Pharmacodynamic and clinical results from a phase 1/2 study of the HSP90 inhibitor onalespib in combination with abiraterone acetate in prostate cancer.

Running Title: Onalespib and abiraterone in CRPC

Keywords: AT13387, onalespib, GU/prostate, abiraterone acetate, HSP90 inhibitor, Phase 1/2, clinical trials, genitourinary cancers/prostate cancer

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Statement of Translational Relevance:

HSP90 is an ATP-dependent molecular chaperone that is critical to the folding and function of a wide range of client proteins involved in prostate cancer progression, including the androgen receptor (AR), HER-2, glucocorticoid receptor (GR), and AKT. Onalespib is a potent, fragment-derived HSP90 inhibitor with good tissue distribution and long tumor half-life in preclinical models leading to prolonged knockdown of HSP90 client proteins. Onalespib demonstrated anti-tumor activity in preclinical models of CRPC, causing depletion of full-length AR and the AR splice variant AR-V7. This study represents the first clinical trial of an HSP90 inhibitor in combination with abiraterone acetate (AA) in patients with castration-resistant prostate cancer (CRPC) no longer responding to AA. The tolerability, pharmacokinetic, pharmacodynamic and anti-tumor activity of two dosing schedules of onalespib and abiraterone acetate were investigated. Pharmacodynamic effects and AR modulation by onalespib were evaluated in circulating tumor cells and paired pre- and post-tumor biopsies.

Abstract

Purpose: Onalespib is a potent, fragment-derived second-generation HSP90 inhibitor with preclinical activity in castration-resistant prostate cancer (CPRC) models. This Phase 1/2 trial evaluated onalespib in combination with abiraterone acetate (AA) and either prednisone or prednisolone (P) in men with CRPC progressing on AA/P.

Experimental Design: Subjects with progressing CRPC were randomized to receive one of two regimens of onalespib combined with AA/P. Onalespib was administered as intravenous (IV) infusion starting at 220 mg/m2 once weekly for 3 of 4 weeks (Regimen 1); or at 120 mg/m2 on Day 1 and Day 2 weekly for 3 of 4 weeks (Regimen 2). Primary endpoints were response rate and safety. Secondary endpoints included evaluation of androgen receptor (AR) depletion in circulating tumor cells (CTCs) and in fresh tumor tissue biopsies.

Results: Forty-eight patients were treated with onalespib in combination with AA/P. The most common ≥ Grade 3 toxicities related to onalespib included diarrhea (21%) and fatigue (13%). Diarrhea was dose-limiting at 260 mg/m2 and 160 mg/m2 for Regimen 1 and Regimen 2, respectively. Transient decreases in CTC counts and AR expression in CTC were observed in both regimens. HSP72 was significantly up-regulated following onalespib treatment, but only a modest decrease in AR and GR was shown in paired pre- and post-treatment tumor biopsy samples. No subjects showed an objective or PSA response.

Conclusions: Onalespib in combination with AA/P, showed mild evidence of some biological effect, however this effect did not translate into clinical activity, hence further exploration of this combination was not justified.

INTRODUCTION

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer deaths in males worldwide(1). Androgen deprivation therapy (ADT) remains the mainstay of treatment for patients with advanced disease. However, in almost all patients, response to initial ADT is unfortunately followed by the emergence of resistance, so-called castration-resistant prostate cancer (CRPC). The efficacy of abiraterone acetate (AA) (2) and enzalutamide (3) in patients progressing after multiple prior hormonal manipulations indicates that CRPC generally remains dependent on the androgen receptor (AR) signaling axis (4). However, resistance to second-generation hormonal treatment is now common and a major challenge in the management of prostate cancer.

Resistance to enzalutamide and AA in CRPC has been associated with AR copy number gain, somatic point mutations and the expression of alternatively spliced AR (5-8). Truncated AR-V7 expression in circulating tumor cells (CTC), and CRPC biopsies has been linked to resistance to AA and enzalutamide highlighting an urgent need for alternative treatment strategies in CRPC effectively targeting both persistent AR-FL and AR splice variant signaling.

Onalespib is a synthetic, non-ansamycin, small molecule inhibitor of Heat Shock Protein 90 (HSP90) (Kd 0.71 nM) identified by fragment screening and subsequent structure-based drug design (9-12). HSP90 is required for the functional stabilization of numerous client proteins involved in cell growth and differentiation, including the full-length AR (AR-FL)(13, 14). Inhibition of HSP90 by onalespib results in proteasomal degradation of client proteins (12, 15) and inhibition of multiple signal transduction pathways, including the AR-FL signaling in both hormone-sensitive and CRPC models (15, 16).

We have previously shown that onalespib can inhibit growth of a range of tumor cell types, including the CRPC cell line 22Rv1, expressing the AR-V7, and VCaP cell line overexpressing the AR-FL (16). HSP90 inhibition effectively depletes both wild-type and promiscuous mutant AR-FL. In addition, HSP90 inhibition leads to the depletion of AR-V7 splice variant protein by downregulating AR-V7 mRNA splicing.

Modulation of HSP90, a ubiquitously expressed and highly abundant molecular chaperone, is an attractive therapeutic strategy in CRPC, as it offers the prospect of simultaneously inhibiting the following: (1) Multiple kinase-dependent signaling pathways that control cell growth, resistance to apoptosis, and post-translational modification of AR, including AKT and RAF; (2) The expression of both AR-FL and AR-V7 splice variant; 3) The expression of GR, which has also been implicated in CRPC(17). Various preclinical studies support this hypothesis (16, 18-21). Moreover, durable antitumor activity was shown in a patient suffering from CRPC in a Phase 1 study of the geldanamycin derivative alvespimycin (17-DMAG) (22), although tanespimycin (17-AAG) was not active (23). Since HSP90 inhibition offers the prospect to block both AR-FL signaling and AR splicing we hypothesized that AA/P in combination with onalespib would have antitumor activity in patients progressing on AA/P by overcoming multiple potential mechanisms of resistance to AA/P. We therefore conducted a Phase 1/2 study to evaluate the tolerability and the antitumor and pharmacodynamic activity of onalespib in combination with AA/Pin subjects with CRPC progressing on AA/P.

Subjects, Materials, and Methods

Study Centers and Subject Population

A total of 33 study centers (21 in the US, 10 in the UK, 1 in Canada, and 1 in Spain) participated in the study.

Inclusion criteria included histologically or cytologically confirmed adenocarcinoma of the prostate without neuroendocrine differentiation or small cell histology; prior castration by orchiectomy and/or luteinizing hormone-releasing hormone agonist with or without antiandrogen and documented serum testosterone <50 ng/dL; Eastern Cooperative Oncology Group Performance Status (ECOG PS) ≤2; no AR antagonist treatment within 6 weeks prior to first dose of study drug; receiving AA/P therapy for ≥1 month; documented disease progression on AA/P defined by one or more of the following: (1) prostate-specific antigen (PSA) progression according to PCWG2 criteria with 3 consecutive rising PSA measurements, all collected at least 1 week apart (24), (2) radiographic progression in soft tissue or bone by revised Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) (25) for subjects with measurable disease, or (3) bone disease progression defined by 2 or more new lesions on 2 consecutive bone

scans in the absence of falling PSA; CTC count ≥1 detected at screening (Part A only); adequate bone marrow function; adequate hepatic function; adequate renal function; willing to provide pre-existing diagnostic or resected tumor samples. Complete list of Inclusion and Exclusion Criteria is provided in Supplementary Methods.

Exclusion criteria included prior anti-cancer treatment with any Heat Shock Protein 90 (HSP90) inhibitor; screening QTc >450 msec; known symptomatic brain or central nervous system involvement such as a result of cord compression; contraindication to corticosteroids use or history of pituitary or adrenal dysfunction; prior dose reduction of abiraterone acetate as a result of increased transaminases. Standard washout period from previous chemotherapy or radiotherapy treatment was required. The study was conducted in accordance with Good Clinical Practice guidelines, in compliance with local and/or national regulations, and in accordance with the ethical principles that have their origin in the Declaration of Helsinki. All patients gave written informed consent, and approval was obtained from the Institutional Review committees at each participating institution. The study is registered on ClinicalTrials.gov (NCT01685268). Study schema and CONSORT diagram is provided in Supplementary Materials.

Study Design and Dosing Regimens

This study was designed as a 2-part, Phase 1/2, open-label, parallel-group, randomized trial in subjects with CRPC progressing on AA/P

Part A consisted of an assessment of the following using the combination of onalespib with AA/P in two different regimens: safety and tolerability, pharmacokinetics, antitumor activity, pharmacodynamics in fresh tumor biopsies and circulating tumor cells including and AR depletion. Subjects were randomized to receive one of two different treatment regimens of onalespib in combination with AA 1000 mg by mouth (PO) daily (QD) and prednisone or prednisolone 5 mg PO twice daily (BID), as follows:

- Regimen 1 (once weekly): onalespib given as a 1-hr intravenous (IV) infusion at a starting dose of 220 mg/m^2 once weekly for 3 weeks in a 4-week cycle.

- Regimen 2 (twice weekly): onalespib administered as a 1-hr IV infusion at starting dose of 120 mg/m^2 on Day 1 and Day 2 weekly for 3 weeks in a 4-week cycle.

Dose-limiting toxicity (DLT) was defined as (1) Grade 4 neutropenia or thrombocytopenia persisting for more than 1 week or was associated with neutropenic fever or bleeding, or (2) Grade 3 or 4 non-hematologic toxicity. The DLT window encompassed the first 28 days of treatment.

The maximum tolerated dose (MTD) was defined as the highest dose with ≤1 DLT in 6 subjects and was confirmed by enrollment of an additional ≥14 subjects. These additional subjects were randomized to one of the two regimens at the identified MTD. The absence of at least 1 response in 14 evaluable subjects in either of the treatment arms was to indicate antitumor activity in that arm of <20% with 95% confidence; that arm was not to proceed to Part B. A data review committee was to select the best treatment regimen and dose of onalespib in combination with AA/P to continue evaluation in Part B, the selection was to be based on combined assessment of safety, antitumor activity, and biological activity.

In Part B of the trial, subjects were to be randomized to the selected treatment regimen and dose of onalespib in combination with AA/Pfrom Part A, or to onalespib alone. Treatment with onalespib alone was to be administered at the monotherapy MTD using the same treatment regimen that was selected for use in the combination treatment arm. However, due to the absence of meaningful antitumor activity and minimal evidence of client protein knockdown at the MTD for both regimens in Part A, Part B of the study was not performed.

Treatment assignments for individual subjects were determined through a computer-generated randomization list prepared by Medpace (Cincinnati, OH) and accessed by using an interactive voice response system.

Efficacy Assessment

Response rate was based on one or more of the following:

- Complete response (CR) or partial response (PR) with 30% decrease in change of sum of longest diameters of target lesions, according to RECIST 1.1 (25)
- PSA response defined as ≥50% decrease in PSA at 12 weeks according to PCWG2 criteria (24) in the absence of disease progression

- Conversion of CTC count, defined as decline in CTC count from ≥5 cells/7.5 mL of blood to <5 cells/7.5 mL of blood, or a 30% decrease in CTC count from baseline at Week 12 in the absence of disease progression.

Blood samples for CTC enumeration were collected at screening, pre-dose Day 1 and 48-72 hours after Day 15 in Cycle 1, pre-dose Day 1 of each cycle up to Cycle 4, and every 8 weeks post Cycle 4. Blood samples for CTC characterization were collected at screening, pre-dose Day 1 and 48-72 hours after Day 15 in Cycle 1. CTCs were enumerated using the CellSearch® System (San Diego, CA).

Pharmacodynamics

Minimally invasive tumor biopsies (bone biopsies, soft tissue biopsy, or any other tissue that could be safely biopsied) were performed at Screening and 48-72 hours after Day 15 of Cycle 1 to assess for client protein depletion due to onalespib. Protein levels in formalin-fixed and paraffin embedded (FFPE) tissue were determined by immunohistochemistry (IHC) (Supplementary Methods). Nuclear and cytoplasmic staining intensity were semi-quantitatively assessed using the H-score formula: 3 x percentage of strongly staining nuclei + 2 x percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0 to 300 (26). Evaluation of all IHC sections was done by a pathologist blinded to the subjects' clinical characteristics and treatment data. Analysis was performed if at least 50 cancer cells were identified in the section. Ki67 results were recorded as the percentage of immunoreactive cells. Levels of AR, a relevant client in prostate cancer, were also evaluated in CTCs using an immunofluorescent assay on the CellSearch System (27).

Pharmacokinetics

Blood samples for assessment of abiraterone plasma concentrations were taken over an 8-hr period at screening at the following timepoints: pre-dose, 0.5 (±5 min), 1 (±5 min), 2 (±5 min), 3 (±5 min), 4 (±5 min), 6 (±10 min), and 8 hours (±1 hr) following AA/P administration. Blood samples for assessment of onalespib plasma concentrations were taken over an 8-hr period beginning on Cycle 1, Day 1 (C1D1)(Regimen 1) or on C1D2 (Regimen 2) at the following timepoints: pre-dose, 0.5 (±5 min), 1 (to coincide with the end of AT13387 infusion ±5 min), 2

(±5 min), 3 (±5 min), 4 (±5 min), 6 (±10 min), and 8 hours (±1 hr) from the start of infusion of onalespib. .. The pharmacokinetic profiles of abiraterone and onalespib were characterized by analysis of Lithium heparin-treated plasma by validated liquid chromatography-mass spectrometry/mass spectrometry methods, with a dynamic assay range of 0.5 - 500 ng/mL for abiraterone and of 1.0- 1000 ng/mL for onalespib. Pharmacokinetic non-compartmental analysis and statistical analyses were performed using Pharsight® Knowledgebase ServerTM (PKS) version 4.0.2 and WinNonlin® 5.3

Safety Assessments

Safety was assessed by subject-reported and investigator-observed AEs, along with concomitant medications, physical examination, clinical laboratory tests, vital signs, ECOG performance status, ECGs, and ECHO/MUGA scans. All AEs were graded according to the NCI CTCAE, v4.1. Visual symptoms were assessed by use of a Visual Assessment Questionnaire. In Part A ECGs were performed in triplicate at screening, before and at the end of infusion (+1 hr) on all treatment days for Regimens 1 and 2 in Cycle 1, then before treatment on all treatment days in subsequent cycles. Fridericia's formula was used to calculate the QTc interval throughout the study. Central ECG monitoring was used in Part A

Statistical Methods

The estimated response rate and 95% confidence intervals (CIs) were computed for each regimen using the Clopper-Pearson exact CI, if appropriate. The Fisher's exact test was also used to compare response rates for the two regimens. A p value of ≤ 0.10 was taken as evidence of a difference. The 95% CI for the difference between proportions was also computed using the normal approximation, if appropriate. PSA response rate, response rate per RECIST 1.1, and CTC count conversion rate were analyzed in a similar manner. PFS was defined the number of days from the day the subject received study medication to the date of disease progression or death. Progression was defined according to PCWG2 criteria (24). Progression-free survival for subjects who withdraw from the study without documented progressive disease (PD) by PCWG2 criteria were censored on the day of study withdrawal. PFS and overall survival (OS) were evaluated using the Kaplan-Meier estimate and summaries of the number and percentage of subjects with an event. Median time to progression, median time to death, and 95% CIs were

determined. Comparison of Regimen 1 and Regimen 2 was made by log-rank test. Subjects were included in the pharmacodynamic analyses if they had provided sufficient samples (i.e. pre- and post-treatment biopsy samples) for the pharmacodynamic tests. T-tests were used to compare continuous variables. All tests were two sided and a p-value of 0.05 or less was considered statistically significant.

Results

Patient demographics. A total of 49 subjects were randomized and 48 were treated in the dose-finding stage of the study (n = 23 in Regimen 1, and n =25 in Regimen 2). Median age was 68 years (range, 55-90 years). Demographics and baseline characteristics of subjects are provided in Table 1.

Dose Escalation and MTD. All subjects in each regimen received at least 1 cycle of study treatment at the starting dose. In Regimen 1, 3/23 subjects were dose-escalated from 220 mg/m^2 to 260 mg/m^2, and these 3 each received 1 cycle of study treatment at the higher level. In Regimen 2, 3/25 subjects were dose-escalated from 120 mg/m^2 to 160 mg/m^2, and 1 subject received 4 cycles of study treatment at the higher dose. Four subjects had DLTs in the study (2 in Regimen 1 at 260 mg/m^2; and 2 in Regimen 2 at 160 mg/m^2). All 4 DLTs were Grade 3 diarrhea, persisting despite optimal symptomatic treatment, and all were considered related to onalespib. As a result, the doses of 220 mg/m^2 in Regimen 1 and 120 mg/m^2 in Regimen 2 were considered the protocol-defined MTDs.

Adverse Events

All 48 subjects (100%) experienced at least 1 AE, and all 48 subjects experienced at least 1 treatment-emergent adverse event (TEAE). TEAEs that occurred in at least 10% of subjects and that were considered related to onalespib are shown in descending order in Table 2. Overall, related TEAEs with highest incidence were diarrhea (94%, with 21% Grade 3), fatigue (63%, with 13% Grade 3), decreased appetite (52%; none were Grade 3), and nausea (50%; with 2% Grade 3). No shifts ≥2 CTCAE Grades in QTc from baseline were observed in the study. Three related TEAE were reported as Grade 4 (increased amylase in Regimen 1; thrombocytopenia and increased amylase in Regimen 2).

Fifteen subjects (31.3%) experienced SAEs during the study; 2 subjects (4.2%) died on study as a result of an SAE, but the SAEs were considered not related to study treatment. Seven other subjects (14.6%) had an SAE considered related to study treatment; these were Grade 3 diarrhea (n=4), Grade 3 dehydration (n=1), and Grade 2 left chest pain (n=1).

Pharmacodynamics

The pharmacodynamic signature for HSP90 inhibition has been established and used in previous clinical trials (22, 28, 29). This signature includes depletion of client proteins such as CDK4, and CRAF, and elevation of certain heat shock proteins (eg, HSP72, the inducible isoform of HSP70) (22, 28, 30). In the current study CTCs and tumor biopsies were collected to examine pharmacodynamic biomarkers to pursue the Pharmacological Audit Trail (PhAT) (31, 32). HSP90 target engagement was evaluated by measuring the degree of depletion of protein clients relevant to prostate cancer (AR, GR) and the induction of HSP72 protein in paired pre- and post-treatment tumor tissue biopsies. Achievement of biological activity was evaluated by measuring the proliferation biomarker Ki67 and the apoptotic biomarker cleaved caspase 3. Levels of AR were also evaluated in CTCs using an immunofluorescence assay on the CellSearch System (27).

Paired tumor tissue biopsies were collected from 7 subjects. One of the matched biopsies contained <50 cancer cells, resulting in a total of 6 paired pre- and post- dose biopsies available for comparison (as described in 34). AR protein expression was evaluated using an antibody against the N-terminus domain (AR-NTD) binding the AR-FL and AR variants; and an antibody directed against the C-terminus (AR-CTD), specific for the AR-FL. The AR-NTD was highly expressed in CRPC biopsies taken before starting onalespib treatment both in the nucleus and in the cytoplasm. Comparison of paired biopsies obtained pre- (Screening) and post-dose (Cycle 1 Day 17) demonstrated a reduction in nuclear AR-NTD in 5/6 patients (Figure 1A) (paired t-test p= 0.01). AR-CTD was also reduced in 3/6 patients (Figure 1B). Cytoplasmic GR was also depleted following onalespib treatment (paired t-test p= 0.003) (Figure 1C). HSP72 was significantly up-regulated following onalespib treatment in keeping with HSP90 inhibition (paired t-test p= 0.005) (Figure 1D). Changes in tumor cell proliferation following onalespib treatment were evaluated by measuring the proliferation marker Ki67 in paired tumor tissue biopsies. Overall no significant changes were observed (data not shown). Increases in apoptosis

following onalespib treatment were evaluated by measuring the apoptotic biomarker cleaved caspase 3. Overall no significant changes were observed (data not shown). Representative images of IHC staining are shown for one subject in Figure 1E.

Thirty-two patients had samples collected before starting treatment (at screening and/or C1D1 pre-dose) and on C1D17. Sixteen subjects had at least 5 CTCs/7.5 mL in the baseline sample, and AR results available at baseline and on-treatment and were included in the analysis. Three of 7 subjects (42%) that received weekly onalespib (Regimen 1) had a decline in proportion of AR positive CTCs >30% on-treatment. Four of 9 subjects (44%) that received twice-weekly onalespib had a >30% decline on-treatment (C1D17) (Figure 2).

Pharmacokinetics

PK evaluations were performed on plasma samples obtained from 44 subjects (n=20 for onalespib 120 mg/m^2/dose [Regimen 2], n=2 for 160 mg/m^2/dose [Regimen 2], n=18 for 220 mg/m^2/dose [Regimen 1], and n=3 for 260 mg/m^2/dose [Regimen 1]). Onalespib showed a biphasic decline with a two-compartmental disposition. Onalespib exposures increased in a dose-proportional manner and were similar to those observed in the single agent Phase 1 dose escalation study (29), suggesting no PK interaction. The PK profile of onalespib appeared reproducible and similar between Regimens 1 and 2, with moderate inter-individual variability across all dose levels in each regimen. Onalespib mean $AUC_{0\text{-}t}$ and C_{max} estimates ranged from 1480 to 3550 ng*hr/mL, and from 636 to 1850 ng/mL, respectively.

The exposures of abiraterone generally decreased when abiraterone was administered in combination with onalespib, although due to PK variability of AA the significance of these findings are uncertain since no direct metabolic drug-drug interactions was expected, although this could be due to reduced gastrointestinal transit time as a result of onalespib AEs (diarrhea) observed on onalespib dosing days.

Efficacy

Overall, no subjects in either regimen showed an objective tumor response according to RECIST v1.1, and likewise no subjects in either regimen showed a response in PSA (ie, no response in PSA ≥50% decrease at Week 12 per PCWG2). Eight subjects (35%) in Regimen 1 and 7 subjects

(28%) in Regimen 2 had a conversion of CTCs from ≥5 cells/7.5 mL of blood to <5 cells/7.5 mL or a decrease of more than 30% from the baseline CTC value on-treatment (Figure 3). However, these declines were transient, and CTC values increased rapidly after treatment. Maximum percentage changes in PSA and CTCs by regimens are shown in Figure 3A and 3B, respectively.

The median PFS in Regimen 1 (n=23) was 77 days (95% CI: (71.0, 83.0)) and in Regimen 2 (n=24) was 84 days (95% CI: (77.0, 158.0). PFS was not different between the regimens (p>0.23). The median OS in Regimen 1 (n=23) was 10.6 months (95% CI: (3.8, NA)) and in Regimen 2 (n=24) was 8.9 months (95% CI: 4.8, NA)). Median OS was not different between the regimens (p=0.52).

DISCUSSION

Despite the promise of HSP90 inhibitors as a class of therapeutic agents, these agents have, to date, shown variable results in clinical trials. Encouraging single-agent activity has been seen in specific molecular backgrounds, e.g., HER2 amplification in breast cancer and EGFR mutations and ALK rearrangements in NSCLC, suggesting that client protein sensitivity to HSP90 inhibition is likely to be a key contributor to HSP90 inhibitor success (13). In other cancer types HSP90 inhibition has been disappointing, despite the fact that the oncogenic constituents of such cancers are among HSP90's clientele. In general, to date, single-agent activity of HSP90 inhibitors has not been adequate to justify market approval. Few combination trials of HSP90 inhibitors have been conducted.

This study represents the first clinical trial of an HSP90 inhibitor in combination with AA/P in patients with metastatic CRPC. HSP90 treatment results in depletion of AR-FL and AR-V7, as well as GR, in preclinical models, hence treatment with an HSP90 inhibitor such as onalespib was hypothesized to address resistance to agents such as AA or enzalutamide that act via the AR-FL (27). Resistance to these agents may be mediated by AR alterations, including the expression of AR-V7 splice variant (5, 6). This study documented progression on AA/P treatment as a condition for study entry and explored the ability of HSP90 inhibition to overcome resistance to AA/P.

The combination of the HSP90 inhibitor with AA appeared to be tolerated at onalespib doses below the single-agent MTD (220 mg/m^2 weekly vs 260 mg/m^2 for the single agent weekly regimen (10, 29); 120 mg/m^2 vs 160 mg/m^2 for the twice weekly on two consecutive day regimen (11) with gastrointestinal toxicity in this population, most notably diarrhea being dose limiting. AA/P has a 5% reported incidence of diarrhea, but despite the reported single-agent MTDs, many subjects were unable to complete three full dosing cycles due to either AEs, study withdrawal, or disease progression. This may reflect additive toxicity, and perhaps the study of a more elderly population than studied in onalespib Phase 1 trials, or both.

Pharmacodynamic studies in tumor biopsies collected 24-48 hours post-dose, although limited in numbers, demonstrated a significant induction of HSP72; this was, however, accompanied by only a modest depletion of AR and GR and no changes in tumor proliferation or apoptosis. CTC studies also demonstrated a transient decline in AR-positive CTCs in some patients. This lack of clinical activity accompanied by lack of sustained HSP90 client protein depletion did not justify the further evaluation of onalespib in combination with AA/P at the doses and schedule we evaluated. One possible explanation for the lack of activity in our trial is that the tolerable regimens did not achieve a sustained and durable effect on AR. However, the need for IV infusions and the toxicity reported prevented us from exploring more aggressive onalespib dosing schedules. Preclinical studies, both in vitro and in vivo, have shown that onalespib causes prolonged depletion of client proteins in tumor xenografts (33), but this may not be the case in mCRPC patients. In this study we were unable to demonstrate more than modest depletion of client protein 48 hours after treatment administration.

Induction of HSP72 has been widely used as a biomarker to monitor the pharmacodynamic impact of HSP90 inhibitors. However, increasing evidence suggests that HSP72 induction, while necessary, is not itself sufficient to predict response to these agents. For example, in clinical trials of 17-DMAG, HSP72 levels measured in PBMCs showed no correlation with clinical response (35,36). Direct evaluation of the client and modulation of the pathway(s) of interest coupled with the evaluation of downstream biological effects (eg, increase in apoptosis, reduction in proliferation), as performed in this study, are critical for evaluation of antitumor effects and to optimally develop HSP90 inhibitor therapy.

Preclinical studies have revealed a number of mechanisms whereby cancer cells can be rendered less susceptible to the effects of HSP90 inhibition. Firstly, HSP90 inhibition elicits both the depletion of client proteins and the activation of a heat shock response mediated by HSF1. As a result of HSF1 activation, expression of inducible HSPs (e.g. HSP72, HSP27) is dramatically upregulated, and is often used as a readout of HSP90 target engagement. However, these pro-survival chaperones may limit the anti-tumour activity of HSP90 inhibitors and may play a role in resistance to HSP90 inhibition, rescuing client proteins including AR and its splice variants from degradation. Inhibition of HSF1, HSP70 or HSP27 has been reported to increase cell sensitivity to HSP90 inhibition and induction of apoptosis and may be key to more robust AR blockade (37-40).

In this study, we observed induction of HSP72, confirming target engagement, and some depletion of AR and GR post-onalespib treatment, but evidence of residual nuclear AR coupled with no changes in Ki67 or cleaved caspase 3 suggested that the degree of HSP90 inhibition was not sufficient to translate into an effective blockade of AR signaling pathway and a reduction in cancer cell proliferation and increased apoptosis. In summary, for both the weekly or twice weekly regimens of onalespib in combination with AA/P, there was mild evidence of some biological effect, however this effect did not translate into clinical activity, hence further exploration of this combination is not justified.

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Table 1. Demographics and Baseline Characteristics

Demographic Characteristics		Regimen 1 (N=23)		Regimen 2 (N=25)		Total (N=48)	
Age (yr)	n	23 (100%)		25 (100%)		48 (100%)	
	Mean	71.3		68.0		69.5	
	Min.	57		55		55	
	Max.	90		84		90	
ECOG PS (n, %)	n	23	(100)	25	(100)	48	(100)
	0	7	(30)	12	(48)	19	(40)
	1	14	(61)	12	(48)	26	(54)
	2	2	(9)	1	(4)	3	(6)
	3	0		0		0	
Prior Chemotherapy/Immunotherapy	n (%)	18	(78)	20	(80)	38	(79)
Prior Cancer Surgery	n (%)	8	(35)	11	(44)	19	(40)
Prior Hormone Therapy[a]	n (%)	23	(100)	24	(96)	47	(98)
Prior Radiation Therapy	n (%)	18	(78)	17	(68)	35	(73)
Baseline Gleason Score	n	20		23		43	
	<7	2	(9)	2	(8)	4	(8)
	=7	6	(26)	3	(12)	9	(19)
	>7	12	(52)	18	(72)	30	(63)

[a] Does not include abiraterone.

Table 2. Treatment-Emergent Adverse Events Considered Related to Onalespib (≥10% of Total Subjects)

			Regimen 1 (n, %)				Regimen 2 (n, %)			
Adverse Event Preferred Term	**Overall Total N=48**		**220 mg/m^2 n=20**		**260 mg/m^2 n=3**		**120 mg/m^2 n=22**		**160 mg/m^2 n=3**	
	All	**Grade 3,4**	**All**	**Grade 3-, 4**	**All**	**Grade 3, 4**	**All**	**Grade 3, 4**	**All**	**Grade 3- 4**
Diarrhea	45 (94)	10 (21)	19 (95)	3 (15)	2 (67)	2 (67)	21 (96)	3 (14)	3 (100)	2 (67)
Fatigue	30 (63)	6 (13)	15 (75)	1 (5)	0	0	14 (64)	5 (23)	1 (33)	0
Decreased Appetite	25 (52)	0	10 (50)	0	1 (33)	0	11 (50)	0	3 (100)	0
Nausea	24 (50)	1 (2)	9 (45)	0	1 (33)	0	13 (59)	1 (5)	1 (33)	1 (4)
Vomiting	15 (31)	0	5 (25)	0	0	0	10 (46)	0	0	0
Dry Mouth	10 (21)	0	5 (25)	0	0	0	4 (18)	0	1 (33)	0
Dizziness	8 (17)	0	3 (15)	0	0	0	5 (23)	0	0	0
Infusion Site Pain	7 (15)	0	4 (20)	0	0	0	2 (9)	0	1 (33)	0
Anemia	7 (15)	5 (10)	3 (15)	1 (5)	0	0	4 (18)	4 (18)	0	0
Headache	6 (13)	0	3 (15)	0	0	0	3 (14)	0	0	0
Weight Decreased	6 (13)	0	2 (10)	0	0	0	3 (14)	0	1 (33)	0
Dysgeusia	6 (13)	0	3 (15)	0	0	0	3 (14)	0	0	0
Dry Eye	5 (10)	0	2 (10)	0	0	0	3 (14)	0	0	0
Photopsia	5 (10)	1 (2)	2 (10)	0	0	0	3 (14)	1 (5)	0	0
Insomnia	5 (10)	0	2 (10)	0	0	0	3 (14)	0	0	0

Figure Legends

Figure 1. Changes in **(A)** nuclear AR-NTD, **(B)** nuclear AR-CTD, **(C)** GR and **(D)** HSP72 expression in paired fresh tumor tissue biopsies obtained pre- and post- onalespib treatment. Immunostaining was assessed using an H-score, calculated by multiplying each intensity level (0, for absent, 1 for weak, 2 for moderate, and 3 for intense stain) by the corresponding percentage of positive cancer cells; **(E)** Representative images of AR, GR, Ki67 and HSP72 immunohistochemistry staining in paired fresh tumor tissue biopsies (lymph node biopsy) taken pre-dose Cycle 1 Day 1 and 48 hours post-dose on Cycle 1 Day 17. Micrographs show AR-NTD, HSP72, Ki67, and GR expression by DAB immunohistochemistry method.

Figure 2. **(A)** Waterfall plot showing maximal changes in the percentage of AR positive CTCs in individual subject with ≥5 CTCs/7.5 mL at baseline. Cycle 1 Day 17 sample was compared with Cycle 1 Day 1. When Cycle 1 Day 1 sample was not available, post-dose sample was compared with the screening sample. **(B)** Representative images of AR expression in individual CTCs from two patients at screening, Cycle 1 Day 1 and Cycle 1 Day 7. CTCs were isolated and detected on the CellSearch platform. The enriched cells were stained by immunofluorescence using antibodies specific for Cytokeratin (keratin 8, 18 and 19) conjugated to Phycoerythrin (CK-PE), anti-CD45 conjugated to Allophycocyanin (CD45-APC), the nucleic acid dye 4′,6-diamidino-2-phenylindole (DAPI) and an AlexaFluor 488-conjugated rabbit monoclonal antibody directed against the amino-terminus domain of the AR as previously described (27) .

Figure 3. (A) Waterfall plot showing changes in total number of circulating tumor cells by subject. Increases were capped at 100%; **(B)** Maximum percentage change in prostate specific antigen by subject. Increases were capped at 100%.

Figure 1

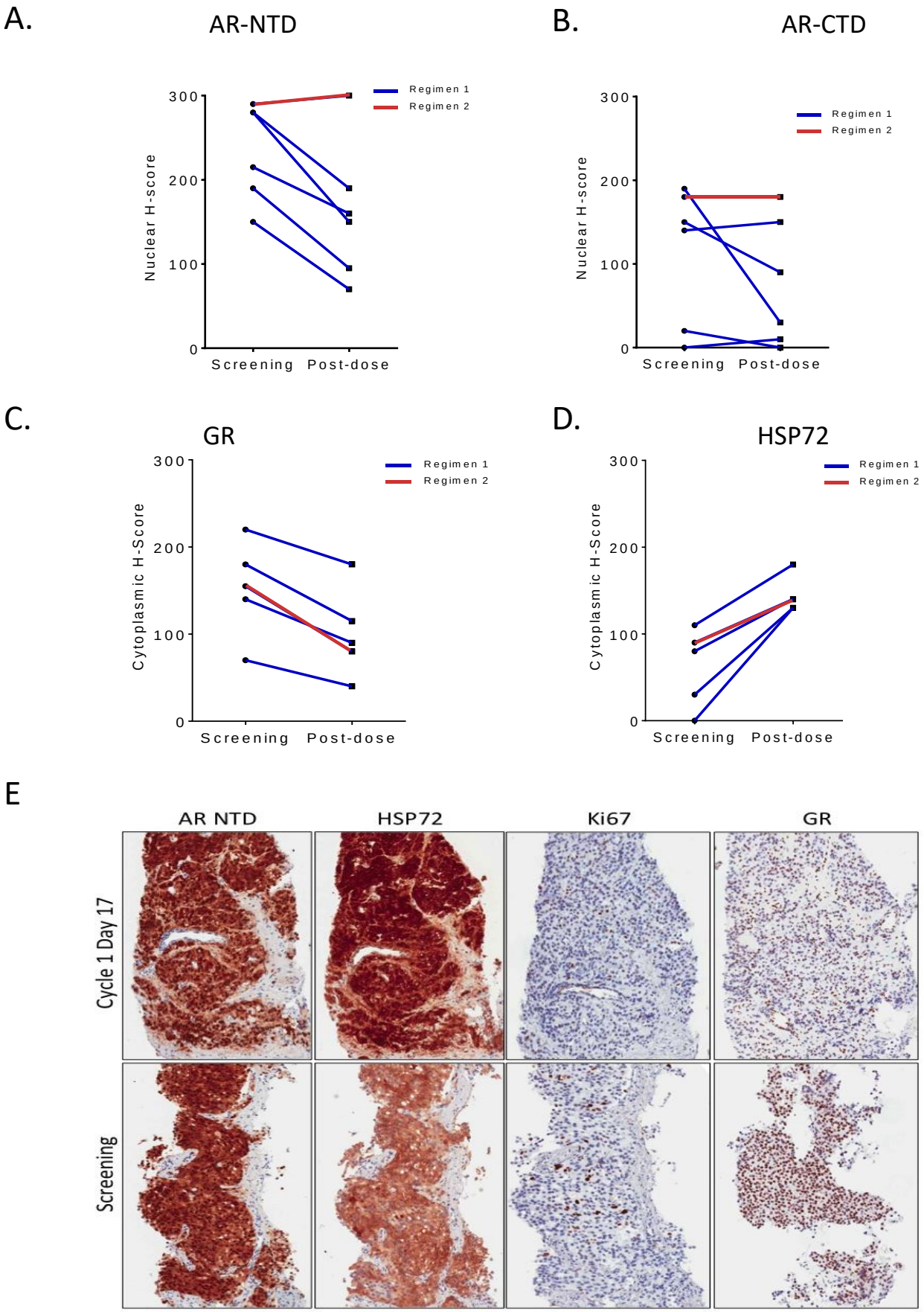
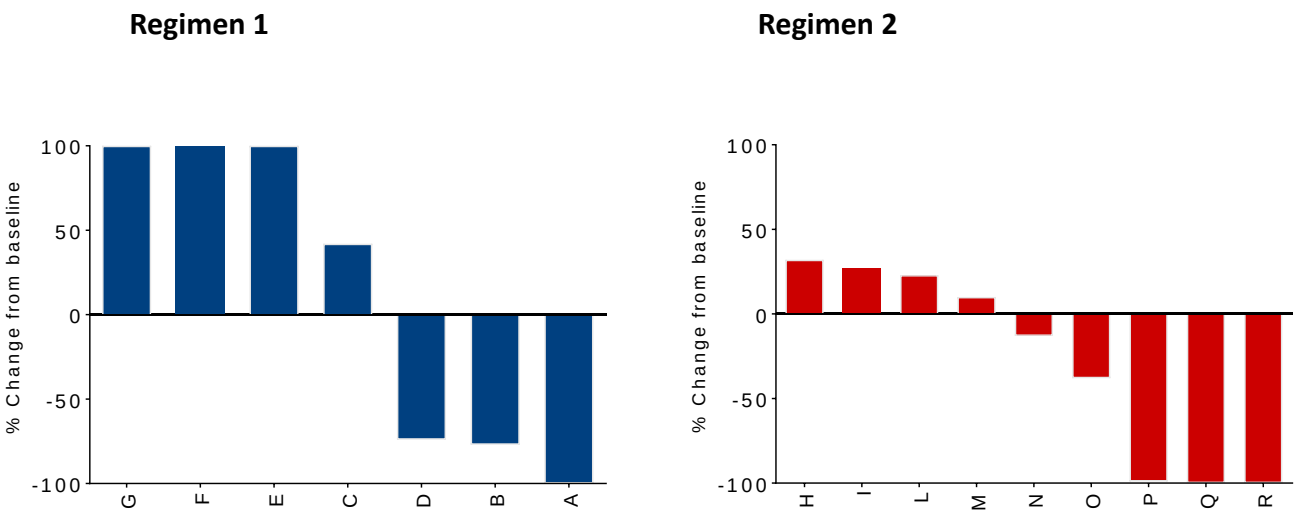


Figure 2

A



B

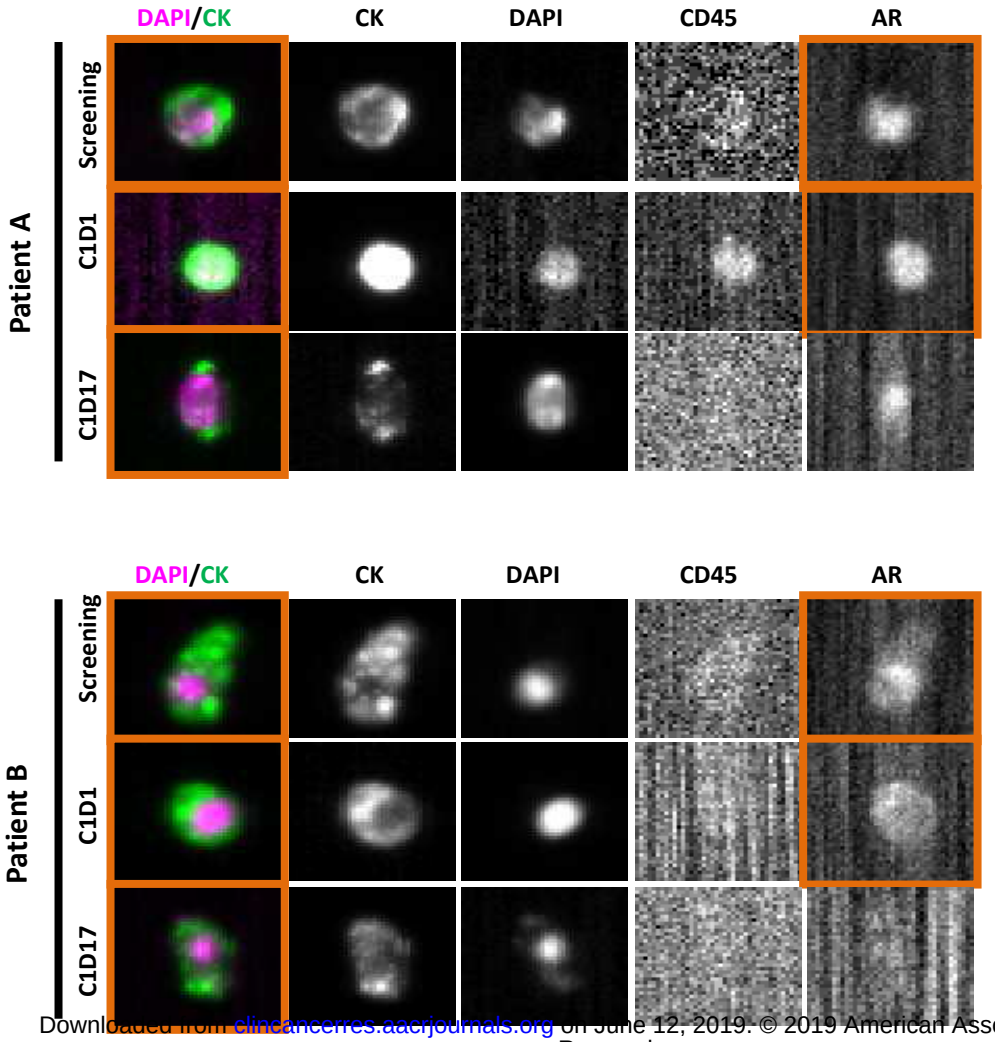
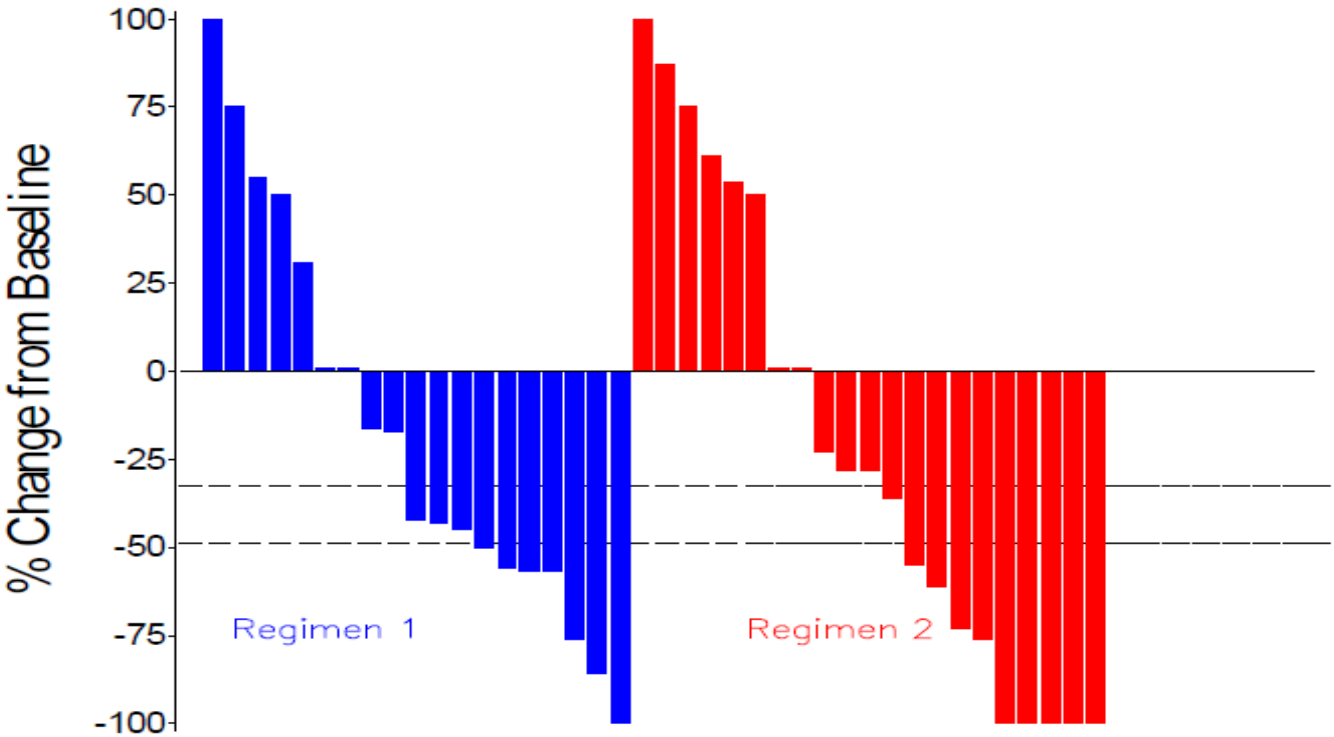
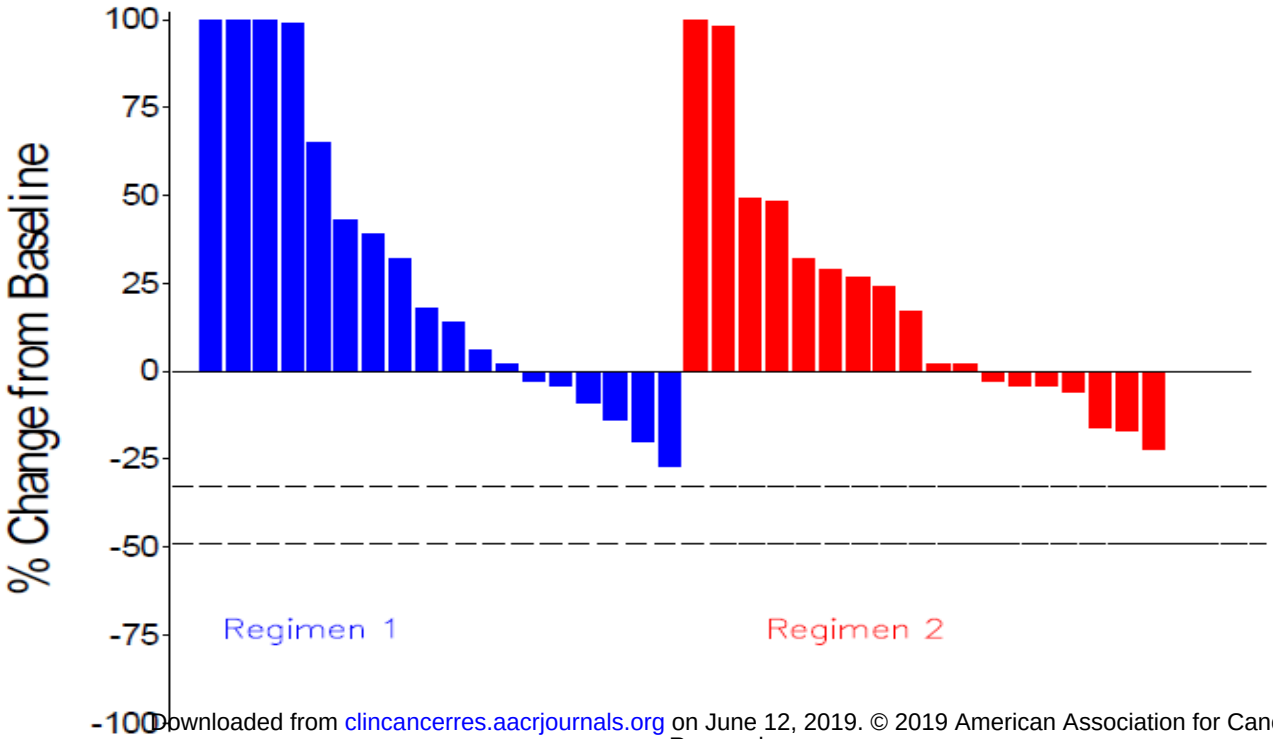


Figure 3

A. Maximal change in circulating tumor cells count



B. Maximal PSA change



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