Novel Trifluoromethylated Enobosarm Analogues with Potent Antiandrogenic Activity In Vitro and Tissue Selectivity In Vivo

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Abstract

Prostate cancer often develops antiandrogen resistance, possibly via androgen receptor (AR) mutations, which change antagonists to agonists. Novel therapies with increased anticancer activity, while overcoming current drug resistance are urgently needed. Enobosarm has anabolic effects on muscle and bone while having no effect on the prostate. Here, we describe the activity of novel chemically modified enobosarm analogues. The rational addition of bis trifluoromethyl groups into ring B of enobosarm, profoundly modified their activity, pharmacokinetic and tissue distribution profiles. These chemical structural modifications resulted in an improved AR binding affinity—by increasing the molecular occupational volume near helix 12 of AR. In vitro, the analogues SK33 and SK51 showed very potent antiandrogenic activity, monitored using LNCaP/ARLuciferase cells where growth, PSA and luciferase activity were used as AR activity measurements. These compounds were 10-fold more potent than bicalutamide and 100-fold more potent than enobosarm within the LNCaP model. These compounds were also active in LNCaP/BicR cells with acquired bicalutamide resistance. In vivo, using the ARLuc reporter mice, these drugs showed potent AR inhibitory activity in the prostate and other ARExpressing tissues, e.g., testes, seminal vesicles, and brain. These compounds do not inhibit AR activity in the skeletal muscle, and spleen, thus indicating a selective tissue inhibitory profile. These compounds were also active in vivo in the Pb-Pten deletion model. SK33 and SK51 have significantly different and enhanced activity profiles compared with enobosarm and are ideal candidates for further development for prostate cancer therapy with potentially fewer side effects. Mol Cancer Ther; 17(9): 1846–58. ©2018 AACR.

Introduction

Prostate cancer is the most commonly diagnosed male cancer in the Western world (1). Tumor growth is initially androgen dependent—driven by the androgen receptor (AR). The AR signaling pathway remains a key driver of prostate cancer, implicated throughout the different stages of the disease, and as such represents an obvious therapeutic target in prostate cancer therapy (2, 3). AR is a member of the family of steroid hormone receptors, which are ligand-dependent transcriptional regulators for diverse sets of genes involved in both reproductive and anabolic actions (4–6). The prototypical model for AR involves ligand binding that induces conformational changes in the ligand-binding domain (LBD), revealing a coactivator dimerization surface composed of helices 3, 5, and 12, as the LBD site is adjacent to helix 12 (H12).

Currently, the mainstays of prostate cancer treatment are androgen ablation (to castrate levels) and/or antiandrogen treatment, which block AR signaling (2, 3). The major antiandrogens in clinical use are bicalutamide and enzalutamide (and historically flutamide, hydroxyflutamide, and nilutamide; Fig. 1A). However, these compounds bind to AR with low affinity and can induce escape mechanisms. Furthermore, under AR amplification or mutation conditions some of these compounds exhibit agonist activity and fail to inhibit AR. This resistance may arise from, among other mechanisms, mutations to several key residues in the LBD (AR-LBD; refs. 7, 8). Common mutations in AR found in prostate cancer patients include T877A, W741L, and W741C. Bicalutamide (Fig. 1A) can act as an agonist in ARW741L and ARW741C while hydroxyflutamide is an agonist in ARW741A (7, 8). Gain-of-function mutations are common in prostate cancers and are correlated with disease progression, the lack of therapeutic response and poor clinical outcomes. Additionally, AR mutations may be selected for during antiandrogen therapy—reviewed in ref. 9 and 10.

Additionally, the use of antiandrogens in patients may elicit moderate side effects, e.g., muscle wastage, bone density loss, and CNS dysfunction—which are exacerbated in elderly men. Therefore, there is a growing need for selective androgen receptor modulators (SARM) that would demonstrate tissue-selective...
activity—being inhibitors of the AR in the prostate with tissue-sparing effects elsewhere (11).

Enobosarm (also referred to as Ostarine, GTx-024 or S-22) is a nonsteroidal SARM with a favorable safety and pharmacokinetic profile (Fig. 1B). It belongs to the class of arylpropionamide, which are structurally derived from the antiandrogen bicalutamide. Enobosarm binds to and activates the AR with entanselective affinity, potency, and efficacy similar to, but somewhat less than, that of DHT (12). Transient transfection studies indicate that enobosarm is remarkably selective for the AR, and it does not cross-react with other nuclear hormone receptors. This receptor specificity differentiates enobosarm’s pharmacology from other steroidal androgens and has likely contributed to observations that the drug has generally been well tolerated in clinical trials. It has selective anabolic effects and is under clinical development for prevention and treatment of muscle wasting (cachexia) in cancer patients (13, 14).

Results

Rational design of novel enobosarm analogues

Nonsteroidal antiandrogens such as bicalutamide act as AR antagonists (Fig. 1A), thus preventing androgen-dependent cell growth. In contrast, AR agonists, e.g., enobosarm (Fig. 1B) may act as agonists in all the AR LBD mutants, because they will fit sterically better with the bulk-reducing AR mutations than in ARwt (15). The difference in activity between agonists such as enobosarm and antagonists such as bicalutamide in ARwt may arise from the linker oxygen’s smaller size relative to the sulfonyl group of bicalutamide (Fig. 1A and B). This bulk in AR antagonist structure, though small, is thought to push the AR helix 12 away from the binding pocket, disturbing the agonist conformation of the androgen receptor (16). The current theoretical key to antagonism states that the differences observed among AR modulators (agonist or antagonist) are not because of their B-ring size or shape but because of their linker size and orientation (17). The improved understanding is being applied to rationally designed treatment options with novel mechanisms of action. It is proposed that AR antagonists should have appending molecular extensions to the core structure of AR agonists that interfere with the placement of H12, thereby disrupting coactivator recruitment (16, 18, 19).

In the enobosarm/ARwt cocystal structure (PDB code; 3RLJ; ref. 17; Fig. 2A and B), the 4-cyanophenyl ether group of enobosarm is situated between residues of H12 and the indolyl side chain of Trp741, suggesting that in the wild-type receptor (and probably the W741L mutant) the larger substitution with the same orientation of the linker would result in a better AR antagonist by pushing against Helix 12. We tested this hypothesis in silico by investigating whether the introduction of 3,5-bis-trifluoromethyl (3,5-bis-CF3) into B ring of enobosarm would provide the geometric bulk needed to keep ring B toward Helix 12 of AR. Preliminary molecular modeling studies showed that the bis-trifluoromethyl phenyl moiety (ring B in SK33 and SK51) has significantly changed the orientation of ring B so that the (3,5-bis-CF3) groups are pointing outward away from Trp741 and toward helix 12, thus preventing AR from adapting the AR agonist conformation, while keeping the small size of the oxygen linker. The combination of the small oxygen linker and the steric hindrance of the (3,5-bis-CF3) substitution on ring B resulted in the optimum size and orientation of ring B to impose conformational restriction away from Trp741 and toward helix 12 (Fig. 2C and D). A small library of AR antagonists having the (3,5-bis-CF3) motif was prepared (20), among which SK33 showed the best antiproliferative and metabolic profiles, hence it was selected for further studies.

The introduction of fluorinated substituents into drug candidates can impart a unique set of properties owing to the unique combination of electronegativity, size, and lipophilicity features of fluorinated groups (21–25). These factors can have a substantial impact on the molecular conformation, which in turn affect the binding affinity to the target protein (26, 27).

Chemical synthesis of SK33 and SK51

Racemic enobosarm derivatives (SK33 and SK51) were prepared by reacting methacryloyl chloride (2) with the corresponding substituted anilines 1a and 1b in dimethylacetamide (DMA) solvent to obtain phenylacrylamides 3a and 3b, which were converted into the corresponding epoxides 4a and 4b in the presence of a large excess of hydrogen peroxide and trifluoroacetic anhydride in dichloromethane (DCM). Opening of the epoxide rings of 4a and 4b with substituted 3,5-bis (trifluoromethyl)phenol 5 in tetrahydrofuran (THF) gave the final compounds SK33 and SK51. The structures of the synthesized compounds were confirmed using analytical and spectroscopic data (1H NMR, 13C NMR, 19F NMR, and mass spectrometry), which were in full accordance with their depicted structures. SK33 and SK51 were purified (>95% as confirmed by high-performance liquid chromatography—HPLC) and tested as racemic mixture (Fig. 1C).

Cellular activity of enobosarm and the trifluoromethylated analogues SK33 and SK51

The antiproliferative and selective activities of these compounds were tested in vitro in a panel of prostate cancer cell lines, namely, LNCaP, VCaP, PC3, and Du145. LNCaP and VCaP represent prostate cells that have retained the expression of the AR (28, 29). However, LNCaP cells overexpress a mutant AR, with a T877A mutation in the ligand-binding site. The VCaP cell line overexpresses wild-type AR. Both cell lines are responsive to androgens and cells will express androgen responsive genes. The PC3 and Du145 cells do not express the AR nor do they express androgen responsive genes.

Cells were exposed to increasing concentrations of antiandrogens for 96 hours and cell viability was analyzed using the MTT assay. In LNCaP ARwt cells, the parental compound enobosarm, showed insignificant activity with the IC50 = 40 μmol/L; however, the trifluoromethylated enobosarm analogue, SK33, demonstrated a significantly potent activity with IC50 = 0.2 μmol/L (Fig. 3A). Moreover, when compared with the antiandrogen bicalutamide, the novel compounds showed an increased efficacy (Fig. 3B). SK51 also displayed an increased activity compared with bicalutamide (IC50 = 0.7 μmol/L; Fig. 3B).

In PC3AR−ve cells, enobosarm showed activity only at relatively high dose (Fig. 3C), while SK33 showed some moderate activity. When compared with bicalutamide, SK33 showed a significantly increased activity. On the other hand, the antiproliferative activity of SK33 in LNCaP ARwt cells was over 10 times higher than that of its activity against PC3AR−ve cell line (Fig. 3D).
A, Chemical structure of the clinically used nonsteroidal antiandrogens: flutamide, hydroxyflutamide, nilutamide, bicalutamide, and enzalutamide showing rings A and B. 

B, Chemical structure of enobosarm (left), SK33, and SK51 (right). 

C, Chemical synthesis of SK33 and SK51 compounds, reagents and conditions: (i) DMA, rt, 24 hours, (ii) H2O2, DCM, rt, 2 hours, (iii) NaH, THF, rt, 24 hours.

Figure 1.

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Furthermore, the panel of prostate cancer cell lines was screened for its expression of the target AR by qPCR. As expected, LNCaP<sup>AR</sup><sup>+</sup> and VCaP<sup>AR</sup><sup>+</sup> cells showed robust expression of AR while AR was not detectable in PC3 AR<sup>-</sup>ve and Du145AR<sup>-</sup>ve cells (Fig. 3E).

Figure 3F depicts the summary of the activity profile of the SK33 and SK51 compounds in the four prostate cancer cell lines where a clear activity difference (selectivity) can be observed between the AR<sup>+</sup> cell lines (LNCaP and VCaP) and the AR<sup>-</sup> cell lines (PC3 and Du145).

SK33 decreases cells entering S-phase in LNCaP cells but not in PC3 cells

To further analyze the activity of these compounds in prostate cancer cell lines, we treated LNCaP (AR<sup>+</sup>) or PC3 (AR<sup>-</sup>) cells with 10<sup>−6</sup> mol/L of antiandrogen (or vehicle DMSO) for 48 hours. These cell lines were collected for FACS analysis, and standard cell-cycle profiling using propidium iodide staining for DNA content was carried out.

LNCaP cells showed a decrease in the number of cells in S phase with all the antiandrogen compounds. SK33 showed the highest reduction of the number of cells in S phase, followed by SK51, enzalutamide, and then bicalutamide, respectively (Fig. 3G). No apparent cell toxicity could be visualized as estimated by the absence of a sub-G<sub>1</sub> population or fragmented necrotic cells (Fig. 3G). In the PC3 cell line, no significant effect was observed with any of the tested compounds (Fig. 3H).

However, a small increase in G<sub>2</sub>–M phase could be detected with SK33 in PC3 cells.

SK33 reduces AR transcriptional activity

We then analyzed the ability of SK33 to inhibit the activity of the AR protein, i.e., the ligand activated transcription factor activity. The LNCaP/Luc cell line has an integrated luciferase reporter gene under the specific control of the AR transcription factor, as shown in the schematic in Fig. 4A (30). Growing these LNCaP/Luc cells in charcoal-stripped serum for 72 hours removes exogenous androgens and reduced the expression of AR-responsive genes down to a baseline level, as monitored by qPCR. Adding a synthetic androgen (R1881) back into the media (0–10 nmol/L) increased both PSA and luciferase gene expression in a dose-dependent manner (Fig. 4B).

Androgen treatment does not affect G418 gene expression (coexpressed from the same integrated plasmid as the luciferase reporter) nor did it affect the expression of housekeeping genes L19, β-actin, and GAPDH. The ability of the antiandrogens to block this androgen-dependent gene upregulation was then further tested. Treating hormonally starved LNCaP cells with R1881 showed a 9-fold upregulation of the endogenous gene PSA. When treated with increasing doses of either bicalutamide or SK33 a dose-dependent inhibition of gene expression was seen. SK33 showed a greater PSA inhibition than bicalutamide at 1 and 10<sup>−6</sup> mol/L (Fig. 4C). Conversely, the parental compound, enobosarm, showed a mild stimulatory activity (Fig. 4D).

We then examined the real-time activity of AR in live LNCaP/Luc cells in response to increasing doses of SK33 compared with the parental compound enobosarm and the antiandrogen bicalutamide. Live imaging and photon flux measurements of LNCaP/Luc cells grown on 96-well plates in the presence of 0 to 100 μmol/L compound for 24 hours resulted in a strong inhibition of AR-mediated luciferase activity (Fig. 4E). The AR inhibitory concentration (50%) for both SK33 and bicalutamide were approximately 1 μmol/L. Bicalutamide showed no further inhibitory effects past this concentration. However, SK33 maintained a dose-dependent inhibition, completely inhibiting the AR-mediated luciferase activity at 50 to 100 μmol/L concentration (Fig. 4F).
Figure 3.
Enobosarm analogues SK33 and SK51 show potent and selective activity in AR-positive prostate cancer cells. MTT cytotoxicity assays of LNCaP cells treated with increasing doses of enobosarm or SK33 (A) or bicalutamide, SK33 and SK51 (B) at 0–100 µmol/L. MTT cytotoxicity assays of PC3 cells treated with increasing doses of enobosarm or SK33 (C) or bicalutamide, SK33 and SK51 (D) at 0–100 µmol/L for 96 hours. E, qPCR analysis of a panel of prostate cancer cells for AR expression. Data represent the mean of three independent experiments. Data were normalized to GAPDH, β-actin, and RPL19 housekeeping genes. F, Bar chart representing a summary of the IC50 values for the 4 cell lines listed according to their AR status. G, FACS analysis of LNCaP cells treated with 10 µmol/L bicalutamide, enzalutamide, SK33, SK51, or vehicle control for 48 hours, and analyzed for S phase (left-hand side). Histograms showing DNA content (PI fluorescence) of treated LNCaP cells (right-hand side). H, FACS analysis of PC3 cells treated with 10 µmol/L bicalutamide, enzalutamide, SK33, SK51, or vehicle control for 48 hours, and analyzed for S phase (left-hand side). Histograms showing DNA content (PI fluorescence) of treated PC3 cells (right-hand side). Gated cells (10,000) were measured for each experiment.
SK33 and SK51 are active in LNCaP cells with acquired bicalutamide resistance

LNCaP cells were grown in increasing concentrations of bicalutamide for 3 to 6 months, until proliferation was seen. This bicalutamide resistant cell line (LNCaP/BicR) then grew well in 20 µmol/L bicalutamide, with cell-cycle kinetics similar to that of the parental cell line. An increased expression level of AR was seen in this cell line upon qPCR analysis (Fig. 5A). Treating this cell line with increasing doses of bicalutamide showed a proliferative response—at 20 to 25 µmol/L—whereas the parental cell line showed sensitivity and an IC₅₀ of 3 µmol/L (Fig. 5B). The LNCaP/BicR cell line also showed a partial cross-resistance to enzalutamide (IC₅₀ ≈ 5 µmol/L approximately; Fig. 5C). We then analyzed if the mechanism of the acquired resistance to bicalutamide extended to the novel agents SK33 and SK51. Treatment of LNCaP/BicR cells with increasing concentrations of SK33 and SK51 for 96 hours resulted in a dose-dependent response and an inhibition of cell growth, rather than being proliferative agents as bicalutamide (Fig. 5D), with IC₅₀ of approximately 2 µmol/L for SK51 and 5 µmol/L for SK33. Although not as active as in the parental sensitive cell line, SK51 did show an improved activity over enzalutamide (and bicalutamide).
In vitro aqueous solubility and microsomal metabolic stability

Subsequently, an early in vitro pharmacokinetic study of the most promising compound, SK33, was performed. The metabolic stability of SK33 was tested in human liver microsomes, where compounds were incubated for 45 minutes with pooled liver microsomes and the intrinsic clearance (CLint) and half-life (t1/2) values were calculated based on 5 time points. Compound SK33 is characterized by an increased metabolic stability in comparison with the standard—bicalutamide (see Table 1). SK33 has microsomal half-life (t1/2) of 32 hours, which compares with that of standard drugs (bicalutamide and enzalutamide) and represents a good starting point for optimization toward a weekly or twice-weekly administrable product. No observed in vitro cardiotoxicity was observed in our preliminary studies (7.6% inhibition of hERG at 25 μmol/L concentration; http://cyprotex.com). Because of these promising in vitro properties and the potent antiproliferative profile in prostate cancer cell lines, SK33 was selected for more detailed preclinical in vitro and in vivo studies.

SK33 is a potent inhibitor of AR in the ARE-Luc reporter mouse and shows tissue specificity

The transgenic ARE-Luc mouse model shows AR-driven luciferase activity in all tissues in response to androgen action and activity can be monitored in live animals in real time (31). Using age-matched male mice (littermates), we analyzed the basal AR-driven luciferase levels in mice prior to treatment. ARE-Luc age-matched male mice (littermates), we analyzed the basal activity can be monitored in live animals in real time (31). Using

In Figure 5, SK33 is active in LNCaP cells with acquired bicalutamide resistance. A, Bar graph indicating the relative expression levels of AR in LNCaP and LNCaP/BicR cells. Data represent the mean of three independent experiments. Data were normalized to GAPDH, β-actin, and RPL19 housekeeping genes. B and C, MTT cytotoxicity assays of LNCaP or LNCaP/BicR cells treated with increasing concentrations of bicalutamide (B) or enzalutamide (C) for 96 hours. D, MTT cytotoxicity assays of LNCaP/BicR cells treated with increasing concentrations of bicalutamide, SK33, and SK51 for 96 hours.

Table 1. t1/2 calculated in human liver microsomes; SK33 is metabolically stable with no loss of parent compound detected for the duration of the assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>t1/2 (minutes)</th>
<th>PPB %</th>
<th>% hERG inhibition at 25 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicalutamide</td>
<td>214</td>
<td>n.d.</td>
<td>96.4</td>
</tr>
<tr>
<td>SK33</td>
<td>1950</td>
<td>94.7</td>
<td>7.64</td>
</tr>
</tbody>
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NOTE: Plasma protein binding (PPB) assay; results are expressed in terms of mean % bound from duplicate experiments. Cardiotoxicity expressed in terms of % hERG inhibition at 25 μmol/L compound concentration. 

Mice were injected with n-luciferin and imaged using an IVIS CCD camera in order to obtain baseline visualization and measurements of androgen receptor tissue activity. ARE-Luc mice were then injected subcutaneously with 50 mg/kg bicalutamide, SK33, or vehicle (DMSO) control and left for 24 hours (n = 3 for each treatment), after which the mice were imaged again under the exact same conditions. Posttreatment mice were imaged again for luciferase activity (Fig. 6A). Luciferase activity was measured from three regions of interest (ROI), including the head, abdominal, and gonadal region (Fig. 6B). Bicalutamide and SK33 treatment resulted in a decreased luciferase activity in all areas. However, mice treated with SK33 showed a greater reduction of luciferase activity in these areas when compared with bicalutamide. DMSO vehicle did not significantly affect luciferase activity in any region (Fig. 6C).

Tissues were then removed from sacrificed animals, and RNA was extracted and analyzed by qPCR for luciferase transcripts in each individual tissue. Bicalutamide and SK33 showed strong reduction in luciferase transcripts compared with vehicle or untreated mouse tissues. Although similar, SK33 reduced luciferase transcripts to a greater extent than bicalutamide—cantly greater effects in the brain. Neither bicalutamide nor SK33 had significant effects on AR activity in the spleen. However, SK33 did not reduce luciferase activity in the leg muscle and showed a significant upregulation of luciferase transcripts in this tissue (Fig. 6D). Additionally, both bicalutamide and SK33 have reduced luciferase enzymatic activity in the prostate, testes and brain. Both antiandrogens showed significant inhibition compared with vehicle control. Although slightly more inhibition was seen in the SK33 treated mice, the antiandrogens did not show statistically significant differences from each other (Fig. 6E).

SK33 is a potent inhibitor of AR in the Pten−/− mouse model for prostate intraepithelial neoplasia (PIN)

Pten loss is one of the most frequent events in prostate cancer both at the initiation stage and during late-stage metastatic...
Figure 6.
SK33 inhibits AR transcriptional activity in vivo and in PIN. A, Bioluminescent imaging of live ARE-Luc mice pre and posttreatment with bicalutamide or SK33 (or DMSO vehicle) for 24 hours. Image represents a grayscale image overlaid with pseudocolor bioluminescent activity data (scale represents flux rate of photons/sec/cm^2). B, Grayscale image of an ARE-Luc mouse, white circles represent the three ROI measurements for each mouse. C, Bar graph representing relative bioluminescent data emanating from the three ROI—head, abdomen, and gonadal regions. Data are normalized to mouse bioluminescence pretreatment with bicalutamide, SK33 (50 mg/kg), or DMSO vehicle. D, qPCR analysis of luciferase expression from various body tissues as indicated. Data are presented as luciferase/housekeeping genes (GAPDH, RPL19, and β-actin). E, Bar graph indicating relative luciferase enzymatic activity from various tissues from ARE-Luc mice treated with either bicalutamide, SK33, or DMSO. F, qPCR analysis of luciferase expression from the mouse PTen^−/− prostate tissues. Data are presented as luciferase/housekeeping genes (GAPDH, RPL19, and β-actin). G, MTT assay of PTen-null mouse prostate cells treated with increasing doses of bicalutamide, enzalutamide, and SK33 for 96 hours. *, P = 0.05; **, P = 0.01.
Prostate cancer, like the prostate gland from which it is derived, is androgen dependent—at least in the initial stages. The inhibition of the AR activity either by antiandrogen therapy or by androgen ablation remains the mainstay of treatment choices. Recently, there has been a movement away from classical antiandrogen ablation remains the mainstay of treatment choices.

The LNCaP cell line is an androgen-sensitive cell line with a T877A mutation in the LBD of the AR. Treating this cell line in culture for an extensive period with bicalutamide gave rise to a resistant subclone (LNCaP/BicR), which proliferated in the presence of high doses of bicalutamide and showed an enhanced expression of AR. This switch to AR agonism is indicative of clinical prostate cancer as it progresses, and this work has been previously reviewed (35, 36). We did not detect other LBD mutations in this cell line. Although not as active in the parental cell line, SK33 and especially SK51 showed moderate activity. At this stage, it is unclear if the concentration of SK33 required to inhibit the acquired resistance phenotype may be achievable in vivo, but their increased metabolic stability, resistance to oxidative metabolism and detoxification together with increased solubility may translate to high levels of drug in circulation. Additionally and of clinical importance, SK51 showed increased activity over enzalutamide in the LNCaP/BicR cells.

It is rare that an inhibitor compound such as an antiandrogen can be tested in a biological system where there is a highly specific real-time reporter for its target protein activity—integrated into the host genome. Utilizing an LNCaP cell line with an integrated AR-driven luciferase reporter (30), we analyzed the effect of these compounds directly on the transactivation function of the AR. SK33 resulted in a stronger inhibition of AR-mediated gene activation of both the endogenous gene PSA and the transgene luciferase, than that mediated by bicalutamide. SK33 could also competitively inhibit the AR in the presence of a strong synthetic androgen (R1881), while enobosarm itself had a mildly stimulating effect.

Further testing in vivo using unique AR-reporter mice, developed by the authors (31), showed the antiandrogen activity of these compounds in the target tissue, i.e., prostate, as well as in peripheral tissues, in real time within the same animal. Here, side effects and bystander effects could be monitored. SK33 showed a stronger or equal antiandrogen effect in most tissues, when compared with bicalutamide at the same dosage concentration. But, interestingly, we observed that the effects of SK33 upon leg muscle AR activity was stimulatory, and the effects in the spleen were minimal.

The anabolic effects of enobosarm have been well documented, although its mechanism of action in specifically stimulating skeletal muscle AR and not prostate or gonadal AR remains elusive. Because SK33 is a modified analogue of enobosarm the effects on skeletal muscle was interesting to observe, especially given its simultaneous high inhibitory activity in the prostate and other tissues. The rational addition of bis-trifluoromethyl groups into ring B of enobosarm seems to have successfully exaggerated the SARM activity of enobosarm. The mechanism of SARM-like compounds remains relatively unknown, but locally expressed AR tissue-specific cofactors have been
postulated to govern the activity of AR modulating compounds such as SARMs. Although our results are limited to the mouse leg skeletal muscle, the use of such a compound in prostate cancer patients may ease side effects such as muscle wasting and fatigue, while maintaining prostate AR inhibition. The effects on other muscle tissues, e.g., the AR-responsive levator ani muscle, in the mouse was unavailable.

It should also be noted that SK33 showed very potent inhibitory effects in the brain—this may reflect the increased lipophilicity of the fluorinated compounds in traversing the blood–brain barrier or in fact their increased antiandrogen potential. The CNS-specific effects could not be determined in the mouse model, but no deleterious effects on motor function or behavior was observed. The effects in humans may well correlate with CNS-related side effects.

Finally, within a developing hyperplasia/adenocarcinoma in situ in mice with homozygous fl fluorescence deletion we observed that SK33 was equal to or better than bicalutamide in reducing AR activity.

Conclusions

We demonstrate that structure-based design can efficiently engineer second-generation AR antagonists that have clinical potential to circumvent antiandrogen resistance by complementing receptor mutations at the molecular level. In principle, there are a limited number of mutations that can cause an AR antagonist to function as an AR agonist; therefore, it is conceivable that such approaches may ultimately lead to the development of antiandrogens that resist mutations that cause antiandrogen withdrawal syndrome.

Utilizing novel in vitro and in vivo screening techniques and models, ideally suited to analyze and evaluate novel antiandrogens, we have identified a novel antiandrogen candidate SK33. This bis-trifluoromethyl-containing enobosarm analogue, displays better activity than bicalutamide in vitro with increased efficacy against acquired antiandrogen resistance. Importantly, SK33 has comparable activity in vivo, but maintains a degree of tissue selectivity associated with its SARMS—enobosarm parental molecule. The increased stability and potential differential side effect profile of these molecules may make them useful drugs for prostate cancer treatment.

Materials and Methods

All chemicals were purchased from Sigma Aldrich or Alfa Aesar and were used without further purification. All reactions were performed under a nitrogen atmosphere. H-NMR (500 MHz), C-NMR (125 MHz), and F-NMR (470 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer at 25°C. Chemical shifts (δ) are expressed in parts per million (ppm) and coupling constants (J) are given in hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet), td (triple doublet), and m (multiplet). Mass spectrometry was run on a Bruker Micromass system in electrospray ionization mode. Thin-layer chromatography: precoated aluminum backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short and long wave UV light (254 and 366 nm). Flash column chromatography was carried out using silica gel supplied by Fisher (60 Å, 35–70 μm). Purity of prepared compounds was determined by Analytical HPLC analysis using either a Thermo Scientific or a Varian Prostar system. All compounds tested in biological assays were >95% pure. HPLC using the eluents water (eluent A) and acetoni­trile (eluent B).

General method for the preparation of intermediates (3a,b)

Methacryloyl chloride 2 (2.63 mL, 27.16 mmol/L) was added over the course of 10 minutes to a stirring solution of the different aniline 1a, b (3.4 mmol/L) in N,N-dimethylacetamide (10 mL) at room temperature for 3 hours. After the reaction was complete, the mixture was diluted with ethyl acetate (100 mL), extracted with sat. aq. NaHCO3 solution (3 × 25 mL) and with cold brine (4 × 50 mL). The organic layer was dried over Na2SO4 and the solvent was removed at reduced pressure. The crude residue was purified by flash column chromatography.

General method for the preparation of intermediates (4a,b)

To a stirred solution of the intermediates (3a,b; 3 mmol/L) in DCM (7 mL) was added 30% hydrogen peroxide (3.6 mL, 32.03 mmol/mL). The reaction mixture was put in a water bath at room temperature and trifluoroacetic anhydride (3.7 mL, 26.7 mmol/L) was added slowly to the mixture, which was then stirred for 24 hours. The reaction mixture was transferred to a separating funnel using DCM (30 mL). The organic layer was washed with distilled water (20 mL), sat. aq. Na2SO4 (4 × 20 mL), sat. aq. NaHCO3 (3 × 20 mL) and brine (20 mL), dried over Na2SO4 and concentrated at reduced pressure.

General method for the preparation of compounds SK33 and SK51

To a mixture of NaI (60% in mineral oil, 0.250 g, 1.23 mmol/L) in anhydrous THF (2 mL) at 0°C under Ar atmosphere was added a solution of the 3,5-bis-trifluoro­methylphenol 5 (1.11 mmol/L) in 1 mL of anhydrous THF. This mixture was stirred at room temperature for 20 minutes. A solution of the different intermediates (4a,b; 0.74 mmol/L) in anhydrous THF (3 mL) was added slowly. The reaction mixture was stirred at room temperature overnight. The mixture was then diluted with ethyl acetate (30 mL), washed with brine (15 mL) and water (30 mL), dried over Na2SO4, and concentrated under vacuum. The crude residue was purified by flash column chromatography.

3-(3,5-bis(trifluoromethyl)phenoxy)-N-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (SK33)

Purified by flash column chromatography eluting with n-hexane/EtOAc 100:0 v/v increasing to n-hexane/EtOAc 90:10 v/v. Obtained in 85 % yield as a white solid.

1H NMR (CDCl3-d) δ 9.20 (bs, 1H, NH), 7.73 (d, J = 7.5 Hz, 1H, ArH), 7.10 (d, J = 7.5 Hz, 1H, ArH), 6.95 (d, J = 7.0 Hz, 1H, ArH), 7.65 (s, 1H, ArH), 7.38 (s, 2H, ArH), 4.59 (d, J = 9 Hz, 1H, CH0), 3.43 (d, J = 9 Hz, 1H, CH2), 3.48 (bs, 1H, OH), 1.67 (s, 3H, CH3). 13C NMR (CDCl3-d) δ 172.03 (C=O), 158.28 (ArC), 141.29 (ArC), 135.92 (ArC), 130.60 (ArC), 132.57 δ 62.72–63.09. 19F NMR (CDCl3-d) δ 172.03 (C=O), 158.28 (ArC), 141.29 (ArC), 135.92 (ArC), 132.57 δ 62.72–63.09.
CF3), 121.84 (ArCH), 117.33 (q, 3JCF = 5.1 Hz, ArCH), 115.56 (q, 3JCF = 3.6 Hz, ArCH), 115.38 (ArC), 115.14 (q, 3JCF = 3.8 Hz, ArCH), 104.92 (ArC), 75.83 (COH), 73.10 (CH2), 23.04 (CH3). MS [ESI, m/z]: 501.1 [M + H], 523.1 [M + Na]. HPLC: retention time = 25.18 minutes.

3-(3,5-bis(trifluoromethyl)phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-2-( trifluoromethyl)phenyl) propanamide (SKS1)

Purified by flash column chromatography eluting with n-hexane/EtOAc 100:0 v/v increasing to n-hexane/EtOAc 90:10 v/v. Obtained in 78% yield as a white solid.

1H NMR (CDCl3-d4) δ 9.68 (bs, 1H, NH), 8.76 (d, J = 9 Hz, 1H, ArH), 8.59 (dd, J = 3 Hz, 1H, ArH), 8.47 (dd, J = 2.5, 9.5 Hz, 1H, ArH), 7.56 (s, 1H, ArH), 7.36 (s, 2H, ArH), 4.59 (d, J = 9 Hz, 1H, CH2), 4.15 (d, J = 9 Hz, 1H, CH2), 3.25 (bs, 1H, OH), 1.69 (s, 3H, CH3); 13C NMR (CDCl3-d4) δ 158.15 (ArC), 143.17 (ArC), 140.38 (ArC), 133.16 (q, 3JCF = 33.8 Hz, ArC), 133.03 (q, 3JCF = 32.8 Hz, ArC), 126.38 (ArCH), 123.22 (q, 3JCF = 268.3 Hz, CF3), 122.75 (q, 3JCF = 271.3 Hz, CF3), 122.62 (ArCH), 122.46 (q, 3JCF = 6.3 Hz, ArC), 115.67 (q, 3JCF = 3.8 Hz, ArCH), 115.11 (ArCH), 75.86 (COH), 73.93 (CH3), 22.94 (CH3). MS [ESI, m/z]: 521.1 [M + H], 543.1 [M + Na]. HPLC: retention time = 26.42 minutes.

Cell culture

All cell lines were purchased from the ATCC cell bank. Cells were obtained from the ATCC cell bank.

MTT assay

The MTT assay was used as a cell viability assay for all the cell lines listed using the antiandrogen compounds. Briefly, cells (5 × 104 cells/mL) were seeded into 96-well plates (200 μL/well), allowed to attach and grow for 24 hours, and subsequently treated with varying concentrations of antiandrogens (0–100 μmol/L) for 96 hours. Optimal seeding densities were measured for linear growth over the 96 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL in PBS) was added to a final concentration of 0.5 mg/mL for 4 hours at 37°C. After 4 hours, purple formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in acidified isopropanol (200 μL/well) and the absorbance was read at 570 nm after 10 minutes incubation. Percent inhibition of viability was calculated as a percentage of untreated control and the cytotoxicity/cell growth inhibition was expressed as IC50.

 Luciferase assays

Cells were washed and lysed in reporter lysis buffer (Promega). Lysate was mixed with α-luciferin substrate (Promega) and light emission was measured using the Promega Glomax multiluminometer.

For live cell imaging α-luciferin substrate was added directly into the cell media, and plates were imaged on a Synergy G Box imager. Tissue was pulsed by grinding in liquid nitrogen and then completely homogenized, using a microfuge pestle, in reporter lysis buffer with protease inhibitors (Promega). Lysate (20 μL) was mixed with luciferin substrate (20 μL) and light emission measured using the Promega Glomax multiluminometer. Light emission was then normalized to protein content as measured by a Bradford Assay.

RNA extraction and RT-PCR

Total RNA samples were prepared using TRIzol reagent (Sigma) and converted to cDNA using the GoScript Reverse Transcription System (Promega).

qPCR

Reactions were performed in triplicate on cDNA samples in 96-well optical plates on an ABI Prism StepOne System (Thermo Fisher). Reactions consisted of 2 μL cDNA, 7 μL PCR-grade water, 10 μL 2 X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL Taqman specific assay probes (Applied Biosystems) for PSA, TMPRSS2, KLK2, and L19. Parameters were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were recorded using Sequence Detector Software (SDS version 2.3; PE Applied Biosystems). Levels were normalized to GAPDH, β-actin, and RPL19.

Animal experiments

All mouse procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under Home Office license. AR-luciferin strains were made and available “in house” (31), and Ptenlox/lox; P53-cre/cre were obtained from The Jackson Laboratory.

Luciferase imaging

Anesthetized mice (3% isoflurane with O2 carrier, Abbott Animal Health UK) were injected i.p. or s.c. with α-luciferin (Caliper Life Sciences Ltd) at 150 mg/kg, 10 minutes before imaging. Light emission from luciferase was detected by the IVIS Imaging System 100 series (Xenogen Corporation), and overlaid as a pseudocolor image with reference scale, upon a grayscale optical image. For ex vivo imaging, mice were sacrificed 10 minutes after luciferin injection and immediately dissected. Target organs were rinsed briefly in PBS and placed under the bioluminescent camera. Tissues were then collected for luciferase assays, RNA extraction or fixed in 10% formaldehyde before wax embedding and sectioned using standard procedures.

Cell-cycle analysis (FACS)

Cells were grown on 10-cm dishes for 24 to 96 hours treatments. Cells were then trypsinized, washed twice in PBS, fixed in 70% ethanol at 4°C, were stained with 5 μg/mL propidium iodide, and RNA was removed using 50 μg/mL RNase A. FACS analysis was carried out using a FACS Calibur (Beckton-Dickinson) using linear scale representation of forward and side scatter during flow analysis, as well as DNA content. Single
cells were gated and the cell-cycle profiles measured using FCS Express software, using the built in kinetics and cell-cycle analysis best-fit model for cell-cycle phase identification. A total of 10,000 events were measured per sample.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.A. Dart, S. Kandil, C.L. Bevan, A.D. Westwell
Development of methodology: D.A. Dart, S. Kandil
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.A. Dart, S. Kandil, S. Tommasini-Gheffi, G. Serrano de Almeida, C.L. Bevan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.A. Dart, S. Kandil, G. Serrano de Almeida, C.L. Bevan
Writing, review, and/or revision of the manuscript: D.A. Dart, S. Kandil, G. Serrano de Almeida, C.L. Bevan, W. Jiang, A.D. Westwell

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.A. Dart, S. Kandil

Study supervision: D.A. Dart, C.L. Bevan, W. Jiang, A.D. Westwell

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The authors would like to pay a tribute to Professor Christopher McGuigan (the Drug Hunter) for all his contributions for the success of this project. Professor McGuigan was an exceptional and dedicated scientist who has remarkable achievements in the anticancer and antiviral drug-discovery field.

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