAG were acquired on the AS-VTI HCS platform and analyzed using the MT algorithm: **(A)** selected object/cell counts per image; **(B)** average 2GFP-AR intensity in the nuclear “circ” mask area; **(C)** average 2GFP-AR intensity in the cytoplasmic “ring” mask area; **(D)** mean circ-ring 2GFP-AR average intensity difference; and **(E)** the mean circ:ring 2GFP-AR average intensity ratio. The data represent the mean ± SD of replicate determinations ($n=12$) from one of numerous independent experiments.

the variability of the low androgen media controls appeared to be slightly higher with the 5×10^3 and 10×10^3 seeding densities (Fig. 4C). On the basis of these data and to reduce the cell culture burden, we selected a 384-well cell seeding density of 3×10^3 N3 C4-2-2GFP-AR cells per well for the remaining assay development experiments. DMSO has two major effects on cells that could significantly impact the 2GFP-AR nuclear localization screening assay: at DMSO concentrations $>1\%$ but $<5\%$, the cell morphology may change from a well-attached spread phenotype to a more rounded smaller less well-attached phenotype; and at DMSO concentrations $>5\%$, there may be significant cell loss due to cytotoxicity and/or loss of attachment.³¹ Cell rounding and cell shrinking would severely diminish the ability

of the MT algorithm to accurately segregate the nuclear and cytoplasm regions of cells, and significant cell loss would reduce the statistical power of the measurements. The 2GFP-AR nuclear localization HCS assay proved to be exquisitely sensitive to DMSO (Fig. 4D) and we selected 0.2% DMSO as the maximum DMSO concentration for the primary screen. In our experience, most cell-based assays do not tolerate DMSO concentrations $\geq 1.0\%$. Nuclear translocation and localization HCS assays are perhaps even more susceptible to interference by DMSO because even at concentrations $<1\%$, there may be cell morphology changes, including cell rounding and cell shrinkage. Although we conduct most of our HCS assays at DMSO concentrations $\leq 0.5\%$, C4-2 cells

Fig. 4. Development and optimization of the 2GFP-AR nuclear localization HCS assay. **(A)** Charcoal-stripped versus complete medium. N3 C4-2-2GFP-AR cells were cultured in charcoal-stripped (CS) media and complete (CM) media and treated overnight in the presence or absence of 300 nM 17-AAG. The mean circ-ring average intensity difference (MCRAID-Ch2) indicator of 2GFP-AR nuclear localization for low androgen (■) and 17-AAG (□) cultured cells is plotted on the left Y-axis, and the cell number per field of view (CNPFV) for low androgen (▨) and 17-AAG (▩) cultured cells is plotted on the right Y-axis. Error bars represent range of duplicate determinations from one of two independent experiments. **(B)** Microtiter plate types. N3 C4-2-2GFP-AR cells were seeded into five different 384-well microtiter plates and cultured overnight in complete media and then treated overnight in the presence or absence of 300 nM 17-AAG: Corning CellBind plates (CellBind); Greiner Bio-one plates manually coated with Poly-L-lysine (Man-PLL); Greiner Bio-one plates manually coated with Poly-L-lysine (Com-PLL); Greiner Bio-one plates manually coated with Poly-D-lysine (Man-PDL); and Greiner Bio-one plates commercially coated with Poly-D-lysine (Com-PDL). The MCRAID-Ch2 for low androgen (■) and 17-AAG (□) cultured cells is plotted on the left Y-axis, and the CNPFV for low androgen (▨) and 17-AAG (▩) cultured cells is plotted on the right Y-axis. The data represent mean $\pm$ SD of replicate ($n=96$) determinations from one of three independent experiments. **(C)** Cell seeding density. N3 C4-2-2GFP-AR cells were seeded at the indicated densities, ranging from 1.25×10^3 to 10×10^3 cells per well, into Com-PLL 384-well microtiter plates, cultured overnight in complete media, and then treated overnight in the presence or absence of 300 nM 17-AAG. The MCRAID-Ch2 values for low androgen (■) and 17-AAG (□) cultured cells are plotted on the left Y-axis, and the CNPFV for low androgen (▨) and 17-AAG (▩) cultured cells is plotted on the right Y-axis. The data represent mean $\pm$ SD of replicate ($n=4$) determinations from one of three independent experiments. **(D)** DMSO Tolerance. N3 C4-2-2GFP-AR cells were seeded at 3×10^3 cells per well into Com-PLL 384-well microtiter plates, cultured overnight in complete media, and then treated overnight in the presence or absence of 300 nM 17-AAG with the indicated concentrations of DMSO. The MCRAID-Ch2 for low androgen, 300 nM 17-AAG-, and 300 nM 17-AAG + DMSO-treated (0.3% to 2.5%) cells is plotted on the left Y-axis (■), and the corresponding CNPFV for the same treatments is plotted on the right Y-axis (□). **(E)** 17-AAG concentration response. N3 C4-2-2GFP-AR cells were seeded at 3×10^3 cells per well into Com-PLL 384-well microtiter plates, cultured overnight in complete media, and then treated overnight with the indicated concentrations of 17-AAG. The MCRAID-Ch2 values are plotted on the left Y-axis (●), and the corresponding CNPFV is plotted on the right Y-axis (○). **(F)** Day 1 of the 3-Day assay signal window and Z-factor coefficient determination. N3 C4-2-2GFP-AR cells were seeded at 3×10^3 cells per well into $4 \times$ Com-PLL 384-well microtiter plates and cultured overnight in complete media, and then, two maximum plates were treated overnight with 0.2% DMSO, and two minimum plates were treated with 3 μ M 17-AAG + 0.2% DMSO. The MCRAID-Ch2 average intensity difference values for the two maximum plates, Max-PL1 (●) and Max-PL2 (○), and the two minimum plates, Min-PL1 (■) and Min-PL2 (□), are presented in the scatterplot. The mean MCRAID-Ch2 values of the 768 maximum and minimum wells are indicated by the dotted lines.

may be particularly susceptible to the effects of DMSO on cell rounding and cell shrinkage. We conducted 17-AAG concentration-response assays using the optimized conditions for the 2GFP-AR nuclear localization assay (Fig. 4E). Under these optimized assay conditions, the N3 C4-2-2GFP-AR

cells appeared more resistant to 17-AAG with respect to cell loss and also required a concentration of 3 μ M to effectively inhibit 2-GFP-AR nuclear localization (Fig. 4E). Therefore, we selected 3 μ M 17-AAG for the minimum plate controls for the 2GFP-AR nuclear localization HCS. In a 3-day variability

Table 2. GFP-AR Nuclear Localization HCS Assay 3-day Variability and Z-Factor Coefficient Determination

Class	Day	Plate	Max ^a /Min ^b	Mean ^c	SD	CV	Z	SB
Intraplate	1	1	Max	175	14.2	8.15	0.55	6.80
		2	Max	171	11.9	6.95	0.53	4.87
		3	Min	25.7	8.31	32.3	0.59	6.64
		4	Min	35.9	7.6	21.2	0.57	4.75
		All plates					0.52	5.61
	2	1	Max	185	17.4	9.41	0.51	6.78
		2	Max	184	19.9	10.9	0.53	7.60
		3	Min	27.2	8.53	31.3	0.46	6.74
		4	Min	24.3	7.74	31.9	0.48	7.56
		All plates					0.49	7.15
	3	1	Max	111	13.7	12.4	0.32	5.98
		2	Max	103	10.5	10.2	0.38	4.95
		3	Min	18.5	7.12	38.5	0.43	5.57
		4	Min	22.4	4.63	20.7	0.44	4.61
		All plates					0.34	5.23
Interplate	1	1 and 2	Max	173	13.3	7.68	0.52	5.61
		3 and 4	Min	30.8	9.45	30.7		
	2	1 and 2	Max	184	18.7	10.2	0.49	7.15
		3 and 4	Min	25.8	8.28	32.1		
	3	1 and 2	Max	107	12.8	12.0	0.34	5.23
		3 and 4	Min	20.4	6.31	30.8		
	Day to day	1 and 2	Max	178	17.2	9.62	0.47	6.31
			Min	28.3	9.24	32.7		
	Day to day	2 and 3	Max	146	41.8	28.7	-0.22	6.30
			Min	23.1	7.82	33.9		

^aMax = 0.2% DMSO.^bMin = 3 μ M 17-AAG, 0.2% DMSO.^cThe mean circ-ring average intensity difference MCRAID-Ch2 data were generated in 3 independent experiments of 2 $\times$ 384-well plates each of the maximum and minimum plate controls conducted on three separate days.

CV, coefficient of variance; SD, standard deviation; SB, signal to background ratio; Z, Z-factor coefficient.

and Z-factor coefficient determination test, the Z-factor coefficients for the 2GFP-AR nuclear localization assay on days 1, 2, and 3 were 0.52, 0.49, and 0.34, respectively (Fig. 4F and Table 2). The signal to background ratios ranged between 4.8-fold and 7.6-fold across all of the 12 $\times$ 384-well plates (Table 2), and together these data indicated that the assay was sufficiently robust and reproducible for screening.

High-Throughput Screening of an NIH Library of 219,055 Compounds

Having developed and optimized the 2GFP-AR nuclear localization HCS assay on the AS-VTI platform, we then proceeded to screen 219,055 small molecules of the NIH MLSCN library at 20 μ M (Fig. 5 and Table 3). Figure 5A is a scatter plot of the 2GFP-AR MCRAID raw data of a single representative 384-well assay plate from the primary HCS campaign. The 2GFP-AR MCRAID values of 0.2% DMSO Max controls were $\sim$ 4.5-fold higher and well separated from the 17-AAG controls, and all 320 compound-treated wells produced signals similar to the Max controls, indicating that they failed to alter or inhibit 2GFP-AR nuclear localization and would therefore be classified as inactive (Fig. 5A). Figure 5B is an overlay scatter plot of the normalized % inhibition data from a single screening operation run of 9,600 compounds that were arrayed and screened at 20 μ M in 30 $\times$ 384-well assay plates. The normalized 2GFP-AR MCRAID responses of the Max and Min plate controls were reproducible and provided a robust assay signal window (Fig. 5B). The majority of the tested compounds failed to alter or inhibit 2GFP-AR nuclear localization, but 17 compounds achieved the activity criterion of $\geq$ 60% inhibition of 2GFP-AR MCRAID values (Fig. 5B). A cutoff of 60% inhibition was selected to ensure that the HCS active rate was $\leq$ 0.5% after cytotoxic or fluorescent outlier compounds had been excluded (Table 3). A frequency distribution plot of the normalized % inhibition data for the compound-treated wells for the 219,055 compound primary HCS campaign illustrates that 99.55% of the compounds exhibited 2GFP-AR MCRAID responses comparable to Max controls, and they were therefore classified as inactive (Fig. 5C and Table 3). The % inhibition data

presented in Figure 5C were before the removal of any cytotoxic or fluorescent outlier compounds. To identify and eliminate cytotoxic and autofluorescent compounds that might be scored as false positives or could interfere with the 2GFP-AR nuclear localization assay format, we calculated z-

scores for several of the parameter outputs by the MT image analysis algorithm (Table 3), as described previously.³¹ We found a number of compounds (828, 0.38%) that were either cytotoxic or reduced N3 C4-2-2GFP-AR cell adherence sufficiently at 20 μ M that they exhibited average cell counts per image z-scores ≤ -4 (Table 3). Compounds that significantly increased the mean average Hoechst-stained nuclear total and/or average intensity values in Ch1 and produced z-scores >4 (2,591, 1.8%) were also eliminated (Table 3), because these compounds fluoresce strongly in Ch1 and interfere with the ability of the MT image analysis algorithm to accurately create the nuclear mask that is used to create the nuclear and cytoplasm regions in Ch2 images. In general, compounds with the mean average Hoechst-stained nuclear total and/or average intensity z-scores ≤ -4 were also flagged for cytotoxicity. A small number of compounds (150, 0.07%) produced z-scores >4 for the Ch2 2GFP-AR total and average “ring” (cytoplasm) or “circ” (nucleus) intensity values, respectively (Table 3). Compounds that fluoresce brightly within cells in Ch2 have the potential to obscure any compound effect on 2GFP-AR nuclear localization. A total of 980 (0.45%) actives remained after we had eliminated cytotoxic and autofluorescent compounds, which interfered with the 2GFP-AR nuclear localization HCS assay format (Table 3). Nearly two hundred (182, 18.6%) of the cherry-picked samples were confirmed active because they reproducibly inhibited 2GFP-AR nuclear localization by $\geq 50\%$ at 20 μ M ($n=3$) (Table 3). All of the confirmed actives were then tested in a 10-point twofold dilution series of concentration-dependent inhibition of 2GFP-AR nuclear localization (IC_{50}) assays starting at a maximum concentration

Fig. 5. Primary HCS to identify inhibitors of 2GFP-AR nuclear localization. **(A)** Scatterplot of the mean circ-ring average intensity difference (MCRAID-Ch2) intensity data from a single representative HCS assay plate. The MCRAID-Ch2 data from a single 384-well assay plate from the HCS campaign are presented; $32 \times 0.2\%$ max controls (●), $32 \times 3 \mu$ M 17-AAG + 0.2% DMSO min controls (■), and $320 \times$ compound-treated wells (○). **(B)** Overlay scatterplot of the normalized % inhibition data from 30×384 -well assay plates from a representative screening operation run. The MCRAID-Ch2 values of the 0.2% DMSO Max plate control wells ($n=32$) and the 3 μ M 17-AAG + 0.2% DMSO Min plate control wells ($n=32$) were used to normalize the MCRAID-Ch2 values of the compound-treated wells and to represent 0% and 100% inhibition of 2GFP-AR nuclear localization, respectively. 0.2% max controls (●), 3 μ M 17-AAG + 0.2% DMSO min controls (■), and compound-treated wells (○). **(C)** Binned results frequency distribution graph of the normalized % inhibition data from the primary HCS. The normalized % inhibition data from 219,055 compounds screened at 20 μ M in the primary HCS campaign are presented. The normalized % inhibition data were binned into discrete ranges indicated on the x-axis and the number of compounds within each % inhibition bin is represented as counts on the y-axis.

Table 3. GFP-AR Nuclear Localization HCS Campaign Summary

HCS phase and category	Number of compounds	% of total
Primary screen		
NIH MLSCN compound library	219055	100
Cytotoxic outliers, SOCb zsc <−4	828	0.38
Ch1 Fl outliers, MNTle and MNAIf zs >4	2,591	1.18
Ch2 Fl outliers, MCTlg, MRTlh, MRAlh zs >4	150	0.07
Primary HCS actives, % inhibition MCRAIDj >60%	980	0.45
Active confirmation		
Mean% inhibition MCRAID >50% (n=3)	182	18.6 (980)
IC ₅₀ hit confirmation		
IC ₅₀ < 1 μM	2	1.1 (182)
IC ₅₀ > 1 but <10 μM	65	35.7 (182)
IC ₅₀ > 10 but <40 μM	96	52.7 (182)
IC ₅₀ > 40 μM	19	10.4 (182)
NIH molecular library screening center network (MLSCN) compound library. Fl, fluorescence intensity; MCRAID, mean circ-ring average intensity difference Ch2; MCTl, mean "circ" total intensity Ch2; MNAI, mean nuclear average intensity Ch1; MNTl, mean nuclear total intensity Ch1; MRAl, mean "ring" average intensity Ch2; MRTl, mean "ring" total intensity Ch2; NIH, National Institutes of Health; SOC, selected object/cell count per image; zs, z-score.		

of 40 μM. Of these compounds, 163 (89.6%) produced calculable IC₅₀s < 40 μM for inhibition of 2GFP-AR nuclear localization (Table 2).

Validation of Selected Hit Compounds

The candidate small molecules identified in the HCS campaign were evaluated for potential biological promiscuity, which could limit their potential for drug development. Small molecules with promiscuous biological activity identified in a cross target query of the PubChem database often interfere with the HTS assay format³⁶ or with multiple signaling pathways and therefore were not considered further. Any compound with <95% purity and IC₅₀ > 10 μM was also eliminated. To further prioritize the remaining hits, we classified and clustered their chemical structures, applied computational filters (PAINS/REOS) to identify and eliminate nuisance compounds and to predict their drug-like characteristics and

potentially adverse ADME/Tox properties,^{37,38} and considered their chemical tractability. A total of 23 small-molecule candidates were selected for further analysis. After overnight exposure, some of the small molecules appeared to reduce the overall expression levels of 2GFP-AR, rather than enhancing the cytoplasmic localization of the 2GFP-AR in C4-2 cells, and a few of the compounds exhibited significant cytotoxicity (data not shown). Two structurally related compounds (SID 14730725 and SID 14742211) inhibited the nuclear localization of 2GFP-AR in C4-2 cells (Fig. 6A–D). The concentration responses for inhibition of 2GFP-AR nuclear localization by SID 14730725 and SID 14742211 are presented in Figure 6B. In the presence of ≥10 μM concentrations of these two compounds, the subcellular localization of 2GFP-AR was predominantly shifted into the cytoplasm region of C4-2 cells (Fig. 6C, D). SID 3712502 (Fig. 6A) exhibited a significant inhibition of nuclear 2GFP-AR levels in C4-2 cells without increasing the levels of cytoplasmic 2GFP-AR (Fig. 6E). This suggests that this compound produced a global down-regulation of endogenous AR expression that might thereby suppress the activity of AR. To test this hypothesis, we treated C4-2 cells with SID 3712502 at the indicated concentrations for 48 h. The effect of SID 3712502 on AR and PSA protein levels was assessed by Western blotting analysis. AR protein levels were significantly reduced at the 10 and 50 μM concentrations of SID 3712502 (Fig. 6E). In contrast, the concentration-dependent effects of SID 3712502 on PSA levels were biphasic (Fig 6F). However, PSA protein levels were significantly decreased at 2, 10, and 50 μM concentrations of SID 3712502, suggesting that this compound can inhibit the AR activity at 2 μM and cause downregulation of AR protein expression at 10 and 50 μM concentrations. This result indicates that SID 3712502 is also capable of inhibiting AR signaling.

DISCUSSION

New approaches to identify novel small-molecule inhibitors of AR function are critically important because of the need to inhibit CRPC cells resistant to first- and second-generation antiandrogens such as flutamide, nilutamide, bicalutamide, and the more recently approved enzalutamide. In this study, we describe a high-throughput HCS campaign to identify small molecules capable of inhibiting the level of nuclear AR using C4-2 CRPC cells stably transfected with 2GFP-AR. Using this AR nuclear localization HCS assay, we identified three novel small-molecule hits from a library of 219,055 compounds that were capable of reducing the level of nuclear AR in C4-2 cells. Two of the three small-molecule hits are structurally related and effectively reduced the nuclear

Fig. 6. Prioritized Hits from the 2GFP-AR nuclear localization HCS campaign (**A**) selected hits. Chemical structures, PubChem substance identifiers (SID), PubChem compound identifiers (CID), IC₅₀ values, and the results of a cross target query of the PubChem database for 3 small-molecule hits capable of inhibiting AR nuclear localization and/or activity in C4-2 cells. (**B**) SID 14730725 and SID 14742211 concentration responses. N3 C4-2-2GFP-AR cells were seeded at 3×10^3 cells per well into Com-PLL 384-well microtiter plates and cultured overnight in complete media and then treated overnight with the indicated concentrations of SID 14730725 (○) or SID 14742211 (●). The normalized% inhibition of the 2GFP-AR MCRAID-Ch2 values from a representative 10-point serial dilution experiment performed in triplicate wells per concentration is presented. (**C–E**) Manual validation of candidate small molecules targeting AR in C4-2 cells. The effect of 3 small molecules at their indicated concentrations on 2GFP-AR subcellular localization in C4-2 cells on regular culture plates. C4-2-2GFP-AR cells were treated with vehicle control or indicated concentrations of small-molecule SID 14730725 (**C**), SID 14742211 (**D**), and SID 3712502 (**E**) overnight before taking images by fluorescent microscopy. (**F**) The effect of small-molecule SID 3712502 on AR and PSA protein levels in C4-2 cells. C4-2 cells on regular culture plates were treated with indicated concentrations of SID 3712502 for 48 h and then harvested for preparation of cell lysates, SDS-PAGE separation and immunoblotting analysis of AR and PSA. GAPDH protein was probed as a loading control. PSA, prostate-specific antigen.

localization of 2GFP-AR in a concentration-dependent manner. The third screening hit did not induce the cytoplasmic localization of 2GFP-AR but inhibited the expression levels of AR proteins in C4-2 cells. The high-throughput HCS assay based on C4-2-2GFP-AR subcellular localization performed well in screening and based on our experience, this HCS assay could be used to screen additional small-molecule libraries.

Using the MT image analysis algorithm of the ArrayScan V^{II} HCS platform, we were able to quantify the relative intensity of

2GFP-AR in the nuclear and cytoplasm regions of the C4-2 CRPC cell line and to calculate the extent of the altered subcellular distribution phenotype produced by exposure to the HSP90 inhibitor 17-AAG. An interesting observation from this quantitative analysis is the finding that 17-AAG inhibited the nuclear intensity of 2GFP-AR without apparently increasing the cytoplasmic intensity of 2GFP-AR (Fig. 3). Since the 17-AAG reduction of average nuclear intensity did not translate into an increase in cytoplasmic intensity of 2GFP-AR in C4-2 cells, the mechanism of 17-AAG inhibition might be

primarily mediated through a degradation of nuclear AR rather than inducing the translocation of nuclear AR to the cytoplasm. Although 17-AAG affects diverse signaling pathways, it reproducibly produced a cytoplasmic subcellular distribution phenotype for 2GFP-AR localization in C4-2 cells. Thus, it provided a reliable positive control for the screening assay. However, 17-AAG can cause cell death since it inhibits multiple signaling pathways. It was important to determine an appropriate concentration and adequate length of exposure such that 17AAG reduced 2GFP-AR nuclear localization without inducing significant cell death. Additional studies will be required to clarify the mechanism by which 17-AAG affects the subcellular localization of AR in CRPC cells. It is possible that other small molecules capable of inhibiting androgen-independent nuclear localization of AR may also function through selective degradation of AR in CRPC cells.

The structures of all three small molecules are very different from androgens or the known AR antagonists. Therefore, the mechanisms of their action are likely to be very different from the existing AR antagonists, which target the LBD of AR. AR inhibition by the three small-molecule hits could be mediated through direct mechanisms targeting AR or by indirect mechanisms such as targeting an AR cofactor or the expression of an AR cofactor. A determination of the mechanism(s) of action of the small-molecule hits could provide new insights into how AR subcellular localization and/or turnover are regulated in CRPC cells. This should be an important future research direction.

In summary, we have developed a high-throughput HCS assay for small molecules capable of reducing the level of nuclear 2GFP-AR in C4-2 cells. We have also identified three small molecules from two structural clusters as inhibitors of 2GFP-AR nuclear localization and/or AR function in CRPC cells. Further characterization of these screening hits may lead to new AR antagonists with the potential to treat CRPC patients.

AUTHOR CONTRIBUTION

All authors have contributed to the manuscript and approved the final version of the manuscript.

DISCLOSURE STATEMENT

No competing financial interests exist.

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Abbreviations Used

17-AAG	= 17-allylaminogeldanamycin
2GFP-AR	= GFP-AR-GFP
AR	= androgen receptor
AS-VTI	= ArrayScan V ^{TI} automated HCS platform
Ch1	= channel 1
Ch2	= channel 2
Circ	= nucleus region
CRPC	= castration-resistant prostate cancer
DMSO	= dimethyl sulfoxide
FBS	= fetal bovine serum
GFP	= green fluorescent protein
HCS	= high-content screening
LBD	= ligand-binding domain
MCRAID-Ch2	= mean circ-ring average intensity difference in channel 2
MLSCN	= molecular library screening center network
MT	= molecular translocation
NIH	= National Institutes of Health
NL1	= nuclear localization signal 1
NL2	= nuclear localization signal 2
PBS	= phosphate-buffered saline
PSA	= prostate-specific antigen
Ring	= cytoplasm region
SCC	= selected object or cell count