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TITLE: Novel Small Molecule Antagonists of the Interaction of the Androgen Receptor and Transcriptional Co-regulators

PRINCIPAL INVESTIGATOR: Clementine Feau, Ph.D.

CONTRACTING ORGANIZATION: St. Jude Research Children's Hospital
Memphis, TN 38105

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Clementine Feau, Ph.D.

E-Mail: clementine.feau@stjude.org

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**14. ABSTRACT**

Androgens, mediated by the Androgen Receptor (AR), play a crucial role in prostate cancer. Current treatments are focused on anti-androgenic drugs competing with natural androgens and antagonizing the transcriptional activity of the AR. Although widely used, these drugs have shown significant side effects and in addition, tumors have become resistant suggesting mutations of the receptor. Regulation of gene expression by AR requires the binding to androgens or to its natural ligand, dihydrotestosterone (DHT), and assembly of coregulatory proteins (CoR). The blockage of the interaction between DHT-liganded AR and CoR by small molecules has been shown to inhibit gene transcription. Thus, novel approaches to inhibit AR activity by means other than ligand binding could have a significant clinical impact. Preliminary data revealed that non-steroidal anti-inflammatory drugs, like flufenamic acid (FLF), block AR transcriptional activity but don't displace androgens. We developed small molecule structural analogs of FLF that inhibit AR transcription activity in the nanomolar range and act on AR target genes. We demonstrated an AR antagonism in vitro (transcription assays, binding assays) that is different from ligand competition. Multiple lines of evidence presented here suggest that compounds function by a distinct mechanism than the current marketed anti-androgens (drug combination studies). An early pharmacological profile of these small inhibitors has been characterized (cytotoxicity, cell permeability). We provide the first class of small molecules able to inhibit AR transcription activity without androgen competition. Those small molecule inhibitors represent powerful assets to study the mechanism of AR transcriptional function and a new potential therapeutic modality for prostate cancer treatment.

**15. SUBJECT TERMS**
Androgen receptor, dihydrotestosterone, co-regulators, gene regulation, small molecule inhibitors, high-throughput screening

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## Introduction

The Androgen Receptor (AR) is crucial for development and maintenance of the sexual characters, bone density and muscle strength.[1] AR is an important mediator for diseases like prostate benign hyperplasia (BPH) and prostate carcinogenesis[1] and belongs to the steroid receptor subclass of nuclear receptor family (NRs) that are intracellular transcriptional factors.[2, 3] In the cytoplasm, inactive AR dissociates from heat shock proteins upon binding to androgens like dihydrotestosterone (DHT), and undergoes a series of conformational changes while translocating to the nucleus.[4] In the nucleus, ligand-activated AR binds as homodimers to specific androgen response elements (AREs), recruits coregulatory proteins, and starts the regulation of a distinct subset of genes.[5-8] Complex interactions with either coactivator or corepressors fine-tune AR mediated gene expression. Like other NRs, liganded AR exhibits extra binding sites on its surface such as activation function 2 (AF2), recruiting coregulators including the steroid receptor coactivator (SRC) family.[9-12]

Although AR mediated signaling transduction might be modulated differently, current pharmacological strategies are focused on developing SARMs (selective Androgen Receptor Modulators) that compete for the hormone binding pocket located within the LBD.[13] Anti-androgens like hydroxyflutamide (OHF) or bicalutamide (Bic) are used successfully against androgen dependent prostate cancer but exhibit strong side effects. Additionally, tumors treated with anti-androgens become resistant within several years of treatment.[14-16] It has been reported that tumor resistance is partly due to mutations that mainly occur in the LBD and lead to a receptor hypersensitive to its natural ligand, other endogenous hormones, and synthetic anti-androgens.[17, 18] The elevated expression of androgen regulated reporter genes indicates, that these mutations directly affects both receptor binding specificity and gene expression. Since AR is still playing an oncogenic role in hormone refractory prostate cancer, the community addresses the importance of targeting AR signaling even in the setting of tumor resistance.[19]

While screening for novel AR inhibitors, our group and collaborators identified that flufenamic acid (FLF), a non-steroidal anti-inflammatory drug (NSAID), is able to disrupt the interaction between AR and coregulatory proteins without displacing DHT.[20] A new AR allosteric binding site, named BF3, was discovered to modulate recruitment of co-regulatory proteins upon binding of small molecules like FLF. The pharmacological activity of NSAIDs is mainly attributed to their inhibition of cyclooxygenase (COX) 1 and 2, key enzymes that convert arachidonic acid to prostaglandin H2. COX-2, in contrast to COX-1,[21] is highly expressed in prostate tissues, modulates cell cycle kinetics, and is abnormally expressed in numerous human cancers.[22-24] FLF has been previously reported to be a chemopreventive agent as it reduces cell-proliferation,[25, 26] especially for androgen-induced cell growth in LNCaP.[27] Investigations elucidating FLF's mode of action are lacking. Herein, we describe the synthesis and biological evaluation of FLF analogs with a new mode of action.

## Body

**Design of novel AR small molecule inhibitors.** Since we identified flufenamic acid **SJ000000101** (FLF) as an inhibitor of the interaction between AR and coregulatory proteins,[20] we synthesized structural derivatives of this lead compound. Substituted anilines were coupled to the 2-chloro-benzoic acid using Ullmann coupling[28] conditions to generate a library of 150 analogs of FLF (Figure 1). We screened them directly in a cell-based transcription assay: the *para*-cyclohexyl (**SJ000000132**) and *para*-phenyl (**SJ000000110**) *N*-phenyl-anthranilic derivatives were found to be the most potent of this first series to reduced AR gene transcription activity in the sub-micromolar range. We then pursue the scaffold optimization by synthesizing a second generation of compounds focused on substitution patterns of the third aromatic ring in para position. This second generation of derivatives presented a strong improvement regarding the inhibitive potency. Activity profiles of the *para*-piperidine (**SJ000311866**), *para*-morpholino (**SJ000000130**) and diverse substituted *para*-phenyl compounds (**SJ000311867-877**) were further investigated.

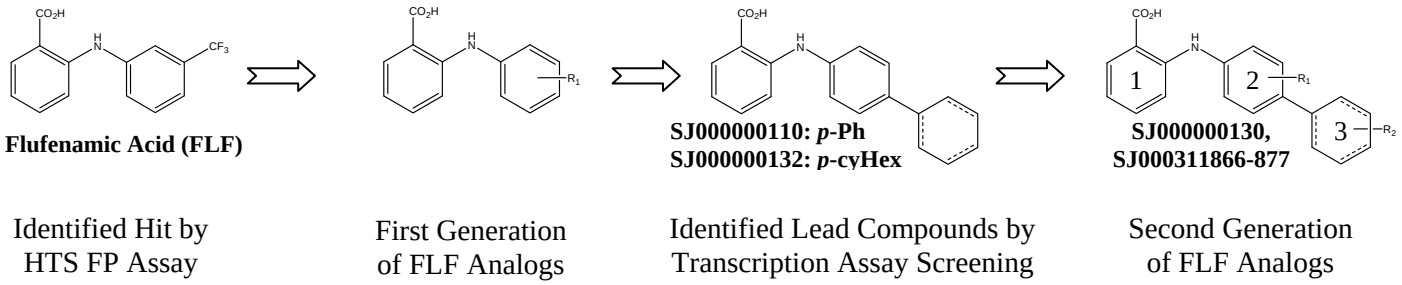


**Figure 1.** General scheme of structural improvement of the flufenamic acid scaffold.

**Novel inhibitors repress AR transcription activity.** At each modification of the flufenamic scaffold, compounds were evaluated regarding their ability to reduce AR gene transcription activity in MDA-kb2 cell line[29] (Figure 2A and Table 2, supp. data). This stable cell line expresses a MMTV promoter where over-expressed AR can bind and activates the transcription of a luciferase reporter gene. After attachment, cells were incubating for 20 hours at 37°C in presence of serial diluted drug with or without DHT (data not shown). After exposure, cells were lysed and $IC_{50}$ values were calculated based on the luminescence read-out. In the optimized conditions, DHT induces AR transcription activity with an $IC_{50}$ of 0.2 nM and an $IC_{90}$ of 8 nM (Figure 2C); these two concentrations were chosen for assay conditions. In parallel, compounds have been also assayed in a luciferase inhibition assay performed with lysed MDA-kb2 cells. Some compounds showed a slight enzyme inhibition with $IC_{50}$ values around 10 µM allowing non-ambiguous transcription signal analysis (data not shown).

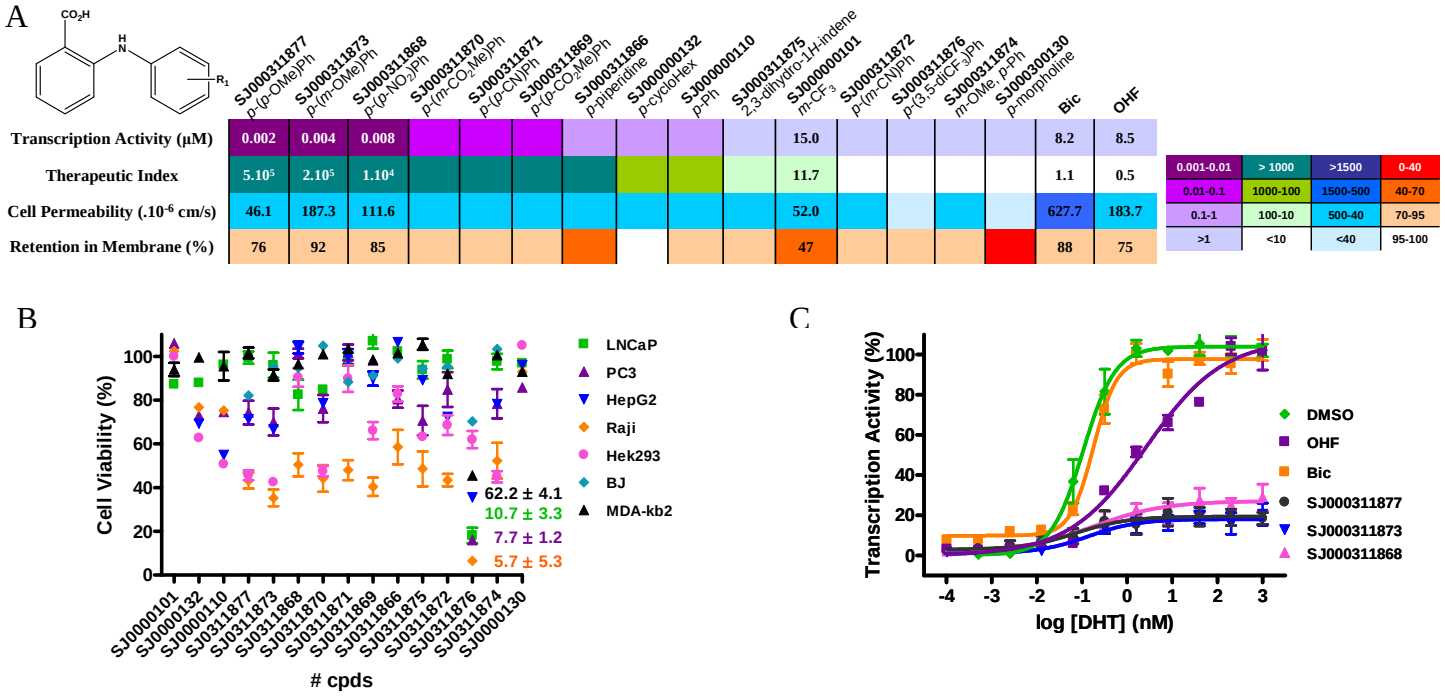


**Figure 2.** A) Heat maps of cellular profiles of flufenamic acid analogs. Transcription activities were measured in MDA-kb2 cells[29] using a luciferase reporter gene assay after 20 hour exposure to serial diluted compounds in presence of 8 nM DHT. Cytotoxicity in MDA-kb2 was measured after 20 hour drug exposure and therapeutic indexes were calculated. Cell permeability and retention in cell membrane were evaluated with the PAMPA technique (pH = 7.4). Calculated constants are gathered in Table 2 (supp. data). B) Cell viability assays were performed after 72 hour of 10 µM drug exposure in different mammalians cell lines and after 20 hours for MDA-kb2. C) DHT-induced AR transcription activity was measured in MDA-kb2 cells using a luciferase reporter gene assay after 20 hour exposure to FLF analogs and DHT competitors, hydroxyflutamide (OHF) and Bicalutamide (Bic) at 100 nM.

The analogs of flufenamic acid from the first generation, **SJ000000110** and **SJ000000132** were able to block AR transcription activity with $IC_{50}$s in the high nanomolar range. When we introduced a piperidine moiety in *para* position, we noticed that the potency of the obtained compound **SJ000311866** remained similar to the cyclohexyl derivative (**SJ000000132)** whereas the change to a morpholine moiety lead us to an inactive molecule (**SJ000000130**). Following up the modification of the *para*-phenyl compound **SJ000000110**, we first synthesized a more rigid molecule such as the fluorene derivative **SJ000311875** which didn't show any ability to inhibit AR signaling. Adding another group on the second aromatic ring, like a methoxy group (**SJ000311874**), resulted in a loss of activity. However, substitution of the third benzene ring gave us more potent compounds with the exception of derivatives **SJ000311872** and **SJ000311873**. In general, analogs from the second generation showed a 10 time stronger inhibition than the unsubstituted phenyl compound **SJ000000110** (**SJ000311869-871**) and the most potent of the series presented $IC_{50}$ values in the low nanomolar range (**SJ000311877**, **SJ000311873**, and **SJ000311868**). Surprisingly, no clear relationship between the position or electronic effect of the substitution pattern could be established with the increase of inhibiting activity.

Cytotoxicity studies were performed in MDA-kb2 using an ATP level detection assay after 20 hour drug exposure. Most analogs were found to be not toxic at 10 μM excepted compound **SJ000311376** that reduced cell viability of 50% at 62 μM. Calculated therapeutic windows ($LD_{50}$ in cytotoxicity assay / $IC_{50}$ in transcription assay) were higher than 3 log of activity for the tested compounds allowing non-ambiguous cell-based assay analysis.

We measured the cell permeability of each synthesized compound and their retention in the lipid layer using a parallel artificial membrane permeation assay (PAMPA)[30] (Figure 2A). The assay was carried out with a 0.5% DMSO content at pH 7.4, reflecting the solution conditions of the cell-based assays. The partition of the derivatives between a donor well and acceptor well separated by a lipid layer was measured by UV absorption. Although FLF analogs were highly soluble in buffer (Table 2, supp. data), we observed that they exhibit medium ($360 > P_e > 40.10^{-6}$cm/s) to very poor ($P_e < 40.10^{-6}$cm/s) cell permeability. Since those compounds are very hydrophobic they tend to accumulate in the lipid layer. They showed high retention rates but their ability to diffuse was very weak but still acceptable compare to the marketed drugs FLF, OHF and Bic. An improvement in cell permeability between the two generations of compounds could explain the improvement of activity observed in cell-based transcription assay. No major change in cellular profiles was found for the analogs of the second generation in comparison to compounds **SJ000000110** and **SJ000000132**.

Each compound was incubated at 10 μM with cultured prostate cancer tissue cell lines (LNCaP, PC3), and other mammalian cell lines (HepG2, Raji, Hek293 and BJ) to determine their cytotoxicity after 72 hours (Figure 2B). The toxicity assays were performed either by using a red-ox indicator (Alamar Blue[31] Biosource / Invitrogen), either by using an ATP level based assay (Cell-Titer Glo, Promega). In general, small molecules didn't show any particular cytotoxicity at high concentration independently of the nature of the cell line. Only the bis(trifluoromethyl) analog **SJ000311876** appeared to be quite toxic and was assayed in a dose response manner. The calculated $IC_{50}$s for **SJ000311876** confirmed that the others analogs were not particularly toxics below 10 μM.

The analogs of the second generation were not significantly more cell permeable, less retained in the lipid membrane, or more toxic than the compounds **SJ000000110** and **SJ000000132** suggesting that the gain in transcription activity was truly due to a structural improvement.

In absence of DHT, the compounds did not activate gene transcription (data not shown). In comparison to the mixed agonist-antagonist OHF that can induce itself up to 25% of the DHT response in this assay, these new small molecule AR inhibitors are pure antagonists. Moreover, the inhibition of marketed drugs OHF and Bic showed an obvious dependency on the DHT concentration used in the assay (Figure 2C). Indeed, when DHT was titrated in MDA-kb2 in presence of 100 nM OHF, we could observe a shift in DHT $IC_{50}$ towards higher values. This behavior is clearly describing the direct competition of this inhibitor for the hormone

binding site. In the case of Bic treatment, barely any effect was detected since Bic is acting at higher concentration than 100 nM (Table 2, supp. data). Concerning the small molecules **SJ000311877**, **SJ000311873** and **SJ000311868**, an important reduction of DHT efficacy up to 80% was observed whereas no shift in DHT $IC_{50}$ value occurred. As for the other described analogs of flufenamic acid (Table 2, supp data), DHT concentration doesn't influence the inhibiting potency of this new class of AR inhibitors suggesting another mode of action different from anti-androgens.

**Novel inhibitors act on AR target genes.** We then studied the effect of the AR inhibitors at the mRNA transcription level in MDA-kb2 and LNCaP (Figure 3). Cells were exposed to drugs with or with out DHT during 20 hours after attachment (no toxicity was detected at that time point: Figure 2B). Real time pcr experiments were performed on 2 house-keeping genes as well as 4 endogenous AR target genes in LNCaP cells. Compound **SJ000311877** was selected for these studies as it is one of the 3 best compounds of the series, and was tested at two different concentrations, 0,1 and 5 µM. Bicalutamide was chosen as a control drug since OHF acts since a mixed agonist in MDA-kb2[29] and LNCaP cells[32]. First in MDA-kb2 (Figure 3A), no dramatic effect was observed on house-keeping gene expression (GAPDH and 18S), confirming that FLF analogs were not general transcription inhibitors. In this particular cell line, we found FK506-binding immunophilin 51 (FKBP51) gene to be DHT well-responsive genes among 4 tested genes. Transmembrane protease serine 2 (TMPRSS2) is shown as an example of DHT non-regulated gene in MDA-kb2 on which the tested drug didn't have any particular effect either. However, the compound **SJ000311877** inhibited the expression of FKBP51 gene in presence of DHT.

Second, we completed this study in LNCaP cells (Figure 3B). Once again, the expression of house-keeping genes was not affected after treatment with the small molecule (data not shown). DHT induced the transcription of FKBP51, TMPRSS2, kalikerin 3 (KLK3) (i.e. prostate specific antigen (PSA)), and NK homeobox family member 3 (Nkx3.1). The compound **SJ000311877** didn't seem to act on gene regulation by it self but reduced DHT effect on AR endogenous target genes. Overall in both cell lines, the flufenamic analog **SJ000311877** when used at 5 µM inhibited the AR gene regulation with the same potency than the marketed Bic. The inhibition due to the compound seems to follow in a dose response manner since at 0.1 µM the effect is similar to DHT alone. Taken together, those results confirm the previous studies in protein level expression in cell-based assay.

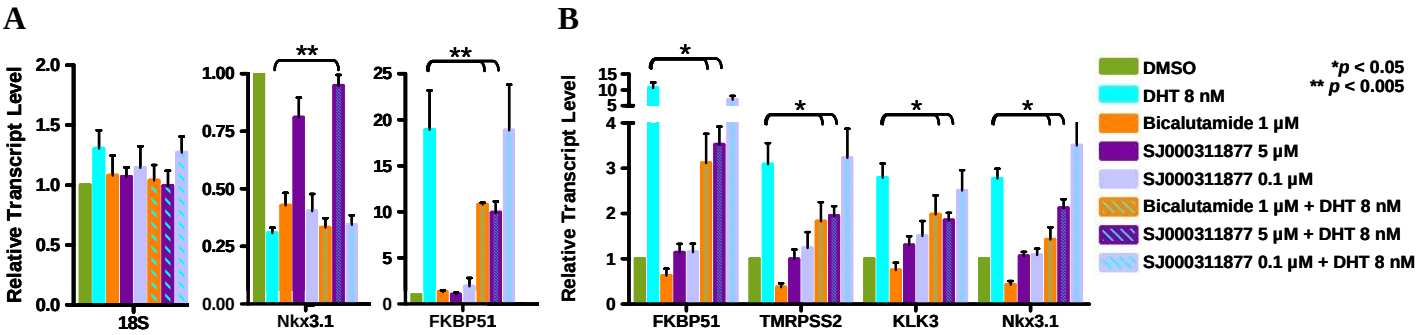


**Figure 3.** Real-time PCR experiments in MDA-kb2 (A) and LNCaP (B) cells. Cells were exposed to drugs during 20 hours after attachment. Transcript level of each tested gene was normalized to GAPDH transcript level and to DMSO control condition. Standard deviations were calculated from 4 biological independent experiments performed in triplicates.

**Hypothetic modes of action of novel AR inhibitors.** We investigated the mode of action of the small molecule inhibitors. Various biochemical assays were performed to test possible binding events up-stream of the gene regulation machinery recruitment governed by AR (Table 1). Since the AR inhibitors have similar chemical structure to marketed carboxylic acid-containing NSAIDs (Diclofenac[33], Lumiracoxib[34]), we first looked at the ability to inhibit COX enzymes. Tested compounds were incubated with the enzymes prior to addition of their substrate, arachidonic acid at 50 µM, following a previously described procedure[35]. Unlike NSAIDs, FLF derivatives didn't block the activity of any of two COX enzymes.

We then studied the eventual binding of each compound to the hormone binding site. A scintillation proximity assay (SPA) was used to monitor the displacement of [$^3$H]-DHT by small molecules from AR-LBD [36]. We assayed each compound in a dose response manner and $IC_{50}$ were calculated. We confirmed that AR inhibitors don't act through the hormone binding site since their binding affinities were much higher than their activity found in a transcription assay.

| # | $R_1$ | $R_2$ | COX inhibition (μM) | | Binding Affinities (μM) | | Solubility Limit (μM) |
|---|---|---|---|---|---|---|---|
| | | | *o*COX-1 | *h*COX-2 | DHT Binding Site (SPA) | Coactivator Binding Site (FP) | |
| **SJ000000101** | *m*-$CF_3$ | | 50 | 230 | No binding | 76.5 ± 7.5 | > 100 |
| **SJ000000132** | *cy*Hex | | 38 | No inhib. | 4.45 ± 0.5 | 46.5 ± 8.5 | 31.6 ± 0.7 |
| **SJ000000110** | *p*-Ph | | >100 | No inhib. | 19.4 ± 9.2 | 34.0 ± 13.0 | 52.9 ± 2.8 |
| **SJ000311877** | *p*-Ph | *p*-OMe | 42 | >100 | 10.9 ± 3.1 | 11.1 ± 5.0 | 12.8 ± 0.3 |
| **SJ000311873** | *p*-Ph | *m*-OMe | 92 | No inhib. | 31.6 ±10.5 | *Fluoresc.* | 62.2 ± 1.0 |
| **SJ000311868** | *p*-Ph | *p*-$NO_2$ | 56 | No inhib. | 5.5 ± 1.1 | 14.7 ± 8.0 | 4.7 ± 0.4 |
| **SJ000311870** | *p*-Ph | *m*-$CO_2$Me | >100 | No inhib. | 19.7 ± 9.3 | 43.0 ± 35.0 | 23.8 ± 1.7 |
| **SJ000311871** | *p*-Ph | *p*-CN | >100 | No inhib. | 15.9 ± 7.7 | 67.5 ± 15.0 | 66.4 ± 1.0 |
| **SJ000311869** | *p*-Ph | *p*-$CO_2$Me | 72 | No inhib. | 12.1 ± 2.7 | 8.2 ± 4.0 | 12.3 ± 0.9 |
| **SJ000311866** | *p*-piperidine | | No inhibition | | No binding | *Fluoresc.* | 85.9 ± 1.6 |
| **SJ000311875** | 2,3-dihydro-1*H*-indene | | >100 | No inhib. | 44.3 ± 14.8 | 11.2 ± 5.0 | 22.3 ± 1.4 |
| **SJ000311872** | *p*-Ph | *m*-CN | >100 | No inhib. | 14.5 ± 4.1 | 29.9 ± 14.0 | 55.0 ± 1.4 |
| **SJ000311876** | *p*-Ph | 3,5-di$CF_3$ | 15 | >100 | 3.9 ± 1.0 | *Fluoresc.* | < 3* |
| **SJ000311874** | *p*-Ph, *m*-OMe | | >100 | No inhib. | 49.6 ± 21.7 | *Fluoresc.* | 77.0 ± 1.0 |
| **SJ000000130** | *p*-morpholine | | No inhibition | | No binding | *Fluoresc.* | > 100 |

**Table 1**. Biochemical binding assays performed with novel AR inhibitors. Enzyme inhibition assays were performed for oCOX-1 and hCOX-2 following the consumption of substrate [1-$^{14}$C]arachidonic acid[35]. A Scintillation Proximity Assay (SPA) was used to measure [$^3$H]-DHT displacement from AR-LBD hormone binding site[36]. A Fluorescence Polarization assay (FP) was used to measure fluorescently labeled SRC2-3 peptide displacement from DHT-bound AR-LBD coregulator binding site[37]. "*Fluoresc.*" indicates when the compound emits itself fluorescence that interferes with the measured signal. Solubility limits with 5% DMSO were evaluated by UV absorption after filtration.

Finally, we looked at binding events at the coregulatory protein binding pocket AF2. We used a fluorescence polarization assay and followed a method previously described for other NRs.[37] In general, the observed $IC_{50}$s were found in the micromolar range reflecting overall the binding affinities of the compounds for the hormone binding site previously measured. The measured binding constants rely on the hormone displacement but don't reflect the $IC_{50}$s found in transcription cell-based assays. Compounds may compete with the hormone in the micromolar range but are obviously able to act through a different pathway to inhibit AR gene transcription activity.

The solubility of each FLF analog was determined in PBS buffer containing 5% DMSO at pH 7.4, reflecting the solution conditions of the biochemical binding assays. This assay was carried out by allowing equilibrium solubility to establish, separating insoluble material by filtration and measuring by UV absorption. Overall, the described derivatives showed solubility limits superior to their binding affinities previously observed; consequently, no solubility issues interfered with the binding assay measurements.

**Novels AR inhibitors act independently of ligand binding.** Since the previous data suggest that this family of inhibitors seems to act through a different pathway than the current anti-androgens, we performed some synergistic experiments. The fixed ratio method[38-40] was chosen and different combinations of compound **SJ000311877** and Bic were serial diluted and tested in a transcription assay in MDA-kb2. From the calculated $IC_{50}$s we could determine the fractional inhibitory concentrations (FIC) and draw the corresponding isobologram (Figure 4).

**A**

| **SJ000311877** / Bic | $IC_{50}$ (nM) | ∑FIC |
|---|---|---|
| 1/0 | 5.2 ± 0.7 | - |
| 1/10 | 55.6 ± 14.4 | 1.17 |
| 1/17 | 129.8 ± 23.4 | 1.71 |
| 1/50 | 210.5 ± 73.7 | 1.24 |
| 1/150 | 309.2 ± 50.0 | 1.05 |
| 1/250 | 274.2 ± 51.9 | 0.79 |
| 0/1 | 468.7 ± 132.8 | |

**B**

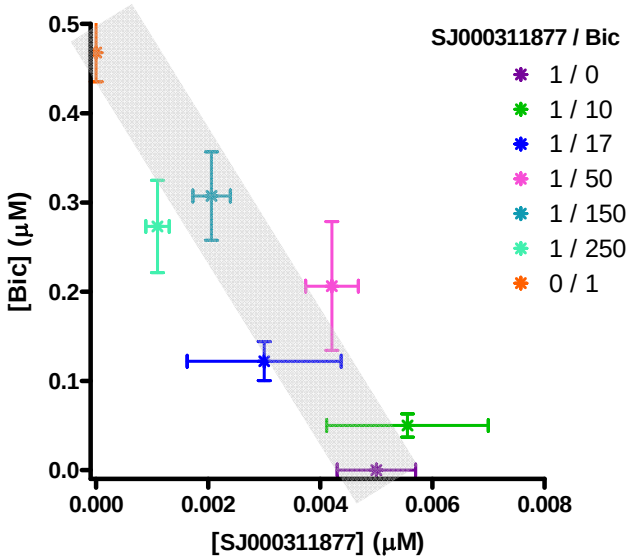


**Figure 4.** Synergistic experiments in MDA-kb2. Transcription assays were performed in presence of 0.2 nM DHT with serial diluted combinations of compound **SJ000311877** and bicalutamide using the method of the fixed ratio. Standard deviations were calculated from two independent experiments. Fractional inhibitory concentrations (∑FIC) were calculated for each $IC_{50}$s (A) and corresponding molecular fractions were plot in an isobologram (B). The defined grey zone represents the empirical "line of additivity".

In theory, ∑FCI is inferior to 0.7 for a synergistic drug combination, superior to 3 for antagonists compounds but we chosen to determine empirically those cut-offs corresponding to our particular study.[41, 42] In control experiments where the drug is combined with itself, we found ∑FCI at $IC_{50}$ to be between 0.90 and 1.31 (data not shown). We also defined empirically the "line of additivity" for the tested combinations (Figure 4B) based on the measured 95% confidentiality of $IC_{50}$s of each individual drug. The calculated ∑FCI as well as the isobologram agreed to define the two drugs as having additive modes of action. We can conclude that biphenyl analogs of FLF and anti-androgenic drugs have totally different signaling pathways that are additionally independent from each other. Since Bic is described as recruiting co-repressors once AR is bound to DNA[43] and no antagonism was observed with Bic pathway, we can hypothesize that those novel AR inhibitors might act downstream of AR binding to DNA response elements.

**FLF derivatives show specificity for the AR.** In order to assess whether the synthetic AR inhibitors were able to inhibit other nuclear receptors transactivation, we checked if our compounds had some inhibitive activity on the Estrogen Receptor (ER) (Figure 5). We used a cell-based reporter luciferase gene assay in U2OS cells. Different FLF derivatives with various potencies regarding AR transcription were incubated and luciferase production induced by estrogens was measured. Overall, the compounds showed inhibition in the 10-100 micromolar range. We can consider that they exhibit specificity towards the AR versus the ER and won't interfere with the ER signaling pathway.

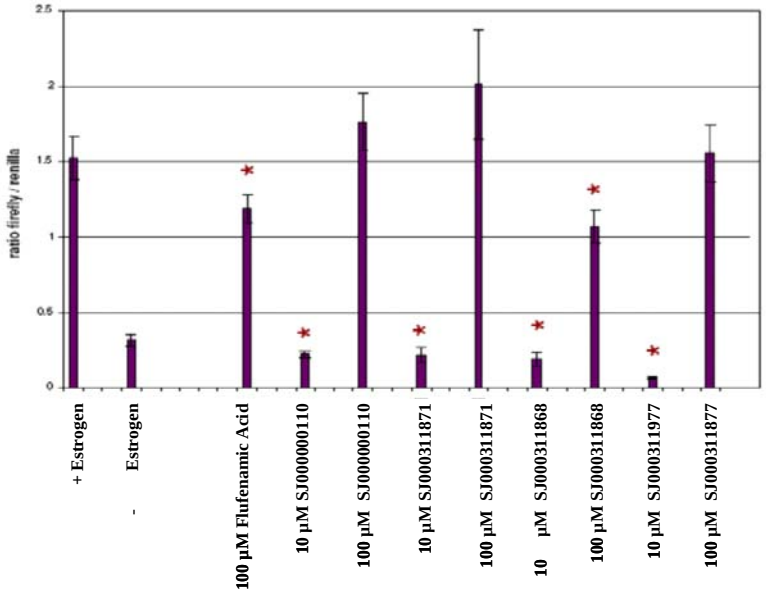


**Figure 5.** Luciferase activity of human cells lysates transfected by plasmids containing ER and Estrogen Response Elements (ERE)-reporter construct (Dr. M. Carraz, Dr L. Brunsveld, Eindhoven University)

## Key Research Accomplishments

- ✓ Design and synthesis of 150 novel flufenamic acid derivatives
- ✓ Full structural characterization and quality control for each compound
- ✓ Development of a novel HTS SPA ligand binding assay for the Androgen Receptor and applicable to other nuclear receptors
- ✓ Determination of binding affinities (hormone binding site, coregulator binding site) for each compound
- ✓ Optimization of a transcription assay in MDA-kb2 cell line in 96 well microplate
- ✓ Establishment of a transcription activity profile for each compound
- ✓ Determination of solubility profile in biologic environment for each compound
- ✓ Determination of cell permeability and membrane retention for each compound
- ✓ Evaluation of the cytotoxic character of each compound in diverse mammalian cell lines
- ✓ Establishment of SAR models to optimize the structure of the FLF scaffold
- ✓ Hit-to-Lead optimization with improvement of inhibitive potency from 10 µM to 1-10 nM in cell-based systems
- ✓ Confirmation of AR transcription activity inhibition at the gene level (rt-pcr studies) on AR target genes in two different cell lines
- ✓ Validation of the novel mode of action of FLF derivatives different and independent of the current anti-androgen therapies (no displacement of the natural ligand)

## Reportable Outcomes

### 1. Publications

◆ **A High-Throughput Ligand Competition Binding Assay for the Androgen Receptor and other Nuclear Receptors.** Clémentine Féau, Leggy A. Arnold, Aaron Kosinski, and R. Kiplin Guy.
*Manuscript accepted in JBS (see pdf file attached)*
Abstract: Standardized, automated ligand binding assays facilitate evaluation of endocrine activities of environmental chemicals and identification of antagonists of nuclear receptor ligands. Many current assays rely on fluorescently labeled ligands which are significantly different from the native ligands. We describe a radiolabeled ligand competition scintillation proximity assay (SPA) for the androgen receptor (AR) using Ni-coated 384-well FlashPlates® and liganded AR-LBD protein. This highly reproducible, low cost assay is well-suited for automated HTS. Additionally, we show that this assay can be adapted to measure ligand affinities for other nuclear receptors (peroxisome proliferation activated receptor γ, thyroid receptors α and β).

◆ **Non-competitive Androgen Receptor Inhibition *In Vitro* and *In Vivo*.** J. O. Jones, E. C. Bolton, Y. Huang, C. Féau, R. K. Guy, K. R. Yamamoto, B. Hann, M. I. Diamond, *PNAS*, **2008**. (*under review*)
Abstract: Androgen receptor (AR) inhibitors are used to treat a wide array of human diseases, including prostate cancer (PCa). New strategies are needed, because all available anti-androgens target only ligand binding, either by reduction of available hormone or by competitive antagonism. In PCa patients, anti-androgen therapy inevitably results in tumors that grow in the setting of low hormone levels, although most such "androgen independent" growth still depends on AR signaling. An inhibitor that functions by a different mechanism could delay or prevent the development of resistance associated with current treatments. In prior work, we used a cell-based assay of AR conformation change to identify *non-ligand* inhibitors of AR activity. Here, we characterize two compounds identified using this assay, pyrvinium pamoate, an FDA-approved drug, and harmol hydrochloride, a natural product. Each compound functions by a unique, non-competitive mechanism and synergizes with competitive antagonists to disrupt AR activity. Pyrvinium inhibits AR-dependent gene expression in the prostate gland *in vivo*, and induces prostate atrophy.

◆ **Novel Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity.** C. Féau, L. A. Arnold, A. Kosinski, Fangyi Zhu, Michele Connelly, Annie B. Blobaum, Lawrence Marnett, R. K. Guy,
*Manuscript in preparation for Nature Chemical Biology.*

### 2. Trainings

◆ Molecular Operating Environment (MOE), November 2007, St Jude Children's Research Hospital
- _Overview of the software
- _Pharmacophore generation and modeling studies
- _Cheminformatics and Quantitative Structure Activity Relationship

◆ DiscoveryGate, September 2007, St Jude Children's Research Hospital
- _Overview of the software
- _Use of the database

◆ Pharmacokinetics studies, December 2008, St Jude Children's Research Hospital
- _Handling of small rodents
- _Techniques of administration (tail vein injection, oral gavage)
- _Drug monitoring (blood sampling, cardiac stick, organ harvest)

**3. Poster presentations**

◆ October 2008, **Poster Award**, Androgens 2008 Meeting, Rotterdam (The Netherlands) (see appendices).
*Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity for Prostate Cancer Drug Discovery.*
Abstract: Androgens, mediated by the Androgen Receptor (AR), play a crucial role in prostate cancer. Current treatments are focused on anti-androgenic drugs competing with natural androgens and antagonizing the transcriptional activity of the AR. Although widely used, these drugs have shown significant side effects and in addition, tumors have become resistant suggesting mutations of the receptor. Novel approaches to inhibit AR activity by means other than ligand binding could have a significant clinical impact. Preliminary data revealed that non-steroidal anti-inflammatory drugs, like flufenamic acid (FLF), block AR transcriptional activity but don't displace the hormone.
Herein we describe the development of small molecule structural analogs of FLF that inhibit AR transcription activity in the nanomolar range and act on AR target genes. We demonstrate AR antagonism *in vitro* (transcription assays, binding assays) that is different from hormone competition. Multiple lines of evidence presented here suggest that compounds function by a distinct mechanism than the current marketed anti-androgens (drug combination studies). An early pharmacological profile of these small inhibitors has been characterized (cytotoxicity, cell permeability).
Small molecule inhibitors represent powerful assets to study the mechanism of AR transcriptional function and a new potential therapeutic modality for prostate cancer treatment.

◆ June 2007, Combinatorial Chemistry Gordon Conference, Cambridge (New Hampshire, USA)
*Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity as Novel Targets in Prostate Cancer Drug Discovery*

◆ March 2007, Keystone Symposia, Steamboat Springs (Colorado, USA)
*Androgen Receptor/ Transcriptional Coregulator Interactions as Novel Targets in Prostate Cancer Drug Discovery*
Abstract: Androgens, mediated by the Androgen Receptor (AR), play a crucial role in prostate cancer. Current treatments include antiandrogenic drugs competing with natural androgens and antagonize the transcriptional activity of the AR. Although widely used, these drugs have shown significant side effects and in addition tumors have become resistant suggesting mutations of the receptor.
Regulation of gene expression by AR requires the binding to its natural ligand and assembly of a dynamic multi-protein complex including obligate coregulatory proteins (CoR). The blockage of the interaction between liganded AR and CoR by small molecules has shown to inhibit gene transcription. These compounds represent a new class of drugs and might overcome tumor resistance during prostate cancer treatment.
Preliminary data revealed that the non-steroidal anti-inflammatory drugs, like flufenamic acid, bind to AR and inhibit the recruitment of CoR. Herein we describe the development of small molecules, analogs of flufenamic acid, using biochemical and cellular assays to establish careful structure activity relationship (SAR) models.
Small molecule inhibitors of the interaction between liganded AR and CoR represent powerful assets to study the mechanism of AR transcriptional function and a new potential therapeutic modality for prostate cancer.

## Conclusion

Over those two years of funding, we were able to take over the primary results of a HTS campaign providing a hit compound, flufenamic acid (FLF), potential inhibitor of the interactions between transcriptional co-regulators (CoR) and the Androgen Receptor (AR). We developed focused libraries of FLF derivatives and directly test them in a transcription assay. Compounds **SJ000000110** and **SJ000000132** turned to be the most potent inhibitors with sub-micromolar activities. Second generation of compounds inspired from **SJ000000110** and **SJ000000132** lead us to a small set of highly active compounds (**SJ000311877**, **SJ000311873** and **SJ000311868**). Their inhibitive potency in cell-based transcription assays occur in the low nanomolar range. Those compounds were able to reduce AR transcription activity up to 80% independently of the DHT concentration. Several lines of evidence proved that this new family of inhibitors doesn't compete with the hormone like current marketed anti-androgens.

These three biphenyl analogs exhibited similar cellular profiles (solubility, cell permeability, retention in membrane) than the compounds from the first generation. The improvement in activity of those small inhibitors is not due to a potential change in physical properties of the molecules themselves. Furthermore, these derivatives didn't present any toxicity in mammalian cell lines either. Finally, enzymatic biochemical assays proved that activity of COX enzymes was not affected by the FLF derivatives. These drugs are not involved in any critical cell viability pathway. Additionally, their specificity for the AR was proven by their inactivity regarding the Estrogen Receptor transcription activity.

The synthesized inhibitors **SJ000311877**, **SJ000311873** and **SJ000311868** didn't reduce gene transcription by themselves proving that they are not general transcription inhibitors. They can be characterized as pure AR antagonists since they didn't enhance gene transcription either. In addition, these compounds were acting especially on diverse endogenous AR target genes and not on AR unregulated genes in MDA-kb2 and LNCaP. In general, these novels AR modulators present an opposite effect than DHT on gene expression level with an equivalent efficacy to bicalutamide. Since LNCaP cells express a hormone refractory AR, consistent reduction of AR target gene expression by the small inhibitors suggest that those drugs would be able to still act on mutated receptor. Finally, combinations of **SJ000311877** and bicalutamide presented clear additivity of the two drugs. This confirms once again that signaling pathways of androgen competitors and the FLF derivatives are totally different and also independent from each other.

Investigations are currently on the way to better characterize the mode of action of these novel AR small molecule inhibitors. With those high activity transcription inhibitors we should be able to design chemical probes inspired from the biphenyl FLF scaffold and solve unclear aspects of the biology of the AR. Meanwhile, we are exploring the therapeutic profile of those promising drugs with pharmacokinetic studies and *in vivo* efficacy studies.

Most drug development strategies focus on competing androgens or inhibiting AR signal up-stream of its binding to DNA. We were able to design novel small molecule inhibitors that strongly reduce AR transcription activity by a non-competitive and independent pathway from anti-androgens. Described potencies of those FLF derivatives are equivalent to the current marketed drugs. Our studies suggest that these inhibitors may overcome side effects observed after anti-androgen treatment and open new perspectives to therapeutic strategies.

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# Clémentine FÉAU, Ph.D.

142 Clark Place #1, Memphis, TN 38104
Phone (pers.) (504) 906-3375
Phone (work) (901) 595-5791
Fax (work) (901) 595-5715
clementinefeau@ymail.com

French citizen
US Permanent Residency application ongoing

## RESEARCH INTERESTS

- Structural activity relationship studies of molecules with biological interest
- Structure-based drug-like ligand design, construction of compound libraries, lead structure optimization
- Special interest in discovery of therapeutic small modulators of transcription factors
- Development of biochemical assays for small molecule-protein interaction studies
- Design of chemical tools like fluorescently labeled probes to study biomolecules of interest

## PROFESSIONAL SKILLS

***Compound synthesis, purification and analytical characterization***

✓Synthesis of small molecules (10 mg to 100 g), microwave-assisted reactions (Biotage), hydrogenations operated on H-cube (Thales)
✓Parallel chemistry using MiniBlock (Bohdan), Minimapper (Mettler Toledo), HT-4/24, EZ-2 (GeneVac)
✓Flash chromatography (silica gel; SP1/4 Biotage), preparative and semi-preparative HPLC (Waters Systems), preparative TLC, distillation, recrystallization
✓NMR (1D, 2D), HPLC, LC-MS, UPLC, nitrogen calibrator, FTIR, UV-VIS spectroscopy, polarimeter

***Biochemical assays: performed, developed and improved towards HTS applications***

✓Protein binding assays: fluorescence polarization, alpha-screen, TR-FRET (Envision, PerkinElmer), radioactivity (TopCounter, PerkinElmer), protein pull-down (SDS gel, western blot)
✓Cell-based assays: cytotoxicity, cell growth, reporter-gene transcription, rt-pcr experiments
✓Solubility (Millipore), permeability assays (Biomex station, PAMPA *p*-ION technique)

***Pharmacokinetics / In Vivo Studies***

✓Small rodent handling: peritoneal injection, tail injection, oral gavage, retro-orbital blood sampling, isoflurane anesthesia, $CO_2$ euthanasia, cardiac stick, organ harvesting
✓Compound formulation for IV and oral gavage administration
✓Analysis of *in vivo* toxicity, bioavailability and drug metabolism

***Computer skills***

✓Microsoft Windows and Macintosh operating systems and applications (MS Office, Chem Office, GraphPad Prism, MestRec, EndNote …)
✓Use of databases (Scifinder, CrossFire, Beilstein, Discovery Gate, Scopus, NCBI PubMed…)
✓Knowledge in computational chemistry, QSAR, pharmacophore modelling (MOE)

***Languages***

✓French (native), English (fluent), German (basic)

## EDUCATION

**⇨Ph.D., Organic Chemistry** ***2002 - 2005***
Université Louis Pasteur, UMR 7514 CNRS, Strasbourg, France
Advisor: Dr. L. Lebeau
*"Design, Synthesis and Applications of New Lanthanide Complexes as Fluorescent Labels for Immunoassays"*

**⇨M.S., Pharmacology and Pharmacological Chemistry** ***2002 - 2003***
Université Louis Pasteur, Strasbourg, France

**⇨M.S., Molecular and Supra-Molecular Organic Chemistry** ***2001 - 2002***
Université Louis Pasteur, UMR 7514 CNRS, Strasbourg, France
Advisor: Dr. L. Lebeau
*"Design of New Molecular Self-organized Tools, Synthesis of Locally Perfluorinated Lipids"*

**⇨Engineering D., Organic and Analytical Chemistry, Process and Engineering** ***1998 - 2001***
INSA (National Institute of Applied Sciences), Rouen, France
3rd year at Lund University, internship and pharmacology courses, Lund, Sweden

## RESEARCH EXPERIENCE

**⇨Post-Doctoral Research Associate** ***Dec. 2005 -***
St Jude Children's Research Hospital, Memphis, USA
Advisor: Dr. R. K. Guy
*"Development of Small Molecules Inhibitors of Androgen Receptor / Transcriptional Co-Regulator Interactions as Novel Therapeutic Targets"*

**⇨Research Engineer Internship Fellow** ***2001 (6 months)***
Sanofi Aventis, CNS Medicinal Chemistry Research Center, Paris, France
Advisor: Dr. M. Saady
*"Synthesis of Tetrahydropyrimidopyrimidone Analogs, Kinase Inhibitors and Applications for Alzheimer's Treatment"*

**⇨Internship Fellow** ***2000 (6 months)***
Laboratory of Supramolecular Approaches towards Catalysis, Lund University, Sweden
Advisor: Dr. K. Wärnmark
"Towards the Synthesis of a New Isoquinolone Derivative of Tröger's Base"

## AWARDS

➢*Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity for Prostate Cancer Drug Discovery*
**Poster Award**, Androgens 2008 Meeting, Rotterdam (The Netherlands), October 2008

➢*Novel Small Molecules Antagonists of the Interaction of the Androgen Receptor and Transcriptional Co-Regulators*
**Prostate Cancer Research Program Training Award 2006**, Department of Defense, ***grant funding for 2006-2008***

## PUBLICATIONS

◆ **A High-Throughput Ligand Competition Binding Assay for the Androgen Receptor and other Nuclear Receptors.** C. Féau, L. A. Arnold, A. Kosinski, R. K. Guy, *Journal of Biomolecular Screening*, **2008**. (*in press*)

◆ **Synthesis and Properties of Europium Complexes Derived from Coumarin-Derivatized Azamacrocycles.** C. Féau, E. Klein, P. Kerth, L. Lebeau, *Synthetic Metals*, **2008**. (*in press*)

◆ **Non-competitive Androgen Receptor Inhibition *In Vitro* and *In Vivo*.** J. O. Jones, E. C. Bolton, Y. Huang, C. Féau, R. K. Guy, K. R. Yamamoto, B. Hann, M. I. Diamond, *PNAS*, **2008**. (*under review*)

◆ **Synthesis of a Coumarin-Based Europium Complex for Bioanalyte Labeling**. C. Féau, E. Klein, P. Kerth, L. Lebeau, *Bioorg Med Chem Lett*, **2007**, 17 (6), 1499-503.

◆ **Novel Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity.** C. Féau, L. A. Arnold, A. Kosinski, Fangyi Zhu, Michele Connelly, Annie B. Blobaum, Lawrence Marnett, R. K. Guy, *in preparation* for *Nature Chemical Biology.*

◆ **Synthesis of Europium Labels Derived from Coumarin-Derivatized Azamacrocycles for Homogeneous Time-Resolved Fluorescence Bioassays.** C. Féau, E. Klein, P. Kerth, L. Lebeau, *in preparation* for *J Org Chem.*

## PRESENTATIONS

➢*Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity for Prostate Cancer Drug Discovery*, **Poster Award**, Androgens 2008 Meeting, Rotterdam (The Netherlands), 10-2008

➢*Small Molecule Inhibitors of the Androgen Receptor Transcriptional Activity as Novel Targets in Prostate Cancer Drug Discovery*. **Poster presentation**, Keystone Symp., Whistler (Canada), 04-2008

➢*Novel Small Molecules Antagonists of the Androgen Receptor Transcriptional Activity as Novel Targets in Prostate Cancer Treatment*. **Poster presentation**, Combinatorial Chemistry Gordon Conference, Cambridge (New Hampshire), 06-2007

➢*Androgen Receptor/ Transcriptional Coregulator Interactions as Novel Targets in Prostate Cancer Drug Discovery*. **Poster presentation**, Keystone Symp., Steamboat Springs (Colorado), 03-2007

➢*Development of Small Molecule Antagonists of the Interaction of the Androgen Receptor and Transcriptional Co-Regulators*. **Poster presentation**, Combinatorial Chemistry Gordon Conf., Oxford (England), 08-2006

➢*Design and synthesis of haptens and dyes*. **Oral communication**, Strasbourg (France), 04-2004

➢*First generation of fluorescent probes and spectral properties*. **Oral communication**, Freiburg (Germany), 06-2003

ANDROGENS

2008

**POSTER AWARD CERTIFICATE**

The Poster Award Committee of Androgens 2008 certifies that the Best Poster Award of Androgens 2008 was presented to

Clémentine O. Féau

This award recognizes her significant, innovative and outstanding scientific achievements in the field of androgen action and androgen receptor function. She presented her investigations in her poster at Androgens 2008 on 2 October 2008 in Rotterdam, The Netherlands. The poster was entitled:

*Small molecule inhibitors of the androgen receptor transcriptional activity for prostate cancer drug discovery*

Poster Award Committee of Androgens 2008:
Dr. Albert O. Brinkmann, Netherlands (chair)
Prof. dr. Olli Jänne, Finland
Prof. dr. Michael Mancini, USA
Dr. Ian Mills, UK

Rotterdam, 2 October 2008

Albert O. Brinkmann, PhD (chair)

# A High-Throughput Ligand Competition Binding Assay for the Androgen Receptor and other Nuclear Receptors.

**Clémentine Féau, Leggy A. Arnold, Aaron Kosinski, and R. Kiplin Guy.**

Department of Chemical Biology and Therapeutics, St Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA

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## ABSTRACT

Standardized, automated ligand binding assays facilitate evaluation of endocrine activities of environmental chemicals and identification of antagonists of nuclear receptor ligands. Many current assays rely on fluorescently labeled ligands which are significantly different from the native ligands. We describe a radiolabeled ligand competition scintillation proximity assay (SPA) for the androgen receptor (AR) using Ni-coated 384-well FlashPlates® and liganded AR-LBD protein. This highly reproducible, low cost assay is well-suited for automated HTS. Additionally, we show that this assay can be adapted to measure ligand affinities for other nuclear receptors (peroxisome proliferation activated receptor γ, thyroid receptors α and β).

**Key words:** Scintillation Proximity Assay, androgen receptor, high-throughput screening, endocrine disrupting chemicals, nuclear receptors.

## INTRODUCTION

The androgen receptor (AR) mediates androgen functions, including maintenance of male secondary sexual characteristics and development of the prostate gland. Like other nuclear hormone receptors (NRs), AR is a transcription factor that becomes active upon binding to its natural ligand, dihydrotestosterone (DHT).[1] Small molecules that inhibit ligand binding can modulate gene transcription regulated by AR. Environmental exposure to antiandrogens, such as DDT, can cause developmental abnormalities.[2] On the other hand, antiandrogens (flutamide, bicalutamide) currently used to cure prostate cancer present side effects and drug resistance has been observed with these treatments which therefore provides a compelling need to discover new antiandrogens.

High-throughput screening (HTS) techniques are attractive for both of these needs. Two classes of AR assays have been developed: 1) cell-based transcription assays measuring the inhibition of AR transcriptional activity by small molecules and 2) biochemical competition assays measuring blockade of ligand binding AR by small molecules. Historically, biochemical assays have been limited by the lack of necessary amounts of pure and functional AR protein whose purification is complicated by low solubility and instability in the absence of androgen [3-4]. Utilizing a His6-tagged AR-LBD (Ligand Binding Domain) expressed in E. coli in the presence of DHT can overcome these problems.[5]

While measuring ligand binding by fluorescence polarization (FP) with commercially available fluorescently labeled ligands has become popular, this technique shows limitations in HTS.[6] Both interference with the emission signal from the fluorescent ligand by tested compounds and perturbation of ligand binding and protein function by the fluorescent ligand can be problems. For a robust and broadly applicable biochemical method, radioligands are superior as they more closely mimic the natural ligand. However radioligands carry with them issues relating to safety and waste disposal. Among radiolabeled ligand binding assays developed for

NRs, only scintillation proximity assays (SPAs) are truly HTS compatible.[7-9] So far, few radiolabeled ligand binding assays have been described in the 96-well format for AR.[10-11]

Herein we report an AR ligand competition binding assay using SPA 384-well FlashPlates® and liganded AR-LBD protein expressed in *E. coli*. Additionally, we show that this assay can be used to measure ligand affinities for other NRs including the peroxisome proliferation activated receptor (PPARγ) and the thyroid receptors (TRα and TRβ).

## MATERIALS AND METHODS

### *Materials*

Chemicals and materials were purchased from vendors and used without purification: [1,2,4,5,6,7-$^{3}$H(N)]-5α-Androstan-17β-ol-3-one ([$^{3}$H]-DHT) (110 Ci/mmol) and [$^{125}$I]-T3 (779 Ci/mmol) (PerkinElmer, Boston, MA**);** [$^{3}$H]-Rosiglitazone (ARC, St. Louis, MO) (50 Ci/mmol); Uncoated 96-well polypropylene (3359) and 384-well polystyrene (3573) microplates (Corning Life Sciences, Acton, MA); 384 Ni-chelate HTS PLUS Flashplates® (PerkinElmer, Boston, MA).

### *Expression and Purification of Proteins*

cAR-LBD ($His_6$; residues 663-919) was expressed in *E. Coli* and purified in the presence of DHT using a modified version of published protocols.[5] Briefly, (pKBU553) was transformed into OneShot BL21 Star (DE3) *E. coli* (Invitrogen) and streaked onto a LB agar Carbenicillin (100 µg/ml) plate. A single colony from this plate inoculated a seed culture (overnight, 37°C). 2 L of 2x LB + 1x Carbenicillin and 10 µM DHT were seeded at 0.1 OD and grown at 25°C with shaking until OD reached 0.6-0.8. Expression was induced with 60 µM (final concentration) isopropyl-β-D-thiogalactoside, and cultures were left to grow 14-16 h at 17°C. Cells were pelleted (20 min, 5000 g), transferred into a 50 mL conical tube, flash frozen (liquid $N_2$), and stored at -80°C. To purify AR, cells were thawed at 4°C and resuspended in 30 mL of freshly prepared buffer 1 (50 mM Tris pH 7.5, 150 mM NaCl, 10 µM DHT, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/L Lysozyme, and Roche Complete EDTA free protease inhibitor cocktail tablet). Cells were lysed by sonication (4°C, 6 x 2 min cycles with 2 min breaks, 30% amplitude, Branson Digital Sonifier) and clarified by ultracentrifugation (2 x 30 min; 100,000 *g*; 4°C). Talon resin (1 ml per liter cell culture) was add to a 50 ml conical tube and washed twice with 15 ml freshly prepared buffer 2 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT). The protein supernatant was added to Talon resin (40 ml of supernatant for each conical tube) and rotated gently overnight at 4°C. The resin was pelleted by centrifuging for 20 min followed by washing five times with 10 ml buffer 3 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 10 mM imidazole). Additionally, resin was washed five times with 10 ml buffer 4 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 10 mM imidazole, 2 mM ATP, 10 mM $MgCl_2$). Elution was carried out in fractions equal to or less then bed volume using buffer 5 (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 µM DHT, 250 mM imidazole, 100 mM KCl). Protein purity (>90 %) was assessed by SDS-PAGE and analytical size exclusion FPLC. Protein concentrations were measured by Bradford and BCA protein assays. Usually 6-8 mg of protein per liter of cell culture were obtained. The protein was dialyzed overnight against buffer 6 (50 mM HEPES pH 7.2, 150 mM $Li_2SO_4$, 10% glycerol, 0.2 mM TCEP, 20 µM DHT) and stored at -80°C in buffer 6.

hPPARγ was expressed and purified following the procedure above using the following modifications. Cultures were grown up and induced at 22°C for the same amount of time as above. Induction was obtained with 500 µM of isopropyl-β-D-thiogalactoside. Buffer 1 contained 20 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM PMSF, 0.5% Triton X-100, and 10 mg/L Lysozyme. Buffer 2 contained 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM imidazole, and 5 mM DTT. Buffer 3 contained 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM DTT, and 1 mM imidazole and was used to wash the beads seven times instead of five. Buffer 4 was not necessary in the purification of hPPARγ. Buffer 5 contained 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM DTT, and 250 mM

imidazole. Buffer 6 contained 50 mM Tris pH 8.0, 25 mM KCl, 2 mM DTT, and 10% glycerol. PPARγ does not require any ligand to remain stable in buffer 6. The average yield was 15 mg per liter of cell culture.

hTRα and hTRβ were prepared using a published procedure.[12]

***SPA Ligand Competition Binding Assay***

All liquid handling was carried out using an automated liquid handling system (Biomek FX). To each well of a 384-well Ni-chelate coated Flashplate® (PerkinElmer) was added 50 µl of 5 µM NR-LBD in assay buffer. After 30-60 minute incubation the protein solution was discarded (followed eventually by washes with assay buffer). 25 µl of serial diluted small molecules in assay buffer containing 10% DMSO were added into each well followed by addition of 25 µl of a radioligand solution in assay buffer. The final assay solution contained 5% DMSO. The plates were sealed with clear tape (Millipore® tape multiscreen) and allowed to equilibrate for 1-24 hours at room temperature or 4°C. Radiocounts were measured using a TopCount Microplate Scintillation and Luminescence Counter (Packard Instrument Company). All data were analyzed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA); $IC_{50}$ values were obtained by fitting data to equation (Sigmoidal dose-response (variable slope)): y = Bottom + (Top-Bottom)/(1+10^((Log$IC_{50}$– x)*Hillslope)); x is the logarithm of concentration; y is the response. Two independent experiments, in triplicates, were carried out for each compound.

In case of AR binding assay, [$^3$H]-DHT was used at a final concentration of 20 nM and the assay buffer contained 50 mM HEPES, 150 mM $Li_2SO_4$, 0.2 mM TCEP, 10% glycerol, 0.01% Triton X-100, pH 7.2. In case of hPPARγ assay, [$^3$H]-Rosiglitazone was at 40 nM and the assay buffer contained 50 mM Tris pH 8.0, 25 mM KCl, 2 mM DTT, 10% glycerol, 0.01% Triton X-100, pH 7.2. In case of hTR assays, [$^{125}$I]-T3 was at 1 nM and the assay buffer contained 50 mM HEPES, 100 mM NaCl, 1 mM DTT, 0.1% BSA, 10% glycerol, 0.01% Triton X-100, pH 7.2.

## RESULTS AND DISCUSSION

***Optimization of an AR SPA ligand competition assay***

A number of assay parameters were optimized. First, we measured total binding (protein and radioligand), non-specific binding (NSB: protein, radioligand and excess of unlabeled ligand) and calculated specific binding (total – NSB) for different protein concentrations (Fig. 1a). A concentration of 20 nM [$^3$H]-DHT was necessary to minimize background (data not shown). The percentage of bound [$^3$H]-DHT in relationship with the input was low, saturating at 4.5%. Based on this result we used an AR concentration of 5 µM.

Secondly, we observed that performing the assay directly by mixing the protein along with the unlabeled and radiolabeled ligand ("mix-and-read") led to a high background signal (higher than 50% of total binding), a narrow signal window 1000 cpm (Fig. 1b), high standard deviations ($IC_{50}$ = 179.1 ± 111 nM), and a low z' value (0.54). However, removal of the protein solution prior to the addition of unlabeled and radiolabeled ligand increased the signal substantially. The addition of consecutive wash-steps resulted in improved data ($IC_{50}$ = 30.6 ± 10 nM) and assay quality (z' = 0.89). Additionally, we reuse the protein solution and carried out the assay the next day without compromising the assay quality (data not shown).

Third, we determined a $K_d$ of 31.6 nM of this specific ligand receptor interaction by measuring radiocounts for different [$^3$H]-DHT concentrations after incubation with 5 µM AR (Fig. 1c). Thus the [$^3$H]-DHT concentration (20 nM) used was lower than the calculated $K_d$, although it was 10 times higher than reported $K_d$ for DHT. [10-11, 13] A $B_{max}$ of 4.1 pmoles of bound [$^3$H]-DHT per mg of AR protein was calculated.

Fourth, we focused on the influence of Triton X-100 (TX-100) (Fig. 1d) or BSA (data not shown). No effect was observed in presence or absence of 0.1% BSA. The dose response curve obtained in absence of detergent showed high standard deviations among each triplicate and consequently a high variability of the $IC_{50}$ (108.6 ± 65 nM). TX-100 concentrations of 0.01% increase the signal window, gave the best z' value (0.92) and an $IC_{50}$ value of 56.9 ± 6 nM.

Fifth, we analyzed the time dependency of the signal at room temperature and 4°C (Fig. 1e). Normally, we accumulated data after 5 hours but the assay could be read after one hour (z' > 0.5). In both cases, the protein was stable at least for 24 hours.

Sixth, we changed the order of addition with no effect on the results (data not shown); thus, the radioligand can be safely added at the very last step of the assay.

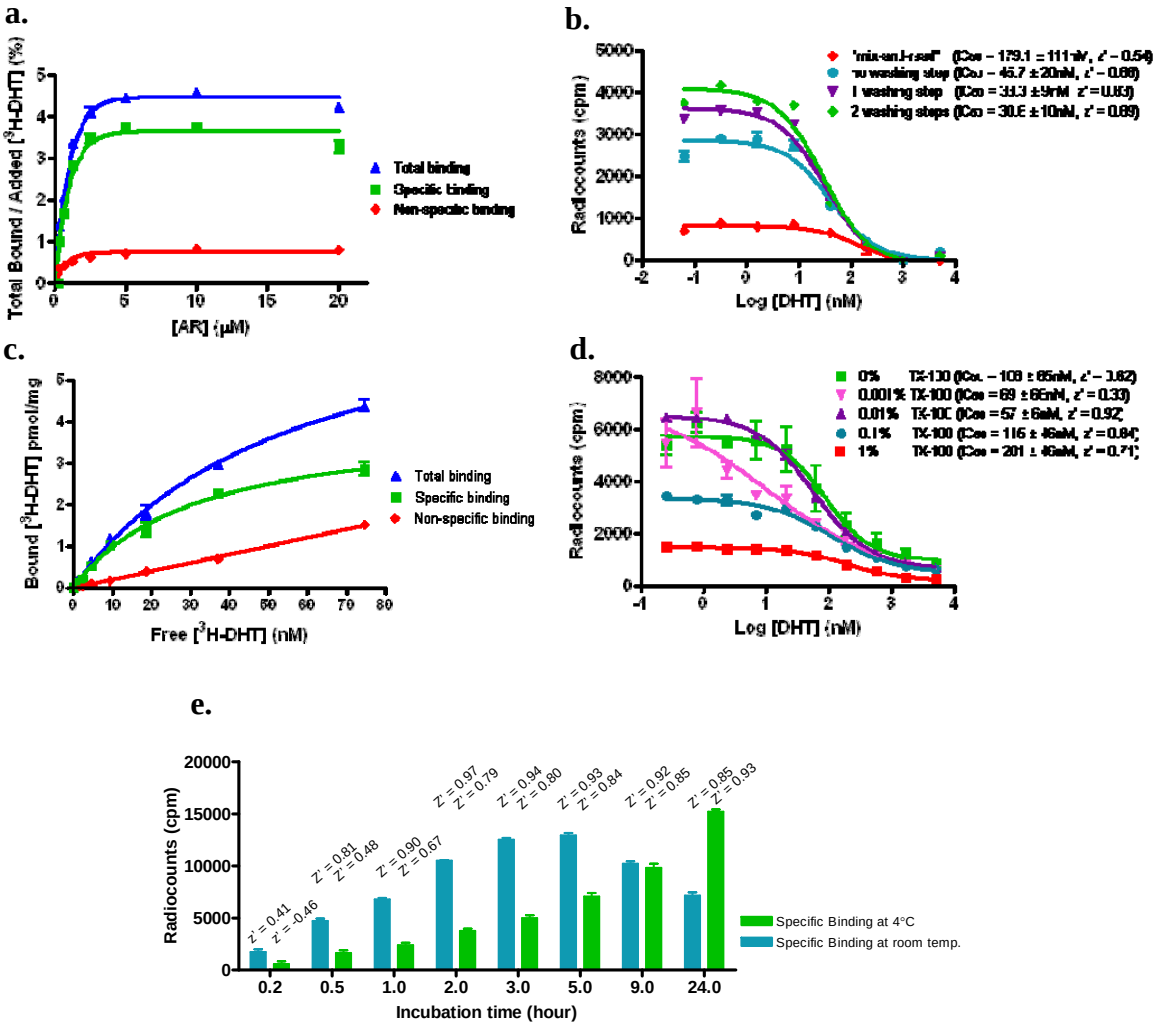


**Figure 1**. Optimization of an AR SPA ligand competition assay. (**a**). Measurements of total binding (AR + 20 nM [$^3$H]-DHT), non-specific binding (NSB: AR + 20 nM [$^3$H]-DHT + 5 µM DHT) and specific binding (SB = total – NSB) for different AR concentrations after 2 washes. (**b**) Effect of washes: SB was measured for experiments carried out with 5 µM AR, serially diluted DHT in the presence of 20 nM [$^3$H]-DHT. (**c**) Saturation binding plot: Measurements of total binding (5 µM AR), NSB (5 µM AR + 5 µM DHT) and SB for different [$^3$H]-DHT concentrations. $B_{max}$ = 4.1 pmol/mg, $K_d$ = 31.6 ± 9.3 nM. (**d**) Influence of Triton X-100 (TX-100): SB was measured for experiments carried out with 5 µM AR for incubation step, serially diluted DHT in the presence of 20 nM [$^3$H]-DHT. (**e**) Incubation time course: measurements of SB after different incubation times at room temperature and 4°C. Corresponding z' values are specified above columns.

### *Evaluation of an AR SPA ligand competition assay*

To evaluate our AR ligand binding assay, we investigated several known competitors of DHT and applied this assay procedure to other nuclear receptors: PPARγ, TRα and TRβ (Table 1). The assay conditions were optimized using [$^3$H]-Rosiglitazone in case of PPARγ and [$^{125}$I]-T3 in case of TRα and TRβ (data not shown).

| AR | | PPARγ | | | TRα | TRβ |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (μM) | | $IC_{50}$ (μM) | | $IC_{50}$ (μM) | |
| **DHT** | 0.057 ± 0.006 | **GW9662** | 0.23 ± 0.02 | **T3*** | 0.03 ± 0.02 | 0.14 ± 0.02 |
| **Methyltrienolone (R1881)** | 0.11 ± 0.02 | **Rosiglitazone** | 0.34 ± 0.08 | **T4*** | 1.4 ± 0.5 | 4.0 ± 2.0 |
| **17β -Estradiol** | 3.2 ± 0.6 | **Troglitazone** | 3.70 ± 0.44 | **GC1** | 1.6 ± 0.3 | 0.29 ± 0.11 |
| **Cyproterone Acetate** | 2.3 ± 0.4 | **Lineolic Acid** | 3.80 ± 0.49 | **TRIAC*** | 0.18 ± 0.1 | 0.07 ± 0.05 |
| **Bicalutamide** | 12.0 ± 2.6 | **Arachidonic Acid** | 4.20 ± 0.53 | **T4Ac*** | 10.0 ± 2.0 | 5.0 ± 2.0 |
| **Progesterone** | 5.0 ± 0.7 | | | **T3Bz*** | 12.0 ± 2.0 | 10.0 ± 4.0 |
| **Hydroxyflutamide** | 33.0 ± 8.0 | | | | | |
| **Flutamide** | 73.4 ± 29.3 | | | | | |
| **Dexamethasone** | 188.5 ± 100.0 | | | | | |

*3,3',5-triiodo-L-thyronine sodium salt (T3); L-thyroxine (T4); 3,3',5-triiodothyroacetic acid (TRIAC); 3,3',5,5'-tetraiodothyroacetic acid (T4Ac); 4-(4'-Hydroxy-3'-iodophenoxy)-3,5-diiodo-benzoic acid (T3Bz).

**Table 1.** Summary of $IC_{50}$ values measured in a SPA ligand competition assay for diverse nuclear receptors.

For the homologous DHT competition assay, we measured an $IC_{50}$ value of 56.9 nM. DHT and R1881 showed the highest affinities followed by miscellaneous steroid hormones. Different classes of PPARγ ligands were investigated. Most active were irreversible antagonist GW9662 and reversible agonist Rosiglitazone. The natural unsaturated fatty acids (linoleic and arachidonic acid) exhibited similar activities. Finally, we tested a panel of known T3 competitors. T3 and its analogue TRIAC showed the highest affinities for TRα and TRβ. The synthetic agonist GC1 and TRIAC exhibited high specificities for TRβ.

Using liganded NRs in these binding assays resulted in general higher $IC_{50}$ values for competitors compared with the literature values. As calculated $K_d$s gave us the same range of binding affinities (data not shown), relative binding affinities (RBAs) remain the best choice to draw comparisons with other binding assays. To show the relevance of our radioassay we plotted log values of measured $IC_{50}$s against log values of reported binding affinities (Fig. 2). We found a statistically significant correlation for the AR ($p = 0.0002$, $N = 6$) and TRs ($p = 0.015$, $N = 4$) receptors, whereas no significant correlation was observed for the PPARγ receptor ($p = 0.214$, $N = 3$).[7, 13-14]

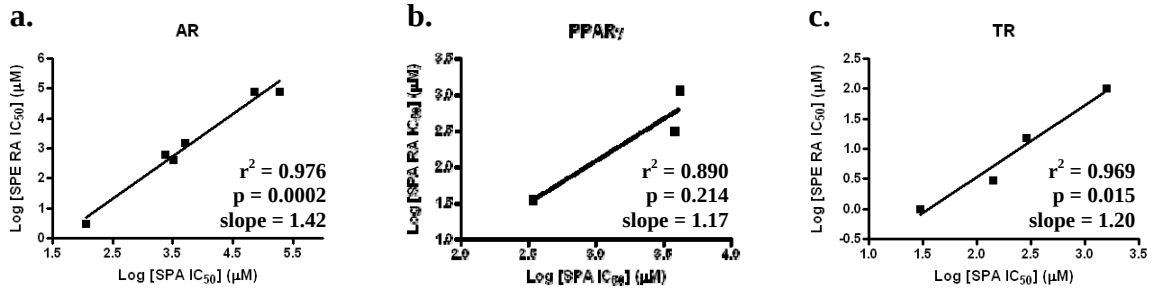


**Figure 2.** Correlation plots of $IC_{50}$ values obtained in NR SPA ligand competition assay using FlashPlate® versus literature values. AR(**a**), PPARγ (**b**) and TR (**c**) SPA ligand competition assays using FlashPlate® vs. reported radioassays. [7, 13-14]

***Validation of an AR SPA ligand competition assay for HTS***

To investigate if the optimized AR SPA ligand competition assay can be automated for HTS, we followed validation protocols from the NIH-NCGC[15]. First, we carried out a plate uniformity assessment. Therefore, after pre-incubation with 5 µM AR solution, three different FlashPlates® were treated with DMSO (high signal), 50 nM DHT (medium signal), and 5 µM DHT (low signal) in the presence of 20 nM [$^3$H]-DHT following the published plate layouts. Radiosignal measurements were read after 5 hours (Fig. 3a and b). Plotting the radiocounts against the well number by row we observed a linear relationship between radiocounts and well number. This behavior excluded the presence of drift or edge effects. Additionally, we plotted radiocounts against the well number by column. The clustering of values indicated no major variation of the measured signal depending on the geographic position. The experiment was repeated twice and z' values were found between 0.85 and 0.92 for all plates confirming the integrity of independent assays (Fig. 3c). Finally, we noticed that DMSO concentrations were tolerated up to 5% without a change in signal (Fig. 3d).

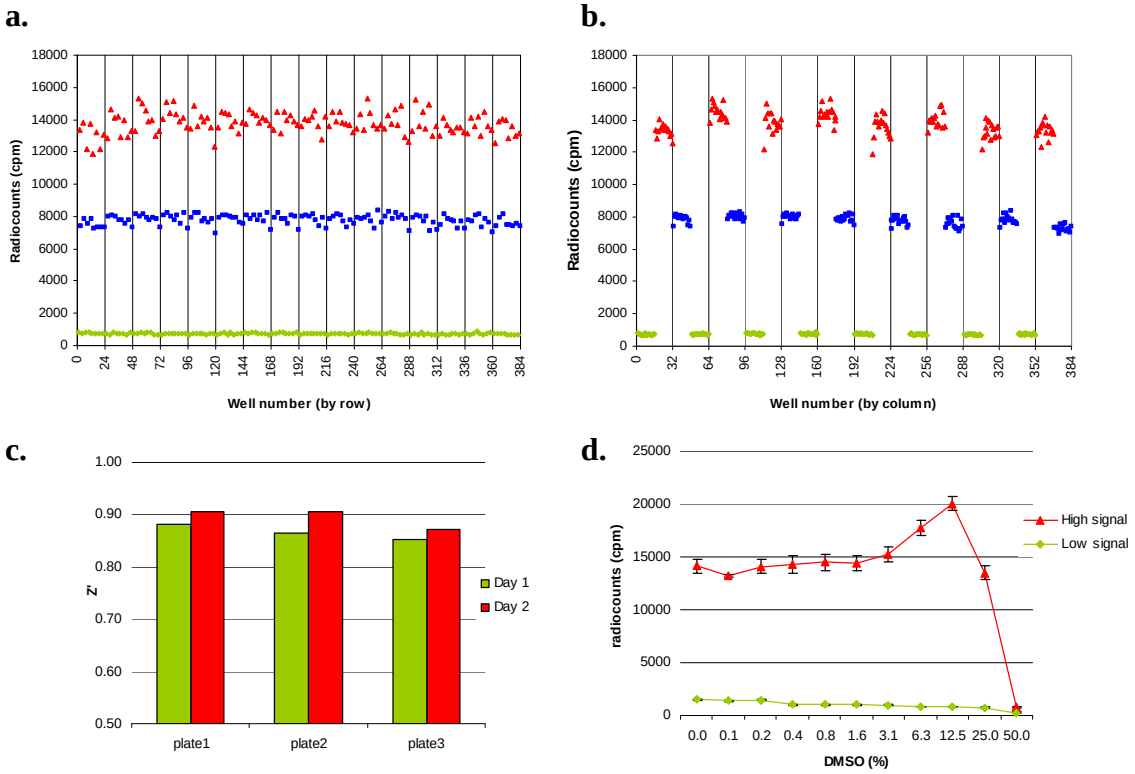


**Figure 3**. HTS validation. Plate geographic effects investigation across rows (**a**)**,** across columns (**b**). DHT was assayed at different concentrations in the presence 20 nM [$^3$H]-DHT (▲ DMSO, ■ [DHT] = 50 nM, ♦ [DHT] = 5 µM). (**c**) Assay reproducibility: comparison of z' values determined by using two independent assays performed in triplicates. (**d**) Assay stability: influence of DMSO content on radiocounts.

In summary, we describe a ligand competition assay for androgen receptor using 384-well FlashPlates® and purified liganded AR-LBD. The "mix-and-read" process leads to very low accuracy and z' values. We recommend the removal of unbound AR-LBD prior to the addition of small molecule and [$^3$H]-DHT. This process allows protein recycling. Additionally, we were able to confirm the robustness of signal from 1 to 24 hours allowing the detection of slow binders. Drift experiments showed excellent homogeneity and reproducibility. All z' values measured in the optimized conditions are higher than 0.85 whereas fluorescence polarization methods tend to perform around 0.6. Although absolute measured $IC_{50}$s of known binders vary from reported values, we observed a strong correlation between $IC_{50}$s determined by both methods, indicating this method provides reliable measurement of relative binding affinities. The addition of the radioligand as the last protocol step followed by sealing with

clear tape decreases significantly the risk of contamination. Finally, the cost per data point was relatively low in comparison with other NR binding assays due to the 384-well format and in-house production and recycling of proteins. Overall, we are convinced that this assay can be fully automated and used for HTS purpose.

## ACKNOWLEDGMENTS

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# Non-competitive Androgen Receptor Inhibition *In Vitro* and *In Vivo*.

Jeremy O. Jones[1,2], Eric C. Bolton[2], Yong Huang[3], Clementine Feau[4], R. Kiplin Guy[4], Keith R. Yamamoto[2], Byron Hann[5], Marc I. Diamond[1,2]

1_Department of Neurology, UCSF, San Francisco, CA
2_Department of Cellular and Molecular Pharmacology, UCSF, San Francisco, CA
3_Department of Biopharmaceutical Sciences, UCSF, San Francisco, CA
4_Chemical Biology & Therapeutics, St. Jude Children's Research Hospital, Memphis, TN
5_Comprehensive Cancer Center, UCSF, San Francisco, CA

Corresponding Author:
Marc Diamond, M.D.
GH-S572B
600 16th Street
San Francisco, CA 94143-2280
Phone: 415-514-3646
Fax: 415-514-4112
Email: marc.diamond@ucsf.edu
21 Pages, 4 Figures

**Abstract**
Androgen receptor (AR) inhibitors are used to treat a wide array of human diseases, including prostate cancer (PCa). New strategies are needed, because all available anti-androgens target only ligand binding, either by reduction of available hormone or by competitive antagonism. In PCa patients, anti-androgen therapy inevitably results in tumors that grow in the setting of low hormone levels, although most such “androgen independent” growth still depends on AR signaling. An inhibitor that functions by a different mechanism could delay or prevent the development of resistance associated with current treatments. In prior work, we used a cell-based assay of AR conformation change to identify *non-ligand* inhibitors of AR activity. Here, we characterize two compounds identified using this assay, pyrvinium pamoate, an FDA-approved drug, and harmol hydrochloride, a natural product. Each compound functions by a unique, non-competitive mechanism and synergizes with competitive antagonists to disrupt AR activity. Pyrvinium inhibits AR-dependent gene expression in the prostate gland *in vivo*, and induces prostate atrophy.

**Introduction**
The androgen receptor (AR) is a member of the nuclear hormone receptor (NR) superfamily, which consists of a large group of ligand-regulated transcription factors. AR is expressed in many tissues and influences an enormous range of physiologic processes such as cognition, muscle hypertrophy, bone density, and prostate growth and differentiation (1). However, AR signaling is directly linked to numerous human conditions including benign prostatic hyperplasia, alopecia, and hirsutism (2). AR signaling also drives the proliferation of prostate cancer, even in the setting of therapies that reduce systemic hormone ligand levels, making AR the major therapeutic target for this malignancy (3). Prior to ligand binding, AR associates with a complex of cytoplasmic factors and molecular chaperones that maintain the receptor in a high-affinity ligand binding conformation (4). AR signaling is initiated by binding of testosterone or the more potent dihydrotestosterone (DHT). This induces an intramolecular conformation change in AR that brings the amino (N) and carboxy (C)-termini into close proximity. This occurs with t1/2~3.5min in cells treated with DHT (5), and does not occur in cell lysates (6), suggesting that conformation change is not protein autonomous, but depends on additional cellular factors. Activation of AR causes its accumulation in the nucleus, where it binds to DNA as a homodimer at specific androgen response elements (AREs) to regulate gene expression. Transcriptional control by AR results from complex interactions with positive (coactivator) and negative (corepressor) factors (1). These coregulatory factors fine-tune the control of AR activity, and AR can even be activated in the absence of

ligand by various cross-talk pathways (7). Although AR activity is highly regulated with many possible points for intervention, all existing approaches to treat the diseases driven by AR signaling ultimately target ligand binding to AR. This includes direct competition with competitive antagonists, and reduction of systemic ligand levels with chemical castration agents, both of which form the basis of androgen deprivation therapy (ADT). ADT initially controls PCa proliferation, resistance develops in patients within 3-5 years, yet the majority of these resistant cancers remain dependent upon AR signaling (3). We hypothesized that by targeting AR signaling by an alternative means we could identify *non-ligand* AR inhibitors that may lead to a novel therapeutic approach to treat androgen-dependent diseases. Furthermore, the characterization of such inhibitors would offer important insights into the molecular mechanisms controlling AR activity. Using a cell-based assay, we identified compounds that inhibited AR conformation change and subsequent transcriptional activity (6). Here, the two most potent AR inhibitors identified by the screen, pyrvinium pamoate (PP), an FDA-approved drug (8, 9), and harmol hydrochloride (HH), a natural product (10) (Fig. 1a) are evaluated for their ability to non-competitively inhibit AR activity *in vitro* and *in vivo*.

**Results**

**PP and HH are potent, synergistic anti-androgens in cultured PCa cells.**

We began by testing the ability of PP and HH (Figure 1a) to inhibit endogenous AR activity in two prostate-derived cancer-derived cell lines: LNCaP, which expresses a “hormone-refractory” AR mutant (11), and LAPC4, which expresses wild-type AR (12). These cells were transfected with androgen-responsive firefly luciferase reporter and androgen-unresponsive renilla luciferase reporter plasmids. PP and HH inhibited AR signaling more potently than the competitive antagonists hydroxy-flutamide (OH-F) or bicalutamide (BiC) when used alone (Figure 1b). Neither drug induced reporter activity in the absence of DHT. When the pamoate counter ion of PP was replaced with a chloride, the resultant compound was equally potent, confirming pyrvinium as the active component (Figure 1b). The potencies of various combinations of PP, HH, and BiC were examined using this assay (Figure 1c). A 1:1 combination of PP and BiC was synergistic in LAPC4 cells, using combination indices as a gauge (13). The same combination appeared to be synergistic in LNCaP cells as well, though a 1:10 combination was more powerful. A 1:1 combination of HH and BiC displayed strong synergy in both LNCaP and LAPC4 cells, as did a 1:10 combination of HH and PP. The synergy observed between these compounds suggests that BiC, HH, and PP each regulate AR signaling by distinct mechanisms. Next we quantified the transcript levels of six endogenous androgen-responsive genes (14-18) in LNCaP cells treated with AR inhibitors. DHT induced the transcription of transmembrane protease serine 2 (TMPRSS2), kallikrein 3 (KLK3, or prostate specific antigen (PSA)), NK homeobox family member 3 (Nkx3.1), and FK506-binding immunophilin 51 (FKBP51). BiC, PP, and HH reduced the expression of most genes by more than 50%, with PP and HH being at least 10-fold more potent than BiC (Figure 1d). DHT suppressed the transcription of metalloproteinase 16 (MMP16) and G-protein coupled receptor RDC1 homolog (RDC-1), which was blocked to varying degrees by each inhibitor. While reaching statistical significance with such a small number of samples is difficult, the trend for each gene was identical in all four experiments, and PP and HH were more potent than BiC at most genes, corroborating results with the luciferase reporter. Neither PP nor HH have chemical structures similar to known AR ligands (Figure 1a). To determine if these drugs act competitively to inhibit AR-regulated transcription, DHT was titrated in LAPC4 cells transfected with an androgen-responsive firefly luciferase reporter in the presence or absence of 1μM OH-F, 300nM HH, or 100nM PP (Figure 2a). The competitive antagonist OH-F shifted the dose response to the right, without preventing the maximal DHT response. In contrast, PP and HH blocked maximal AR activity, and excess DHT could not overcome this inhibition. These responses are most consistent with PP and HH being non-competitive inhibitors. To test directly for ligand competition, LAPC4 cells were incubated with 1nM [3H] DHT and unlabeled competitors (Figure 2b). Unlabeled DHT and the competitive antagonist OH-F effectively competed [3H] DHT binding. PP did not display any significant competition with [3H] DHT, demonstrating its non-competitive nature. HH competed for ligand binding, but only at concentrations ~30x higher than its EC50, suggesting that this is not its mechanism of action. Results were similar in LNCaP cells. Taken together, these data indicate that, at their effective concentrations, PP and HH are not competitive antagonists. Subtle changes in AR conformation are thought to translate to distinct downstream AR activities (19, 20). The synergy between

PP and HH suggested that although both drugs inhibit ligand-induced conformation change, they function by distinct mechanisms to prevent AR transcriptional activity. Neither inhibitor affected AR expression levels, or nuclear localization following DHT treatment (data not shown). Thus, we used chromatin immunoprecipitation (ChIP) to test for effects of each compound on AR occupancy at AR binding sites (ARBSs) located near androgen-responsive genes. The androgen-unresponsive heat shock protein 70 (HSPA1A) promoter was a negative control (Figure 2c). DHT stimulated AR occupancy of binding sites near androgen-induced (ARBS 2.10, 3.25, 6.35, 12.13, 14.04, and ARE III) and androgen-repressed (ARBS 4.15, 8.04) genes in LNCaP cells. HH and BiC each prevented receptor access to ARBSs. In contrast, PP did not appreciably affect DHT-induced AR occupancy of ARBSs. In conjunction with their synergy in transcription assays, these results indicate that PP and HH inhibit AR signaling by independent mechanisms. For *in vivo* testing, we first determined the approximate half-life and toxicity of PP and HH. A single 5mg/kg dose of PP or 2mg/kg dose of HH was administered to male, FVB mice by intraperitoneal (IP) injection or oral gavage (PO). The plasma concentration of PP was greater in mice after IP administration (Figure 3a). HH was too rapidly metabolized to be evaluated further *in vivo*. PP was toxic at doses ≥5mg/kg (IP) when administered for two weeks; 1mg/kg PP caused a mild reduction in body weight in the first three days of treatment; mice given less than 1mg/kg PP showed no adverse effects (data not shown). Weight loss was averted by escalating the dose of PP from 0.1mg/kg to 1mg/kg over the first week of treatment. These data were consistent with the results of the National Cancer Institute's *in vivo* tumor screen (http://dtp.nci.nih.gov). PP serum levels 24hrs after the final dosing were ~5ng/mL, indicating that the drug did not accumulate over the course of this experiment. The data indicate that the concentration of PP in the plasma fell from approximately 150nM to 20nM using this once daily dosing regimen—well within the efficacious range of the drug. These experiments indicated it was feasible to carry out a study of *in vivo* efficacy of PP as an anti-androgen. Androgen deprivation *in vivo* causes global prostate atrophy, and castrated mice have reduced prostate size accompanied by characteristic changes at the cellular level (21). Thus, we examined the effects of PP in mice, using prostate size, gene expression, and morphologic changes as indicators of anti-androgen activity. Cohorts of 9-10 litter-controlled FVB male mice were treated for four weeks with PP (escalated over 10 days to 1mg/kg), BiC (100mg/kg), their combination, or vehicle controls. An additional cohort was castrated at the onset of the study as a positive control. Weights of the mice did not vary significantly among the treatment populations (data not shown). After four weeks, animals were sacrificed, and the prostates were dissected and weighed, including equal portions of each lobe for histochemical analysis and RNA extraction. We used analysis of variance (ANOVA) to test for differences among treatment groups. PP alone caused a 9% reduction in prostate weight compared to the vehicle treated group, which was not statistically significant ($p=0.3$). The remaining treatments all caused significant changes in prostate weight. BiC caused a 35% reduction, and combining BiC with PP caused a 63% reduction in prostate weight. This was not significantly different from the 74% reduction observed in the castrated group (Figure 3b), suggesting that combination treatment was as effective as castration. Hematoxylin and eosin staining of the prostate revealed changes characteristic of androgen deprivation in all prostate lobes, including loss of columnar architecture, decreased secretory protein, thickening of the stromal layer, and dead cell accumulation in the lumen (Figure 3d). We measured AR-regulated transcript levels in the prostate via quantitative PCR. We included the probasin gene (22) instead of PSA, which is not expressed in mice. All four treatments significantly decreased the level of TMPRSS2, Nkx3.1, probasin, PSP94 and FKBP51 transcripts (Figure 3c). PP and BiC had similar effects. In every case their combination was more effective than either drug alone, and in most cases this difference was significant. Castration consistently resulted in the greatest decrease in androgen-responsive transcripts. Importantly, at 4 of 5 genes, this decrease was not significantly different from the combination treatment. These data indicate that combination treatment with a competitive (BiC) and non-competitive (PP) AR inhibitor is nearly as powerful as castration, using the prostate as a physiologically relevant biomarker.

**Discussion**

We previously created and applied a cell-based assay to identify AR inhibitors that function by inhibiting AR ligand-induced conformational change, an activation step distinct from ligand binding. With this assay, we identified two potent, non-ligand AR inhibitors. Here, we have used these inhibitors to demonstrate AR

antagonism *in vitro* and *in vivo* that is not based on competition for ligand binding. Several lines of evidence suggest that HH, PP, and BiC each function by a distinct mechanism, including their synergy with each other, the lack of effect of PP and HH on ligand binding, and the different effects of PP and HH on AR promoter occupancy. The synergy between PP and BiC appears to extend *in vivo*, as PP causes morphologic changes consistent with androgen deprivation, reduces AR-dependent gene expression, and augments the inhibitory activity of BiC at the prostate gland in experimental mice. The mechanisms of the three inhibitors studied here are distinct (Fig. 4). BiC binds the ligand binding pocket of AR, presumably inhibiting AR signaling by preventing DHT from binding. HH and PP clearly do not bind the AR ligand binding pocket or prevent DHT access, yet they are able to prevent normal conformation change and inhibit AR activity. Neither drug affects AR protein stability or nuclear accumulation. ChIP experiments suggest that HH prevents AR conformational change which leads to failure of ARBS binding. In contrast, PP still permits ARBS binding, and instead appears to block AR recruitment of necessary transcription factors (Fig. 4). Although the exact target of these novel anti-androgens remains to be identified, we predict that PP and HH either bind AR to prevent normal conformation change by an allosteric mechanism, or that they target coregulatory proteins or cross-talk pathways that are necessary for ligand-induced conformation change. A clear implication of our work, and that of others (23), is that there are multiple intracellular binding sites that may be targeted to inhibit AR, and potentially other NRs. Identification of intracellular factors that mediate the effects of these compounds could vastly improve our understanding of nuclear receptor biology, and could significantly expand the spectrum of NR inhibitors available to treat human disease. Most drug development for AR antagonists has concentrated on novel ligands or on mechanisms to reduce hormone production. We have demonstrated that AR signaling can be inhibited *in vivo* by a ligand-independent, non-competitive mechanism, using the prostate of healthy mice as a model. The prostate is highly sensitive to changes in AR signaling, making it an ideal model to test novel anti-androgens. Our work suggests that it may be possible to develop effective non-competitive AR antagonists for clinical use, which could have a significant impact in many diseases.

**Figure Legends**

**Figure 1: Novel, efficacious AR inhibitors.** (**a**) Structures of pyrvinium and harmol. (**b,c**) LAPC4 or LNCaP cells were transfected with luciferase reporter constructs and treated with titrations of the indicated compounds for 24hrs. IC50 values were calculated from the renilla-normalized PSA-luciferase reporter activities from 4 independent experiments with single (**b**) or combination (**c**) treatments. Expected and actual IC50 and combinatorial index values (CI at IC50) were calculated from mean-effect plots. CI50= 1 indicates an additive effect, CI < 1 indicates synergy, CI > 1 indicates antagonism. (**d**) LNCaP cells were treated with 1nM DHT and the indicated compounds for 24hrs and androgen-responsive transcript levels were quantified relative to vehicle treated cells. Averaged results from 4 independent experiments indicate that PP and HH inhibit both AR transcriptional activation and repression. Error bars = standard error of the mean.

**Figure 2: Non-competitive inhibition of AR.** (**a**) LAPC4 cells were transfected with luciferase reporter constructs and treated with a DHT dose titration in the presence or absence of 1uM OH-F, 300nM HH, or 100nM PP. Renilla-normalized PSA-luciferase activity was measured 24hrs later. OH-F shifted the response to the right, indicating competitive antagonism. PP and HH did not shift the curve, but did prevent the maximum response, suggesting non-competitive inhibition. (**b**) LAPC4 cells were incubated with 1nM [3H] DHT and the indicated compounds. Competition by each compound for [3H] DHT binding is expressed relative to the no competition value, which was set to 1. Neither PP nor HH competed for binding at their fully effective concentrations. (**c**) Chromatin immunoprecipitation: LNCaP cells were treated with the indicated compounds for 4hrs. AR-bound chromatin was isolated and AR occupancy was quantified by Q-PCR using primers specific to known binding sites and normalized to non-specific occupancy at the HSPA1A promoter (24). Bicalutamide (BiC) and HH displaced AR from regulated promoters, whereas PP had no effect. Error bars = standard error of the mean.

**Figure 3: Properties and efficacy of PP and HH *in vivo*.** (**a**) Mice were administered 5mg/kg PP or 2mg/kg HH by the indicated methods. Plasma concentrations were determined by MS, and reported in ng/ml. nd = compound not detected. (**b,c**) Littermate-controlled FVB male mice were treated for four weeks with 100mg/kg (PO) BiC (n=9), an escalating dose of PP to 1mg/kg (IP) (n=9), or a combination of these treatments (n=9).

Cohorts of mice were castrated at the onset of the study (n=9), or were sham-treated with IP and PO vehicles (n=10). (**b**) Prostate wet weight was measured as a proximal marker of anti-androgen potency. Analysis of variance methods were used to test the difference of the means of treatment groups. (**c**) Q-PCR was performed on reverse-transcribed RNA isolated from mouse prostate tissues. Transcripts for each gene were normalized to RPL19, an androgen unresponsive gene. Analysis of variance methods were used to test for differences among treatment populations for each gene examined. (*=p<.05; **=p<.01; ***=p<.001; ns = no significant difference between indicated populations.) Error bars = standard error of the mean. (**d**) Representative sections of dorsal prostate from each treatment group were stained using hematoxylin and eosin. Substantial prostate atrophy is present in castrated and combination treatment samples. Signs of androgen deprivation are also visible in the samples of mice treated with BiC and PP alone. Note the loss of columnar epithelial architecture (white arrows), decreased secretory protein, increased nuclear staining, a relative thickening of the stromal layer, and dead cell accumulation in the lumen (black arrows).

**Figure 4: Model of mechanisms of non-competitive AR antagonism.** AR resides predominantly in the cytoplasm prior to binding hormone. Ligand binding induces a conformation change, nuclear accumulation, dimerization, and assembly of a transcriptional complex at regulated promoters. BiC directly competes for DHT binding, blocking AR activation at a proximal step. HH prevents DNA binding by nuclear-localized AR. PP permits AR promoter binding, but interferes with assembly of a productive transcription initiation complex.

**Materials and Methods:**

**Cell culture:** LNCaP cells were maintained in RPMI 1640 media supplemented with sodium bicarbonate, glutamine, HEPES, antibiotics, and 10% FBS. LAPC4 cells were maintained in phenol-red free RPMI 1640 media supplemented with antibiotics and 10% FBS. BiC was a generous gift of Ingo Mellinghoff, PP was purchased from MP Biochemicals, and all other compounds were purchased from Sigma.

**Dowex ion exchange**: Dowex 1x8 200-400 Mesh Cl (Sigma) was washed in ethanol followed by 0.5M NaOH to remove ions, and water to neutralize. The Dowex was then washed with 0.5M HCl to charge the matrix, and water to neutralize. 100mg PP in a 2% DMSO solution was incubated with the Dowex for 1hr at RT. The resultant supernatant was retrieved by filtration (2 micron). Pyrvinium with chloride counter-ion was lyophilized and the powder resuspended in water. The purity was examined by MS and found to be >99%.

**Transcription assays and statistics:** For all transfections, pools of cells were transfected using Lipofectamine Plus (Invitrogen) with pRL-SV40 (Promega) and PSA-luciferase (The region from -4882 to +12 relative to the transcription start site of PSA was amplified from human genomic DNA by PCR and inserted into pGL4.10 (Promega). This region has been shown to induce expression of a similar luciferase reporter gene upon treatment with androgen(14)). The following day, the cells were replated and drugs were added and 24hrs later, luciferase production was measured (Dual luciferase assay kit, Promega). Mean-effect plots (log[compound] vs log[fractional effect]) were generated to determine the IC50 values for each compound or combinations of compounds at constant ratios. Microsoft Excel was used to calculate the statistics for a line using the “least squares” method. The F statistic was used to determine whether the observed relationship between the dependent and independent variables occurred by chance. Only data with an r2 value greater than 0.95 and an F value that was greater than that indicated by the F table for alpha=0.05 were used for analysis. The methods of Chou and Talalay were used to determine whether two compounds had antagonistic, additive, or synergistic reactions toward each other (13). Briefly, a combination index (CI) was established for a range of fractional effects, where a CI~1 indicates additivity, CI > 1 indicates antagonism, and a CI < 1 indicates synergy. The CI’s were based upon a non-exclusive assumption, which was indicated by the slope of the line of the combination of drugs from the mean-effect plot. However, CI’s based upon an exclusive assumption were similar.

**RT-PCR and statistics**: LNCaP and LAPC4 cells were grown in media containing charcoal-stripped (C/S) FBS in the presence or absence of test compounds for 24hrs. Total RNA was isolated from cells in culture plates using an RNAeasy kit (Qiagen) and reversed transcribed (MMLV-RT, Invitrogen). Primers used to detect human transcripts in these studies are as follows: RPL19 sense 5’-atgtatcacagcctgtacctg-3’, antisense 5’-ttcttggtctcttcctccttg-3’; KLK3 sense 5’-cccgagcaggtgcttttg-3’, antisense 5’-ggagttcttgaccccaaa-3’; Nxk3.1 sense 5’-ttctcccacactcaggtgatc-3’, antisense 5’-gtgagcttgaggttcttggc-3’; FKBP51 sense 5’-ctgtgacaaggcccttgga-3’, antisense 5’-ctgggcttcacccctccta-3’; TMPRSS2 sense 5’-cagcaagtgctccaactctg-3’, antisense 5’-

acacaccgattctcgtcctc-3'; MMP16 sense 5'-tccttgaggatggatcttgg-3', antisense 5'-tctcctcagggagcatttgt-3'; RDC1 sense 5'-ccagtctgggtggtcagtct-3', antisense 5'-ctcatgcacgtgaggaagaa-3'. Primers used to detect mouse transcripts were as follows: probasin sense 5'-atcatcctcctgctcacactgcatg-3', antisense 5'-acagttgtccgtgtccatgatacgc-3'; RPL19 sense 5'-atgtatcacagcctgtacctg-3', antisense 5'-ttcttggtctcctcctccttg-3'; MMP16 sense 5'-cctgaatcacctcagggagc-3', antisense 5'-atcacagcccataaagtcct-3'; Nkx3.1 sense 5'-ttctctcacactcaggtgatt-3', antisense 5'-gtgagtttgaggttcttggc-3'; FKBP51 sense 5'-ctgcgacaaggcccttgga-3', antisense 5'-ctgggcctcgccccttctg-3'; RDC1 sense 5'-cccgtctgggtggtcagtct-3', antisense 5'-ctcatgcaggcgaggaagaa-3'; TMPRSS2 sense 5'-gaacccaggcatgatgctaga-3', antisense 5'-caccccgaaatccagcatt-3'. For studies on mouse prostates, tissue was frozen in RNA Later (Qiagen) and mechanically homogenized prior to RNA isolation. Qiagen Taq and reagents were used for amplification, and each sample was measured in triplicate in 96-well plates. Real-time PCR was carried out on a 7300 Real Time PCR System (Applied Biosystems), using SYBR green (Invitrogen) as the detecting dye and Rox (Invitrogen) as the reference dye. Differences between experimental (x) and no DHT control (y) samples were normalized to RPL19 transcript levels (androgen unresponsive) and determined with the following calculation: (2^(Ctxgene1-Ctygene1))/(2^(CtxRPL19-CtyRPL19)). To test for differences in means, analysis of variance methods were used for planned comparisons between treatment groups that were defined by linear contrast statements.

**Radioligand competition binding assay:** LAPC4 or LNCaP cells were seeded in 12-well plates in phenol-red free media containing 5% C/S FBS. After 3 days, media was replaced with serum-free media containing 1nM [3H] DHT in the absence or presence of 0.1-1000 fold molar excess of unlabeled competitor ligands for 90min at 37°C. Cells were washed with phosphate buffer, bound ligand was extracted in ethanol for 30min at RT, and detected using a scintillation counter. Curve fitting and IC50 calculations were performed using Prism software (GraphPad Software, Inc.).

**Chromatin immunoprecipitation:** ChIP assays were performed as described (24) with the following modifications. LNCaP cells in medium containing C/S FBS were treated with 0.1% DMSO vehicle, 10nM DHT, 10nM DHT + 10μM BiC, 10nM DHT + 300nM PP, or 10nM DHT + 300nM HH for 4 hr at 37C, 1% formaldehyde for 3 min as the dishes cooled from 37C to 22C, and 125mM glycine for 10 min as the dishes cooled from 22C to 4C. Cells were lysed in IP lysis buffer (50mM HEPES-KOH, pH 7.4, 1mM EDTA, 150mM NaCl, 10% glycerol, 0.5% Triton X-100, supplemented with protease inhibitors) and harvested by scraping. Nuclei were collected by centrifugation (500x*g* for 5 min at 4C), resuspended in 2ml of RIPA buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 150mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, supplemented with protease inhibitors) and sonicated until an average DNA fragment size of 100-500 bp was achieved (assessed by agarose gel electrophoresis). For ~107 LNCaP nuclei, 6μg of anti-AR antibody (PG-21, Upstate) was used for immunoprecipitation. Immunoprecipitated material was washed with RIPA buffer containing 300mM NaCl + 100μg/ml yeast tRNA and resuspended in 80μl of proteinase K solution (TE, pH 8.0, 0.7% SDS, 200μg/ml proteinase K). After reversing crosslinks, DNA fragments were purified using QIAquick PCR Purification kit (Qiagen). Real-time PCR was carried out as described above, and AR ChIP data were normalized to a region 140 bp upstream of the HSPA1A gene, which is not occupied by AR. Differences between experimental (x) and no DHT control (y) samples were determined with the following calculation: (2^(Ctxgene1-Ctygene1))/(2^(CtxHSP1A1-CtyHSP1A1)).

**Mouse studies:** All experiments were performed with UCSF IRB regulatory approval in collaboration with the pre-clinical therapeutics and mouse pathology core facilities at UCSF. All mice used in these studies were male, FVB-background mice, greater than 10 weeks of age. For studies measuring the availability of compounds in plasma, mice were given compounds in 100uL doses of saline with 1% DMSO, either PO or IP, and plasma samples taken at the indicated times. PP and HH were measured by LC/MS/MS method. Briefly, mouse plasma (20ul) was added to 100ul of 70% CH3CN and vortexed for 1 min, then centrifuged at 10,000rpm for 5min. The supernatant was transferred to an auto-sampler vial and 10ul was injected into LC/MS/MS. The LC/MS/MS conditions were set as follows: Column, C8, 4.6 x 50 mm; mobile phase, 60%CH3CN, 0.05% acetic acid and 5 mM ammonium acetate for PP and 14% CH3CN, 1.8% methanol, 0.1% formic acid for HH. The flow rate was set at 1.2 ml/min and ¼ of elution from the column was split to the mass system. Compounds were monitored in ESI positive MRM mode at 382.2>352.3 m/z for PP and 199.1 >171.3 m/z for HH (Micromass Quattro Ultima). The cone voltage and collision energy were set as 40V and 30eV for PP and 40 V and 25 eV for HH.

For efficacy studies, mice were administered PP by IP injection or BiC by PO (capsule crushed and resuspended in saline) once daily (M-F) for four weeks. The dose of PP was escalated over the course of the study (0.1mg/kg for the first week, 0.3mg/kg for the second week, 1.0mg/kg for the final two weeks). Two mice died from fighting at the onset of the study, and one mouse each from the BiC and PP treated groups died from unrelated causes during the study, leaving 10 mice in the untreated group and 9 mice in the others. At the end of the study, mouse liver and prostate were dissected, extraneous tissue was removed, and the wet weights were recorded. The same analysis of variance methods used in the Q-PCR experiments were used to detect differences among treatment groups. The prostate was dissected along an axis such that each of the lobes was equally represented in each half, and one half was fixed in formalin and paraffin-embedded. Tissue sections were stained with hemotoxylin and eosin. The other half was frozen in RNA-later (Qiagen) for RT-PCR.

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**Author contributions**: JOJ designed and carried out all experiments except where noted in the following: ECB performed ChIP experiments and designed many of the primers used for Q-PCR. YH created methods and performed analysis of PP and HH in mouse samples. FC and KG assisted with competition experiments. BH helped in the design and implementation of *in vivo* preclinical testing. MID directed experiments and wrote the paper with JOJ.

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## Supporting Data

| # | $R_1$ | $R_2$ | Transcription Activity in MDA-kb2 (μM) [DHT] = 8 nM | Transcription Activity in MDA-kb2 (μM) [DHT] = 0.2 nM | Cytotoxicity in MDA-kb2 (μM) | Cell permeability ($*10^{-6}$ cm/s) | Retention in membrane (%) | Solubility Limit (μM) |
|---|---|---|---|---|---|---|---|---|
| SJ000000101 | *m*-$CF_3$ | | 15.7 ± 3.7 | - | > 100 | 52.0 ± 10.5 | 47 | > 100 |
| SJ000000132 | *cy*Hex | | 0.340 ± 0.10 | 0.110 ± 0.04 | > 100 | 188.0 ± 13.5 | 95 | 31.6 ± 0.7 |
| SJ000000110 | *p*-Ph | | 0.180 ± 0.03 | 0.310 ± 0.18 | > 100 | 131.4 ± 28.1 | 88 | 52.9 ± 2.8 |
| SJ000311877 | *p*-Ph | *p*-OMe | 0.002 ± 0.002 | 0.003 ± 0.001 | > 100 | 46.1 ± 20.7 | 76 | 12.8 ± 0.3 |
| SJ000311873 | *p*-Ph | *m*-OMe | 0.004 ± 0.003 | 0.006 ± 0.004 | > 100 | 187.3 ± 22.8 | 92 | 62.2 ± 1.0 |
| SJ000311868 | *p*-Ph | *p*-$NO_2$ | 0.008 ± 0.003 | 0.002 ± 0.001 | > 100 | 111.6 ± 11.0 | 85 | 4.7 ± 0.4 |
| SJ000311870 | *p*-Ph | *m*-$CO_2$Me | 0.014 ± 0.01 | 0.020 ± 0.01 | > 100 | 360.1 ± 12.9 | 92 | 23.8 ± 1.7 |
| SJ000311871 | *p*-Ph | *p*-CN | 0.020 ± 0.01 | 0.020 ± 0.01 | > 100 | 209.6 ± 13.7 | 82 | 66.4 ± 1.0 |
| SJ000311869 | *p*-Ph | *p*-$CO_2$Me | 0.049 ± 0.03 | 0.063 ± 0.04 | > 100 | 141.8 ± 39.5 | 80 | 12.3 ± 0.9 |
| SJ000311866 | *p*-piperidine | | 0.099 ± 0.04 | 0.140 ± 0.05 | > 100 | 154.1 ± 24.7 | 44 | 85.9 ± 1.6 |
| SJ000311875 | 2,3-dihydro-1*H*-indene | | 16.1 ± 0.4 | 13.9 ± 1.0 | > 100 | 148.9 ± 25.4 | 93 | 22.3 ± 1.4 |
| SJ000311872 | *p*-Ph | *m*-CN | 56.9 ± 15.2 | 23.9 ± 7.6 | > 100 | 165.3 ± 15.8 | 80 | 55.0 ± 1.4 |
| SJ000311876 | *p*-Ph | 3,5-di$CF_3$ | 77.7 ± 15.5 | 71.5 ± 9.8 | 62.0 ± 14.0 | 0.0 ± 0.0 | 93 | < 3* |
| SJ000311874 | *p*-Ph, *m*-OMe | | 90.4 ± 21.1 | 75.0 ± 14.6 | > 100 | 148.1 ± 43.1 | 90 | 77.0 ± 1.0 |
| SJ000000130 | *p*-morpholine | | 205.0 ± 205 | 34.3 ± 32.8 | > 100 | 6.6 ± 2.8 | 8 | > 100 |
| Hydroxyflutamide | | | 8.50 ± 8.0 | 0.12 ± 0.10 | > 100 | 183.7 ± 29.2 | 75 | - |
| Bicalutamide | | | 8.20 ± 0.8 | 0.42 ± 0.10 | > 100 | 627.7 ± 21.7 | 88 | - |

**Table 2.** Cellular profiles of flufenamic acid analogs in MDA-kb2[29]. Transcription activities were measured in MDA-kb2 cells using a luciferase reporter gene assay after 20 hour exposure to DHT at different concentrations and serial diluted drugs. A cell viability assay based on ATP level detection was performed after 20 hour drug exposure. Cell permeability and retention in cell membrane were evaluated with the PAMPA technique (pH = 7.4). Solubility limits with 5% DMSO were evaluated by UV absorption after filtration.