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ORIGINAL ARTICLE

Cabazitaxel inhibits prostate cancer cell growth by inhibition of androgen receptor and heat shock protein expression

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Abstract

Purpose Cabazitaxel, a semi-synthetic taxane of the third generation, inhibits prostate cancer (PC) cell growth by affecting the microtubule architecture. Since cabazitaxel has also been demonstrated to inhibit androgen receptor (AR) functionality, AR and AR-associated heat shock protein (HSP) expressions in the presence of cabazitaxel were characterized.

Methods AR and HSP expressions were assessed via Western blotting utilizing a PC-cell-line in vitro system incubated with cabazitaxel.

Results Incubation experiments with 0.3 nM cabazitaxel exhibited significantly reduced levels of AR and the AR-associated factors HSP90α, HSP40, and HSP70/HSP90 organising protein. Furthermore, expression of the anti-apoptotic factor HSP60 was suppressed. In contrast to other anticancer compounds, cabazitaxel did not alter the cytoprotective chemoresistance factor HSP27.

Conclusions Despite the deregulation of microtubule organisation, cabazitaxel has been shown to suppress the expression of HSP. Very notably, and may be as a result of down-regulated HSP, cabazitaxel additionally inhibits the expression of the AR in AR-positive PC cells. Thus, cabazitaxel bears an additional anti-proliferative activity which is at least in part specific for PC cells.

Keywords Prostate cancer · Cabazitaxel · Chemoresistance · Androgen receptor · Heat shock proteins

Abbreviations

PC	Prostate cancer
AR	Androgen receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ADT	Androgen deprivation therapy
CRPC	Castration-resistant prostate cancer
PSA	Prostate-specific antigen
HSP	Heat shock protein
HOP	HSP70/HSP90-organising protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Introduction

Prostate cancer (PC) is the most frequently diagnosed cancer and the third cause of death due to cancer among men in developed countries [1]. Its incidence has increased over the last decade, while mortality decreased in most countries [1–3]. Since Charles Huggins [4], androgen deprivation therapy (ADT) was considered as the primary treatment for metastatic, hormone-sensitive PC; it delays the development of symptoms and clinical progression [5–7], but molecular alterations during ADT lead to castration resistance, and consequently to a disease progression after approximately 3 years [8]. Progression of PC despite castrate levels of testosterone has been denominated metastatic castration-resistant prostate cancer (mCRPC) [9] and docetaxel plus prednisone was the standard first-line treatment for mCRPC until 2010 based on results of TAX 327 and SWOG phase 3 trials showing a significant survival benefit versus mitoxantrone plus prednisone [10, 11]. Since 2010, new agents (abiraterone, enzalutamide, cabazitaxel, sipuleucel-T, and alpharadin) have also demonstrated a survival advantage

in mCRPC [10–15], while docetaxel in combination with ADT has become a standard of care for newly diagnosed metastatic hormone-sensitive PCa based on results of CHAARTED and STAMPEDE trials [16, 17]. Docetaxel still remains an essential therapeutic option in mCRPC patients, which is reflected in different international clinical practice guidelines on PC [18, 19], but its clinical utility is restricted by the development of chemoresistance [20], especially in patients previously treated with abiraterone or enzalutamide [21] and in patients with hormone-sensitive PC treated with docetaxel plus ADT [22].

Cabazitaxel is a next-generation taxane specifically developed to overcome resistance to docetaxel [23, 24], which also retains its activity after abiraterone and enzalutamide [25]. It shows a greater cell penetration than docetaxel [26]. The primary cytostatic effect of taxanes results from the binding to β-tubulin and the subsequent inhibition of mitosis as well as intracellular transport processes. In PC cells, cabazitaxel is 10 times more effective than docetaxel [24]. Furthermore, it has been reported that taxanes also affect androgen receptor (AR) signaling, inhibiting AR nuclear translocation and downstream transcriptional activity [27]. The AR plays a crucial role in the tumor biology of the PC. Its growth and progression require active AR signaling, which occurs following translocation of AR from the cytoplasm to the nucleus, where it acts as a transcription factor, binding to and activating AR target genes such as the prostate-specific antigen (PSA) gene [27]. The function of AR is primarily dependent on the activity of heat shock proteins (HSP); the receptor is found in the cytosol of PC cells, complexed to HSP70-, HSP90-, HSP40-, and HSP70/HSP90-organising protein (HOP), which maintains it in an inactive state and protects it from degradation. HSP are upregulated in PC and other solid tumors and their expression is often associated with the inhibition of apoptosis in PC cells [28, 29]. HSP are also involved in transcriptional regulation, and they have cytoprotective properties and are thereby implicated in resistance to radiation- and chemotherapy-induced apoptosis [28, 30]. In general, they are essential for cancer cell survival and several HSP, most notably the HSP27, are associated with poor prognosis of PC [29–31]. The aim of this research was to investigate cellular and molecular effects of cabazitaxel in PC cells, with special regards to different types of HSP to gain insight in factors and mechanisms of induced chemoresistance.

Materials and methods

Antibodies and chemicals

Primary antibodies directed against AR, HSP27, HSP40, HSP60, HSP70, HSP90α, HSP90β, HOP, p53, mouse-anti-rabbit-, horse-anti-mouse-, and anti-rat-specific secondary antibodies (Cell-Signaling Technology, Danvers, USA) were used. Cabazitaxel was kindly provided by Sanofi-Aventis (Frankfurt/Main, Germany).

Cell culture

The human PC-cell lines LNCaP (AR positive) and PC-3 (AR negative) were obtained from American-Type Culture Collection (ATCC, Manassas, USA) and cultured in 1640 RPMI with phenol red, 5% penicillin/streptomycin (PAN Biotech, Aidenbach, Germany), and 10% fetal bovine serum (Invitrogen, Darmstadt, Germany) at 37 °C, 5% CO_2, and saturated humidity. PC-3 cells stably overexpressing HSP27 (PC-3-HSP27) were propagated in the presence of 400 µg/ml G418 (Carl Roth, Karlsruhe, Germany) as described previously [32].

Proliferation assay

Live cell counting was performed using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). 3.0×10^4 cells were, therefore, seeded in a 24-well plate, treated with cabazitaxel, and harvested each 24 h with 200 µl trypsin (Promega, Madison, USA) and 2 × 600 µl RPMI1640 (PAN Biotech) obtaining 1.4 ml of cell suspension. Subsequently, 100 µl of cell suspension was transferred to 10 ml of CASYton solution, mixed manually before analyzing it in CASY cell counter. Measurement was performed using a capillary of 150 µm in diameter and cell-line-specific gate settings to discriminate between living cells, dead cells, and cellular debris (LNCaP: 12.25 µm/6.0 µm; PC-3: 15.45 µm/7.2 µm). The number of living cells was determined in duplicates for each passage.

Western blotting

Proteins were extracted from their source using RIPA lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM K_2HPO_4, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.05% sodium dodecysulfate, 1 mM Na_3VO_4, 20 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM 2-phosphoglycerate, and complete protease inhibitor cocktail from Roche Applied Science), separated within a gel using polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) (SERVA Electrophoresis, Heidelberg, Germany) and transferred to a membrane via a semi-dry-blot to a PROTAN nitrocellulose membrane (Whatman, Dassel, Germany). Immobilized protein was specifically detected by antibodies and visualized by Super Signal West Dura substrate (Thermo Scientific, Rockford, USA) in a Chemi-Doc™ XRS+ System (Bio-Rad) with Image Labsoftware. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

(Sigma-Aldrich, Steinheim, Germany) was used as loading control. To evaluate the protein expression, the mean values in the presence of cabazitaxel were set in relation with the expression values of the cabazitaxel-free control, and the latter was normalized to 1.0.

Statistics

Statistical analysis was performed using Microsoft Excel 10. Data shown in the diagrams are shown as means and standard deviations (SD) of at least three independent experiments. The data were tested by means of an unpaired Student´s *t* test, $p < 0.05$ (*) was considered significant, $p < 0.01$ (**) very significant and $p < 0.001$ (***) highly significant.

Results

Determination of cabazitaxel's inhibitory activity

The inhibitory activity of cabazitaxel at approximately 50% (comparable to IC_{50}) was tested in cell model systems LNCaP and PC-3 at various concentrations (0.1–1.0 nM), as indicated in Fig. 1. Both cell lines showed to be sensitive to cabazitaxel, showing a statistically significant attenuation of proliferation over 120 h. 0.3 nM was used as inhibitory concentration in the following incubation experiments.

Effects of cabazitaxel on AR expression and AR-associated HSP

The starting point of our investigations has been the observation, that AR is an important driver of cell proliferation and resistance mechanisms in PC cells. We, therefore, examined the effect of cabazitaxel on AR and on the HSP associated to the receptor, namely, HSP70, HSP90α/β, HSP40, and HOP. The AR expression in cabazitaxel-treated cells was determined only in an AR-positive LNCaP cell line by Western blotting after 24–72 h. The attenuation of AR expression was highly significant over a period of 72 h ($p < 0.0024$) (Fig. 2). The expression of AR-associated HSP such as HSP70, HSP90α, HSP90β, HOP, and HSP40 in both LNCaP and PC-3 cells treated with cabazitaxel was determined by Western blotting (Fig. 3). As shown in Fig. 3a, e, the expression of HSP70 and HSP90β was not altered

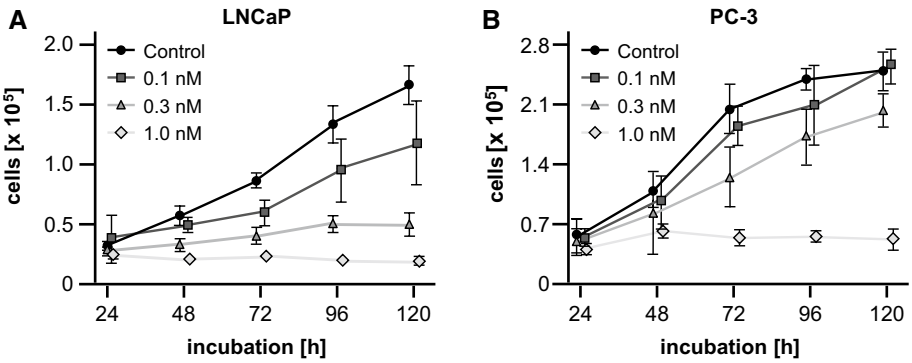

Fig. 1 Inhibition of PC-cell growth during cabazitaxel incubation of LNCaP and PC-3 cells. Living cell number of LNCaP cells (**a**) and PC-3 cells (**b**) treated with 0.1, 0.3, and 1.0 nM cabazitaxel with vehicle treatment as control. Cell number was examined utilizing a CASY Cell Counter and Analyzer Modell TT at indicated timepoints over a period of 120 h. Results are expressed as the mean ± SD of cell count

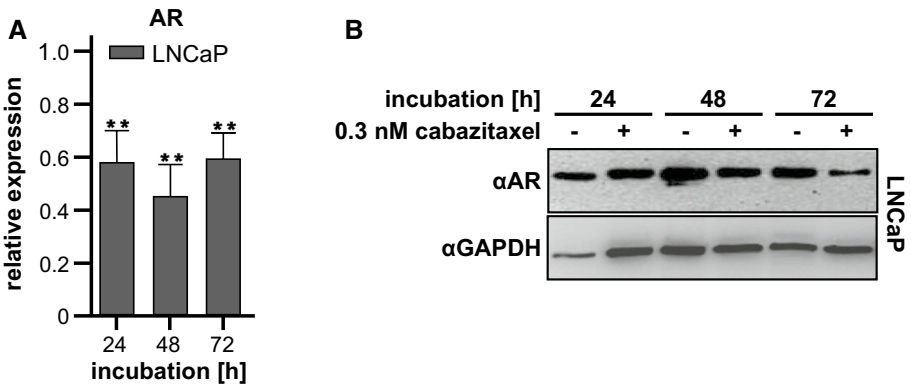

Fig. 2 Cabazitaxel suppresses AR expression in LNCaP cells. Western blot analysis of relative AR expression in the presence of 0.3 nM cabazitaxel at indicated timepoints. Data were standardized to vehicle-treated cells (control = 1.0) (**a**) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as loading control (**b**). Error bars indicate ± SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, as determined by Student's *t* test

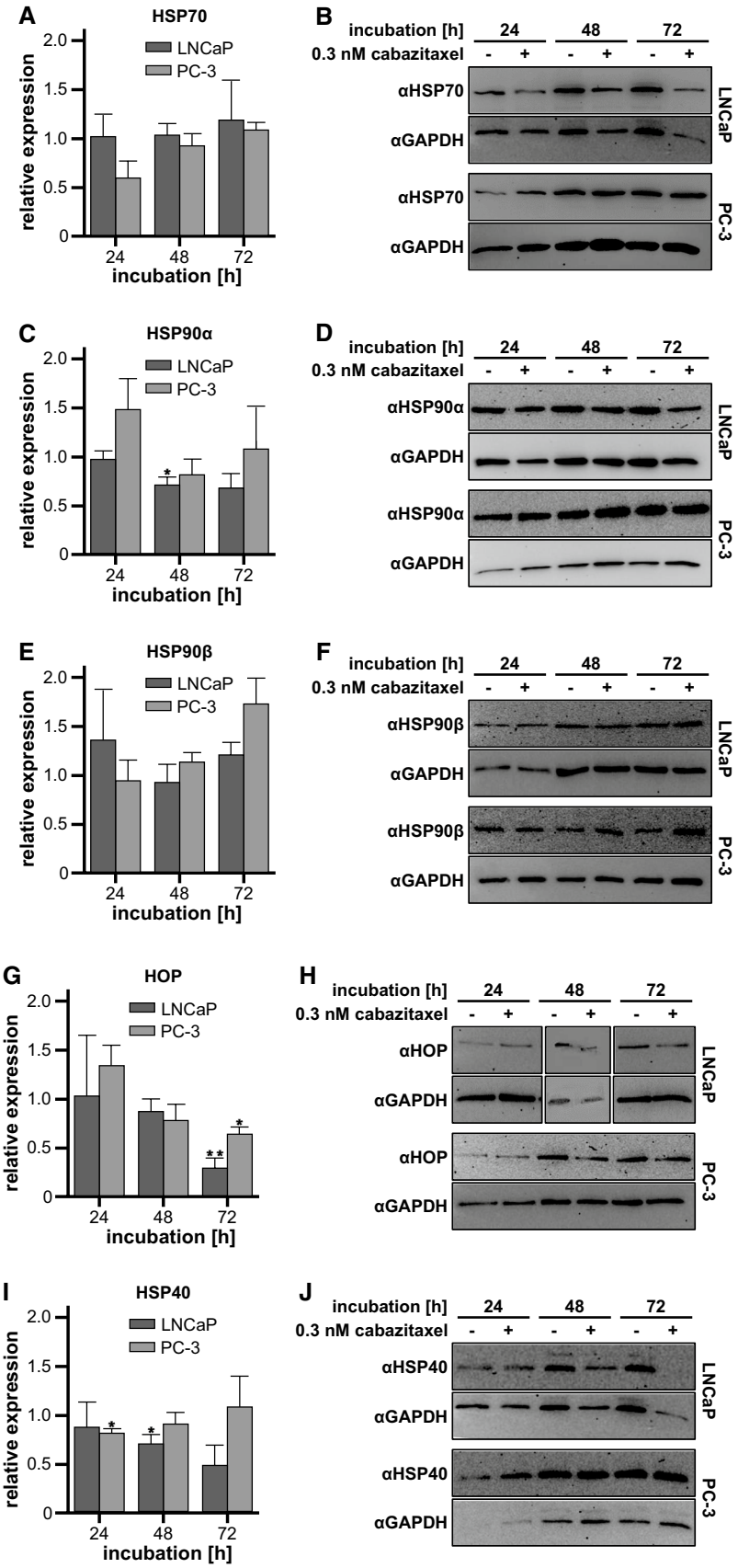

Fig. 3 Modulation of AR-associated HSP in the presence of cabazitaxel in LNCaP and PC-3 cells. Western blot analysis of relative expression of HSP70 (**a**), HSP90α (**c**), HSP90β (E), HOP (**g**), and HSP40 (**i**) in the presence of 0.3 nM cabazitaxel at indicated timepoints Data were standardized to vehicle-treated cells (control = 1.0) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as loading control (**b**, **d**, **f**, **h**, **j**). Error bars indicate ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, as determined by Student's *t* test

by cabazitaxel in LNCaP and PC-3 cells. In contrast, the expression of HSP90α was significantly reduced in LNCaP cells (Fig. 3c), and HSP40 and HOP were reduced in LNCaP as well as PC-3 cells (Fig. 3g, i). While the effect on HSP90α expression in the LNCaP cell line after 48 h ($p = 0.0277$) was significant, no significant changes in PC-3 cells could be observed. HSP40 expression was reduced significantly after 24 h in PC-3 cells ($p = 0.241$) and in LNCaP cells after 48 h ($p = 0.044$) (Fig. 3i). The most significant effects of cabazitaxel could be observed on the expression of HOP, which was reduced by ~40% in PC-3 cells ($p = 0.0133$) and by ~60% in LNCaP cells ($p = 0.020$) after 72 h (Fig. 3g).

Suppression of anti-apoptotic HSP60 in AR-positive LNCaP

HSP60 is strongly expressed in PC [29, 33] and associated with poor prognosis and hormone resistance [34, 35], as well as defective apoptosis [36, 37]—we, therefore, investigated the induction of HSP60 by cabazitaxel in LNCaP and PC-3 cells. In this context, we also examined the induction of the tumor protein p53, which also plays an important role in inducing apoptosis. The used cell lines were incubated with cabazitaxel for 72 h. It significantly suppressed HSP60 expression in LNCaP cells after 72 h ($p = 0.0046$), but showed no effect on PC-3 cells (Fig. 4a). The tumor suppressor p53 was strongly induced by the presence of cabazitaxel, and the result was significant after 24 h ($p = 0.0065$), 48 h ($p = 0.0202$), and 72 h ($p = 0.0303$) (Fig. 4c).

Expression of cytoprotective HSP27 in LNCaP and PC-3 cells

HSP27 is a protein associated to AR, but it is also known for its cytoprotective properties [31], which is relevant for docetaxel induced, secondary resistance in LNCAP and PC-3 cells [32, 38]. The cytoprotective effect of HSP27 after cabazitaxel incubation was tested in LNCaP and PC-3 cells. The treatment with cabazitaxel did not reduce HSP27 expression in PC-3 cells; in contrast, it tended to be suppressed in LNCaP cells after 72 h, even if the difference was not significant ($p > 0.05$) (Fig. 5a). The cytoprotective effect on PC-3 cells and PC-3 cells stably overexpressing HSP27 (PC-3-HSP27) was also tested in a long-term experiment for 120 h after incubation with 1.0 nM cabazitaxel, measuring the cellular growth (Fig. 5c, d). This higher concentration of cabazitaxel was used to distinguish a higher cytostatic effect; however, no significant difference in cellular growth could be observed.

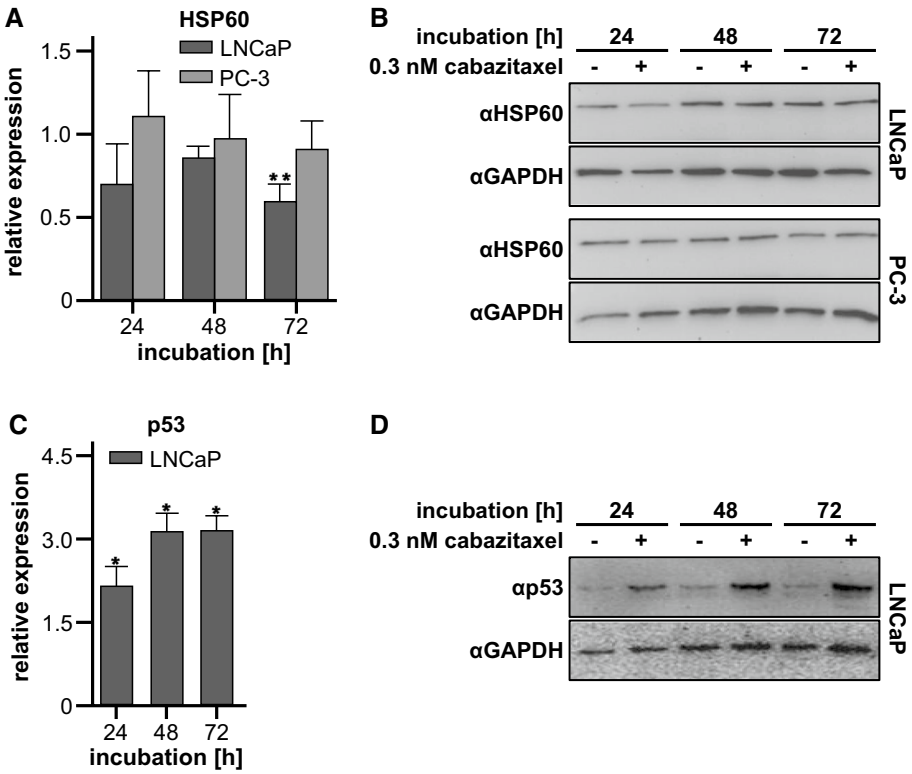

Fig. 4 Suppression of the apoptotic factor HSP60 in LNCaP and PC-3 cells and induction of the apoptotic factor p53 in LNCaP cells in the presence of cabazitaxel. Western blot analysis of relative expression of HSP60 (**a**), and p53 (**c**) in the presence of 0.3 nM cabazitaxel at indicated timepoints. Data were standardized to vehicle-treated cells (control = 1.0) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as loading control (**b**, **d**). Error bars indicate ± SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, as determined by Student's *t* test

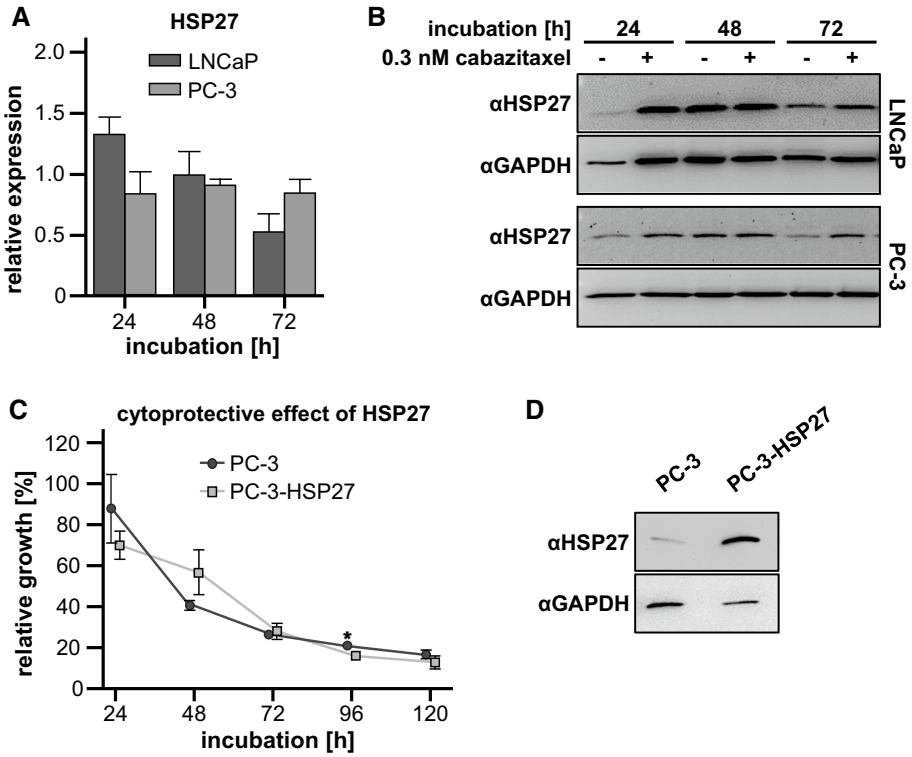

Fig. 5 Expression of the cytoprotective factor HSP27 is unaffected by cabazitaxel in LNCaP and PC-3 cells and HSP27 stably overexpressed in PC-3 cells do not confer resistance to cabazitaxel efficacy. **a** Western blot analysis of relative expression of HSP27 in the presence of 0.3 nM cabazitaxel at indicated timepoints. Data were standardized to vehicle-treated cells (control = 1.0) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as loading control (**b**). **c** Relative living cell number of PC-3 cells and PC-3 cells stably overexpressing HSP27 (PC-3-HSP27) **d** treated with 0.3 nM cabazitaxel standardized to vehicle treatment (control = 100%). Cell number was examined utilizing a CASY Cell Counter and Analyzer Modell TT at indicated timepoints over a period of 120 h. Results are expressed as the mean ± SD of cell count. Error bars indicate ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, as determined by Student's *t* test

Discussion

The next-generation taxane cabazitaxel is approved for the treatment of patients with mCRPC previously treated with a docetaxel containing regimen since 2011, and offers the advantage to be active in tumors resistant to docetaxel [23, 24] and new AR-targeted agents [25]. In this work, we characterized the effect of cabazitaxel on different PC-cell lines. Data presented in this report show that LNCaP as well as PC-3 cell lines are sensitive to cabazitaxel (Fig. 1). The IC_{50} concentration of cabazitaxel found for both cell lines was 0.3 nM, which is much lower than the concentration reported in the literature [24, 39], even if they are not entirely comparable due to different cell lines and incubation times as well as to different types of proliferation assays.

It has been reported that drugs of the taxane group impair the AR activity in prostate cancer cells. Taxanes such as docetaxel and paclitaxel inhibit microtubule dynamics, leading to both mitotic impairment and inhibition of intracellular transport processes. In PC cells, the translocation of the AR into the nucleus is suppressed in a concentration-dependent manner. Cabazitaxel also belongs to the group of taxanes, but is used in such low concentrations that the inhibition of AR translocation hardly plays a role in anticancer activity, and the inhibition of other cellular processes is in the foreground [40, 41].

Our results show that cabazitaxel leads to a significant reduction of intracellular AR levels in AR-positive LNCaP cells (Fig. 2). Although the suppression of AR expression by cabazitaxel cannot be excluded, the main cause seems to be the destabilization of the AR protein by decreasing concentrations of AR-associated HSP like HSP70, HSP90α/β, HSP40, and HOP. Interestingly, it appears that cabazitaxel has an effect on the expression of AR-associated HSP rather in LNCaP cells than in PC-3 cells (Fig. 3). These findings suggest that the suppression of AR-associated HSP may lead to a reduction of AR stability. It is also possible, however, that those effects are due to feedback mechanisms.

HSP60 expressed in PC cells is usually down-regulated in the course of tumor progression [38, 42] and both cytoprotective [43, 44] as well as apoptotic effects [36, 37] have been described for it. The expression of HSP60 in LNCaP cells is weakly suppressed in the presence of cabazitaxel (Fig. 4). This may indicate that the cytostatic

effect of cabazitaxel is based on the induction of apoptosis. This notion is consistent with the induction of the tumor suppressor protein p53, which is an important factor in the apoptotic signaling pathway (Fig. 4). The finding that HSP60 is suppressed by cabazitaxel in PC cells and the correlating induction of p53 speaks in favor of an anti-apoptotic role of HSP60 in PC; further experimental studies are needed to clarify this.

The expression of HSP27 is suppressed by cabazitaxel after 72 h, and no enhanced cell survival after overexpression with HSP27 and cabazitaxel induction over 120 h could be found (Fig. 5). This strongly suggests that cabazitaxel inhibits the cytoprotective effect of HSP27, contrary to docetaxel, which induces HSP27 and in this way contributes to cytoprotection in PC cells [45]. This could explain the efficacy of cabazitaxel after drug resistance to docetaxel. From a molecular point of view, it is very notable that two close compounds of the taxane group show intensively varying or contrary effects in PC cells.

In conclusion, the results in this study show the suppression of cellular growth of PC cells. In AR-positive LNCaP cells, but not in AR negative PC-3 cells, this may occur primarily by downregulation of AR expression and by impairing AR functionality at least in part by inhibition of AR–HSP complexes. In addition, Cabazitaxel suppresses HSP60 expression in LNCaP cells and subsequently induces the expression of p53. Attenuation of HSP60 expression could play a part in induction of apoptosis of tumor cells; however, the role of HSP60 in PC cells remains unclear. Finally, cabazitaxel is also an inhibitor of the cell survival factor HSP27 in LNCaP cells, which means that HSP27's cytoprotectivity is inhibited by cabazitaxel, contrary to the observations made with docetaxel. This may explain the efficacy in patients with resistance towards docetaxel, and the relevance of cabazitaxel as a second-line treatment option for PC in clinical practice. Since the effect of cabazitaxel on the expression of HSPs was detectable in PC-3 cells as well as in LNCaP cells, it seems that cabazitaxel has another AR-independent impact on PC cells; this effect is not fully clear yet and needs further investigation. In any case, it appears that cabazitaxel has an additional molecular mode of action apart from the known mechanisms of taxanes in PC; comparable to abiraterone, which—aside from the known inhibition of androgen synthesis—also suppresses apoptosis and cell cycle pathways in PC cells lacking AR signaling [46, 47].

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Author's contribution AMR data collection and protocol development. HA data collection. BM data collection. RW data analysis and manuscript editing. UZ manuscript editing and data analysis. MB data analysis and manuscript editing. MBS protocol development, project management, data analysis, manuscript writing, and editing.

Compliance with ethical standards

Conflict of interest By way of disclosure of conflict of interest, the compound cabazitaxel was kindly provided by Sanofi-Aventis Deutschland GmbH (Frankfurt/Main, Germany). The preparation of the manuscript for the present publication was financially supported by the company Sanofi-Aventis Deutschland GmbH. Sanofi-Aventis Deutschland GmbH has no influence on the published content. The authors alone are responsible for the scientific content of this publication.

Ethical standards The manuscript does not contain clinical studies or patient data.

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