Cytochrome P450 17A1 inhibitor abiraterone attenuates cellular growth of prostate cancer cells independently from androgen receptor signaling by modulation of oncogenic and apoptotic pathways

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Abbreviations: PC, prostate cancer; TGFβ, transforming growth factor β; AR, androgen receptor; Bax, Bel-2-associated X protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Key words: prostate cancer, abiraterone, cytochrome P450 17A1, apoptosis, cell cycle, transforming growth factor β

Abstract. Abiraterone provides significant survival advantages in prostate cancer (PC), however, the current understanding of the molecular mechanisms of abiraterone is still limited. Therefore, the abiraterone impact on androgen receptor (AR)-positive LNCaP and AR-negative PC-3 cells was assessed by cellular and molecular analyses. The present study demonstrated, that abiraterone treatment significantly decreased cell growth, AR expression, and AR activity of AR-positive LNCaP cells. Notably, AR-negative PC-3 cells exhibited comparable reductions in cellular proliferation, associated with DNA fragmentation and pro-apoptotic modulation of p21, caspase-3, survivin, and transforming growth factor β (TGFβ). Our observations suggest that the attenuation of AR signaling is not the only rationale to explain the abiraterone anticancer activity. Abiraterone efficacy may play a more global role in PC progression control than originally hypothesized. In this regard, abiraterone is not only a promising drug for treatment of AR-negative PC stages, even more, abiraterone may represent an alternative for treatment of other malignancies besides prostate cancer.
of advanced PC. Most likely based on its steroidal structure, abiraterone has been found bound to the AR and thus competitively antagonizes subsequent AR signaling cascades, however, not as potent as pure antagonists, e.g. bicalutamide (7,8). On the contrary, there are lines of evidence that abiraterone is an inducer of steroidogenic enzymes including CYP17A1 and may increase expression of steroid synthesis machinery (9-11). Our present understanding of molecular properties of abiraterone is still limited. In particular, combinatorial or sequential combinations of abiraterone with other drugs as well as the usage of abiraterone in different settings of PC remain unsolved. Due to multiplicity and complexity of recently described abiraterone effects, an inhibitory activity exclusively based on a blockade of androgen synthesis appears unlikely. With this study we assessed abiraterone effects on AR-positive and AR-negative PC cells to gain insight into the molecular mode of action.

Materials and methods

**Chemicals and antibodies.** Abiraterone acetate was kindly provided by Janssen-Cilag GmbH (Neuss, Germany) and was used as a 10 mM (LNCaP cells) and 30 mM (PC-3 cells) stock solution, respectively, with dimethyl sulfoxide (Carl Roth, Karlsruhe, Germany) as solvent. Docetaxel was purchased from Sigma-Aldrich (Munich, Germany) and was used as 10 µM stock solution. Antibodies directed against the AR and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as well as peroxidase-coupled secondary antibodies directed against mouse and rabbit were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for the transforming growth factor β (TGFβ) receptor type I and II were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and antibodies directed against homolog of Caenorhabditis Sma and Drosophila Mad 3 (Smad3) and Smad4 were obtained from Zymed Laboratories (San Francisco, CA, USA).

**Cell culture.** The PC cell lines LNCaP and PC-3 from Cells Service (CLS, Heidelberg, Germany) were propagated in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin/streptomycin (all from PAN Biotech, Aidenbach, Germany) at 5% CO₂ atmosphere and 37°C. Cells were passaged twice per week using a trypsin/ethylenediaminetetraacetic acid solution (0.1/0.04%) dissolved in phosphate-buffered saline (PBS) (both from PAN Biotech). For experiments, cells were plated in poly-L-lysine (Sigma-Aldrich, Deisenhofen, Germany)-coated 24-well (proliferation assay) and 6-well [quantitative reverse transcription-polymerase chain reaction (RT-PCR), western blot analysis] cell culture plates, respectively.

**Proliferation assay.** Cell growth of abiraterone treated cells was examined by cell counting utilizing a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany) and compared to vehicle treated cells. Therefore, 30,000 cells/well were seeded in 24-well cell culture plates and treated as indicated. Number of living cells was determined by tryptophan/ethylenediaminetetraacetic acid detachment of adherent cells and subsequent analysis. The cell suspension (100 µl) was diluted in 10,000 µl CASYton (Roche Applied Science) and analysis of 400 µl dilution was performed in triplicates using a capillary of 150 µm in diameter. Gate settings of 7.20/15.45 µm were used to ensure the discrimination between living cells and dead cells, as well as cellular debris.

**Annexin V assay.** Apoptosis detection of abiraterone-incubated PC-3 cells was performed using a FITC Annexin V Apoptosis Detection kit I (BD Bioscience, Heidelberg, Germany) as recommended by supplier instructions. After washing the cells once with PBS, 6x10³-1x10⁴ cells were harvested with a cell scraper and were resuspended in 1 ml PBS. After centrifugation and discarding the supernatant, the pellet was resuspended in binding buffer and stained with propidium iodide and FITC Annexin V for 15 min. Data were assessed by a FACSCanto A Cytometer and were analyzed using BD FACSDiva™ software (both from BD Bioscience).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** TUNEL apoptosis analysis of abiraterone-incubated PC-3 cells was performed using the HT TiterTACS assay kit (Treven, Gaithersburg, MD, USA) following supplier recommendations. After indicated time-points of incubation adherent cells were detached by 0.1% trypsin/0.04% EDTA. Data were acquired using an Infinite 200 PRO multimode reader and were analyzed using the i-control 1.9 software (both from Tecan, Männedorf, Switzerland). Unlabeled and nuclease treated samples served as negative and positive control, respectively.

**Quantitative RT-PCR.** Total RNA was extracted using 500 µl peqGold TriFast reagent (PeqLab, Erlangen, Germany) and 50 µl 1-bromo-3-chloropropane (Sigma-Aldrich) according to manufacturer's instructions. Prepared RNA was resuspended in diethyl pyrocarbonate (Carl Roth) treated water and RNA concentration was determined in a spectral photometer (NanoDrop 2000c; Peqlab). One microgram of total RNA was applied in RT using MMLV Reverse Transcriptase (Promega, Mannheim, Germany), Ribolock RNase Inhibitor (Thermo Fisher Scientific, Rockford, IL, USA) and an oligo(dT)₁₀ oligonucleotide. Subsequently, mRNA amounts were quantified by real-time PCR using the following pairs of oligonucleotides: PSA forward, 5'-CCGGAGAGCTGTGTCCACAT-3' and reverse, 5'-GTGACGACAAATCCAACTGC-3'; caspase-3 forward, 5'-GCTCTAGGGAGTGGTGC-3' and reverse, 5'-GATTCCAAGGGCGACGAAC-3'; cyclin-dependent kinase inhibitor 1 (p21) forward, 5'-TGGAGACTCTCAGGGTGC-3' and reverse, 5'-GCGCGTTGAGGTGTTGAGAATC-3'; Bel-2-associated X protein (Bax) forward, 5'-TCCGCCGAGGCTTTT-3' and reverse, 5'-CGGCTTTGGGTGTTGAGAATC-3'; Bcl-2-associated X protein (Bax) forward, 5'-TCCGCCGAGGCTTTT-3' and reverse, 5'-CGGCTTTGGGTGTTGAGAATC-3'; survivin forward, 5'-TGCCGGCCAGCTTGCC-3' and reverse, 5'-CAGTCTCTTTAATGTAGATGGCTTG-3'; ribosomal protein large P0 (RPLP0) forward, 5'-CAATGGGCAGCATCTACAACC-3' and reverse, 5'-ACTCTCTCCCTGGCTTCCAACC-3'; real-time PCR was carried out with SensiMix SYBR Hi-Rox (Bioline, Luckenwalde, Germany) in a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, München, Germany). For quantification, target specific signals were standardized to RPLP0 mRNA as reference.
Western blot analysis. Cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM K2HPO4, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 1 mM Na3VO4, 20 mM NaF; 0.1 mM phenylmethylsulfonyl fluoride, 20 mM 2-phosphoglycerate, and complete protease inhibitor cocktail (Roche Applied Science), total amount of protein was determined by using Bradford reagent (12), and equal amounts of protein were conducted to gel electrophoresis. After transfer onto a nitrocellulose membrane (GE Healthcare Europe, Freiburg, Germany) and blocking (Roti-Block; Carl Roth), protein detection was done by incubation with target specific primary antibodies followed by species specific secondary antibodies and visualized by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific) in a ChemiDoc XRS System (Bio-Rad Laboratories). Protein signals were quantified by Image Lab 3.0 software (Bio-Rad Laboratories) and standardized to GAPDH signals as reference.

Statistical analysis. Results of at least three independent experiments were statistically analyzed. Analysis was performed using the Student's t-test with \( P \leq 0.05 \), \( P \leq 0.01 \) and \( P \leq 0.001 \) given as significant. Data are expressed as mean ± SD.

Results

Cellular growth of AR-positive and hormone-sensitive LNCaP cells is attenuated in the presence of abiraterone by diminishing AR expression and activity. To investigate the effects of abiraterone on cellular growth of PC cells, AR-positive LNCaP cells were propagated in the presence of various concentrations of abiraterone, ranging from 4 to 30 µM. We found abiraterone diminished cellular growth in a concentration-dependent manner compared to vehicle treated control cells. At intermediate dosage of 10 µM abiraterone, cell proliferation was inhibited to ~50% after an exposure of 120 h (Fig. 1A). To verify these data, growth kinetics of cells treated with 10 µM abiraterone were done and statistical analysis revealed that growth suppression was significant (72 h, 1.5-fold, \( P=0.0082 \); 120 h, 1.5-fold, \( P=0.0010 \)) (Fig. 1B). Subsequent western blot analysis of the AR expression after 72 h lasting abiraterone incubation showed significantly reduced levels of AR protein (24 h, 0.13±0.09, \( P<0.0001 \); 48 h, 0.20±0.10, \( P<0.0001 \); 72 h, 0.16±0.10, \( P=0.0001 \)) (Fig. 2A). The downregulation was accompanied by strongly attenuated transcriptional activity of the AR (24 h, 0.12±0.12, \( P=0.0096 \); 48 h, 0.06±0.04, \( P<0.0001 \);
participation of AR protein and AR properties in the molecular mode of action of abiraterone have not been extensively investigated. Therefore, AR-negative PC-3 cells treated with 10 to 40 µM abiraterone were analyzed and, very notably, mRNA levels were verified by quantitative RT-PCR and normalized to control treated cells. During propagation of PC-3 cells in the presence of 30 µM abiraterone, the expression of the anti-apoptotic cell survival factor survivin was suppressed (24 h, 0.33±0.40, P=0.0438; 48 h, 1.01±1.05; 72 h, 0.25±0.08, P<0.0001) (Fig. 5A). Moreover, alterations of survivin, cyclin-dependent kinase inhibitor 1 (p21), caspase-3 and Bcl-2-associated X protein (Bax) mRNA levels were verified by quantitative RT-PCR and normalized to control cells. During propagation of PC-3 cells in the presence of 30 µM abiraterone, the expression of the anti-apoptotic cell survival factor survivin was suppressed (24 h, 0.33±0.40, P=0.0438; 48 h, 1.01±1.05; 72 h, 0.25±0.08, P<0.0001) (Fig. 6A). Accordingly, mRNA levels of the pro-apoptotic regulators p21 (24 h, 1.56±2.26; 48 h, 4.00±1.52, P=0.0269; 72 h, 5.19±2.20, P=0.0088) (Fig. 6B) and caspase-3 (24 h, 5.62±2.63, P=0.0382; 48 h, 4.08±4.84; 72 h, 1.02±0.69) (Fig. 6C) were significantly elevated. Notably, synthesis of the pro-oncogenic factor Bax was slightly but significantly reduced within 72 h of abiraterone incubation (24 h, 1.11±0.55; 48 h, 1.00±0.82; 72 h, 0.37±0.09, P<0.0001) (Fig. 6D).

**Discussion**

Molecular properties of CYP17A1 as a key enzyme in androgen biosynthesis provided the rationale for development of the CYP17A1 inhibitor abiraterone. Former studies with regard to the multiple cytochrome P450 enzyme inhibitor ketoconazole have served as proof-of-principle: repression of androgen biosynthesis and AR signaling, which is the primary antitumor activity of the drug. However, abiraterone inhibits androgen biosynthesis and AR signaling, androgenic receptor isoforms, TGFβ receptor type I (24 h, 0.52±0.43, P=0.0152; 48 h, 0.44±0.18, P=0.0009; 72 h, 0.49±0.22, P=0.0034) (Fig. 5A) and type II (24 h, 0.54±0.08, P<0.0001; 48 h, 0.70±0.08, P=0.008; 72 h, 0.50±0.24, P=0.24) (Fig. 5B), compared to control treated cells given as 1.0. Furthermore, the TGFβ intracellular downstream signaling proteins Smad3 (24 h, 0.53±0.18, P=0.0096; 48 h, 0.49±0.12, P<0.0001; 72 h, 0.27±0.07, P<0.0001) (Fig. 5C) and Smad4 (72 h, 0.56±0.28, P=0.0071) (Fig. 5D) were significantly suppressed. Moreover, alterations of survivin, cyclin-dependent kinase inhibitor 1 (p21), caspase-3 and Bcl-2-associated X protein (Bax) mRNA levels were verified by quantitative RT-PCR and normalized to control cells. During propagation of PC-3 cells in the presence of 30 µM abiraterone, the expression of the anti-apoptotic cell survival factor survivin was suppressed (24 h, 0.33±0.40, P=0.0438; 48 h, 1.01±1.05; 72 h, 0.25±0.08, P<0.0001) (Fig. 6A). Accordingly, mRNA levels of the pro-apoptotic regulators p21 (24 h, 1.56±2.26; 48 h, 4.00±1.52, P=0.0269; 72 h, 5.19±2.20, P=0.0088) (Fig. 6B) and caspase-3 (24 h, 5.62±2.63, P=0.0382; 48 h, 4.08±4.84; 72 h, 1.02±0.69) (Fig. 6C) were significantly elevated. Notably, synthesis of the pro-oncogenic factor Bax was slightly but significantly reduced within 72 h of abiraterone incubation (24 h, 1.11±0.55; 48 h, 1.00±0.82; 72 h, 0.37±0.09, P<0.0001) (Fig. 6D).
Figure 4. Abiraterone (AA) treatment is followed by induction of apoptosis. (A and B) PC-3 cells were treated with vehicle or with 30 µM abiraterone for the indicated time-points and were stained with propidium iodide and FITC Annexin V. Data were assessed by a FACSCanto A Cytometer. (C) PC-3 cells were treated with vehicle or with 30 µM abiraterone for the indicated time-points. Docetaxel of 10 nM treated PC-3 cells served as control. Unlabeled control cells served as negative control and nuclease treated cells as positive control, respectively. Cells were harvested and processed by specific labeling of nuclear DNA fragmentation with a HT TitertACS assay kit. Data are expressed as the mean ± SD (compared and normalized to vehicle treated control cells). *p<0.05 and ***p<0.001, as determined by Student’s t-test.

Figure 5. Abiraterone diminishes factors of the TGFβ signaling pathway. (A-D) PC-3 cells were treated with vehicle or with 30 µM abiraterone for the indicated duration and total cell extracts were subjected to western blot analyses normalized to GAPDH as internal reference. (A) TGFβ receptor I; (B) TGFβ receptor II; (C) Smad3; (D) Smad4. Columns, mean; bars, ± SD; *P≤0.05, **P≤0.01 and ***P≤0.001 (compared to vehicle treated control cells).
cytochrome P450 enzymes including CYP17A1 were shown to harbor anticancer activity in PC, however, ketoconazole administration was of minor benefit and was accompanied by significant toxicity (13). Recent studies of Brossard et al utilizing abiraterone derivates with a broad spectrum of inhibited cytochrome P450 enzymes have confirmed this hypothesis (14). Therefore, current understanding of molecular abiraterone efficacy in PC is primarily based on attenuated androgen biosynthesis/conversion and subsequent diminished AR signaling by the potent and specific inhibition of CYP17A1. The data presented here provide evidence that abiraterone treatment resulted in significantly reduced proliferation rates even for PC cells lacking AR signaling. We previously reported that abiraterone significantly decreased the viability of AR expressing LNCaP cells most likely by the antagonistic properties of abiraterone (7,8). Moreover, growth attenuation was accompanied by a dramatically diminished AR protein expression and activity. These observations are in accordance with a study of Soifer et al, which considered downregulation of the AR in the presence of 1.0-15.0 µM abiraterone (15).

Remarkably, AR-negative PC-3 cells exhibited comparably inhibited growth characteristics in the presence of abiraterone. The applied drug concentration of 30 µM corresponds to abiraterone concentrations which were determined in our study (10 µM in LNCaP cell incubation experiments) and in a study of Bruno et al (10 µM in PC-3 cell incubation experiments) (16). Notably, attenuation of PC-3 proliferation point to additional and AR-independent pathways which were targeted by abiraterone. Accordingly, further experiments suggested a broad spectrum of anti-tumor activities of abiraterone displayed by targeting regulatory factors of TGFβ, apoptosis and cell cycle pathways.

Besides the numerous AR signaling events, it is well established that there are several proliferative signal cascades which are qualified to become active in PC growth, e.g. phosphatidylinositide 3-kinase (17), epidermal growth factor (18), and estrogen receptor (19) pathways. The precise role of the pleiotropic growth factor TGFβ, which is also part of this multiple and fine-tuned regulatory network, is controversial. On the one hand, TGFβ has been reported to govern differentiation and anti-proliferation in non-malignant prostate epithelium as well as in early stage PC tissue. On the other hand, TGFβ signals are linked to a tumorigenic phenotype in advanced PC stages (20-23). Even though abiraterone did not affect TGFβ1 and TGFβ2 mRNA levels (data not shown), analysis of TGFβ pathway proteins revealed a clear suppression of TGFβ cascades. Abiraterone inhibited both types of TGFβ receptors, type I and II, as well as the downstream signaling molecules Smad3 and Smad4; thus, it is reasonable to hypothesise a strong reduction of TGFβ signaling by abiraterone treatment. However, since TGFβ signals play a dual role in PC reflected by its opposing properties of pro- and anti-oncogenic effects, it is difficult to estimate the TGFβ-dependent impact of abiraterone. Though, elevated serum levels of TGFβ have been shown to predict tumor recurrence and metastasis, and the PC cells became less responsive to TGFβ growth inhibition in later stages of PC (20,24), therefore, abiraterone efficacy was probably due to attenuation of TGFβ-dependent oncogenic
signal cascades. However, these results further exemplify the difficulties of interpretation of TGFβ pathways in PC cell biology and may suggest further investigations in this field.

Chemotherapy widely exerts its anticancer effect by triggering apoptotic mechanisms of tumor cells. A primary regulator of apoptosis is the tumor suppressor p53, which plays a key role in cell cycle control, genomic stability, and apoptosis. Due to a frame shift within the p53 gene, PC-3 cells express a truncated and therefore inactive form of the protein (25,26), however, the induction of apoptosis pathways in absence of p53 as a response to drug treatment has been extensively described (27-29). Notwithstanding, in PC-3 cells lacking functional p53 the initiation of intrinsic apoptosis pathway factors occurs in the presence of abiraterone. Increased DNA fragmentation as determined by TUNEL assay, as well as downregulation of the cell survival factor survivin and elevated levels of the cell cycle inhibitor p21 and the effector caspase-3 clearly indicated an execution of apoptotic mechanisms. These data confirm former studies which have shown that anticancer drugs affect the biosynthesis of these intrinsic apoptotic factors in PC-3 cells (28,30-32). Surprisingly, our results could not be definitely verified by Annexin V staining. Since externalisation of Annexin V to the outer plasma membrane is a very early effect in apoptosis, it could be speculated that Annexin V signals have already expired before the first selected time point. However, it is notable that Annexin V staining of PC-3 cells failed also previously (33). Curiously, we monitored significantly reduced levels of pro-apoptotic Bax after 72 h of abiraterone incubation. This finding is not consistent with earlier reports in which Bax has been shown being drug-induced in PC-3 cells (28). The intrinsic apoptosis pathway is controlled by a balance of pro-apoptotic (e.g. Bax, p21 and caspase-3) and anti-apoptotic (e.g. survivin) factors. Apoptosis in the absence of p53, however, appears complex. Therefore, differing cell death cascades compared to the classical intrinsic apoptosis pathways by interfering with other regulatory pathways may occur. For instance, there have been reports that apoptosis induced by the hypoxia response system can provoke a downregulation of Bax in cancer cells (34,35).

Taken together, our observations suggest that abiraterone-mediated attenuation of AR signals is not the only rationale to explain anticancer activity. Besides an expected inhibition of LNCaP cell growth by targeting the AR, very notably, abiraterone comparably inhibited proliferation of AR-negative PC-3 cells. Thus, abiraterone's molecular mode of action is proposed to be mediated through an accessory inhibition of pro-oncogenic TGFβ signals and through the alteration of apoptotic factors.

Experimental and preclinical data continue to enlarge our understanding of abiraterone molecular mode of action and facilitate the opportunity to improve PC treatment regimes as well as to overcome resistance to abiraterone. Interestingly, current data showed no, or no detectable expression of CYP17A1 enzyme in LNCaP and PC-3 cell lines (35) and, in this context, abiraterone efficacy may play a more global role in PC progression control than originally hypothesized. Consequently, abiraterone is not only a promising drug for treatment of AR-negative PC stages, even more, abiraterone may represent a therapeutical alternative for treatment of other malignancies besides PC.

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