Targeting NF-kappa B Signaling by Artesunate Restores Sensitivity of Castrate-Resistant Prostate Cancer Cells to Antiandrogens1,2

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Abstract
Androgen deprivation therapy (ADT) is the most preferred treatment for men with metastatic prostate cancer (PCa). However, the disease eventually progresses and develops resistance to ADT in majority of the patients, leading to the emergence of metastatic castration-resistant prostate cancer (mCRPC). Here, we assessed artesunate (AS), an artemisinin derivative, for its anticancer properties and ability to alleviate resistance to androgen receptor (AR) antagonists. We have shown AS in combination with bicalutamide (Bic) attenuates the oncogenic properties of the castrate-resistant (PC3, 22RV1) and androgen-responsive (LNCaP) PCa cells. Mechanistically, AS and Bic combination inhibits nuclear factor (NF)-κB signaling and decreases AR and/or AR-variant 7 expression via ubiquitin-mediated proteasomal degradation. The combination induces oxidative stress and apoptosis via survivin downregulation and caspase-3 activation, resulting in poly-ADP-ribose polymerase (PARP) cleavage. Moreover, preclinical castrate-resistant PC3 xenograft studies in NOD/SCID mice (n = 28, seven per group) show remarkable tumor regression and significant reduction in lungs and bone metastases upon administering AS (50 mg/kg per day in two divided doses) and Bic (50 mg/kg per day) via oral gavage. Taken together, we for the first time provide a compelling preclinical rationale that AS could disrupt AR antagonist–mediated resistance observed in mCRPC. The current study also indicates that the therapeutic combination of Food and Drug Administration–approved AS or NF-κB inhibitors and AR antagonists may enhance the clinical efficacy in the treatment of mCRPC patients.

Neoplasia (2017) 19, 333–345

Introduction
Prostate cancer (PCa) is the second most common cancer in men and the fifth leading cause of cancer-associated death (~6.6% of the total deaths) among men [1]. Although androgen deprivation therapy (ADT) remains the mainstay for the management of metastatic PCa, majority of men develop resistance to primary ADT, leading to an aggressive stage termed “metastatic castration-resistant prostate cancer” (mCRPC). It has been established that CRPC is not an androgen-independent disease; rather, it continues to rely on androgen signaling [2]. Therefore, various second-generation anti-androgen drugs, such as abiraterone acetate [3,4] and enzalutamide [5,6], have emerged for the treatment of mCRPC. Increased expression of androgen receptor-variant 7 (AR-V7) has been detected in circulating tumor cells and mCRPC tissues, and is associated with shorter biochemical recurrence, shorter survival, and resistance to ADT [7,8]. Interestingly, AR-V7 lacks the ligand-binding domain, which is the direct target of enzalutamide and the indirect target of abiraterone, thus maintaining the receptor in constitutively active conformation in a ligand-independent manner [7]. Not surprisingly, most men with mCRPC inevitably develop resistance to ADT, thus necessitating an urgent need for the development of new chemotherapeutic interventions and combinatorial approaches for the treatment of mCRPC [9–11].

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1 Financial support: This work is partially supported by the Wellcome Trust/DBT India Alliance grant (IA/I(S)/12/2/500635 to B.A.) and an intramural funding from the Indian Institute of Technology, Kanpur (PDF-53 to S.K.P.).
2 Conflict of interest: The authors declare no conflicts of interest or disclosures.
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Received 24 December 2016; Revised 1 February 2017; Accepted 6 February 2017
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1476-5586
http://dx.doi.org/10.1016/j.neo.2017.02.002
The cross talk between inflammation and cancer progression is a well-established phenomenon and an emerging field of research [12]. Recent studies have shown that the nuclear factor (NF)-κB family of transcription factors is an important player in the development and progression of several human malignancies including mCRPC [13,14]. It has been known that inflammatory cytokines present in the tumor microenvironment may drive mCRPC by activating NF-κB signaling and upregulating cytokines, thus restricting the efficacy of ADT [15,16]. Activation of the noncanonical NF-κB pathway (NF-κB2/p52) can enhance expression of AR variants (AR-Vs) including AR-V7, causing resistance to androgens, whereas inhibition of this pathway sensitizes the CRPC cells to androgens by decreasing the expression of AR-Vs [17]. Hence, combination of potent and safe NF-κB inhibitors with androgens might be an effective strategy for the management of CRPC [18].

Artemisinin derivatives (ADs) are semisynthetic compounds derived from Artemisia annua and are approved first-line antimalarial drugs. The clinically important ADs are artesunate (AS), arteether (AM), arteether (AE), and dihydroartemisinin (DHA) [19]. The antiproliferative, antiangiogenic, anti-inflammatory, and antimetastasis properties of these derivatives have been reported in several cancer types including prostate [20–23]. ADs act through their remarkable endoperoxide bridge, which gets cleaved in the presence of iron to generate cytotoxic free radicals and reactive oxygen species (ROS) [22]. The higher iron requirement and enhanced susceptibility to ROS may damage cancer cells (due to lower expression of antioxidant enzymes in cancer cells as compared to normal cells), elucidating the selective action of artesinin on cancer cells [21]. Moreover, ADs are also known to inhibit NF-κB signaling, thereby inducing cell cycle arrest [22,23]. We therefore reasoned that a combinatorial approach using ADs to block NF-κB signaling and androgen could reinstate the responsiveness to AR antagonists in CRPC.

Here, we explored the combinatorial effect of ADs with AR antagonist bicalutamide (Bic) for the treatment of CRPC. We show that ADs enhance the antiproliferative effect of Bic on the castrate-resistant PC3 (AR null), 22RV1 (AR full length and AR-V7 positive), and androgen-responsive LNCaP cells. Interestingly, AS in combination with Bic demonstrated the most effective antiproliferative effect in PC3 cells. This drug combination significantly reduces cell invasion, migration, and foci formation in both androgen-responsive and -nonresponsive cells when compared with individual drug treatments. This is the first report that provides the mechanistic insights into AS and Bic combination-mediated increase in oxidative stress and inhibition of NF-κB signaling, which leads to decrease in AR and/or AR-V7 expression via ubiquitin-mediated proteasomal degradation. Consequently, these cumulative effects of the combinatorial drug treatment lead to enhanced apoptosis through caspase-3 activation, survivin downregulation, and poly-ADP-ribose polymerase (PARP) cleavage. Most importantly, AS and Bic combinatorial therapy in PC3 xenografted mice demonstrates enhanced tumor regression and reduced distant metastases.

**Materials and Methods**

**Cell Lines and Reagents**

Prostate cancer cell lines PC3, 22RV1, LNCaP, and PNT2 were procured from the American Type Culture Collection (ATCC) (Manassas, VA) between the years 2011 and 2013 and were maintained as per ATCC recommendation. Frozen stocks in early passages were established immediately after receiving each cell line, and only these early passaged frozen cell lines were used in the study. Cell lines were routinely tested for mycoplasma contamination. The ATCC-recommended medium was supplemented with 10% fetal bovine serum (Thermo-Fisher, Waltham, MA), and cell lines were cultured at 37°C in an incubator with 5% CO₂.

**Proliferation Assay and Assessment of Drug Synergism**

ADs (AS, AE, AM, and DHA) were procured from the malaria research group at CSIR-CDRI, Lucknow, India (Themis/IPCA Pharmaceuticals, Mumbai, India). ADs were used for evaluating the antiproliferative effect on PCa cells. WST-1 assay (Roche, Basel, Switzerland) was performed as described previously [24]. Cells were treated with selected concentration of test drugs or their combinations, and absorbance was measured at 450 nm after incubating cells with WST-1 reagent. IC₅₀ values were calculated by using preprogrammed Logit regression analysis for the dose-response curves. For assessing the synergism between AS and Bic, PC3, 22RV1, and LNCaP cells were treated with serial dilutions of the selected lead combinations and individual drugs for 48 hours. IC₅₀ values were determined, and combination index (CI) of the drug interaction was calculated by following mathematical equation: CI = Dₓ₁/Dₓ₁ + Dₓ₂/Dₓ₂. Here, D₁ and D₂ are the IC₅₀ of drug 1 and drug 2 in the combination respectively, whereas Dₓ₁ and Dₓ₂ are the IC₅₀ values of individual drug 1 and 2, respectively. CI value of less than 1 was considered as synergism, CI equivalent to 1 as additive effect, and CI less than 1 was considered as antagonism. Isobolographic analysis was carried out to understand the nature and quantitative assessment of drugs’ interaction by Loewe’s method [25].

**Invasion and Migration Assay**

Invasion assay was performed with AS and/or Bic (Sigma-Aldrich, St. Louis, MO) treated cells (100,000 cells) along with DMSO control by placing on the Matrigel-coated Boyden chambers (Corning, NY) as described previously [24]. Same protocol was followed for cell migration assay, except cells were plated in the Transwell chambers without Matrigel.

**Foci Formation Assay**

PCa cells (2000 cells) were plated in the presence of reduced serum (2%) medium in six-well culture dishes. Drugs were replenished every second day, and assay was carried out for 15 days as described before [24]. After staining with crystal violet, foci (>5 μm) were counted under phase contrast Olympus microscope (Tokyo, Japan).

**DCFDA Fluorometric Assay**

For CM–2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) assay, about 10,000 cells were plated and treated with individual drugs or combinations for 24 hours, followed by treatment with CM-H₂DCFDA (10 μM; Sigma Aldrich) in serum-free media for 2 hours after washing with PBS. After lysising the cells, fluorescence readings were taken at an excitation/emission wavelength of 492/517 nm on the BioTek Synergy fluorescence reader (Winooski, VT).

**Western Blotting and Immunoprecipitation**

Western blotting for all protein samples was performed as previously described [24]. Antibodies (phospho-p65 (#3033), total-p65 (#4764), AR (#5153), β-actin (#4970), survivin (#2808), cleaved-PARP (#9541), Bcl-2 (#2870), and Bcl-xL (#2764)) were procured from...
Cell Signaling Technology (Danvers, MA). For immunoprecipitation, 22RV1 cells were treated with AS (2.5 μM) and Bic (25 μM) for 24 hours followed by serum starvation for 6 hours. Hereafter, cells were treated with MG132 (20 μM; Sigma-Aldrich) for 4 hours and lysed in cold radioimmunoprecipitation assay buffer. Total cell lysates (~1 mg) were precleared with proteinA/G sepharose beads (Sigma-Aldrich), and immunoprecipitation was performed as previously described [26]. The eluted protein was subjected to immunoblotting with anti-polyubiquitination (BML-PW8805-0500; Enzo Life Sciences, Farmingdale, NY). ImageJ software was used for quantification, and one-tailed Student’s t test was employed for statistical significance.

Caspase-3 Colorimetric Assay
Caspase-3 colorimetric assay kit was procured from Invitrogen (Carlsbad, CA). Cells were seeded in a six-well plate and treated with the drugs for 48 hours, and assay was performed according to the manufacturer’s protocol.

Luciferase Reporter Assays
The pNiFty-M vector (Invivogen, San Diego, CA) and pGL4.10-PSA vector (Promega, Madison, WI) were kind gifts from Dr. Shyam Nyati, University of Michigan, and Dr. Jindan Yu, Northwestern University, USA, respectively. Cells were seeded in a 24-well plate. For NF-κB reporter assay, PC3 cells were transfected with pNiFty-M-luc vector and pRL-CMV Luciferase control vector (Promega) using FuGENE-HD transfection reagent (Promega) according to manufacturer’s instructions. Cells were treated with individual drugs and combination 24 hours posttransfection. For PSA promoter reporter assay, 22RV1 cells were transfected with pGL4.10-PSA vector and pRL-Null luciferase control vector followed by drug treatment for 24 hours and subsequent R1881 stimulation for 12 hours. Dual-Glo Luciferase Assay was performed according to manufacturer’s instructions (Promega) and normalized using Renilla luciferase activity.

Quantitative Real-Time PCR
Total RNA was extracted using TRIzol (Ambion, Waltham, MA) and reverse-transcribed using SuperScript II Reverse-Transcriptase (Invitrogen). qPCRs were performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on the StepOne Plus (Applied Biosystems) version 1.2.2. The relative expression of the target gene was calculated for each sample using the ΔΔCt method by comparing the mean Ct of the gene to the average Ct of the housekeeping gene. Primer sequences are listed in the Supplementary Table 4.

Immunofluorescence and Immunohistochemistry
LNCaP cells were treated with DHT, AS, Bic, or combination for 24 hours and fixed with 4% paraformaldehyde, and immunofluorescence was performed using primary AR and Alexa Fluor-488 conjugated secondary antibodies (Cell Signaling Technology) as described previously [24]. Immunohistochemical staining for Ki-67 was performed using mouse monoclonal antibody (Cell Signaling Technology) as described earlier [27].

Mouse Xenograft Experiment
Male NOD/SCID mice (6–8 weeks old, Jackson Laboratory, Bar Harbor, ME) were anesthetized using ketamine/xylazine (50 and 5 mg/kg, respectively, intraperitoneal). PC3 cells (1.2 × 10⁶) were suspended in 100 μl of saline with 20% Matrigel (v/v) and implanted subcutaneously on both sides of the dorsal flank (seven per group). Tumor growth was recorded using digital calipers, and volumes were calculated using formula \(V = \frac{1}{2}L \times W^2\) (L = length; W = width). Treatment with AS (50 mg/kg per day in two divided doses) and Bic (50 mg/kg per day) was administered orally once average tumor volume reached ~100 mm³. Spontaneous metastasis in the lungs and bone marrow specimens was analyzed by performing qPCR for human Alu-sequences as described previously [24]. All procedures involving mice were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals and conform to all regulatory standards.

Results
AS Sensitizes Castrate-Resistant PCa Cells to Antiandrogen Therapy and Abrogates Their Oncogenic Potential
To investigate the antiproliferative potential of the ADs (AS, AE, AM, and DHA) in PCa cells, we first determined the half maximal inhibitory concentration (IC₅₀) for ADs in castrate-resistant PC3, 22RV1, and androgen-responsive LNCaP cells by using WST-1 reagent. All ADs showed marginal antiproliferative effect (IC₅₀ > 70 μM) in PC3 and 22RV1 cells, whereas LNCaP cells demonstrated higher sensitivity toward AS and DHA (IC₅₀ 4.42 and 12.20 μM, respectively) (Supplementary Table S1). Similarly, the IC₅₀ value for Bic was higher in PC3 and 22RV1 cells (80 and 63 μM, respectively) as compared to LNCaP (53 μM) (Supplementary Table S1). The percent inhibition in cell proliferation for PC3, 22RV1, and LNCaP cells was assessed using sub-IC₅₀ concentrations for each derivative (AS, AE, AM, and DHA) either alone or in combination; interestingly, AS and Bic combination showed remarkable response for all three cell lines (Supplementary Table S2). The most effective combination of individual AD and Bic in PC3, 22RV1, and LNCaP cells was determined by employing two-way ANOVA test (Supplementary Table S3). Here, we show that AS at much lower concentration can improve the efficacy of antiandrogen drugs such as Bic. Combinatorial treatment with AS (5 μM) and Bic (50 μM) at much lower concentration than their respective IC₅₀ exhibited the most remarkable inhibition in PC3 (82%, \(P < 0.0001\)) and 22RV1 cell proliferation (72%, \(P < 0.0001\)) as compared to individual compound, whereas combinatorial treatment of AS (1 μM) and Bic (25 μM) demonstrated ~70% decrease in proliferation in LNCaP cells \(P < 0.0001;\) Figure 1A and Supplementary Figure S1). Moreover, DHA, AE, and AM in combination with Bic also showed antiproliferative effect on PC3, 22RV1, and LNCaP cells; however, the effect was more pronounced as compared to individual drug treatments (Supplementary Figure S2A–C; Supplementary Tables S2–3). Furthermore, the combinatorial treatment of AS (5 μM) and Bic (50 μM) on the immortalized prostate epithelial PNT2 cells failed to show significant antiproliferative effect (Supplementary Figure S2D). Further, to assess the nature of drug interaction between AS and Bic (5 μM AS +50 μM...
BIC in PC3 and 22RV1 cells; 1 μM AS + 25 μM Bic in LNCaP), isobologram analysis by Loewe’s method was carried out. The calculated CI values for the selected drug combinations suggest strong synergistic interaction in PC3 (CI = 0.2) and 22RV1 (CI = 0.1), whereas weak synergistic interaction was observed in LNCaP cells (CI = 0.9) (Supplementary Figure S2E).

To investigate the effect of AS and Bic alone or in combination on the invasive properties of PCa cells, Boyden chamber Matrigel invasion assay was performed using sublethal concentrations of the drugs. As shown in Figure 1B, Bic (50 μM) alone has no significant effect on cell invasion in PC3 cells, whereas in combination with AS, a significant reduction in cell invasion was observed (P = 1.8e-7). The

Figure 1. AS attenuates cell proliferation and abrogates oncogenic properties by sensitizing PCa cells to antiandrogen. (A) Cell proliferation assay using tetrazolium salt–based WST-1 reagent. PC3 and 22RV1 cells were treated with AS (5 μM) and Bic (50 μM) alone or in combination for 24 to 96 hours, whereas LNCaP cells were treated with AS (1 μM) and Bic (25 μM). Error bars in panel A represent mean ± S.E.M. P values were calculated from two-way ANOVA. *P < 0.01 versus control; **P < 0.0001 versus control. P values < 0.05 were considered significant. (B) Boyden chamber Matrigel invasion assay using PC3 and 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM) alone or in combination for 24 hours. LNCaP cells were treated with AS (1 μM) and Bic (12.5 μM). (C) Transwell cell migration assay. All experimental conditions were same as B. (D) Foci formation assay using PC3 and 22RV1 (treated with AS 1.25 μM and Bic 12.5 μM) and LNCaP cells (treated with AS 0.5 μM and Bic 6.25 μM). In panels B to D, error bars represent mean ± S.E.M. P values calculated from two-sided Student’s t test. P values < 0.05 were considered significant.
**Figure 2.** AS inhibits NF-κB signaling and augments sensitivity to AR antagonist in castrate-resistant PCa cells. (A) Immunoblotting for AR, phospho-p65, total p65, and β-actin in 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM) alone or in combination in the presence of TNF-α (10 nM). (B) Immunoblotting for phospho-p65 and total p65 in PC3 and 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM) alone or in combination. (C) Schema showing NF-κB luciferase reporter assay (left panel); NF-κB luciferase reporter assay in PC3 cells treated with AS (2.5 μM) and Bic (25 μM) alone or in combination (right panel). Error bars represent mean ± S.E.M. *P* values calculated from two-sided Student’s *t* test. *P* values < 0.05 were considered significant.
combinatorial treatment showed remarkable decrease (~60%) in cell invasion of PC3 cells as compared to AS alone (~21%). Similarly, the combinatorial treatment of AS and Bic attenuated cell invasion in 22RV1 (~48% reduction) and LNCaP cells (~49% reduction) (Figure 1B; \( P = 0.0007 \) and \( P = 0.04 \), respectively), whereas individual drugs have no effect. Next, to explore the effect of AS and Bic combination on cell migration, Transwell migration assay was performed. As anticipated, combinatorial treatment of AS and Bic attenuated migration of PC3, 22RV1, and LNCaP cells. Interestingly, PC3 cells showed most pronounced effect (>50% inhibition) on
Next, we investigated the foci-forming potential of PC3, 22RV1, and LNCaP cells upon AS and Bic treatment. We observed that administration of AS along with Bic vastly reduced the foci-forming property of PC3, 22RV1, and LNCaP cells than treatment with Bic alone. Interestingly, combinatorial treatment of AS and Bic in PC3 cells showed dramatic reduction in foci formation (~97% inhibition, \( P = 5.7 \times 10^{-14} \)), 22RV1 (~89% inhibition, \( P = 9.3 \times 10^{-11} \)), and LNCaP cells (~64% inhibition, \( P = 1.8 \times 10^{-5} \)) as depicted in Figure 1D. Moreover, AS alone could also significantly reduce foci formation in all the three PCa cell lines, whereas the effect was more pronounced in PC3 cells (~86% reduction; \( P = 5.6 \times 10^{-10} \); Figure 1D).

**AS and Bic Mediate Inhibition of NF-κB Signaling in PCa Cells**

Activation of NF-κB signaling has been known to increase the expression of AR and AR-Vs in mCRPC, and inhibition of NF-κB pathway sensitizes the castrate-resistant PCa cells to antiandrogens [13,18]. We stimulated 22RV1 cells with tumor necrosis factor alpha (TNF-α), and as anticipated, an upregulation in the NF-κB signaling

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**Figure 3.** AS and Bic combinatorial treatment decreases AR and AR-V7 levels via ubiquitin-mediated proteasomal degradation. (A) Quantitative PCR data showing expression of AR and PSA in LNCaP cells treated with AS (1 μM) and Bic (12.5 μM) alone or in combination for 24 hours in the presence or absence of synthetic androgen R1881 (10 nM for 12 hours). (B) qPCR and luciferase reporter assay data for PSA in 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM) alone or in combination for 24 hours in the presence or absence of synthetic androgen R1881 (10 nM for 12 hours). (C) Immunoblotting for AR-FL in 22RV1 and LNCaP cells. Note expression of both AR-FL and AR-V7 in 22RV1 cells. 22RV1 cells were treated with AS (2.5 μM) and Bic (25 μM) alone or in combination, whereas LNCaP cells were treated with AS (1 μM) and Bic (12.5 μM). (D) Immunoprecipitation assay using 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM) in the presence of MG132 (left panel); immunocomplexes were pulled down using AR or IgG antibodies and immunoblotted with an antibody against ubiquitin marks; immunoblotting with an antibody against AR (right panel). Error bars represent mean ± S.E.M. \( P \) values were calculated from two-sided Student’s \( t \) test. \( P \) values < 0.05 were considered significant.

**Figure 4.** AS and Bic treatment decreases AR expression in DHT-stimulated LNCaP cells. Immunofluorescence images show AR localization in LNCaP cells treated with AS (1 μM) and Bic (12.5 μM) alone or in combination upon DHT stimulation for 24 hours. Nuclei were stained with DAPI (blue; first panel), AR was immunostained with an antibody against AR (green; second panel), and F-actin filaments were stained with TRITC-Phalloidin (red; third panel); the last panel shows the merged images. Scale bar represents 20 μm.
Figure 5. AS treatment induces apoptosis by eliciting oxidative stress and caspase-3–mediated PARP cleavage. (A) DCFDA-fluorometric assay using PC3 and 22RV1 cells treated with AS (2.5 μM) and Bic (25 μM); LNCaP cells treated with AS (1 μM) and Bic (12.5 μM) for 24 hours. H2O2 (50 μM) treatment for 4 hours was included as a positive control. (B) Colorimetric caspase-3 activity assay in PC3, 22RV1, and LNCaP cells; experimental conditions for drugs treatment were the same as in A. (C) Immunoblotting for cleaved PARP and survivin in PC3, 22RV1, and LNCaP cells. PC3 and 22RV1 were treated with AS (2.5 μM) and Bic (25 μM) alone or in combination, whereas LNCaP cells were treated with AS (1 μM) and Bic (12.5 μM) for 48 hours. Doxorubicin (500 nM for PC3 and 22RV1 cells and 50 nM for LNCaP cells) was used as a positive control. Error bars represent mean ± S.E.M. *P values calculated from two-sided Student’s t test. *P values < 0.05 were considered significant.
Figure 6. AS and Bic combinatorial treatment attenuates CRPC tumor xenograft growth and distant metastases. (A) Tumor growth of the castrate-resistant PC3 cells xenograft immunodeficient NOD/SCID mice. Mice were treated with AS (50 mg/kg per day in two divided doses) and Bic (50 mg/kg per day) via oral route daily for 14 days. Drug treatment was initiated once average tumor volume in each group reached ~100 mm³. Results are presented as the mean tumor volume ± S.E.M. (B) Immunohistochemical staining of Ki-67 was performed to evaluate the cell proliferation index in the excised tumor specimens. The cells positive for Ki-67 staining were counted manually from 10 different fields for each tumor tissue section. (C) qPCR for human Alu-sequences using genomic DNA extracted from the lungs of the tumor-bearing mice. (D) Same as C, except bone marrow samples were used. Error bars represent mean ± S.E.M. *P values calculated from two-sided Student’s t test compared with control. *P < 0.0001 versus control. P values < 0.05 were considered significant. (E) Schema showing mechanism of AS-mediated inhibition in NF-κB signaling pathway; consequently, there was a decrease in AR and AR-V7 expression via ubiquitin-mediated proteasomal degradation.
supported by an increase in phospho-p65 level was observed (Figure 2A; \( P = 0.02 \)). Further, treatment with AS and Bic combination abrogated this increase significantly (Figure 2A; \( P = 0.03 \)). Notably, the expression of AR and AR-V7 also increased upon TNF-\( \alpha \) stimulation (Figure 2A; \( P = 0.006 \) and \( P = 0.01 \), respectively), and the combinatorial treatment with AS and Bic significantly downregulated the expression of AR and AR-V7 (Figure 2A; \( P = 0.03 \) and \( P = 0.002 \), respectively), indicating that NF-\( \kappa \)B signaling positively regulated AR and AR-V7 expression. To further elucidate the effect of AS and Bic on NF-\( \kappa \)B signaling, we performed immunoblot analysis for phosphorylated NF-\( \kappa \)B (pNF-\( \kappa \)B or phospho-p65) in PC3 and 22RV1 cells. Interestingly, AS alone or in combination with Bic significantly reduced the pNF-\( \kappa \)B levels in PC3 and 22RV1 cells (Figure 2B; \( P = 0.03 \) and \( P = 0.04 \), respectively). Moreover, LNCaP cells show low levels of NF-\( \kappa \)B activity; therefore, we were unable to detect significant pNF-\( \kappa \)B protein (data not shown).

Since AS is known to interfere with the canonical NF-\( \kappa \)B pathway by targeting RelA/p65 [28], we next determined whether AS treatment alone or in combination with Bic abrogates NF-\( \kappa \)B signaling by performing NF-\( \kappa \)B reporter assay in PC3 cells. As anticipated, a marked reduction in the NF-\( \kappa \)B reporter activity was observed upon AS treatment alone or in combination with Bic (Figure 2C; \( P = 0.01 \) for both). Taken together, our results suggest that treatment with AS alone as well as in combination with Bic blocks NF-\( \kappa \)B signaling in PC3 cells.

**Combinatorial Treatment of AS and Bic Promotes Degradation of AR and AR-Vs via Ubiquitin-Mediated Proteasomal Degradation**

Constitutively active AR and AR-Vs are the key drivers of the AR-regulated transcription in PCa and mCRPC, thus promoting tumor progression and metastases even under castration or ADT conditions. Therefore, we evaluated the effect of AS alone or in combination with Bic on the expression of androgen-driven genes AR and PSA by qPCR using synthetic androgen R1881-stimulated LNCaP cells. As anticipated, R1881-treated LNCaP cells showed significant increase (\( \sim \)six-fold) in AR expression (Figure 3A; \( P = 0.002 \)). We found a remarkable decrease in AR levels upon treatment of AS with Bic (\( \sim \)89%) compared to Bic alone (47%) (Figure 3A; \( P = 0.01 \) and \( P = 0.02 \), respectively). Interestingly, AS alone too gave comparable albeit lower reduction in AR levels in androgen-responsive LNCaP cells (\( P = 0.002 \)). However, no significant decrease in the AR transcript was observed in PC3 and 22RV1 cells upon AS alone or combinatorial treatment with Bic (Supplementary Figure S3, A–B).

Next, the effect of AS alone or in combination with Bic on the expression of PSA, an AR target gene in LNCaP cells was evaluated. As expected, R1881 stimulation significantly increased PSA expression (\( \sim \)13-fold), which upon treatment with AS and Bic, was significantly reduced (Figure 3A; \( P = 0.005 \)). Moreover, AS or Bic alone or combined together significantly reduced the basal expression of PSA in LNCaP cells even in the absence of R1881 (Figure 3A; \( P = 0.001 \), \( P = 0.002 \), and \( P = 0.004 \), respectively). Next, we investigated the effect of AS or/and Bic treatment on the expression of PSA in R1881-stimulated 22RV1 cells. As anticipated, a significant decrease in PSA expression was observed in the 22RV1 cells upon combinatorial treatment with AS and Bic (Figure 3B; \( P = 0.02 \)). Furthermore, a marked reduction in the PSA promoter reporter activity in 22RV1 cells was observed upon treatment with the drug combination (Figure 3B; \( P = 0.004 \)).

To further investigate the effect of AS alone or in combination with Bic on the AR protein level, we performed immunoblotting using 22RV1 and LNCaP cells. A significant reduction in AR full-length (AR-FL; \( \sim \)110 kDa) and AR-V7 expression (\( \sim \)75-80 kDa) was observed (\( \sim \)44% and \( \sim \)53%, respectively) in 22RV1 cells upon treatment with AS and Bic (Figure 3C; \( P = 0.03 \) and \( P = 0.02 \), respectively), suggesting that the expression of AR at the transcript level was not affected by the AS and/or Bic treatment, whereas AR protein was downregulated at the posttranscriptional level. Moreover, LNCaP cells also showed decrease in AR level upon AS treatment alone or in combination with Bic (Figure 3C; \( \sim \)57% reduction in combination; \( P = 0.02 \) and \( P = 0.01 \), respectively). To explore the mechanism of AR downregulation upon AS and Bic treatment in PCa cell lines, we next examined the possibility of ubiquitin-mediated AR degradation through 26S proteasome complex [29]. Immunoprecipitation for AR was performed using AS and Bic treated 22RV1 cells. Interestingly, a significant increase in ubiquitination of AR was observed upon AS and Bic combination treatment in the presence of a proteasome inhibitor MG132 as compared to DMSO control (Figure 3D).

Next, we sought to determine whether AS and Bic induced oxidative stress and caspase-3–mediated PARP cleavage.

Next, we sought to determine whether AS and Bic induced oxidative stress and PARP cleavage. Thus, we employed \( \text{H}_2\text{DCFDA} \) assay for detecting ROS in AS or Bic treated PCa cells. A significant increase in the generation of ROS was observed in PC3 (\( \sim \)two-fold increase; \( P = 0.006 \)), 22RV1 (\( \sim \)two-fold increase; \( P = 0.02 \)), and LNCaP cells (\( \sim \)1.5-fold increase; \( P = 0.04 \)) upon combinatorial AS and Bic treatment (Figure 5A). Next, we sought to determine whether AS and Bic treatment results in Caspase-3 activation, a known key mediator of apoptosis activated in response to ROS generation and an upstream activator of PARP cleavage. Therefore, colorimetric assay was performed to test Caspase-3 activity in PCa cells treated with AS and Bic alone and/or in combination. Interestingly, the LNCaP cells treated with combination showed highest Caspase-3 activity (six-fold increase), whereas 22RV1 and PC3 cells showed \( \sim \)2.5-fold increase as compared to DMSO control (Figure 5B; \( P = 0.02 \), \( P = 0.01 \), and \( P = 0.03 \), respectively). Subsequently, we investigated the caspase-3–mediated PARP cleavage by performing immunoblot analysis using AS and Bic treated PCa cells. AS
treatment alone could significantly increase PARP cleavage in 22RV1 (three-fold increase) and LNCaP (two-fold increase) (Figure 5G; \( P = 0.009 \) and \( P = 0.04 \), respectively). Intriguingly, the combinatorial effect demonstrated five-fold increase in PARP cleavage in 22RV1 cells (\( P = 0.004 \)) and only three-fold increase in LNCaP cells (Figure 5G; \( P = 0.009 \)). However, no significant change in PARP cleavage was observed in PC3 cells. Moreover, no significant change in antiapoptotic proteins (Bcl-2 and Bcl-xL) was observed in PC3, 22RV1, and LNCaP cells (Figure 5C).

Interestingly, treatment with the drug combination led to a significant decrease (one-fold) in survivin expression in PC3, 22RV1, and LNCaP cells (Figure 5G; \( P = 0.0001 \), \( P = 0.007 \), and \( P = 0.0001 \), respectively). These findings indicate that AS and Bic treatment may trigger oxidative stress by generating ROS, along with induction of caspase-dependent cell death and survivin downregulation, in both castrate-resistant and androgen-responsive PCa cells.

**AS and Bic Combination Efficiently Inhibits CRPC Tumor Growth and Distant Metastasis**

To investigate the antitumorigenic potential of AS and Bic combination, PC3 xenografts were generated in NOD/SCID mice. Drug treatment was initiated once the tumor volume reached around 100 mm\(^3\) in all the experimental groups. Combinatorial treatment of AS (50 mg/kg per day) and Bic (50 mg/kg per day) showed drastic regression (>95% reduction) in tumor growth (Figure 6A; \( P = 9e-8 \)), whereas monotherapy with either AS or Bic using matched doses (50 mg/kg per day) of the combinatory therapy failed to exhibit any significant reduction in the tumor burden. To check the cell proliferation index, immunohistochemical staining for Ki-67, a cell proliferation marker, was performed on the tumor samples collected from the xenografted mice. As anticipated, a significant decrease in the Ki-67 positive cells was observed in AS alone (~20% reduction) and in the combinatorial treatment group (~75% reduction) (Figure 6B; \( P = 0.0003 \) and \( P = 5.4e-17 \), respectively).

Spontaneous micrometastases to lungs and bones were investigated by performing qPCR assay for the human \( \alpha \)-sequences. A significant reduction in the lung metastatic burden was observed in the experimental group that received AS and Bic combinatorial therapy (Figure 6C; \( P = 0.01 \)). Similarly, significant decrease in the bone metastases was observed in the groups treated with AS alone and in combination with Bic (Figure 6D; \( P = 0.03 \) and \( P = 0.007 \), respectively). Taken together, our results suggest that AS and Bic combinatorial treatment in castrate-resistant PC3 xenograft--bearing mice demonstrated drastic tumor regression by reinstating sensitivity toward AR antagonists. However, out in vitro data suggest that Bic alone blocks NF-κB signaling in PC3 cells; hence, it could be plausible that Bic in combination with AS regresses tumor growth in AR-null PC3 xenografts by suppressing NF-κB signaling.

**Discussion**

ADT has been the main treatment strategy for the metastatic PCa; however, after initial response to androgens, the disease subsequently progresses to a highly aggressive state of mCRPC. Several mechanisms have been proposed for the maintenance of AR activity and resistance to ADT, for example, AR amplification, point mutations or phosphorylation of AR, gain-of-function mutation in androgen-synthesizing enzyme \( HSD3B1 \), and expression of constitutively active AR-V7, which are important driving forces in the development of mCRPC [7,30,31]. Androgen receptor variants are more frequently expressed in CRPC patients than in patients with hormone-responsive localized PCa. AR antagonist Bic is usually administered in locally advanced nonmetastatic PCas as an adjuvant therapy; however, it fails to be effective upon progression of disease to CRPC [32,33]. Recent studies demonstrated that AR-mediated signaling and downstream transcriptional activation are critical for the progression of CRPC despite castration [2], which led to the discovery of several novel therapies, namely, enzalutamide, a second-generation selective AR antagonist [3,6]; abiraterone acetate, a potent cytochrome p450-17A1 (CYP17A1) inhibitor [3,4]; and sipuleucel-T immunotherapy [34]. Interestingly, many of these agents have been effective as single agents in the pre- and postdocetaxel regimen for mCRPC [35]. A recent study has shown an association between the presence of AR-V7 and resistance to enzalutamide and abiraterone [7]. It is known that treating mCRPC with single-agent therapies in a sequential order eventually fails owing to inherent tumor heterogeneity and development of drug resistance [7,36]. Significantly, our data demonstrate that AR-FL and AR-V7 expression was remarkably reduced in castrate-resistant PCa cells upon treatment of AS with Bic, suggesting that AS-mediated reduction in AR-V7 in the castrate-resistant PCa cells restored CRPC responsiveness to antiandrogen treatment (Figure 6F).

Therefore, our study suggests that employing AS and AR antagonist combination regimens might enhance therapeutic efficacy and abate resistance toward ADT in mCRPC patients.

Several independent studies indicated that NF-κB signaling has an important role in PCa progression and is constitutively activated in CRPC [13,37,38]. Nuclear localization of NF-κB has been associated with an increased risk of biochemical relapse [39]. In addition, an increase in the cytoplasmic NF-κB expression has also been associated with shorter time to disease recurrence, increased PSA levels, and disease-associated mortalities [40]. ADs have been effective as anticancer agents and are known to inhibit NF-κB signaling [22,23,41]. In this study, we have shown that AS sensitizes castrate-resistant PCa cells to AR antagonist and abrogates their oncogenic potential by inhibiting NF-κB signaling; further, it also results in enhanced cell death possibly due to increase in ROS levels and oxidative stress. Presence of high concentration of intracellular iron in the form of ferritin in cancer cells is the key factor for the ART-mediated potent anticancer activity; it has been shown that Fe(III) binds to transferrin (TF), an iron-transport protein, to form holo-TF, which get transported to TF receptor (TFRC) and subsequently internalized into cancer cells via receptor-mediated endocytosis. Intriguingly, most cancer cells including PCa show elevated levels of TFRC [20,42,43], which could be a possible reason for the enhanced sensitivity to ARTs [42,44,45]. Furthermore, TFRC expression was found to be upregulated in PCa metastases, whereas PCa cells stimulated with holo-TF show increase in DHA-mediated effects [20].

Our data suggest that combinatorial treatment with AS and Bic reinstates the responsiveness of CRPC cells to the antiandrogen treatment by increasing ubiquitination of AR and its variants (AR-V7), which consequently results into AR degradation by targeting it to the 26S proteasomal complex (Figure 6E). Recently, Jin et al. showed that BMS345541, a specific inhibitor of the NF-κB pathway, sensitizes the CRPC cells to antiandrogens and bortezomib, an inhibitor of the 26S proteasome complex which blocks the NF-κB signaling by degradation of IkB [18]. Another independent study showed Galeterone and 3β-carbamate analog VNPT55 treatment results in decreased AR and AR-V7 expression by implicating Mdm2/CHIP enhanced ubiquitination and targeting them for the proteasomal degradation [46]. In corroboration to these studies,
our findings strongly indicate that AS-mediated inhibition of NF-κB signaling pathway restores the responsiveness of the castrate-resistant PCa cells toward AR antagonists by decreasing the expression of AR and its variants. We are also aware that there could be potential cross talk between the AR and NF-κB pathways that may explain some of the discrepancies such as phospho-p65 levels in PC3 (AR-null) and 22RV1 (AR-V7 positive) cells upon AS and Bic treatment.

A single-arm phase II trial with bortezomib in combination with ADT for 3 months showed a rapid clinical response as evidenced by decrease in the PSA level when compared with hormone blockade alone. However, clinical response had a short duration, and bortezomib treatment was associated with significant neural toxicity in those patients [47]. Unlike bortezomib and BMS345541, which are not fully suitable for the clinic due to serious side effects, ADs are the first-line antimalarial drugs recommended by the World Health Organization and are comparatively safe, and no side effects are reported as of yet. To the best of our knowledge, no combinatorial therapy with ADs and AR antagonists is in development phase or in clinical practice for the treatment of mCRPC. In this context, it is encouraging to note that a recent case report on a PCa patient showed that sequential oral administration of A. annua capsule after Bic treatment resulted in significant decrease in the PSA level and tumor remission [36]. Nonetheless, the mechanistic exploration and the rationale supporting this sequential therapy with ADs and Bic were not fully explored. Increased levels of AR and AR-Vs are one of the major mechanisms of ADT resistance in mCRPC. Recently approved second-generation antiandrogens abiraterone and enzalutamide have been reported to increase the expression of AR-Vs in PCa cell lines and clinical settings [7,48–50]. Furthermore, AR antagonists currently used for the treatment of PCa do not cause significant apoptosis and may contribute to their failures in the clinic [51].

Intracellular iron in the cancer cells has also been correlated with NF-κB signaling [52]. Furthermore, PC3 cells treated with ferric nitrotriacetate stimulate the expression of uPA levels by generating intracellular reactive oxygen intermediates and activate NF-κB signaling pathways [53]. Concomitantly, in the current study, we observed high levels of NF-κB in PC3 cells, which were downregulated upon treatment with the drug combination. We also observed that the drug combination mediates apoptosis via caspase-3 activation and PARP cleavage, which corroborates with the previous studies where artemisinin-tagged TF induced apoptosis via mitochondrial pathway along with PARP cleavage [42,45]. Yet another study explored the possibility of a caspase-independent non-apoptotic cell death which depends on the intracellular levels of iron, termed ferroptosis, wherein ferric ions induce oxidative stress along with cell death [44,46]. Moreover, generation of free radicals by ADs has been known to trigger oxidative stress and apoptosis [21]. Thus, combinatorial treatment of AS and Bic in the present study induces oxidative stress as well as apoptosis via caspase-3 activation, a key executioner in caspase-mediated cell death [55] and survivin downregulation. Finally, our preclinical mouse studies demonstrated that the combinatorial treatment with AS and Bic significantly attenuated PC3 tumor xenograft growth as well as distant metastases to the lungs and bones, thus indicating that AS restores the responsiveness of the castrate-resistant PCa cells toward AR antagonists. Taken together, our findings strongly recommend the use of targeted therapies against androgen-AR axis and NF-κB signaling pathway, which could provide a novel therapeutic approach for the treatment of mCRPC by alleviating ADT resistance. Overall, this study has high translational relevance and provides strong preclinical rationale for the use of ADs in the treatment of mCRPC and further necessitates the need for well-planned clinical trials using AS and AR antagonists in mCRPC patients.

**Authors’ contributions**

J. J. N., S. K. P., and B. A. designed the experimental studies. J. J. N., S. K. P., A. Y., and S. G. performed the experiments. J. J. N., S. K. P., A. Y., and B. A. performed the PCa xenograft experiments. J. J. N., S. K. P., and B. A. wrote the manuscript. B. A. directed the overall project.

**Acknowledgements**

B. A. is an Intermediate Fellow of the Wellcome Trust/DBT India Alliance and a Young Investigator of the DST-FAST Track scheme. We thank malaria research group at the CSIR-CDRI, Lucknow (Dr. Saman Habib, Dr. Renu Tripathi, and Dr. Kumkum Srivastava) for providing artemisinin derivatives. We are grateful to Prof. S. Ganesh for providing polyubiquitination antibody and Prof. D. S. Katti for the H2DCFDA reagent. We also thank Dr. Anjali Bajpai for critically reading the manuscript.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.neo.2017.02.002](http://dx.doi.org/10.1016/j.neo.2017.02.002).

**References**

[1] Fterajy L, Soerjomataram I, Evrik M, Dikshit R, Eser S, Mathers C, Rebolo M, Parkin DM, Forman D, and Bray F (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. International Agency for Research on Cancer: Lyon, France. Available from: [http://globocan.iarc.fr](http://globocan.iarc.fr), accessed on 16/02/2017.

[2] Longo DL (2010). New therapies for castration-resistant prostate cancer. *N Engl J Med* 363, 479–481.

[3] de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman Jr OB, and Saad F, et al (2011). Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 364, 1995–2005.

[4] Ryan CJ, Smith MR, de Bono JS, Molina A, Logothetis CJ, de Souza P, Fizazi K, Mainwaring P, Piulats JM, and Ng S, et al (2013). Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 368, 138–148.

[5] Beer TM and Tombal B (2014). Enzalutamide in metastatic prostate cancer before chemotherapy. *N Engl J Med* 371, 1755–1756.

[6] Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P, Chi KN, and Shore ND, et al (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 367, 1187–1197.

[7] Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roester JC, Chen Y, Mohammad TA, Fedor HL, and Lotan TL, et al (2014). AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med* 371, 1028–1038.

[8] Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Kong X, Melamed J, Tepper CG, and Kung HJ, et al (2009). A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen deprivation-resistant growth. *Cancer Res* 69, 2305–2313.

[9] Shvivelman E, Beer TM, and Evans CP (2014). Molecular pathways and targets in prostate cancer. *Oncotarget* 5, 7217–7259.

[10] Areqq B, Kunju LP, Cankadon SL, Pandey SK, Singh G, Pradeep I, Tandon V, Singhai A, Goel A, and Amit S, et al (2015). Molecular profiling of ETS and non-ETS aberrations in prostate cancer patients from northern India. *Prostate* 75, 1051–1062.

[11] Cookson MS, Lowrance WT, Murad MH, and Kibel AS (2015). Castration-resistant prostate cancer: AUA guideline amendment. *J Urol* 193, 491–499.
[12] Cruz SM and Balkwill FR (2015). Inflammation and cancer: advances and new agents. Nat Rev Clin Oncol 12, 584–596.

[13] Jin R, Yi Y, Yull FE, Blackwell TS, Clark PE, Koyama T, Smith JA, and Matusik RJ (2014). NF-kappaB gene signature predicts prostate cancer progression. Cancer Res 74, 2763–2772.

[14] Zhang L, Shao L, Creighton CJ, Zhang Y, Xin L, Itrmann M, and Wang J (2015). Function of phosphorylation of NF-kB p65 ser536 in prostate cancer oncogenesis. Oncotarget 6, 6281–6294.

[15] Keller ET, Chang C, and Ershler WB (1996). Inhibition of NFkappaB activity through maintenance of IkappaBAlpHa levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J Biol Chem 271, 26267–26275.

[16] Sharma J, Gray KP, Harshman LC, Evan C, Nakabayashi M, Fichorova R, Rider J, Mucci L, Kantoff PW, and Sweeney CJ (2014). Elevated IL-8, TNF-alpha, and MCP-1 in men with metastatic prostate cancer starting androgen-deprivation therapy (ADT) are associated with shorter time to castration-resistance and overall survival. Prostate 74, 820–828.

[17] Nadiminy N, Tumrala R, Liu C, Yang J, Lou W, Evans CP, and Gao AC (2013). NF-kappaB2/p52 induces resistance to enzalutamide in prostate cancer: role of androgen receptor and its variants. Med Cancer Ther 12, 1629–1637.

[18] Jin R, Yamashita H, Yu X, Wang J, Franco OE, Wang Y, Hayward SW, and Matusik RJ (2015). Inhibition of NF-kappaB signaling restores responsiveness of castrate-resistant prostate cancer cells to anti-androgen treatment by decreasing androgen receptor-variant expression. Oncogene 34, 3700–3710.

[19] White NJ (2008). Qinghaosu (artemisinin): the price of success. Science 320, 330–334.

[20] Morrissey C, Gallis B, Solazzi JW, Kim BJ, Gulati R, Vakar-Lopez F, Goodlett DR, Vesella RL, and Sasaki T (2010). Effect of artemisinin derivatives on androgen-receptor-variant expression. Anticancer Drugs 21, 423–432.

[21] Crespo-Ortiz MP and Wei MQ (2012). Antitumor activity of artemisinin and its analogues in human prostate carcinoma. Mol Cancer Ther 11, 303–310.

[22] Das AK (2015). Anticancer effect of antimalarial artemisinin compounds. Antiviral Res 109, 101–105.

[23] Tran KQ, Tin AS, and Firestone GL (2014). Artemisinin triggers a G1 cell cycle arrest of human Ishikawa endometrial cancer cells and inhibits cyclin-dependent kinase-4 promoter activity and expression by disrupting nuclear factor-kappaB transcriptional signaling. Anticancer Drug Des 25, 270–281.

[24] McCall P, Bennett L, Ahmad I, Mackenzie LM, Forbes IW, Leung HY, Sansom OJ, Grayhack JT, and Penther T (2007). Role of transferrin receptor and the ABC transporters ABCB6 and ABCB7 for resistance and differentiation of tumor cells towards artesunate. PLoS One 2, e798.

[25] Keer RN, Kozlowski JM, Tsai YC, Lee C, McEwan RN, and Grayhack JT (1990). Elevated transferrin receptor expression in human prostate cancer cell lines assessed in vitro and in vivo. J Urol 143, 381–385.

[26] Ateeq B (2015). SPINK1 promotes colorectal cancer progression by downregulating metallothioneins expression. Oncogene 34, e162.

[27] Keer RN, Kozlowski JM, Tsai YC, Lee C, McEwan RN, and Grayhack JT (1990). Elevated transferrin receptor expression in human prostate cancer cell lines assessed in vitro and in vivo. J Urol 143, 381–385.

[28] Keer RN, Kozlowski JM, Tsai YC, Lee C, McEwan RN, and Grayhack JT (1990). Elevated transferrin receptor expression in human prostate cancer cell lines assessed in vitro and in vivo. J Urol 143, 381–385.