



Inhibition of autophagy significantly increases the antitumor effect of Abiraterone in prostate cancer

Ashkan Mortezaei¹ · Souzan Salemi¹  · Benedikt Kranzbühler¹ · Oliver Gross¹ · Tullio Sulser¹ · Hans-Uwe Simon² · Daniel Eberli¹

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Abstract

Purpose Abiraterone acetate (AA) plus prednisone is an approved treatment of advanced prostate cancer (PCa). Autophagy is linked to drug resistance in numerous types of cancers. We hypothesized, that upregulation of autophagy is one of the mechanisms by which PCa cells survive AA anti-tumor treatment and therefore evaluated the potential effect of a combination with autophagy inhibition.

Methods Human PCa LNCaP cell lines were cultured in steroid-free medium and treated with AA. Autophagy was inhibited by 3-methyladenine, chloroquine and *ATG5* siRNA knock-down. Cell viability and apoptosis was assessed by flow cytometry and fluorescence microscopy, and autophagy was monitored by immunohistochemistry, AUTOdots and Western blotting.

Results Western blot revealed upregulation of *ATG5* and LC3 II with a reduction of p62 protein expression in AA-treated cells, indicating upregulation of autophagy. These data were supported by results obtained with immunocytochemistry and AUTOdots assays. Using flow cytometry, we showed that combining AA with autophagy inhibition significantly impaired cell viability (1.3–1.6-fold, $p < 0.001$) and increased apoptosis (1.4–1.5-fold, $p < 0.001$) compared to AA treatment alone.

Conclusions AA activates autophagy as a cytoprotective mechanism in LNCaP prostate cancer cells and targeting of autophagy enhances the antitumor effect of the compound.

Keywords Autophagy · PCa · Abiraterone

Introduction

Prostate cancer (PCa) is a major cause of cancer morbidity and mortality in the western world [1]. Localized tumors are treated by radical prostatectomy or radiation therapy. Androgen-deprivation therapy (ADT) is the standard first-line

treatment for metastatic PCa, but after a primary tumor response, nearly all tumors progress to a castration-resistant state that is consistently fatal [2]. Docetaxel and cabazitaxel were the first systemic cytotoxic nonhormonal therapies available for patients with metastatic castration-resistant PCa (CRPC) [3, 4]. Mechanisms of castration resistance include an increased expression of androgen receptor or the ligand or the splice variants [5].

Abiraterone acetate (AA) plus prednisone is the first endocrine therapy proving a significant survival benefit for patients with CRPC in randomized prospective clinical trials [6, 7]. AA is a prodrug of Abiraterone a selective inhibitor of androgen biosynthesis that blocks 17α -hydroxylase/C17,20 lyase (CYP17A1) [8]. By blocking the action of CYP17A1, AA not only inhibits testosterone production in the testes but also in other testosterone-producing tissues such as the adrenal glands and within the prostate tumor [9]. This agent offers an effective therapy option in advanced prostate cancer when used in combination with prednisone. Analogue to other anti-tumor agents, PCa eventually becomes resistant

Ashkan Mortezaei and Souzan Salemi have contributed equally to this work.

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✉ Daniel Eberli
daniel.eberli@usz.ch

¹ Laboratory for Tissue Engineering and Stem Cell Therapy, Department of Urology, University Hospital Zürich, Frauenklinikstrasse 10, 8091 Zurich, Switzerland

² Institute of Pharmacology, University of Bern, Inselspital, INO-F, 3010 Bern, Switzerland

to AA or some do not respond at all [6, 7]. Therefore, these mechanisms of resistance are subject of several investigations [10, 11].

We hypothesized that upregulation of autophagy is a mechanisms by which PCa cells survive anti-tumor treatment with AA. Studies revealed that increased autophagy in tumor cells exposed to chemotherapy or radiation led to therapeutic resistance [12]. In contrast, inhibition of autophagy in androgen-sensitive PCa cells (e.g., LNCaP) under conventional ADT resulted in reduced cell viability [13]. We recently showed that proteins specific to autophagy were significantly upregulated in metastatic and CRPC [14]. Therefore, we investigated the role of autophagy in AA-treated PCa cell lines and evaluated potential effects of a combination with autophagy inhibition to enhance the therapeutic effect in an in vitro model.

Materials and methods

Detailed experimental procedures are in supplementary and available online.

Cell culture

The PCa cell lines LNCaP, DU145 and PC3 were obtained from American Type Culture Collection and were cultured in RPMI or DMEM/F-12 supplemented with 10% FBS.

Pharmacological agents and autophagy inhibition

To mimic ADT, human PCa cell lines were cultured in steroid-free medium without phenol red with 5% charcoal filtered. After 1 day, cells were treated with AA (AA5, 10 and 15 μM , Janssen Pharmaceutica NV, Belgium), the autophagy inhibitor 3-methyladenine (3MA, 5 mM), chloroquine (Chl, 20 μM) or rapamycin (1 μM).

WST-1 cell proliferation assays

Cell proliferation of treated and untreated cells (control) was measured by WST-1 cell proliferation assay on day 1, 2 and 4.

Cell death assays

Cell death was assessed by ethidium bromide uptake and flow cytometry analysis. For analysis of cell viability in LNCaP cells, cells were stained with 10 μM CellTrace Calcein and detected using a fluorescence microscope. To determine whether cell death was apoptotic, redistribution

of phosphatidylserine (PS) was measured using Annexin V kit and propidium iodide, detected by flow cytometry.

Protein simple WES immunoblotting

Total protein was measured with the BCA protein assay kit and proteins were separated in WES with a capillary cartridge according to the manufacturer's protocol.

Results

Abiraterone acetate induces death in LNCaP cells but not in PC3 and DU145 cells

To investigate the impact of AA treatment in PCa cell lines, we assessed proliferation of LNCaP, DU145 and PC3 cells after treatment with different concentrations of AA in an androgen-depleted medium (charcoal filtered FBS). The treatment led to decreased proliferation of LNCaP, but not in DU145 and PC3 cells (Fig. 1). DU145 and PC3 did not show any response to 10 and 15 μM AA after 2 and 4 days of treatment (Fig. 1a–c). In addition, no changes in Atg5 protein expression was observed after treatment of PC3 cells with AA, 3MA, Chl and combinations (Fig. S1). Although a slightly decrease in cell proliferation was observed in the 3MA treated PC3 cell line, these results were not confirmed by cell death assay measured by FACS and immunoblotting (Fig. S1a–c). In conclusion, AA blocked proliferation of androgen-sensitive but not in androgen-insensitive cell lines. Therefore, we did not include the cell lines DU145 and PC3 in our subsequent experiments. To test whether inhibition of cell proliferation by AA was accompanied by induction of cell death, we investigated cell viability in LNCaP cells in the presence of AA in a concentration and time-dependent manner. A concentration-dependent reduction of cell proliferation was observed when LNCaP cells were treated on day 1 with AA 5 μM (97.28 ± 2.59 , SEM), 10 μM (72.02 ± 6.18) and 15 μM (56.81 ± 2.081). On day 4, AA treatment with 5 μM (51.5 ± 5.8), 10 μM (36.36 ± 1.3) and 15 μM (37.48 ± 0.7) led to further reduction compared to vehicle control (DMSO, 100%) (Fig. 1d). Interestingly, although AA induced death of LNCaP cells, it also appeared to induce autophagy in these cells. For instance, AA treatment elevated the ATG5 and Beclin 1 protein levels and showed a concentration-dependent increase of the conversion of LC3 I to LC3 II. In addition, a decrease in p62 protein expression was observed (Fig. 1e). Furthermore, cells were examined microscopically using Calcein-AM. The most effective loss in viability was observed at day 4 when cells were treated with 10 μM AA as compared to 5 and 15 μM (Fig. 1f). Taken together, efficient cell death in

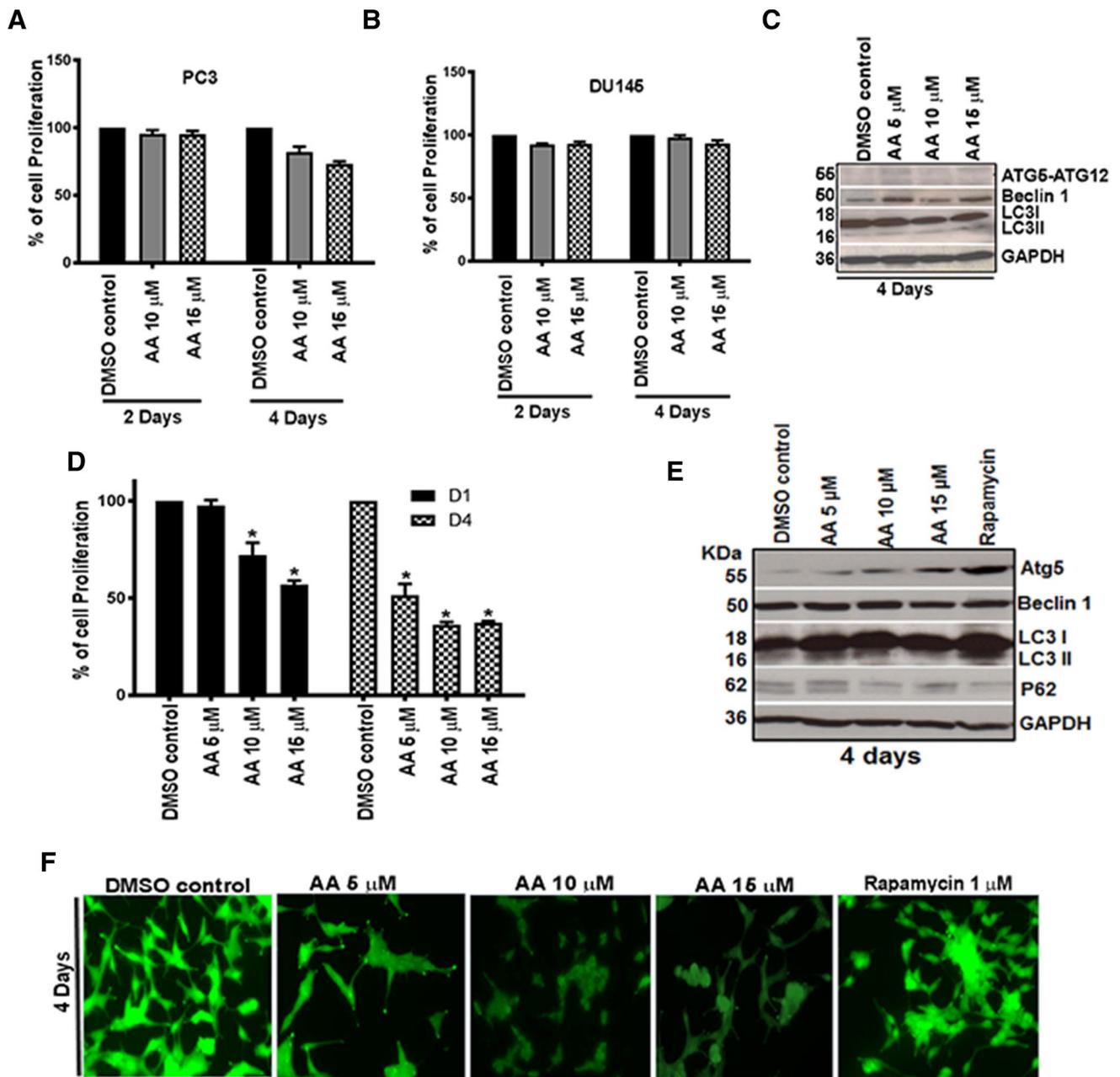


Fig. 1 Cellular sensitivity in response to AA correlates to autophagy induction in LNCaP cells. **a, b** WST cell proliferation assay: PC3 and DU145 cells treated with AA 10 and 15 μ M for 2 and 4 days. Absorbance was measured daily at 450 nm. Bars represent mean \pm SEM ($n=3$). **c** Immunoblotting analysis (days 4) of DU145 cells. Very weak expression or absence of 55 kDa ATG5–ATG12 indicating no autophagy activation in response to different concentrations of AA. Higher expression of LC3 I (18 kDa), weak expression of LC3 II (16 kDa) with no difference in treatments groups was observed. **d** WST viability assay. LNCaP cells treated with AA 5, 10 and 15 μ M for 1 and 4 days. Absorbance was measured daily at 450 nm. Bars represent mean \pm SEM ($n=3$), in triplicates. * $p<0.01$ indicate statis-

tically significant differences compared to DMSO control. **e** Western blot analysis (day 4) of LNCaP cells. Gradual increase of ATG5 indicating autophagy activation in response to different concentrations of AA. Western blotting of LC3 reveals two bands: LC3 I and LC3 II. Increased LC3 II band was observed in 10 μ M treated AA. P62 degradation in 10 μ M AA treated cells indicating increased autophagic flux. **f** Calcein AM cell viability assay of LNCaP cells. LNCaP cells were treated with different concentrations of AA. Images were obtained using Leica microscope at 20 \times magnification field. The bright green color indicates cell viability, which was reduced after 10 and 15 μ M AA treatments

LNCaP cells was induced by 10 μM AA, a concentration which we used in subsequent experiments.

Abiraterone increases autophagy in LNCaP cells

We subsequently investigated the involvement of autophagy in the survival of PCa cells. As assessed by immunofluorescence, low basal expression levels of ATG5 and a diffuse and relatively weak LC3 staining were observed in

both untreated and DMSO-treated controls (Fig. 2a). Upon AA treatment, LNCaP cells exhibited an increased cytoplasmic expression for ATG5 and a punctuated pattern for LC3 confirming the accumulation of autophagosomes. In combination treatments of AA with 5 mM 3-methyl adenine (3MA), a reduced ATG5 expression and LC3 punctuations resulted in a reduction of cell growth and disrupted cell morphology. Chl-treated cells (alone or with AA) showed intense clumped LC3 immunoreactivity, which points to an

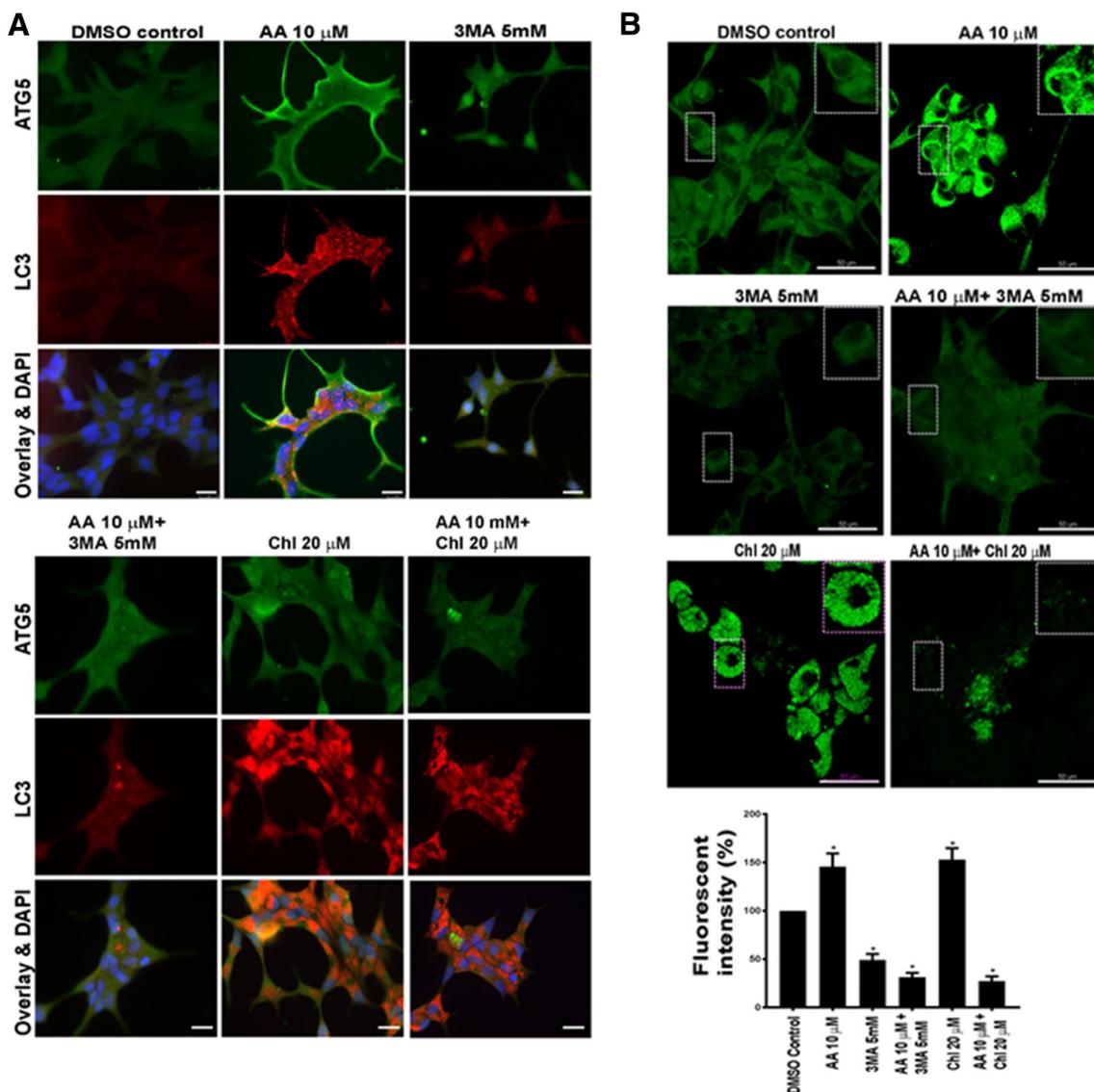


Fig. 2 Upregulation of ATG5 in AA-treated cells and increase in autophagosomes. **a** Confocal images of LNCaP cells treated cells with 10 μM AA, 5 mM 3MA, 20 μM Chl or in combination of AA and 3MA or AA and Chl, stained with anti-ATG5 and LC3 antibodies. The green colour indicates expression of ATG5 and red shows LC3 expression. The LC3 punctuation indicates autophagosome formation and high autophagic activity in AA and Chl treated cells. Samples were detected using Cy3 (red) conjugated secondary antibody or FITC (green) and DAPI (blue, 4',6-diamidino-2-phenylin-

dole). Scale bars: 25 μm . **b** Confocal microscopy. LNCaP cells treated with AA for 6 days before exposing to Autodot staining for 15 min to visualize the formation of autophagosomes. Significant increase in autophagosomes and punctuation was observed in AA and Chl treated cells but not in control, 3MA, or AA in combination with 3MA or chl. Scale bars, 50 μm . Graph indicates quantitative measurement of fluorescent intensity using Image J software. * $p < 0.01$ indicate statistically significant differences compared to DMSO control ($N = 8$ per condition of 63 \times microscopic field images)

association of LC3 II with autophagosomes, thereby indicating a Chl-induced accumulation of autophagic vacuoles.

Autophagosome formation was also monitored by AUTOdol (a monodansylcadaverine-like, lysosomotropic agent), which detects autophagosomes via an ion-trapping mechanism and by interacting with lipids concentrated in the autophagosome membrane (Fig. 2b). The LNCaP cells exposed to AA demonstrated a significantly increased autophagosome formation compared to untreated cells or DMSO-controls, as detected by enhanced green colour punctuation in the cells. In addition, AA-treated cells showed typical features of autophagy, such as cell enlargement and autophagic vacuoles. Moreover, combination

treatments of AA with 3MA showed reduced punctuation, leading to increased cell death and a reduced cell number. Chl-treated cells again showed a Chl-induced accumulation of autophagic vacuoles as indicated by enhanced green punctuation.

At protein level by immunoblotting, we observed higher expression levels of both main autophagy regulators ATG5 and Beclin 1 in AA-treated cells compared to DMSO-treated control (vehicle) (Fig. 3a). These results were also supported by the significant conversion from cytosolic LC3 I to membrane-bound LC3 II observed upon AA treatment compared to control.

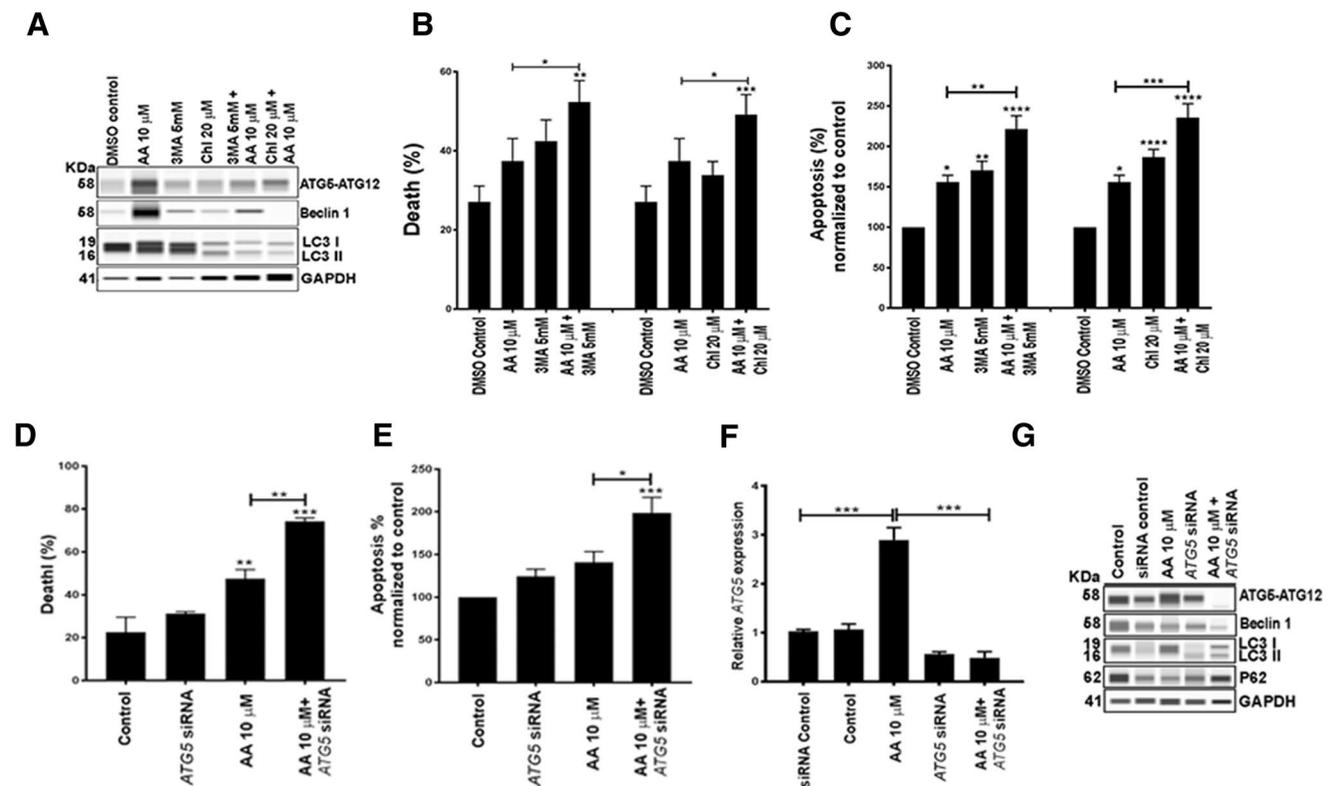


Fig. 3 Treatment with AA and pharmacological blocking of autophagy induces cell death. **a** Immunoblotting. Upregulation of ATG5 and Beclin 1 protein in LNCaP cells treated with 10 μ M AA compared to DMSO control. AA-treated cells showed higher levels of LC3 II compared to control. Cells treated with autophagy inhibitors 3MA, Chl and combination of AA and 3MA or AA and Chl showed reduced levels of ATG5 and Beclin and low LC3 I and II protein expression. **b** Cell death assay. Cells were cultured in the presence and absence of AA 10 μ M, 5 mM 3MA, 20 μ M Chl and in combination of AA and 3MA and AA and Chl for 4 days. Cell viability was assessed using ethidium bromide and measured by FACS. Values are mean \pm SEM of at least three independent experiments. **c** Cell apoptosis assay. Flow cytometry analysis of LNCaP cells using annexin V/PI staining. Cells were treated with the indicated compounds as above for 4 days. The data represent the mean \pm SEM ($n=8$). Asterisk above the bars indicate statistically significant differences compared to DMSO control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

d Increasing of AA induced cell death by silencing ATG5. LNCaP cells were treated with ATG5 siRNA, AA and in combination of AA and ATG5 siRNA and following assays were performed after 7 days of growth upon treatments. Cell death was measured using ethidium bromide and analysed by FACS. Values are mean \pm SEM of at least three independent experiments. ** $p<0.001$, *** $p<0.0001$. **e** Cell apoptosis assay. Annexin V/PI staining of untreated and treated LNCaP cells. Results are presented as percentage of positive cells compared to control. The data represent the mean \pm SEM ($n=3$). Asterisk above the bars indicate statistically significant differences compared to DMSO control; * $p<0.05$ and *** $p<0.0001$. **f** The efficient knockdown of ATG5 gene in LNCaP was confirmed by real time PCR. The results are presented as mean \pm SEM ($n=3$), performed in triplicate. *** $p<0.0001$. **g** Immunoblotting. The effects of the ATG5 siRNA on ATG5 (ATG5-ATG12), Beclin 1, LC3 I and II, P62 and GAPDH levels were analysed by immunoblotting using protein simple WES

Treatment with Abiraterone and autophagy inhibitors induces cell death in LNCaP cells

We next investigated the effect of pharmacological inhibition of autophagy in LNCaP cells. Treatment of cells with 3MA, Chl or a combination of AA plus 3MA or Chl, caused a downregulation of the expression of both ATG5 and Beclin 1, which was associated with a reduction of LC3 I and LC3 II, indicating that both autophagy blockers prevented AA-induced induction of autophagy (Fig. 3a).

To assess whether upregulation of autophagy can contribute to resistance and survival of the cancer cells, LNCaP cells were treated with 10 μ M AA alone and in combination with the autophagy inhibitors 20 μ M Chl or 5 mM 3MA, and measured cell death and apoptosis. An increased rate of cell death was detected in cells treated with AA (35.24% \pm 6.0), 3MA (44.4% \pm 5.4), and Chl (33.8% \pm 3.4) compared to DMSO-control (27.12% \pm 4.0). Importantly, cells treated with AA plus 3MA (55.8% \pm 4.8, $p=0.001$) or AA plus Chl (53.11% \pm 3.6, $p=0.0008$) exhibited significantly increased cell death compared to DMSO-treated cells (Fig. 3b). In addition, the reduction of cell viability with the combination treatment was significantly more distinct as compared with AA alone (AA + 3MA $p=0.025$, AA + Chl $p=0.026$). Increased cell death was also associated with a significant increase in apoptosis as assessed by phosphatidylinositol redistribution assay and necrosis in cells treated with AA (155.9% \pm 8.3, $p=0.01$), 3MA (170.5% \pm 11.2, $p=0.008$) and Chl (186.9% \pm 9.5, $p<0.0001$) compared to DMSO-treated control (100%). This effect was enhanced when cells were treated with the combination of AA plus 3MA (221.7% \pm 16.36, $p<0.0001$) or AA plus Chl (235.8% \pm 17.0, $p<0.0001$; 1.4 \times and 1.5 \times , Fig. 3c). Importantly, when cells were treated with combinations of AA plus 3MA ($p=0.0019$) or AA plus Chl ($p=0.0002$) the cell death was significantly enhanced relative to treatment with AA only.

Lowering ATG5 expression increases cell death in PCa cells

To further understand the role for autophagy for cell survival after AA treatment, ATG5 levels were reduced using short interfering RNA (siRNA, Fig. 3d–g). In parallel to pharmacological treatments, we tested the influence of ATG5 suppression on LNCaP cells and monitored the percentage of cell viability and apoptosis/necrosis in the cells. Cell death was lower in control (22.5% \pm 7.0, SEM) and in cells treated with 30 nM siRNA (31.2% \pm 0.5) but significantly higher upon treatment with AA (47.5% \pm 2.4) (Fig. 3d). Moreover, ATG5-deficient LNCaP cells exhibited a markedly increased sensitivity towards the cytotoxic effect of AA (74.33% \pm 0.8, 1.6 \times , $p=0.002$) compared to AA treatment alone.

In addition, we also obtained evidence showing that downregulation of ATG5 leads to an apoptosis-sensitizing effect (Fig. 3e). Compared with untreated cells (defined as 100%) and siRNA treated cells (124% \pm 8.4, SEM), an increased apoptosis susceptibility was observed in cells treated with AA (141% \pm 12.0). Cell death via apoptosis and necrosis was significantly enhanced when cells were treated with the combination of 10 μ M AA plus 30 nM siRNA (198.8% \pm 18.0, 1.4 \times , $p=0.013$) compared to AA-treated cells only. Furthermore, the ATG5 mRNA expression was significantly increased in AA-treated cells (2.9 \pm 0.2, $p<0.0001$) compared to siRNA (containing all the transfection components without siRNA primer) (1.03 \pm 0.03) and untreated controls (1.06 \pm 0.1). In line with the cell death assays, AA-treated cells with high levels of ATG5 gene expression showed a significant reduction of ATG5 expression when combined with ATG siRNA (Fig. 3f). Moreover, AA treatment led to increased ATG5 protein expression and increased autophagosome specific LC3 II, which could be downregulated by ATG siRNA (Fig. 3g). When cells were treated with AA and ATG5 siRNA, ATG5 protein expression was completely abolished and both LC3 I and LC3 II were expressed equivalently. The degradation of p62 protein confirmed a high autophagic activity and autophagic flux in AA-treated cells; this effect was reversed when cells were treated with ATG5 siRNA or in untreated control, confirming the involvement of autophagy and the role of ATG5 protein.

Discussion

Based on results of recent clinical trials, treatment with Abiraterone acetate plus prednisone is becoming a standard of care in all stages of advanced PCa [6, 7, 15, 16]. Therefore, enhancing the effect of AA may have a significant impact on the survival of men diagnosed with this disease. In the present investigation, we show a significant upregulation of autophagy in LNCaP cells treated with AA, an effect serving as a mechanism of resistance. By blocking the autophagy pathway we were able to enhance the antitumor effect of AA and to intensify cell death and apoptosis. To our knowledge, this is the first study identifying upregulation of autophagy as an escape mechanism to AA treatment in PCa.

Metastatic PCa remains a deadly disease even with high initial response to standard hormone treatment [17]. Mechanisms contributing to androgen-receptor reactivation supported the development of AA, a cytochrome P450-17A1 (CYP17A1) inhibitor capable of suppressing intratumoral androgen synthesis [18]. Abiraterone acetate plus prednisone was the first endocrine therapy showing a significant survival benefit in the castration resistant state pre- and post-chemotherapy [6, 7]. However, not all patients respond to Abiraterone acetate treatment [19].

We now identified autophagy not only as a new possible mechanism of resistance against treatment with AA, but also as a possible target for a combination therapy. This effect was not observed in the androgen-independent cell lines PC3 and DU145. However, analogue to our treatment of hormone sensitive LNCaP cells, two clinical phase III trials recently combined AA with ADT in the treatment of patients with a high risk and metastatic hormone-sensitive PCa and observed distinct prolongation of survival in these men [15, 16]. A combination of a maximum hormone-deprivation (by ADT + Abiraterone acetate) with the inhibition of autophagy, as implemented in our preclinical study, may further increase the efficacy of the treatment and prolong the survival in a group of men with unfavourable prognosis.

Evidence shows that inhibition of autophagy alone or in combination with anticancer therapies is effectively compromising tumorigenesis in cancers [13, 20–22]. The enhanced efficacy of the combination treatment in the present investigation was achieved by therapeutic intratumoral concentrations of Abi together with an autophagy inhibitor. The concentrations of AA required to achieve a direct intracellular antitumor effect in PCa cells are reported to range between 1 and 10 $\mu\text{mol/L}$ [23], which corresponds to the observed viability results in the present study. Studies reported plasma levels of AA ranging from 1.2 to 5 $\mu\text{mol/L}$ after the recommended treatment dosage of 1000 mg Abiraterone acetate in fasting patients, which proves to be lower than the optimal concentration observed in our investigation [18]. However, we decided to continue with the higher concentration based on phase I data showing a good tolerance to higher doses of up to 2000 mg daily Abiraterone acetate [18]. At this concentration, AA-treated LNCaP cells showed an upregulation of autophagy detected by p62 degradation and a significant upregulation of LC3 II and ATG5 expression required for autophagosome formation and autophagy process [24, 25]. The observed, more distinct upregulation in AA-treated cells in comparison to cells treated with ADT alone is indicative for cellular stress. This seems to be a crucial survival mechanism for LNCaP cells, since inhibition with either 3MA, Chl or siRNA led to a constant 1.3–1.6-fold increase of cell death and apoptosis in multiple experiments. The only clinically approved inhibitors of autophagy are the antimalarial drugs Chl and hydroxychloroquine at doses ranging from 200 to 1200 mg/day [26, 27]. The reported concentrations in blood ranged between 3 and 10 μM , which is lower than the concentration we found necessary (20 μM) to sufficiently suppress autophagy [28].

Conclusion

In this study, we provide evidence that the CYP17 inhibitor Abiraterone activates autophagy as a cytoprotective mechanism in LNCaP prostate cancer cells and that targeting

autophagy enhances the antitumor effect of the compound. A combination with established autophagy modulators such as Chl or novel agents may improve cancer therapy in advanced prostate cancer.

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Authors' contributions AM and SS contributed equally to this work: project development, carried out all the experiments, data collection, interpretation and manuscript writing. BK and OG: data collection and revising the manuscript. TS: revising the manuscript. HUS: data interpretation and revising the manuscript and DE: project development, data interpretation and manuscript writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all the individual participants included in the study.

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