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ARTICLE

Novel Flufenamic Acid Analogues as Inhibitors of Androgen Receptor Mediated Transcription

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ABSTRACT The androgen receptor (AR), which mediates the signals of androgens, plays a crucial role in prostate-related diseases. Although widely used, currently marketed anti-androgenic drugs have significant side effects. Several studies have revealed that non-steroidal anti-inflammatory drugs, such as flufenamic acid, block AR transcriptional activity. Herein we describe the development of small molecule analogues of flufenamic acid that antagonize AR. This novel class of AR inhibitors binds to the hormone binding site, blocks AR transcription activity, and acts on AR target genes.

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The androgen receptor (AR) is crucial for development and maintenance of the sexual characteristics, bone density, and muscle. AR is also an important mediator for diseases such as prostate benign hyperplasia (BPH) and prostate cancer (*1*). AR belongs to the steroid receptor subclass of the nuclear receptor family (NRs), intracellular transcription factors that respond directly to their ligands (*2, 3*). In the absence of androgens like dihydrotestosterone (DHT), AR resides in the cytoplasm, bound to chaperone heat shock proteins (HSPs). Upon ligand binding, inactive AR dissociates from HSPs, undergoes a series of conformational changes, and translocates to the nucleus (*4*). In the nucleus, ligand-activated AR binds to specific androgen response elements (ARE), recruits coregulatory proteins, and starts the regulation of a distinct set of genes (Figure 1) (*5–8*). Complex interactions with either coactivators (CoA) or corepressors (CoR) fine-tune AR-mediated gene expression (*9, 10*). Like other NRs, ligand-bound AR exhibits extra binding sites on its surface, such as the activation function 2 (AF2) that recruits coregulators such as the steroid receptor coactivator (SRC) family (*11–14*). We have previously identified another allosterically regulated binding site, BF3, on the surface of AR that can affect AF2 function (*15*).

Antiandrogens such as hydroxyflutamide or bicalutamide are used successfully against androgen-dependent prostate cancer but exhibit strong side effects including gynecomastia, impotence, osteoporosis, and cardiovascular diseases. Additionally, tumors treated with anti-androgens become resistant to that therapy within several years of treatment (*16–18*). For these reasons current pharmacological strategies targeting AR are focused on the development of SARMs (selective androgen receptor modulators) that interact with the ligand binding pocket of AR and regulate AR-

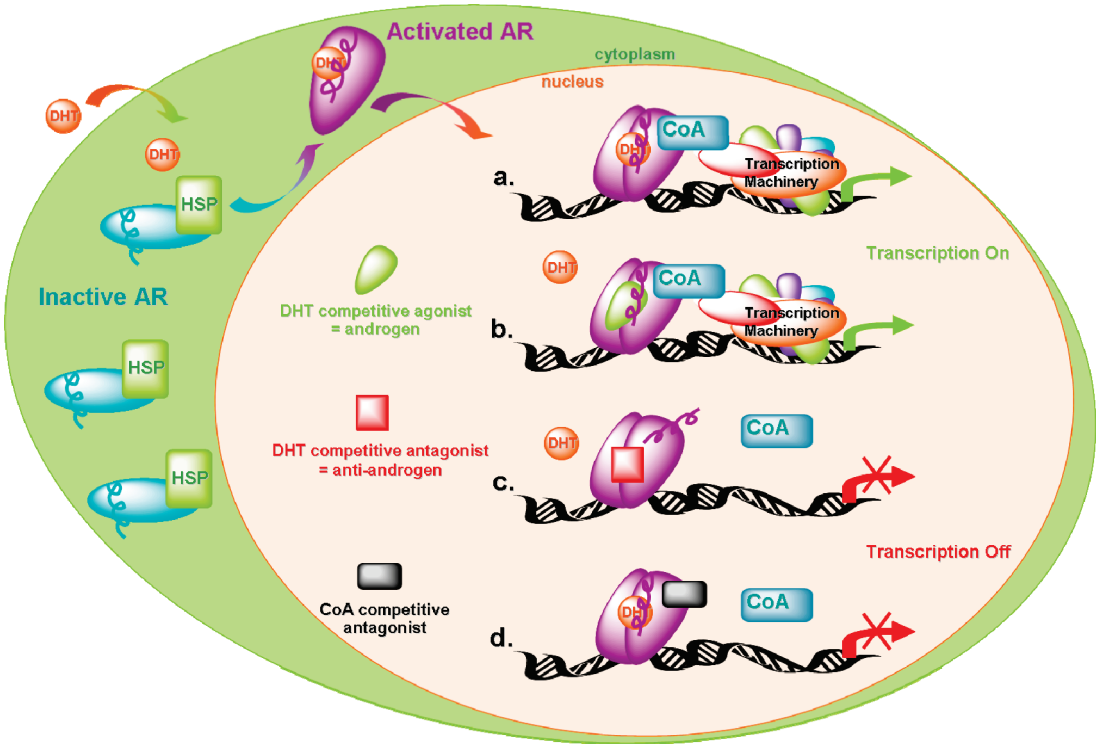


Figure 1. Alternative modes of binding to the androgen receptor (AR). In the absence of endogenous ligand, dihydrotestosterone (DHT), inactive AR resides in the cytoplasm bound to chaperone heat shock proteins (HSPs). Upon ligand binding, inactive AR dissociates from HSP, undergoes a series of conformational changes, becomes active, and translocates to the nucleus. DHT (a) or competitive agonists (b) bind to the hormone binding site and allow AR in an activated conformation to recruit coactivators (CoA), which in turn exert gene regulation by recruiting the transcription machinery. DHT competitive antagonists or anti-androgens (c) bind to the hormone binding site but lock the AR in an inactive conformation where CoA cannot bind and gene transcription is blocked. CoA competitive antagonists (d) bind to the surface of ligand-bound AR preventing the recruitment of CoA and transcription machinery without affecting androgen binding.

mediated gene transcription in a tissue-selective manner (*19, 20*). Because AR plays an important oncogenic role in hormone-refractory prostate cancer, targeting AR signaling in the setting of tumor resistance remains particularly crucial (*21*). It has been reported that tumor resistance can be due to AR mutations occurring in the ligand binding domain (LBD) that produce a receptor hypersensitive to its natural ligand, other endogenous hormones, and synthetic anti-androgens (*22, 23*). One can envision that drugs acting through mechanisms complementary to ligand antagonism might prove useful in combination with SARMS.

We identified flufenamic acid (*1*) (FLF) and other novel AR inhibitors using high-throughput screening and showed that this non-steroidal anti-inflammatory drug (NSAID) is able to block AR transcription activity (*15*). Other studies have shown that FLF inhibits cell growth (*24*) and the expression of the AR in LNCaP (*25*) and aldo-keto reductases (AKR), which can affect androgen signaling at the level of hormone metabolism (*26, 27*). Herein, we describe the synthesis and evaluation of FLF analogues.

RESULTS AND DISCUSSION

Biochemical Structure–Activity Relationships of Flufenamic Acid Analogues. On the basis of our identification of FLF (*1*) as an inhibitor of AR transcriptional activity (*15*), we carried out a structure–activity relationship (SAR) study by synthesizing structural analogues of FLF (Figure 2, panel A). To address the importance of the carboxylic acid group, we prepared the corresponding methyl ester (*2*) and amide (*3*). To evaluate the role

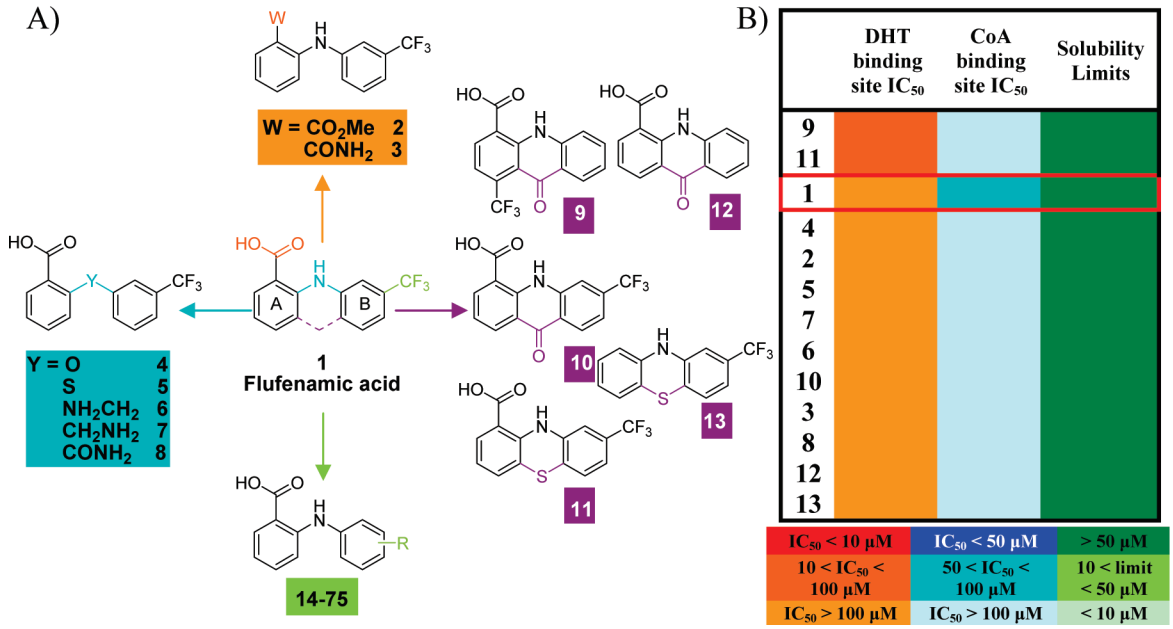


Figure 2. Preliminary SARs of FLF (*1*) derivatives. A) Synthesized analogues of FLF. B) Heat maps reflecting the binding affinities of FLF analogues 1–13 for the AR-LBD (IC_{50} values available in Supplementary Table 3): 1) DHT binding site, a SPA measuring [^{3}H]-DHT displacement from AR-LBD (*28*). 2) CoA binding, a fluorescence polarization assay (FP) measuring fluorescently labeled SRC2-3 peptide displacement from DHT-bound AR-LBD (*15*). 3) Solubility limits were quantified by UV absorbance under conditions reflecting binding assays conditions. Values are means of two independent experiments in triplicates. The general error limits are ±5%.

of the amine function, we replaced the secondary nitrogen with oxygen (*3*) or sulfur (*4*) atoms. Additionally, we elongated the spacer function Y between the two aromatic rings by introducing an additional methylene (**5** and **6**) or a carbonyl group (*7*). Finally, the effects of structural rigidification were explored using both commercially available and synthesized tricyclic analogues of FLF (**9**–**13**). All FLF derivatives were tested to determine their ability to bind to the AR and independently their ability to disrupt binding of transcriptional cofactors.

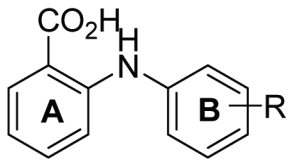
These FLF derivatives were tested for their ability to directly displace the endogenous radioactive ligand [^{3}H]-DHT from AR-LBD using a scintillation proximity assay (SPA) (Figure 2, panel B; IC_{50} values available in Supplementary Table 3) (*28*). The synthesized FLF analogues were tested for their ability to compete with a fluorescently labeled coregulatory peptide, mimicking the coregulatory protein SRC2-3, for binding to DHT-bound AR-LBD (*15*). These two biochemical assays distinguish the ability of the small molecules to competitively inhibit ligand binding, thus allosterically regulating coregulatory protein recruitment, or directly inhibit the recruitment of coregulatory proteins (Figure 1).

The carboxylic acid and amine moieties of FLF were required for binding to the AR as demonstrated by the finding that the derivatives **2**–**5** were inactive in both assays (Figure 2, panel B; Supplementary Table 3). The introduction of a longer spacer between the two aromatic rings yielded inactive compounds as well (**6**–**8**). More rigid structures such as the tricyclic analogues **9**–**13** did not show any particular improvement in binding to the AR-LBD. In addition, we confirmed the findings of Bisson *et al.* (*29*) that phenothiazines are able to inhibit AR since phenothiazine **11** showed a weak affinity for the hormone binding site.

With these basic elements of SAR set, a focused library was prepared to give better understanding of the role of substituents on the B-ring of FLF analogues (Figure 3; IC_{50} values available in Supplementary Table 3).

A focused library of FLF derivatives was produced by Ullmann coupling between the *o*-chlorobenzoic acid and locally synthesized or commercially available anilines

A)



IC_{50} < 10 µM	IC_{50} < 50 µM	> 50 µM
10 < IC_{50} < 100µM	50 < IC_{50} < 100µM	10 < limit < 50µM
IC_{50} > 100µM	IC_{50} > 100µM	< 10µM

#	R	DHT binding site IC_{50} (µM)	CoA binding site IC_{50} (µM)	Solubility Limit (µM)
14	*p*-O(*p*-NO_2)Ph	2.8 ± 0.4		
15	*p*-SPh	3.0 ± 0.3		
16	*p*-O(*p*-CF_3)Ph	3.2 ± 0.5		
17	*p*-*n*OHex	4.4 ± 1.2		
18	*p*-*n*Hex	4.5 ± 0.8		
19	*p*-O(*p*-Me)Ph	5.6 ± 1.1		
20	*p*-Bn	5.8 ± 1.4		
21	*p*-O(*p*-F)Ph	6.5 ± 1.5		
22	*p*-$CO_2C_5H_{11}$	6.7 ± 1.6		
23	*p*-OPh	7.4 ± 1.1		
24	*p*-NO_2, *m*-CF_3			
25	*o*-NO_2			
26	m-CF_3, *p*-OPh			
27	*p*-O(3-Pyridine)			
28	*p*-NO_2			
29	*p*-S-CF_3			
30	*o*-SPh			
31	3,5-diOPh			
32	*o*-Me-*m*-Cl			
33	*m*-CF_3, *p*-O(*p*-OMe)Ph			
34	*m*-Ph			
35	*o*-OBn			
36	*m*-OBn			
37	*p*-CF_3			
38	*m*-OPh			
39	*o*-OPh			
40	*m*-Bn			
41	*o*-Bn			
42	*p*-*t*Bu			
1	*m*-CF_3			
43	*m*,*p*-Ph			
44	*m*-CF_3, *p*-O(*o*-OMe)Ph			
45	*o*-Ph			
46	*m*-O(3-Pyridine)			
47	*p*-CN			
48	*p*-CO_2H			
49	*m*-NO_2			
50	*p*-morpholino			
51	*p*-*i*Pr			
52	*m*,*p*-cyclopentyl			
53	o-F			
54	*o*-Me			
55	*o*,*m*-diMe			
56	*m*-*t*Bu			
57	*o*-*i*Pr			
58	*p*-SO_2-Ph			
59	3,5-diMeO			
60	*m*-Me			
61	*m*-OMe			
62	*p*-OMe			
63	*o*-CF_3			
64	*p*-Me			
65	*o*-OMe			
66	-			
67	*o*-CO_2H			
68	*o*-NH_2, *m*-Cl			
69	*m*-F			
70	*p*-F			
71	*m*-*i*Pr			
72	*o*-morpholino			
73	*m*-O(2-Pyridine)			
74	*m*-CF_3, *m*-CO_2H			
75	*p*-CO_2Me			

B)

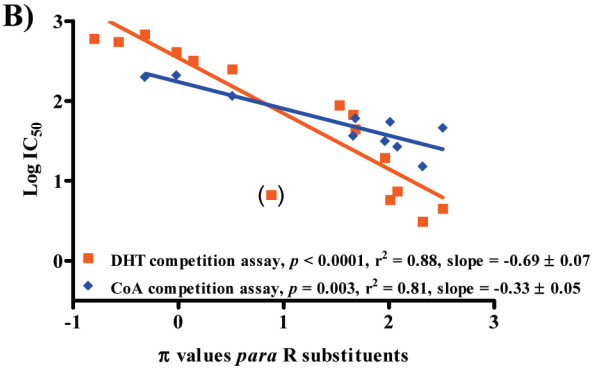


Figure 3. Structure–activity relationships of the distal B phenyl ring of FLF derivatives. A) Heat maps reflecting the binding affinities of FLF analogues 14–75 for the AR-LBD (IC_{50} values available in Supplementary Table 3): 1) DHT binding site, a SPA measuring [^{3}H]-DHT displacement from AR-LBD (*28*). 2) CoA binding, FP assay measuring fluorescently labeled SRC2-3 peptide displacement from DHT-bound AR-LBD (*15*). 3) Solubility limits were quantified by UV absorbance under conditions reflecting binding assays conditions. Values are means of two independent experiments in triplicates. The general error limits are ±5%. B) Correlation plots of log IC_{50} obtained in DHT and CoA competition binding assays against hydrophobic character of each *para* R substituents represented by π values (*30*).

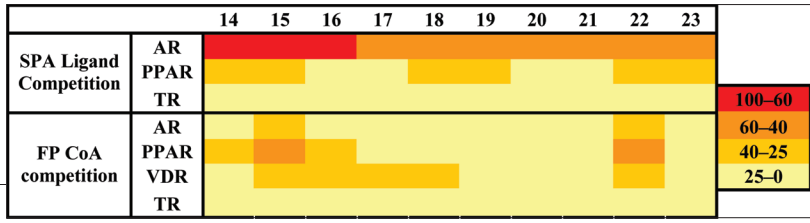


Figure 4. Summary of selectivity of FLF acid analogues for AR. Each ligand was tested at a single concentration of 10 μM against various nuclear receptors to determine effects on ligand binding (SPA assay) (*28*) or coactivator binding (FP assay) (*31, 32*). Results are shown as % inhibition relative to controls (values available in Supplementary Figure 8). Values are means of two independent experiments in triplicates. The general error limits are ±5%.

(Supplementary Scheme 1). This library was tested in biochemical assays described above (Figure 3, panel A). Two subsets of compounds emerged from this study. One set was able to displace the coregulator peptide without affecting DHT binding. Compounds **36**–**39** had a slightly improved ability to displace peptide from the AF2 pocket relative to FLF. The second set of FLF derivatives inhibited both interactions between coactivator peptide and the AR and between AR and DHT. Although the canonical model states that antagonists that displace the hormone will allosterically block binding of the coregulatory peptide, we observed a very poor correlation between the two assays ($r^2 = 0.187$, Supplementary Figure 6).

Hydrogen bonding interactions are often important contributors to the binding between small molecules and proteins. Flufenamic acid has a weakly basic diphenylamine functionality and an acidic aromatic carboxylic group, and both of these functionalities are essential for binding to the AR. The ability of FLF to generate an intermolecular hydrogen bond with AR could be influenced by different electron-donating or -withdrawing groups on the aromatic rings. Comparing FLF analogues with different B-ring substituents in the *para* position to the nitrogen reveals similar binding affinities for electron-donating substituted derivatives (compounds **50**, **62**, and **64**) and electron-withdrawing substituted derivatives (compounds **37**, **47**, **48**, and **75**). Additional substitution on the B-ring in the presence of a *m*-CF_3 group, with electron-withdrawing groups (*p*-NO_2 **24**) or with electron-donating groups (*p*-OPh **26**, *p*-OPh(*m*-OMe) **33**, p-OPh(*p*-OMe) **44**) also had little effect on potency. Correlation plots of the electronic effect of introduced substituents (characterized by σ values (*30*)) and both competition assays showed no evidence of a Hammett relationship ($r^2 < 0.5$, Supplementary Figure 7). Interestingly, analogue **24** that incorporates a 4-nitro-3-trifluoromethylaniline moiety, a structural feature found in the anti-androgen hydroxyflutamide, did not show any activity in either assay.

However, the hydrophobic character of the substituents (π values) was found to influence the activity of the FLF derivatives (Figure 3, panel B). Plotting the π value of each *para* substituent against log IC_{50} gave significant correlations for both the SPA ($r^2 = 0.88$, $p < 0.0001$, $n = 15$) and FP ($r^2 = 0.81$, $p = 0.0003$, $n = 10$) assays. We observed a stronger relationship for the hormone displacement assay when comparing the slopes of the two linear plots ($slope_{SPA} = -0.69 \pm 0.07 > slope_{FP} = -0.33 \pm 0.05$). Compounds with long alkyl chains (*O*-*n*-hexyl **17**, *n*-hexyl **18**) had stronger affinities for the AR than compounds with shorter hydrophobic groups such as methyl (**65**), isopropyl (**51**), and *tert*-butyl (**42**). A similar trend was seen in comparing pentyl ester **22** to methyl ester **75**. Additionally, phenyl-substituted derivatives such as **15** (thiophenyl), **20** (benzyl), or **23** (phenoxy) gave potency in the low micromolar range in contrast to phenylsulfonyl compound (**58**). Finally, the pyridinyl compound **27** showed activity lower than that of the phenoxy analogue **23**. In a subsequent small directed library, introduction of electron-withdrawing (NO_2 **14**, CF_3 **16**, F **21**) or electron-donating (Me **19**) groups on the third aromatic ring had little effect on the potency of the compounds.

There were relatively strong positional effects for the substituents on the B-ring: *para*-substituted derivatives had generally better potencies than *meta*- or *ortho*-substituted compounds. For instance, the *p*-benzyl analogue **20** is more potent than both the *m*-benzyl analogue **40** and the *o*-benzyl analogue **41**. Indeed, the most active compounds are all *para*-substituted FLF derivatives (**14**–**23**). No active compounds were observed among the disubstituted FLF analogues such as 3,5-Me **55**, 3,5-OMe **59**, 3,5-OPh **31**, (*o*-Me, *m*-Cl) **32**, (2-NH_2, 3-Cl) **68**.

Since the most potent analogues had additional aliphatic or aromatic substituents, their increased hydrophobic character may reduce their solubility in aqueous media. Thus the solubility limit of each FLF analogue was determined in a buffer containing 5% DMSO (pH = 7.4), reflecting the assay conditions. Overall, the derivatives showed solubility limits significantly higher than their observed potencies. Therefore, solubility is not believed to interfere with the assays.

To investigate the specificity of these FLF analogues toward the androgen receptor, we selected the 10 most potent compounds **14**–**23** and determined their activities with respect to other nuclear receptors. Ligand displacement was monitored for the AR, peroxisome proliferator-activated (PPARγ) and thyroid (TR) recep-

tors (*28*) and coactivator displacement for the AR, PPARγ, vitamin D (VDR), and TR receptors (*15, 31, 32*) (Figure 4, percent inhibition values available in Supplementary Figure 8).

This set of compounds had no effect whatsoever on the TR. However some of the analogues showed some activity at 10 μM against both the VDR and PPARγ receptors. Overall, we could conclude that compounds **14** and **16** exhibit a reasonable but not strong specificity for the AR.

Transcriptional Structure–Activity Relationships of Flufenamic Acid Analogues. The most potent FLF derivatives **14**–**23** were tested to determine their effects upon AR-mediated gene transcription. The inhibition of DHT-induced transcription in the presence of FLF analogues was determined in MDA-kb2, a cell line stably expressing an AR-responsive luciferase reporter gene (*33*) driven by a MMTV promoter (Table 1 and Supplementary Table 3). The cells were treated with compounds in the presence of DHT, and the transcriptional AR-mediated signal was measured by luminescence after 20 h using BrightGLo (Promega). Two concentrations (0.2 and 8 nM) of DHT were chosen for the assay, representing the previously determined EC_{50} and EC_{90} of DHT under identical conditions (data not shown). Additionally, we evaluated the cell permeability and membrane retention of FLF analogues using a parallel artificial membrane permeation assay (PAMPA) (*34, 35*). This assay was carried out with a 0.5% DMSO content at pH 7.4, reflecting the conditions of the cell-based assays.

In the presence of 0.2 nM DHT, all FLF analogues (except **17**) inhibited AR transcription with IC_{50} below 10 μM. The most potent full inhibitor **22** had an IC_{50} of 700 nM. Three of these compounds (**19**, **21**, and **23**) exhibited partial antagonism. When tested in presence of 8 nM DHT (EC_{90} of DHT in this assay), we observed increased IC_{50} values for all compounds. This indicates that direct competition for DHT is a significant component of the mechanism of action for the tested FLF analogues. A similar IC_{50} shift was observed for control antiandrogens, hydroxyflutamide and bicalutamide. In parallel, we determined that none of the FLF derivatives directly inhibited the luciferase enzyme obtained from lysed MDA-kb2 cells grown in the presence of DHT, thus ensuring unambiguous analysis (Supplementary Table 2). Additionally, the cytotoxicities of these compounds were determined under identical conditions. The LD_{50} values observed were in general higher than 150 μM. Other mammalian cell lines (HepG2, Raji, Hek293, BJ) were used to determine general and specific toxicity (Supplementary Tables 2 and 3). None of the compounds exhibited any significant cytotoxicity independently of any cell line. The permeabilities of the FLF analogues tested were within a range indicating reasonable cell access by passive permeation ($360 > P_e > 40 \times 10^{-6}$ cm s^{-1}), although they did have relatively elevated membrane retention, congruent with their hydrophobicity. Theses values are comparable with marketed antiandrogens.

Specific Regulation of FKBP51 Gene Transcription by Flufenamic Acid Analogues. FKBP51 is a FK506-binding protein with isopropyl peptidyl isomerase activity that associates with heat shock proteins (HSP90, HSP70, and HSP40) and plays a role in the proper folding, binding, and intracellular trafficking of steroid hormone receptors (*5, 36*). This AR target gene is upregulated in the presence of androgens in various cell lines including CWR22 (*5*), LNCaP (*6*), PC3, and DU145 (*37*). In control experiments with MDA-kb2 cells, FKBP51 was induced by 4- to 5-fold in the presence of 0.2 nM DHT. The quantification of the *m*RNA transcription levels of FKBP51, 18S, and GAPDH in MDA-kb2 cells treated with DHT and FLF analogues was carried out using rt-PCR after an exposure time of 20 h (no toxicity was detected at that time point, see Table 1). The results are presented in Figure 5.

Bicalutamide was chosen as a control drug since OHF acts as a mixed agonist in MDA-kb2 (*33*). No dramatic effect was observed on house-keeping gene expression (GAPDH and 18S), confirming that FLF analogues were not general transcription inhibitors. All compounds were able to decrease transcription levels of FKBP51 in the presence of 0.2 nM DHT after 20 h exposure when tested at a concentration of 10 μM. The most active compound was compound **20**, exhibiting a similar efficacy to bicalutamide in presence of 0.2 nM of DHT.

On the basis of the initial discovery that FLF could bind to the androgen receptor and modulate its function, we synthesized a focused library of FLF analogues and tested their ability to block binding of endogenous ligand and coactivator. Like FLF, a small set of these compounds (**36**–**39**) were found to preferentially displace the coactivator rather than compete with the endogenous ligand DHT. However, unlike FLF, these compounds did not inhibit AR-mediated transcription or

TABLE 1. Summary of transcriptional activities and cytotoxicities of FLF analogues in MDA-kb2 (*33*) and cellular permeabilities and membrane retentions determined by a PAMPA assay (*34*, *35*)

		Transcriptional inhibition (IC_{50}) in MDA-kb2[a]			
no.	R	0.2 nM DHT (μM)	8 nM DHT (μM)	Cytotoxicity (IC_{50}) in MDA-kb2 (μM) [b]	Permeability (10^{-6} cm s^{-1})[c] [retention in membrane (%)][d]
14	*p*-O(*p*-NO_2)Ph	1.9 ± 0.9 [100]	38.6 ± 4.7 [100]	Nontoxic	191 ± 18 [87]
15	*p*-SPh	6.5 ± 5.7 [75]	14.4 ± 5.5 [30]	Nontoxic	180 ± 16 [93]
16	*p*-O(*p*-CF_3)Ph	3.7 ± 1.8 [100]	53.0 ± 5.1 [100]	Nontoxic	118 ± 29 [92]
17	*p*-*n*OHex	20.5 ± 18.7 [75]	>100	Nontoxic	55 ± 10 [68]
18	*p*-*n*Hex	7.2 ± 3.9 [75]	77.2 ± 17.7 [50]	Nontoxic	160 ± 116 [88]
19	*p*-O(*p*-Me)Ph	0.4 ± 0.2 [50]	>100	Nontoxic	106 ± 8 [87]
20	*p*-Bn	2.2 ± 1.0 [75]	>100	Nontoxic	98 ± 31 [89]
21	*p*-O(*p*-F)Ph	3.5 ± 2.4 [25]	>100	Nontoxic	113 ± 12 [88]
22	*p*-$CO_2C_5H_{11}$	0.7 ± 0.3 [100]	83.8 ± 20.7 [100]	Nontoxic	294 ± 40 [91]
23	*p*-OPh	0.9 ± 0.3 [50]	>100	181.4 ± 99.4	153 ± 29 [84]
	Hydroxyflutamide	0.06 ± 0.03 [100]	7.2 ± 5.5 [100]	Nontoxic	183 ± 29 [75]
	Bicalutamide	0.5 ± 0.2 [100]	11.1 ± 4.8 [100]	Nontoxic	628 ± 22 [88]

[a]Transcription assay was performed in MDA-kb2 (*33*), stably transformed cell line expressing a MMTV-Luc reporter gene. The assay was conducted in presence of either 0.2 or 8 nM of DHT corresponding to the EC_{50} and EC_{90} values determined for DHT. Percent inhibition is reported in brackets. [b]Cytotoxicity was measured with Cell-Titer Glo. [c]Permeability and retention in cell membrane were evaluated by a PAMPA assay (*34*, *35*). Values are means of two independent experiments in triplicates. The general error limits are ±5%. [d]Same as above.

exhibit general toxicity (Supplementary Table 3). Overall, none of these FLF derivatives exhibits significantly tighter affinity than FLF for the coactivator binding site.

Remarkably, a larger set of the FLF analogues competitively inhibited ligand binding, rather than competing directly for the coactivator binding site or binding to the BF3 site. Many of these compounds were potent inhibitors of DHT-mediated transcription. The novel antiandrogens we identified have a totally different structure than the currently marketed anti-androgenic drugs. Indeed, compound **24**, structurally similar to hydroxyflutamide, was not active in either biochemical or cell-based assays. However these FLF derivatives (**14**–**23**) have several common structural features that are crucial for interacting with the AR, including a free carboxylic acid, a secondary amine linking the two aryl rings, and a single hydrophobic group in the *para* position on the B-ring. Based on correlation plots (Figure 3, panel B) of our SAR study on the FLF analogues, hydrophobicity is a leading component for the preferential binding of these compounds to the hormone binding site rather than to the coactivator binding site.

In general, these analogues, which are able to displace the hormone in low micromolar range, were also able to displace the coactivator peptide. This may suggest that for this particular class of compounds long alkyl chain or articulated phenyl groups in this *para* position are well sited and bulky enough to prevent the formation of the AF2 or other external sites where coregulators are being recruited.

Except for compound **17**, we noted a good correlation between biochemical inhibition and inhibition of transcription among this set of compounds. Indeed, we measured for this particular analogue a higher IC_{50} (20.5 ± 18.7 μM) in the transcription assay in comparison with an IC_{50} value of 4.5 μM in the DHT competition assay. Compound **17** showed the lowest cell permeability of this series (55×10^{-6} cm s^{-1}), which could explain its reduced efficiency to perform in cells than in biochemical conditions. Additionally, analogues **19**, **21**, and **23** were not able to fully inhibit AR transcription signal in cells, whereas they did compete completely for ligand binding under biochemical conditions. Those *p*-phenoxy derivatives showed

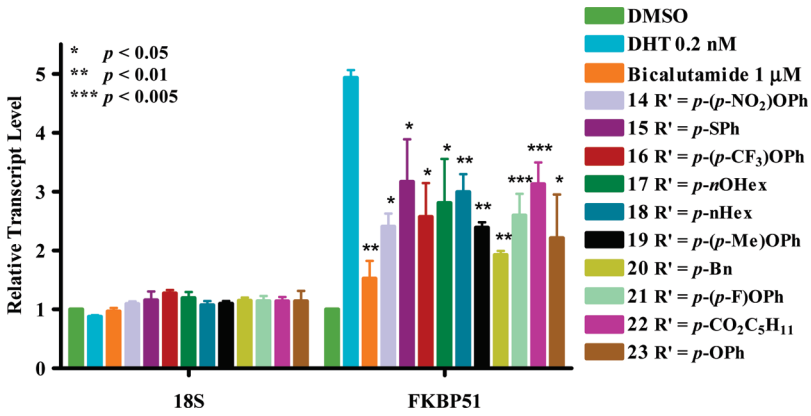


Figure 5. Effects of FLF analogues on 18S and FKBP51 transcription in MDA-kb2 cells. Cells were exposed to drugs (10 μM) for 20 h, and 18S and FKBP51 transcription was monitored by qRT-PCR. The levels for the tested genes are normalized to GAPDH transcript level and to DMSO control condition. The ΔΔ*Ct* method was used to measure the fold change in expression of genes. Standard deviations were calculated from three biological independent experiments performed in triplicates.

partial antagonism, whereas compounds **14** and **16** showed full antagonism. We hypothesize that electron-withdrawing groups introduced on this third aromatic ring lead to full antagonists.

The competitive mode of action of those compounds was also confirmed in cells since a loss of activity was observed when hormone concentration was increased. Finally, the FLF analogues act on AR target genes and are not general transcription inhibitors.

In conclusion, FLF derivatives exhibit a range of biological activities with respect to the androgen receptor. A subgroup of compounds, which were DHT antagonists, were active in cells and inhibited transcription of an AR target gene. Overall, this series contains novel nontoxic antagonists of AR with potencies comparable to those of currently marketed anti-androgens. For these reasons, the continued examination of this compound series is warranted.

METHODS

General Considerations. Synthetic procedures and copies of NMR spectra for each material described are collected in the Supporting Information. Hydroxyflutamide was purchased from LKT Laboratories, Inc. Bicalutamide was purchased from Toronto Research Chemicals, Inc. Radiolabeled dihydrotestosterone ([^{3}H]-DHT) ([1,2,4,5,6,7-^{3}H(N)]-(5α-androstan-17β-ol-3-one), 110 Ci mmol^{-1}, was obtained from PerkinElmer.

All cell lines are purchased from ATCC. All media were supplemented with 10% FBS, 2 mM L-glutamine, and other indicated reagents. Rajii cells were maintained at 37 °C in RPMI 1640/HEPES media (Gibco BRL) supplemented with sodium pyruvate (1 mM) and HEPES (10 mM). HepG2, Hek293, and BJ cells were maintained at 37 °C in D-MEM media (Gibco BRL) supplemented with NEAA (0.1 mM) and sodium pyruvate (1 mM). MDA-kb2 cells were maintained at 37 °C, without CO_2 in L-15 media (Gibco BRL) supplemented with geniticin (500 μg mL^{-1}), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).

Fluorescence polarization and luminescence experiments were performed with an EnVision (PerkinElmer). Radiation experiments were performed with a TopCount microplate scintillation and luminescence counter (Packard Instrument Company).

All data were analyzed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA) and IC_{50} values were obtained by fitting data to the following equation (Sigmoidal dose−response (variable slope) or "four parameter logistic equation"): y = bottom + (top − bottom)/(1 + $10^{(\log EC_{50} - x)Hillslope}$); x is the logarithm of concentration; y is the response. Values are means of two independent experiments in triplicates. The general error limits are ±5%.

Biochemical Assays. *Protein Expression and Purification.* cAR-LBD (His_6; residues 663–919) was expressed in *E. coli* and purified to homogeneity in the presence of DHT (10 μM) using a modified version of published protocols (*28*). The protein was stored with twice the amount of DHT (20 μM) to ensure fully liganded AR-LBD. Protein concentrations were measured by Bradford and BCA protein assays. Usually 6−8 mg of protein per liter of cell culture was obtained. The protein was dialyzed overnight against buffer (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.

Radiolabeled Ligand Competition Binding Assays. The assays were performed following a previously published procedure (*28*).

NR/Coactivator Peptide Competition FP Assays. AR-LBD/SRC2-3 Competition Assay. The small molecules were serially diluted from 10,000 to 2.44 μM in DMSO into a 96-well microplate (Costar 3359). Then 5 μL of diluted compounds was added to 45 μL of assay buffer (50 mM HEPES, 150 mM Li_2SO_4, 0.2 mM TCEP, 10% glycerol, pH 7.2, and 20 μM DHT), and the microplate was shaken at 600 rpm for 30 min at RT (IKA microtiter plate shaker). In 384-well microplates (Costar 3573), 20 μL of diluted compounds (1000−0.24 μM) was added to 20 μL of a protein cocktail (50 mM HEPES, 150 mM Li_2SO_4, 0.2 mM TCEP, 10% glycerol, pH 7.2, 1 μM liganded AR-LBD, and 0.01 μM fluorescent labeled peptide) yielding final compound concentrations of 500−0.12 μM and DMSO to 5%. The samples were allowed to equilibrate for 3 h. Binding was measured using fluorescence polarization (excitation λ 485 nm, emission λ 530 nm) and given in polarization (mP). The mP value (millipolarization level) is defined by: polarization (mP) = 1000 $(S - GP)/(S + GP)$, where S and P are background-subtracted fluorescence count rates and G (grating) is an instrument- and assay-dependent factor.

PPARγ/DRIP2 Competition Assay. pET15b-PPAR-LBD expression plasmid, encoding the PPARγ-LBD (amino acids 173−475) was a generous gift from Gabor J. Tigyi, University of Tennessee, Memphis. PPARγ was expressed in BL21 (DE3) (Invitrogen), and the peptide DRIP2 (CKNHPMLMNLLKDNP) was labeled with Texas Red C_2-maleimide (Invitrogen). The assay buffer was constituted of 20 mM TRIS (pH 7.50), 100 mM NaCl, 0.01% NP-40, 20 μM roziglitazone, 2 μM PPARγ-LBD, 10 nM DRIP-2 Texas Red, 5% DMSO.

VDR/SRC2-3 Competition Assay. This assay has been described in detail previously (*32*). MBP-VDR-LBDmt was expressed in BL21 (DE3) (Invitrogen), and the peptide SRC2-3 (CKKKENALLRYLLDKD-DTKD) was labeled with Alexa Fluor 647 (Invitrogen). The assay buffer was constituted of 25 mM PIPES (pH 6.75), 50 mM NaCl, 0.01% NP-40, 6 μM LG190178, 1 μM MBP-VDR-LBD, 5 nM SRC2-3 Alexa Fluor 647, 5% DMSO.

TR/SRC2-2 Competition Assay. This assay has been described in detail previously (*31*). hTRβ-LBD (His6 T209-D461) was expressed in BL21 (DE3) (Invitrogen), and the peptide SRC2-2 (CLKEKHKILHRLLQDSSSPV) was labeled with 5-iodoacetamido-fluorescien (Molecular Probes). The assay buffer was constituted of 20 mM Tris (pH 7.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.01% NP-40, 1 μM T3, 1 μM hTRβ-LBD, 25 nM SRC2−2 fluorescein, 5% DMSO.

Cell-Based Assays. *Transcription Reporter Assays. Androgen Receptor Assays in MDA-kb2 (33).* MDA-kb2 cells were cultured to 80% confluence, collected, resuspended, and seeded at a concentration of 300,000 cells mL^{-1} in 96-well cell culture treated microplates (Costar 3903) at 100 μL well^{-1}. The cells were allowed to attach at 37 °C for at least 15 h, without CO_2. Compounds were serially diluted in DMSO into a 96-well microplate (Costar 3359), and 0.25 μL of compound solution and 0.25 μL of DHT solution were added to the cells, yielding a final DMSO concentration of 0.5%. After 20 h of incubation at 37 °C and without CO_2, cells were cooled at RT, 80 μL of Bright-Glo reagent (Promega) was added, and luminescence was read directly.

Luciferase Inhibition Assay. MDA-kb2 cells at 80% confluence were incubated with 8 nM DHT for 20 h at 37 °C and without CO_2. After incubation, cells were lysed with a passive RIPA buffer (ThermoScientific) and centrifuged at 1000*g* for 3 min, and supernatant was collected, resuspended in media, and dispensed in 96-well microplates (Costar 3903). Next, 0.25 μL of compound solution was added to me-

dia and allowed to incubate at RT for 1 h. Then, 80 μL of Bright-Glo reagent (Promega) was added, and luminescence was read directly.

Cytotoxicity Assays. Rajii, HEPG2, BJ, and Hek293 cells were grown to 80% confluence, collected, and resuspended at a concentration of 50,000 cells mL^{-1} in 384-well microplates (Costar 3712) at 35 μL $well^{-1}$. Compounds were added to exponential growth phase cultured cells and incubated for 72 h at 37 °C. MDA-kb2 cells were grown to 80% confluence, collected, and resuspended at concentrations of 300,000 cells mL^{-1} in 96-well microplates (Costar 3903) at 100 μL $well^{-1}$. The plates were incubated for 20 h at 37 °C. The cytotoxicities were evaluated by using a Cell-Titer Glo reagent (Promega) and by reading luminescence. Compounds were tested at final concentrations of 40 and 1 μM. If more than 50% of the cells were killed at 40 μM, the compound was titrated in a dose–response matter.

Real Time rt-PCR. MDA-kb2 cells were incubated at 37 °C with tested compounds in the presence or absence of 8 nM DHT for 20 h. Total RNA was isolated from cells using an RNAeasy kit (Qiagen). Genomic DNA was removed and cDNA was generated using equal amounts of RNA (QuantiTect Reverse Transcription Kit, Qiagen). The cDNA was then diluted 50-fold, and the QuantiFast SYBR Green PCR Kit (Qiagen) was used for the real time PCR following manufacturer's recommendations. Primers used in these studies are as follows: GAPDH Forward Primer 5′-accacagtccatgccatcac-3′, Reverse Primer 5′-tccaccaccctgttgctgta-3′; 18S FP 5′-atcctcagtgagttctcccg-3′, RP 5′-ctttgccatcactgccatta-3′; FKBP51 (FK506-binding immunophilin 51) FP 5′-ctgtgacaaggcccttgga-3′, RP 5′-ctgggcttcacccctccta-3′. Real-time rt-PCR was carried out on a 7900HT Fast rt-PCR system (Applied Biosystems). We used the $\Delta\Delta Ct$ method to measure the fold change in gene expression of target genes. Standard deviations were calculated from 3 biological independent experiments performed in triplicates.

Physicochemical Assays. *Solubility Assay.* Thirty microliters of a DMSO stock of each compound (10 mM) was added to 600 μL of phosphate buffered saline in a 96-well plate (Costar 3359) and mixed. The plate was sealed and incubated in RT for 18 h. After mixing, 200 μL of sample suspension was transferred to a filter plate (catalog no. 110322, pION Inc., Woburn, MA) and prefiltered. Another 200 μL of sample was filtered with the same filter plate. The filtrate was collected and injected to UPLC-MS (Waters, Milford, MA), and the concentration was determined according to UV absorbance standard curves.

PAMPA Assay. The PAMPA procedure was conducted using a published method (*34, 35*). All liquid-handling steps for the PAMPA assay were performed on a Biomek FX Laboratory Automation Workstation (Beckman-Coulter) and analyzed by pION's (London, U.K.) PAMPA evolution 96 Command Software. The distribution of the compounds in the donor and acceptor buffers (100 μL aliquot) was determined by measuring the UV spectra from 200 to 500 nm using SpectraMax reader (Molecular Devices). Standards used were verapamil ($Pe = 1505 \times 10^{-6}$ cm s^{-1}) as a high permeability standard, carbamazepine ($Pe = 150 \times 10^{-6}$ cm s^{-1}) as a medium permeability standard, and ranitidine ($Pe = 2.3 \times 10^{-6}$ cm s^{-1}) as a low permeability standard.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Chang, C. (2002) *Androgens and Androgen Receptor: Mechanisms, Functions, and Clinical Applications*, Kluwer Academic Publishers, Norwell.
2. Gelmann, E. P. (2002) Molecular biology of the androgen receptor, *J. Clin. Oncol. 20*, 3001–3015.
3. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) The nuclear receptor superfamily: the second decade, *Cell 83*, 835–839.
4. Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. (1992) Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones, *J. Biol. Chem. 267*, 968–974.
5. Amler, L. C., Agus, D. B., LeDuc, C., Sapinoso, M. L., Fox, W. D., Kern, S., Lee, D., Wang, V., Leysens, M., Higgins, B., Martin, J., Gerald, W., Dracopoli, N., Cordon-Cardo, C., Scher, H. I., and Hampton, G. M. (2000) Dysregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R1, *Cancer Res. 60*, 6134–6141.
6. DePrimo, S. E., Diehn, M., Nelson, J. B., Reiter, R. E., Matese, J., Fero, M., Tibshirani, R., Brown, P. O., Brooks, J. D. (2002) Transcriptional programs activated by exposure of human prostate cancer cells to androgen, *Genome Biol.* 3, RESEARCH0032.
7. Nantermet, P. V., Xu, J., Yu, Y., Hodor, P., Holder, D., Adamski, S., Gentile, M. A., Kimmel, D. B., Harada, S., Gerhold, D., Freedman, L. P., and Ray, W. J. (2004) Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland, *J. Biol. Chem. 279*, 1310–1322.
8. Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L., and Lin, B. (2002) The program of androgen-responsive genes in neoplastic prostate epithelium, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11890–11895.
9. McKenna, N. J., and O'Malley, B. W. (2002) Minireview: nuclear receptor coactivators—an update, *Endocrinology 143*, 2461–2465.
10. McKenna, N. J., and O'Malley, B. W. (2002) Combinatorial control of gene expression by nuclear receptors and coregulators, *Cell 108*, 465–474.
11. Berrevoets, C. A., Doesburg, P., Steketee, K., Trapman, J., and Brinkmann, A. O. (1998) Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2), *Mol. Endocrinol. 12*, 1172–1183.
12. Burd, C. J., Morey, L. M., and Knudsen, K. E. (2006) Androgen receptor corepressors and prostate cancer, *Endocr.-Relat. Cancer 13*, 979–994.
13. Estebanez-Perpina, E., Moore, J. M., Mar, E., Delgado-Rodrigues, E., Nguyen, P., Baxter, J. D., Buehrer, B. M., Webb, P., Fletterick, R. J., and Guy, R. K. (2005) The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor, *J. Biol. Chem. 280*, 8060–8068.
14. Wammark, A., Treuter, E., Wright, A. P., and Gustafsson, J. A. (2003) Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation, *Mol. Endocrinol. 17*, 1901–1909.
15. Estebanez-Perpina, E., Arnold, L. A., Nguyen, P., Rodrigues, E. D., Mar, E., Bateman, R., Pallai, P., Shokat, K. M., Baxter, J. D., Guy, R. K., Webb, P., and Fletterick, R. J. (2007) A surface on the androgen receptor that allosterically regulates coactivator binding, *Proc. Natl. Acad. Sci. U.S.A. 104*, 16074–16079.
16. Klotz, L., and Schellhammer, P. (2005) Combined androgen blockade: the case for bicalutamide, *Clin. Prostate Cancer 3*, 215–219.
17. Marques, R. B., Erkens-Schulze, S., de Ridder, C. M., Hermans, K. G., Waltering, K., Visakorpi, T., Trapman, J., Romijn, J. C., and van Weerden, W. M. (2005) Androgen receptor modifications in prostate cancer cells upon long-term androgen ablation and antiandrogen treatment, *Int. J. Cancer 117*, 221–229.
18. Schellhammer, P. (1999) An update on bicalutamide in the treatment of prostate cancer, *Expert Opin. Invest. Drugs 8*, 849–860.

19. Gao, W., and Dalton, J. T. (2007) Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs), *Drug Discovery Today 12*, 241–248.
20. Narayanan, R., Mohler, M. L., Bohl, C. E., Miller, D. D., and Dalton, J. T. (2008) Selective androgen receptor modulators in preclinical and clinical development, *Nucl. Recept. Signaling 6*, e010.
21. Scher, H. I., and Sawyers, C. L. (2005) Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis, *J. Clin. Oncol. 23*, 8253–8261.
22. Buchanan, G., Yang, M., Harris, J. M., Nahm, H. S., Han, G., Moore, N., Bentel, J. M., Matusik, R. J., Horsfall, D. J., Marshall, V. R., Greenberg, N. M., and Tilley, W. D. (2001) Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function, *Mol. Endocrinol. 15*, 46–56.
23. Gottlieb, B., Beitel, L. K., Wu, J. H., and Trifiro, M. (2004) The androgen receptor gene mutations database (ARDB): 2004 update, *Hum. Mutat. 23*, 527–533.
24. Jung, F., Selvaraj, S., and Gargus, J. J. (1992) Blockers of platelet-derived growth factor-activated nonselective cation channel inhibit cell proliferation, *Am. J. Physiol. 262*, C1464–1470.
25. Zhu, W., Smith, A., and Young, C. Y. (1999) A nonsteroidal anti-inflammatory drug, flufenamic acid, inhibits the expression of the androgen receptor in LNCaP cells, *Endocrinology 140*, 5451–5454.
26. Bauman, D. R., Rudnick, S. I., Szewczuk, L. M., Jin, Y., Gopishetty, S., and Penning, T. M. (2005) Development of nonsteroidal anti-inflammatory drug analogs and steroid carboxylates selective for human aldo-keto reductase isoforms: potential antineoplastic agents that work independently of cyclooxygenase isozymes, *Mol. Pharmacol. 67*, 60–68.
27. Steckelbroeck, S., Jin, Y., Gopishetty, S., Oyesanmi, B., and Penning, T. M. (2004) Human cytosolic 3α-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3β-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action, *J. Biol. Chem. 279*, 10784–10795.
28. Féau, C., Arnold, L. A., Kosinski, A., and Guy, R. K. (2009) A high-throughput ligand competition binding assay for the androgen receptor and other nuclear receptors, *J. Biomol. Screening 14*, 43–48.
29. Bisson, W. H., Cheltsov, A. V., Bruey-Sedano, N., Lin, B., Chen, J., Goldberger, N., May, L. T., Christopoulos, A., Dalton, J. T., Sexton, P. M., Zhang, X. K., and Abagyan, R. (2007) Discovery of antiandrogen activity of nonsteroidal scaffolds of marketed drugs, *Proc. Natl. Acad. Sci. U.S.A. 104*, 11927–11932.
30. Hansch, C., and Fujita, T. (1964) $\rho-\sigma-\pi$ Analysis. A method for the correlation of biological activity and chemical structure, *J. Am. Chem. Soc. 86*, 1616–1626.
31. Arnold, L. A., Estebanez-Perpina, E., Togashi, M., Shelat, A., Ocasio, C. A., McReynolds, A. C., Nguyen, P., Baxter, J. D., Fletterick, R. J., Webb, P., and Guy, R. K. (2006) A high-throughput screening method to identify small molecule inhibitors of thyroid hormone receptor coactivator binding, *Sci. STKE 2006*, pl3.
32. Teichert, A., Arnold, L. A., Otieno, S., Oda, Y., Augustinaite, I., Geistlinger, T. R., Kriwacki, R. W., Guy, R. K., and Bikle, D. D. (2009) Quantification of the vitamin D receptor-coregulator interaction, *Biochemistry 48*, 1454–1461.
33. Wilson, V. S., Bobseine, K., Lambright, C. R., and Gray, L. E., Jr. (2002) A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists, *Toxicol. Sci. 66*, 69–81.
34. Balimane, P. V., Han, Y. H., and Chong, S. (2006) Current industrial practices of assessing permeability and P-glycoprotein interaction, *AAPS J. 8*, E1–13.
35. Yu, S., Konstantin, T., Zhu, Z., Graham, T. (2005) Automation of a double-Sink PAMPA permeability assay on the Biomek→ FX Laboratory Automation Workstation, *Pharm. Discovery*.
36. Febbo, P. G., Lowenberg, M., Thorner, A. R., Brown, M., Loda, M., and Golub, T. R. (2005) Androgen mediated regulation and functional implications of fkbp51 expression in prostate cancer, *J. Urol. 173*, 1772–1777.
37. Periyasamy, S., Warrier, M., Tillekeratne, M. P., Shou, W., and Sanchez, E. R. (2007) The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and -independent mechanisms, *Endocrinology 148*, 4716–4726.