Lysophosphatidic acid receptor isoforms expression in prostate cancer cells is differentially regulated by the CYP17A1 inhibitor abiraterone and depends on the androgen receptor

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Abstract: Prostate cancer (PC) treatment with steroid synthesis inhibitor, abiraterone acetate, (AA) provides substantial survival advantages in advanced PC therapy. Owing to AA's anti-proliferative efficacy, even in the absence of androgen receptor (AR), the molecular mode of action is suspected to be a result of simultaneously targeted cellular factors. The present study demonstrated a differentially regulated expression of proliferative lysophosphatidic acid receptor (LPAR) isoforms in androgen receptor (AR)-positive LNCaP cells incubated with AA. Since LPAR regulation in AR-negative PC-3 cells is unaffected, it could be assumed that AA's anticancer activity on LPAR expression depends on AR signaling cascades.

Keywords: prostate cancer; lysophosphatidic acid receptor; abiraterone; CYP17A1; steroid synthesis

Introduction

Prostate cancer (PC) is the most common malignancy diagnosed and one of the leading causes of oncological mortality in men in the Western hemisphere[1]. Treatment with the recently licensed anticancer drug abiraterone acetate (AA) has provided significant survival advantages in PC therapy, even in advanced and castration-resistant settings[2]. AA elicits cytochrome p450, family 17, subfamily A, polypeptide 1 (CYP17A1) inhibition, thus preventing the conversion of pregnenolone/progesterone to 17α-hydroxypregnenolone/17α-hydroxyprogesterone, as well as the subsequent cleavage of 17α-hydroxypregnenolone/17α-hydroxyprogesterone to dehydroepiandrosterone/androstenedione[3]. Therefore, AA-mediated inhibition of CYP17A1 effectively prevents the biosynthesis of steroid hormones such as mineralcorticoids, estrogens and androgens. In PC therapy, the AA anticancer activity essentially arises from the inhibition of androgen receptor (AR) signaling, which is the primary growth-stimulating pathway in prostatic epithelial cells[4,5].

Lysophosphatidic acid (LPA) is a growth factor-like biolipid with functions in cancer initiation and progression[6,7]. In PC cells, LPA controls proliferation, cell motility and survival via the modulation of various downstream targets (e.g. transcription factors, growth factors and mitogen-activated protein kinases)[8-11]. In contrast, almost nothing is known about upstream regulators modulating the liberation of LPA and the expression of LPA receptors (LPAR). The six LPAR family members, LPAR1 to LPAR6, are G protein-coupled transmembrane...
receptors stimulated by the binding of the natural ligand LPA. Two studies indicated that LPAR signaling in PC cells may depend on steroids. Bex et al. demonstrated that G protein inhibition by the pertussis toxin is connected to PC cell growth attenuation, suggesting that this effect may be androgen-dependent.

The goal of the present study is to characterize AA’s efficacy on LPAR expression. Since AA allows inhibitory activity even on castration-resistant patients with androgen-insensitive PC tissues, we hypothesized that additional proliferative pathways can be targeted during AA treatment.

Materials and Methods

Cell culture and incubation experiments

PC cell lines, PC-3 and LNCaP (Cell Lines Service, Eppelheim, Germany), from human prostate carcinoma epithelium were propagated in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/10,000 µg/mL streptomycin (PAN Biotech, Aidenbach, Germany) at 37 °C in 5% CO₂. For the AA (Janssen-Cilag, Neuss, Germany) incubation experiments, cells were propagated in the presence of 30 µM (PC-3) and 10 µM (LNCaP) AA using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Deisenhofen, Germany) as a vehicle control. These concentrations inhibited cellular growth to approximately 50% as compared to vehicle-treated control cells over an incubation time of 72 h. Cell line-specific dosage of AA was determined by cell growth kinetics in the presence of various AA concentrations as previously described.

Proliferation assay

Cellular growth was analyzed in 24-well cell culture plates (1 mL/well) utilizing a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). Therefore, 30,000 cells per well were plated into 24-well cell culture plates and incubated over a period of 24–72 h. For measurement, the wells were detached by 0.1% trypsin/0.04% ethylenediaminetetraacetic acid (EDTA) and 100 µL of cell suspension was re-suspended in 10 mL of CASYton solution (Roche Applied Science, Mannheim, Germany). Cell line-specific gate settings were used to differentiate living cells, dead cells and cellular debris. Cell number determination was done in duplicates for each sample.

Quantitative mRNA Analysis

After incubation in 10 µM (LNCaP) and 30 µM (PC-3) of AA for 6 and 24 h, respectively, the total RNA was extracted using 500 µL of peqGold TriFast reagent (PeqLab, Erlangen, Germany) according to the manufacturer’s instructions. For the cDNA synthesis, 0.5 µg of total RNA was incubated using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Darmstadt, Germany) and random hexamer primers. The subsequent real-time polymerase chain reaction (PCR) was carried out with SYBR Green PCR master mix (Applied Biosystems, Darmstadt, Germany) on an ABI Prism 7300 system (Applied Biosystems, Darmstadt, Germany) as described previously. The specific oligonucleotide sequences are given in Table 1. The data were normalized against β-actin as a reference and analyzed using the 2ΔΔCT method.

Table 1 Sequences of oligonucleotide primers used in quantitative RT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>LPAR1 FOR</td>
<td>5′-TTGGCTACGGGGCTCTAGTA-3′</td>
</tr>
<tr>
<td>LPAR1 REV</td>
<td>5′-CGTAATGTGCTCTCGATTGC-3′</td>
</tr>
<tr>
<td>LPAR2 FOR</td>
<td>5′-GGCTGTGAGTCTCGAAATGT-3′</td>
</tr>
<tr>
<td>LPAR2 REV</td>
<td>5′-TCTCGACATCTCGGAAATGT-3′</td>
</tr>
<tr>
<td>LPAR3 FOR</td>
<td>5′-ATGGCGCTTCACATCATGTT-3′</td>
</tr>
<tr>
<td>LPAR3 REV</td>
<td>5′-CCCCACTTGTATGGCCAGACA-3′</td>
</tr>
<tr>
<td>LPAR4 FOR</td>
<td>5′-TCTACATCATGCCCCACATCGA-3′</td>
</tr>
<tr>
<td>LPAR4 REV</td>
<td>5′-GGCTGAAAGGGAAGGCTTTTG-3′</td>
</tr>
<tr>
<td>LPAR5 FOR</td>
<td>5′-GGTGAGCTCAGAACGGAGGA-3′</td>
</tr>
<tr>
<td>LPAR5 REV</td>
<td>5′-GGTGAGCTCAGAACGGAGGA-3′</td>
</tr>
<tr>
<td>LPAR6 FOR</td>
<td>5′-AACGACAGCTCTGAGGAGGA-3′</td>
</tr>
<tr>
<td>β-actin FOR</td>
<td>5′-CTGGCGACCCACGCAAAAT-3′</td>
</tr>
<tr>
<td>β-actin REV</td>
<td>5′-GCGATCCACACGGAGACTT-3′</td>
</tr>
</tbody>
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Statistics

Statistical analyses were performed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were evaluated using the graphics and statistics software program GraphPad Prism (version 5.01) and presented as column diagrams. Statistical comparisons were performed using the unpaired Student’s t-test and results with P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001 were considered significant.

Ethics Statement

The manuscript does not contain clinical studies or patient data.
Results

AA is a highly potent anticancer drug in PC therapy and it even inhibits the cellular growth of advanced stages of PC. As expected, AA treatment in a PC cell model system led to a strong attenuation of cell proliferation of AR-positive LNCaP cells (Figure 1B). Notably, AA possesses anti-proliferative properties even on AR-negative PC-3 cells (Figure 1A), which points to an alternative, AR-independent molecular mode of action in AA anti-cancer activity. Owing to a hypothesized steroid dependency of the proliferation-stimulating LPAR, we assessed the expressions of LPAR1 to LPAR6 in the presence of AA.

Quantitative PCR analysis of LPAR in AR-negative PC-3 cells revealed no changes in the LPAR isoform’s mRNA levels (Figure 2A). In contrast, in AR-positive LNCaP cells, the analysis of LPAR isoforms showed differentially regulated levels of LPAR mRNA after a 24-h incubation (Figure 2B). In the case of LPAR2, no significant modulation of mRNA was detectable in the presence of AA (0.96-fold ± 0.09 after 6 h; 0.84-fold ± 0.25 after 24 h). However, three LPAR isoforms were up-regulated (LPAR1: 1.68-fold ± 0.67, P = 0.1525; LPAR4: 1.71-fold ± 0.42, P = 0.0486; LPAR5: 1.42-fold ± 0.17, P = 0.0174) and two LPAR isoforms were down-regulated (LPAR3: 0.27-fold ± 0.06, P < 0.0001; LPAR6: 0.59-fold ± 0.12, P = 0.0110) after 24 h of AA treatment.

Discussion

In the present study, we confirmed the potent efficacy of AA against PC cells in an in vitro cell culture model. Unsurprisingly, LNCaP cells expressing AR were found to be inhibited in the presence of the steroid synthesis...
inhibitor, AA. This observation affirmed the established hypothesis that PC cells exhibit an endogenous de novo synthesis of steroids, which might be targeted by AA’s CYP17A1-inhibiting activity, and consequently provided the rationale for the molecular mode of action of the compound\textsuperscript{18,19}. Even though Zhang et al. could not detect any CYP17A1 expression in LNCaP and PC-3 cells, it is not improbable that AA’s anticancer activity may result from the targeting of currently unknown factors in cellular progression\textsuperscript{20}.

Our results showed AA’s inhibitory effect even on AR-negative PC-3 cells, and are therefore androgen-independent. Recently, we found that AA targets the transforming growth factor β pathway, as well as factors of apoptosis and cell cycle control, in PC-3 cells\textsuperscript{15}. This may underline a more global role of AA in PC cell physiology, and that AA’s molecular mode of action may be a result of simultaneously targeted steroid-dependent factors. Certainly, owing to the marginal literature on AA’s molecular biology, we could not exclude the possibility of different mechanisms being responsible for AA’s efficacy in LNCaP and PC-3 cells.

LPA is a pivotal growth-stimulating factor in hormone-regulated tissues, e.g. mammary, endometrial, ovarian and prostatic tissues\textsuperscript{10,12,16,21}. The secreted lipase autotaxin hydrolyzes lysophospholipids to LPA, which subsequently binds to its receptors LPAR1–LPAR6. Therefore, LPAR stimulation controls numerous cellular responses ranging from morphological change, adhesion, and cytokine secretion to cell survival, motility and proliferation\textsuperscript{22–24}. In various malignancies including PC, the autotaxin enzyme is over-expressed compared to the adjacent non-malignant tissues\textsuperscript{22}. In patients with no history of malignancies, administration of synthetic steroid prednisolone leads to a significant decrease of autotaxin’s plasma levels, thus entailing the assumption that the autotaxin expression is at least partly regulated by steroid hormones\textsuperscript{13}. Enzyme-linked immunosorbert assay (ELISA) analysis of PC cell culture supernatant, however, revealed very low levels of secreted autotaxin and demonstrated no differences between AA treatment and a control (data not shown). Therefore, AA-driven modulation of LPAR isoforms is more likely due to the control of LPAR expression than to the regulation of LPAR ligand synthesis by autotaxin catalysis. Studies of a breast cancer model confirmed this assumption, since LPAR expression has been shown to be linked to steroid hormone production and estrogen receptor activity\textsuperscript{12,26}.

The LPA receptors’ mRNA expression pattern in PC cell lines PC-3 and LNCaP was shown for LPAR1–4. This could be confirmed and extended to LPAR5 and LPAR6 for PC-3 cells, and is in accordance with our data\textsuperscript{27,28}. In AR-negative PC-3 cells, LPAR isoforms exhibited no significant differences in LPAR1 to LPAR6 transcripts in the presence and absence of AA. Notably, in AR-positive LNCaP cells, we observed up-regulated (LPAR1, LPAR4, LPAR5), down-regulated (LPAR3, LPAR6) and unaltered (LPAR2) LPAR mRNA levels after 24 h of AA incubation. This was partially confirmed by Gonzáles-Arenas et al\textsuperscript{12}. In breast cancer cells, the estrogen receptor agonist estradiol causes a declined expression of LPAR1, which could be reversed by incubation with the antagonist ICI 182780. In contrast to that study, our quantitative LPAR mRNA analysis for breast cancer cells MCF-7 (estrogen/progesterone/HER2neu receptor-positive) and MDA-MB-231 (estrogen/progesterone/HER2neu receptor-negative) failed to confirm a modulation of LPAR isoforms by AA treatment (data not shown). Thus, diminished levels of steroid hormones achieved by AA treatment: (1) affected the differential expression of LPAR isoforms specifically in PC cells, and (2) may depend exclusively on the expression of AR but not on other steroid receptors, e.g. estrogen receptor, progesterone receptor and HER2neu.

**Conclusion**

AA is a potent inhibitor of steroid hormone anabolism. In addition to the well-known reduction of AR activity in PC cells, AA treatment also leads to the targeting of proliferative factors LPAR1, LPAR3, LPAR4, LPAR5 and LPAR6. Although LPAR modulation by AA treatment may be specific to AR-expressing PC cells, AA’s multi-targeting characteristics could offer further anticancer treatment options in different settings of PC. Moreover, the cell type as well as cell variant-specific efficacy of AA treatment may explain the multifaceted effectiveness seen in clinical PC therapy.

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