Elucidation of the Multiple Activities of Abiraterone by a Synthetic Chemistry Approach

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Abstract

Objective: Novel agents to treat metastatic prostate cancer include a class of drugs which function primarily by inhibiting the action of the CYP17 enzyme, which results in decreased androgen synthesis. Abiraterone and related molecules have been shown to decrease expression of the androgen receptor (AR) and to inhibit its transcriptional activity in addition to CYP17 inhibition. The structure-activity relationships (SAR) governing AR down regulation are less well understood than CYP17 and AR inhibition, so we designed abiraterone derivatives with the goal of better understanding the SAR of AR down regulation.

Methods: We synthesized 17 abiraterone derivatives with unsaturated, cyclic and acyclic substituents containing hydrogen bond acceptors at C16 and C17 positions. We examined the ability of these compounds to inhibit CYP17 and AR, and to decrease AR expression.

Results: While abiraterone was the most potent AR down regulator, we found that a hydrogen bond acceptor on an unsaturated nitrogen or oxygen, 4 to 6 angstroms from C17 is required for AR down-regulation, but a heterocyclic ring is not required, as shown by the activity of compounds 4, 8, and 10. The size and shape of substituents on active AR down regulators indicates that the binding site near C17 is significantly larger than the pyridine ring in abiraterone. We found no correlation between the ability of a compound to down regulate AR and its ability to inhibit CYP17 or AR transcriptional activity. AR transcriptional activity was inhibited most potently by molecules with hydrogen bond donors 2-3 angstroms from C17. Molecules with C16 substituents could inhibit AR transcription but were inactive as AR down regulators, as indicated by compounds 5, 13, and 14. CYP17 inhibitors had an unsaturated nitrogen (pyridine or hydrazine) oriented away from the steroid backbone and perpendicular to the C16-C17 bond.

Conclusion: We determined the SAR for AR down regulation. The complete lack of correlation between SARs for AR down regulation and AR or CYP17 inhibition suggests distinct mechanisms of actions for these activities. The SAR for AR down regulation suggests that additional hydrogen bond acceptor side chains 4-6 angstroms from C17 could enhance the activity of abiraterone.

Keywords: CYP17 inhibitor; Abiraterone; Prostate cancer; Androgen receptor

Introduction

Despite incredible advances in detection and treatment, prostate cancer remains the second leading cause of cancer death in American men [1]. Signaling by the androgen receptor (AR) controls normal prostate growth and homeostasis but also drives the proliferation of malignant prostate cells. In the setting of metastatic prostate cancer, removal of testicular androgens by surgical or chemical castration initially leads to cancer regression; however, new tumors almost universally recur despite very low levels of systemic androgens and most of these castration-resistant prostate cancers (CRPCs) remain dependent on AR function for growth [2,3]. Several molecular mechanisms have been described to account for continued AR signaling in CRPC, including the amplification of AR [2], gain-of-function mutations in AR that confer greater sensitivity to androgens or increased recruitment of AR coactivator proteins [4], LBD-independent N-terminal activation by growth factors, neuropeptides, and inflammatory mediators [5-7], expression of constitutively active AR splice variants (ARv’s) [8-11], tumoral conversion of adrenal androgens [12], and intratumoral androgen production [13]. These basic discoveries have led to a renewed interest in AR as a target in CRPC and have led to the development of several new drugs that block the AR/androgen signaling axis. The first to gain FDA approval was the CYP17 inhibitor abiraterone acetate, which prevents androgen synthesis from adrenal glands and cancer tissue (5). Several other compounds that target androgen synthesis are in advanced clinical trials, including orteronel and galeterone [14,15]. In addition to inhibiting androgen synthesis, several CYP17 inhibitors have been reported to decrease AR levels [15,16], including the levels of ARv’s [17]. The decreased AR expression appears to occur through a suppression of cap-dependent translation by blocking assembly of the eIF4F and eIF4G complex to the mRNA 5’ cap [16]. Several CYP17 inhibitors also reduce AR transcriptional activity, likely through competitive antagonism of ligand binding [16,17]. Decreasing AR levels could provide a valuable strategy for inhibiting the growth of prostate cancers, especially when combined with androgen synthesis inhibition and competitive antagonism. In an attempt to understand what chemical moieties control the various pharmacologic actions of these multi-activity drugs, we modified the structure of abiraterone and examined the ability of the derivatives to decrease AR expression and inhibit AR transcriptional activity as our main objectives. The ability of these molecules to inhibit testosterone synthesis through CYP17 inhibition was also determined as a secondary objective.

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Results and Discussion

Design strategy and chemistry: In this study, 17 novel derivatives were synthesized around the abiraterone (1) core with the goal of identifying moieties that affect the ability of abiraterone to inhibit CYP17 activity, down-regulate AR expression, and inhibit AR transcriptional activity. The modifications involved replacing the 17-aryl group and the 16-hydrogen with other substituents in both 16-17 unsaturated and 16-17 saturated molecules (Figure 1). The substituents were chosen to better understand the SAR, specifically how the shape of substituents and position of hydrogen bond donors and acceptors affected AR down regulation activity.

AR down-regulation

Abiraterone has been shown to decrease both AR protein and mRNA levels, likely by interfering with cap-dependent translation [16]. To test the ability of derivatives to down-regulate AR expression, LNCaP prostate cancer cells were treated with each derivative at concentrations ranging from 0.5 to 25 uM for 48 hrs and AR was detected in lysates by Western blot. 48 hrs was determined to be the optimal time-point for assay from pilot time-course studies with abiraterone (1). Derivatives were ranked in order of their ability to decrease AR protein levels compared to a vehicle control (Figure 1A). Abiraterone (1) and its 4-isomer (2) were the most potent, but the activities of 8, 11, and 18 show that an aromatic ring is not necessary for activity. A hydrogen bond acceptor on an unsaturated CN or CO bond, 4 to 6 angstroms from C17 is a common feature for 1, 2, 8, 9, 10, 11, and 16. The importance of this hydrogen bond acceptor is further supported by the substantially greater AR down regulation activity of 8 compared to 12 and 13. Compound 8 (C=N stereochemistry unknown) is isosteric with either 12 or 13, differing in that 8 is a carbamoylhydrazone while 12 and 13 are alkanoylhydrazones. Compound 3 has an oxygen atom adjacent to the carbonyl, making the carbonyl in 3 more electronegative than the carbonyls in 12 and 13. Structures 9 and 10 also have more electron rich carbonyl groups and great AR down regulation activity than 12 and 13. Compound 2, with an electron-poor aroylhydrazine carbonyl and a pyridine nitrogen >7 angstroms from C17, is completely inactive. The maintenance of activity in molecules with large C17 groups (7, 10, and 11) all demonstrate that the binding pocket occupied by the pyridyl ring in abiraterone is significantly larger than pyridyl, but does not extend as far as the pyridine ring in the inactive compound 2. Molecules with C16 substituents were inactive. The results suggest the possibility of designing molecules with greater potency than abiraterone by adding additional hydrophobic and hydrogen- bond acceptor groups to the aromatic ring.

AR transcriptional activity

AR is a transcription factor and abiraterone is an inhibitor of transcriptional activity as well as a down regulator of AR. We employed a firefly luciferase reporter driven by the human PSA promoter to assess the effect of derivatives on AR transcriptional activity in LNCaP cells. Although AR transcriptional activity inhibition and AR down regulation involve the same receptor, there is no correlation between these activities (Figures 1B and 1D), suggesting that very distinct mechanisms are responsible for AR transcriptional activity inhibition and AR down regulation. The oxime 6 is the most potent inhibitor of AR transcriptional activity (IC_{50}=0.1 uM), but one of the least potent AR down regulators. Compounds 5, 13, and 14 are potent inhibitors of AR transcription (IC_{50}=<1 uM), but lack AR down regulatory activity. Compound 8 is one of the least potent AR transcriptional inhibitors (IC_{50}=6.5 uM), but is the 3rd most potent AR down regulator. The potent AR transcriptional inhibition of 6, 10, 11, 12, 13 (IC_{50}=0.6 uM), when contrasted with the weak activity of 18 (IC_{50}=>8 uM) suggest that a hydrogen bond donor within 2-3 Angstroms of C17 is consistent with activity. An electron-deficient aromatic ring (1, 4) or halogens (5) at C17 are also consistent with AR transcriptional inhibition. Bulky substituents well tolerated at both C17 (5, 10, 11, with IC_{50}<0.8 uM) and C16 (14, 15, 16, 17 with IC_{50}<2.3 uM). However, very large, rigidly attached C17 substituents lead to much weaker transcriptional inhibition (2 and 7, with IC_{50}>9 uM).

CYP17 inhibition

Although the structures of molecules used in this study were designed or selected to understand the structure/activity relationship for AR down regulation, we were also interested in CYP17 activity because it contributes to the therapeutic utility of abiraterone by inhibiting androgen synthesis. To determine the ability of derivatives to inhibit CYP17 enzymatic activity, we employed the NCI-H295R cell assay, a well-established model of steroidogenesis [18,19]. These cells express CYP17 and have been shown to synthesize T from steroidal precursors, which can be blocked by specific inhibitors [20]. Media was changed and cells were treated with 10 uM of each compound for 48 hrs, at which point, T was quantified in the media using an ELISA specific for T (Figure 1C). The most active CYP17 inhibitors (>29% inhibition at 10 uM) contain an unsaturated C=N bond, either in a pyridine ring nitrogen (1, 4) or a hydrazone with Z stereochemistry (10, 12). A loan pair oriented perpendicular to the C16-C17 bond and pointed away from the steroid framework is important: Z- hydrazones 10 and 12 (32 to 47% inhibition 10 uM) are more potent than E-isomers 9, 11 and 13 (0 to 20% inhibition at 10 uM). For the E isomers, the nitrogen loan pair on C=N is oriented parallel to the C16-C17 bond as opposed to perpendicular for the Z isomers. These results are in line with previous findings which suggest that CYP17 inhibition in abiraterone and related molecules depends upon an appropriately oriented heteroatom [21,22].

Materials and Methods

Chemical syntheses

Hydrazones 10, 11, 12 and 13 were synthesized using an established procedure (27). 16-Dehydropregnenolone was purchased from AK Scientific. Butyric acid hydrazide, Benzyl carbazate, and t-Butyl carbazate were purchased from Combi-Blocks, San Diego, CA, USA

Z-16-Dehydropregnenolone butyroylhydrazine (12) and E-16-Dehydropregnenolone butyroylhydrazine (13)

16-Dehydropregnenolone (115 mg, 0.366 mmol) was suspended in HOAc (1.15 ml). Butyric acid hydrazide (133 mg, 1.30 mmol, 3.56 equivalents) was added, and the mixture which was stirred at 25 degrees for 3 days. The mixture became homogenous during the reaction period. TLC (100% diethyl ether) showed two products, Rf 0.30 (which formed quickly), and Rf 0.15 (which formed more slowly). Water (3 ml) was added to the resulting yellow solution, and the precipitate was collected by centrifugation, then rinsed again with water (3 ml), and was dried to afford (12) all demonstrate that the binding pocket occupied by the pyridyl ring in abiraterone is significantly larger than pyridyl, but does not extend as far as the pyridine ring in the inactive compound 2. Molecules with C16 substituents were inactive. The results suggest the possibility of designing molecules with greater potency than abiraterone by adding additional hydrophobic and hydrogen- bond acceptor groups to the aromatic ring.

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HOAc (1 ml). Benzyl carbazate (102 mg, 0.61 mmol, 1.92 equivalents) was added, and the mixture was stirred at 25-30 degrees for 1.75 h. The mixture initially became homogenous, and then the product precipitated and was collected by vacuum filtration, rinsed with HOAc, and dried in vacuo to afford a white solid (40 mg, 27% yield). TLC in diethyl ether showed a single spot at Rf 0.4. The melting point was 220 -222 degrees.

E-16-Dehydroepiandrosterone-butyloxy carbonylhydrazone (10)

16-Dehydroepiandrosterone (141 mg, 0.45 mmol) was suspended in HOAc (1.15 ml). t-Butyl carbazate (141 mg, 1.07 mmol, 237 mol%) was added and the mixture was stirred at 30 degrees for 4 days. During the reaction the mixture became homogenous and turned yellow. Water (3 ml) was added and the ppt was collected by centrifugation, then rinsed again with water (3 ml), and chromatographed on silica gel with EtOAc-Pet Ether (75:25) to afford a white solid (40 mg, 27% yield). TLC (EtOAc-Pet Ether, 75:25) showed a single spot with an Rf of 0.35.

Testosterone synthesis assay

Human adrenal NCI-H295R cells (ATCC) were cultured in six well plates under standard conditions in Dulbecco's modified Eagle's/Ham's F-12 medium supplemented with 5% Nu-Serum, 0.1% selenium/insulin/transferrin plus, and pen/strep. Twenty-four hours after subculturing, medium was replaced, and drugs were added at 10 μM in normal growth medium for 48 h to triplicate samples. Control cells were treated with 0.1% (v/v) DMSO vehicle. 100μl of media was removed and processed for testosterone quantification according to the manufacturer's instructions (Diagnostic Automation Inc, Calabasas, CA). The colorimetric assay was quantified on a M1000 Infinity plate reader (Tecan), and testosterone concentrations estimated by use of a standard curve. Percent inhibition was calculated compared to the vehicle control.

Western blotting

LNCaP cells were cultured under standard conditions with phenol red-free RPMI supplemented with 10% FBS and pen/strep. Cells were treated with drugs for 4-72 h and then lysed in TBS with 0.1% Tween with protease inhibitors. Lysates were resolved by SDS-PAGE, transferred, and Western blot was performed with an anti-AR antibody (PG21, Millipore) or anti-GAPDH antibody (Santa Cruz, cat no. 47724) as a loading control.

AR transcription assay: Two days prior to transfection, LNCaP cells were placed in media containing charcoal-stripped serum. For all transfections, pools of cells were transfected using Lipofectamine Plus (Invitrogen) with PSA-luciferase and pRL-SV40 (Promega) as a control. The following day, the cells were plated in quadruplicate with drug [23] treatments in 96 well plates. 24 h later luciferase production was measured using the Dual luciferase assay kit (Promega) on the M1000 Infinity plate reader. Firefly signal was normalized to control Renilla luciferase signal and IC_{50} values were determined by linear regression.

Conclusions

AR down regulation requires a hydrogen bond acceptor on an unsaturated N or O, 4 to 6 angstroms from C17, while for inhibition of AR transcription, molecules with hydrogen bond donors 2 to 3 angstroms from C17 can be more potent than abiraterone. The complete lack of correlation between SARs for AR down regulation and inhibition of AR transcription suggest distinct mechanisms of actions for these
2 activities (Figure 1D). Further synthesis will be necessary to dissect these functions. While no derivatives were synthesized with a greater ability than abiraterone to down regulate AR expression or inhibit CYP17, our studies will inform the synthesis of the next generation of derivatives with improved functionality. In particular, our SAR for AR down regulation suggests that the binding site for the aromatic sidegroup in abiraterone can accept larger molecules, such that a properly positioned hydrogen bond acceptor (e.g. carbamate) could enhance the activity of abiraterone.

References