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RESEARCH PAPER

SRF inhibitors reduce prostate cancer cell proliferation through cell cycle arrest in an isogenic model of castrate-resistant prostate cancer

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ABSTRACT

Castrate-resistant prostate cancer (CRPC) is challenging to treat, despite improvements with next-generation anti-androgens such as enzalutamide, due to acquired resistance. One of the mechanisms of such resistance includes aberrant activation of co-factors of the androgen receptor (AR), such as the serum response factor (SRF), which was associated with prostate cancer progression and resistance to enzalutamide. Here, we show that inhibition of SRF with three small molecules (CCG-1423, CCG-257081 and lestaurtinib), singly and in combination with enzalutamide, reduces cell viability in an isogenic model of CRPC. The effects of these inhibitors on the cell cycle, singly and in combination with enzalutamide, were assessed with western blotting, flow cytometry and β-galactosidase staining. In the androgen deprivation-sensitive LNCaP parental cell line, a synergistic effect between enzalutamide and all three inhibitors was demonstrated, while the androgen deprivation-resistant LNCaP Abl cells showed synergy only with the lestaurtinib and enzalutamide combination, suggesting a different mechanism of action of the CCG series of compounds in the absence and presence of androgens. Through analysis of cell cycle checkpoint proteins, flow cytometry and β-galactosidase staining, we showed that all three SRF inhibitors, singly and in combination with enzalutamide, induced cell cycle arrest and decreased S phase. While CCG-1423 had a more pronounced effect on the expression of cell cycle checkpoint proteins, CCG-257081 and lestaurtinib decreased proliferation also through induction of cellular senescence. In conclusion, we show that inhibition of an AR co-factors, namely SRF, provides a promising approach to overcoming resistance to AR inhibitors currently used in the clinic.

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1. Introduction

The year of 2021 marked the 80th anniversary from the use of androgen deprivation therapy (ADT) for the treatment of metastatic prostate cancer (PCa), which greatly improved the overall survival of patients with PCa [1]. Patients with metastatic PCa and those who relapse after localized therapy (surgery and/or radiation) are treated with ADT, including second-generation anti-androgens such as enzalutamide and abiraterone acetate, which target the androgen receptor (AR) and androgens biosynthesis, respectively. Moreover, the combination of ADT with these second-generation drugs has been recently recommended as a new standard treatment for patients with high-risk non-metastatic PCa [2]. However, although ADT is initially effective, singly and/or in combination with radiotherapy [3], the majority of patients become resistant to this treatment, developing castrate resistant prostate cancer (CRPC), a more aggressive form of the disease, where the cancer cells can survive with very low levels of testosterone. Among the many possible mechanisms of resistance to ADT and development of CRPC, a key route involves the co-regulators of AR which indirectly affect its transcriptional activity [4,5]. Therefore, an attractive therapeutic approach to overcome resistance to targeting of AR, would be to target these co-regulators.

Our group and others have identified the serum response factor (SRF) as a co-regulator of AR that is important in PCa development and progression [6–10]. SRF is a transcription factor involved in the regulation of a myriad of cellular mechanisms, including cytoskeleton organization, cell proliferation and

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cell cycle regulation, which are key contributors to cancer hallmarks [11]. Previously, we discovered that inhibition of SRF with the small-molecule compound CCG-1423, reduced cell viability in LNCaP Abl cells, an *in vitro* model of CRPC [10]. Importantly, transient knockdown of SRF leads to increased AR transcriptional activity following androgen stimulation in LNCaP Abl cells, suggesting a possible crosstalk between the SRF and AR pathways [10]. In addition to this, protein kinase N1 (PKN1), an effector of RhoA signaling, mediates the transcriptional activity of SRF during androgen stimulation *in vitro* and *in vivo* [12], further dissecting the potential cofactors that drive the SRF/AR crosstalk. We also showed that inhibiting SRF with CCG-1423 prevented AR translocation to the nucleus *in vitro* and *in vivo* and that elevated SRF expression in patients with CRPC was associated with resistance to enzalutamide [13]. Taken together, these studies suggest that SRF is an ideal candidate for therapeutic intervention, potentially overcoming resistance to AR targeting in CRPC.

The aim of this study was to test three SRF inhibitors, singly and in combination with enzalutamide, as possible therapeutics in PCa. The effect of SRF-inhibition on cell viability and proliferation, singly and in combination with enzalutamide, was analyzed in an isogenic pair of PCa cell lines [14], taken as a cellular model of androgen-dependent/castrate-resistant PCa. To inhibit SRF transcriptional activity, three small-molecule inhibitors were used: CCG-1423, CCG-257081 and the multi-kinase inhibitor lestaurtinib. The CCG-1423 and CCG-257081 compounds inhibit SRF by disrupting its transcriptional activity through inhibition of the SRF essential co-factor MRTF [15,16]. CCG-1423 was the first compound of this family to be synthesized. CCG-257081 works through the same mechanism of CCG-1423 but has improved pharmacokinetics properties [17]. Lestaurtinib is a multi-kinase inhibitor which conveys androgen responsiveness to SRF [12] and was already under clinical investigation for the treatment of acute myeloid leukemia [18], prostate cancer [19] and acute lymphoblastic leukemia [20].

Here, we show that SRF inhibition, through two classes of compounds with different mechanisms of action, reduces cancer cell proliferation through cell cycle arrest and is more effective than enzalutamide in CRPC.

2. Methods

2.1 Cell culture

The LNCaP parental cells were obtained from American Type Culture Collection (ATCC) and cultured in Advanced RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 µL/mL streptomycin, 100 U/mL penicillin and 1% Hepes. LNCaP Abl cells were generated from the parental cell line as previously described [14] and cultured in Advanced RPMI-1640 medium supplemented with 10% charcoal-stripped FBS, 100 µL/mL streptomycin, 100 U/mL penicillin and 1% Hepes. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium Bromide (MTT) cell viability assay

Cell viability was assessed using MTT assay as previously described [13]. LNCaP Parental and Abl cells were treated with either the vehicle (DMSO), increasing concentrations of CCG-257081 (kindly donated by Dr Erika Lisabeth, Michigan University) or increasing concentrations of lestaurtinib (MedChem Express) singly or in combination with increasing concentrations of enzalutamide (MedChem Express). Cells were treated for 5 d, followed by MTT analysis as previously described [21]. IC_{50} values were calculated using GraphPad Prism 7 program (GraphPad, San Diego, California). Combination Index (CI) values were evaluated by the Chou-Talalay method [22] on the Compusyn software (ComboSyn Inc., Paramus, NJ, USA) where CI values between 0.7 and 0.9 represent synergy, values between 0.9 and 1.1 represent an additive effect and greater than 1.11 represent an antagonistic effect. Statistical analysis was carried out using one-way Anova followed by Tukey's test.

2.3 Western blotting analysis

Cells were seeded at a density of 3.5×10^5 per well in 6-well plates (Sigma-Aldrich, Ireland) and treated with IC_{50} values of either enzalutamide, CCG-1423, CCG-257081, or lestaurtinib, singly or in combination for 48 h. Whole-cell lysates were extracted using a cocktail of 1:100 RIPA buffer (Sigma-Aldrich) and

protease and phosphate inhibitors (Cell Signaling Technology, Danvers, Massachusetts, USA). Western blots were carried out as previously described [10]. The following antibodies were used to detect cell cycle checkpoint protein expression: Retinoblastoma (1:2000, Cell Signaling Technology, #9309), phosphorylated Retinoblastoma (1:1000, Cell Signaling Technology, #9301), Cyclin D3 (1:1000, Cell Signaling Technology, #2936), Cyclin E1 (1:2000, Cell Signaling Technology, #4129), CDK2 (1:500, Cell Signaling Technology, #2546), CDK4 (1:500, Cell Signaling Technology, #12790), CDK6 (1:500, Cell Signaling Technology, #13331) and GAPDH (1:5000, Millipore, Massachusetts, USA, #MAB374). HRP-Chemiluminescence (EMD Millipore Corporation, Burlington, MA, USA) was used to detect antibody-antigen complexes on the membrane. Images were taken using the Amersham Imager 600. Densitometric analysis of the protein bands was performed using the ImageJ software. Statistical significance to quantify the differences in the mean values between groups and One Way Anova, followed by Tukey's test, was performed with GraphPad Prism 7. P-value <0.05 was considered significant.

2.4 Flow cytometry

Cells were seeded at a density of 3×10^5 per well in six well plates. Prior to treatment, cells were serum starved for 4 h in serum-free advanced RPMI media to synchronize their cell cycle. Cells were treated with IC_{50} values of either enzalutamide, CCG-1423 and lestaurtinib, singly or in combination for 48 h. Cells were then labeled with 10 µM 5-ethynyl-2′-deoxyuridine (EdU) and incubated in the dark for 1 h. Following incubation, cells were fixed and prepared as stated by the manufacturer (Edu Alexa Flour azide 647 kit by ThermoFisher). DNA was counterstained using 2 µg/µL DAPI (Invitrogen, Life Technologies Corporation, USA) for 15 min. Cell cycle analysis was performed using the CytoFlex LX flow cytometry machine by collecting at least 15,000 forward scatter gated events for sample (Beckman Coulter, California, USA). Data were analyzed using the CytoFlex software.

2.5 Senescence analysis

Fifty thousand cells were seeded per well in six well plates. Cells were treated with IC_{50} values of CCG-1423, CCG-257081 and lestaurtinib, singly and in combination with enzalutamide for 48 h. Treatment with 10 µM H_20_2 was used as a positive control. After treatment, the cells were stained with X-galactosidase using the Senescence detection kit (AssayGenie #BN00587, Dublin, Ireland) as per the manufacturer's protocol. A negative control using non-treated Abl cells was treated with staining solution void of the X-Gal compound. The cells were then incubated in a 37°C non-CO_2 incubator, protected from light for 24 h. Post incubation, wells were washed with 2 mL of DPBS and imaged using an Olympus EP50 Microscope. Wells were divided into quadrants, with each quadrant being imaged to obtain an overview of the whole well. Images were taken at 10X magnification. The total number of senescent cells was counted in each quadrant and expressed as a percentage of total cells. Counting was manually carried out by two independent researchers (SC and SM). Statistical significance was calculated using One-Way ANOVA (Tukey's multiple comparisons test). P-value <0.05 was considered significant.

3. Results

3.1 Concomitant inhibition of SRF (with CCG-1423, CCG-257081 and lestaurtinib) and AR (with enzalutamide) results in decreased cell viability in the isogenic cell line pair LNCaP parental and Abl

We have previously shown that combined inhibition of SRF and AR with CCG-1423 and enzalutamide in an isogenic model of CRPC, namely LNCaP Parental (ADT sensitive) and LNCaP Abl (castrate-resistant) cells, resulted in decreased cell viability. Specifically, the half-maximal inhibitory concentration (IC_{50}) values of enzalutamide were significantly decreased when combined with increasing concentrations of CCG-1423 [13]. Here, the LNCaP parental/Abl isogenic cell line pair was treated with three SRF-inhibitors (CCG-1423, CCG-27081 and lestaurtinib) for 5 d, singly and in combination

with enzalutamide. Cell viability was assessed with MTT assays as described in the methods. All three SRF inhibitors showed to be effective as single treatments in reducing cell viability in both cell lines. For the LNCaP parental cell line, SRF inhibition with CCG-1423 and CCG-257081 showed similar IC_{50} values to enzalutamide (IC_{50} values: 8.8 ± 3.4 μM for enzalutamide vs. 13.4 ± 2.5 μM for CCG-1423 and 7.2 ± 1.1 μM for CCG-257081), while lestaurtinib showed a significantly lower IC_{50} of 0.1 ± 0.02 μM. For the LNCaP Abl cell line, which is resistant to ADT, the IC_{50} values of the SRF inhibitors were significantly lower than the enzalutamide IC_{50}, with lestaurtinib still showing the lowest IC_{50} (Enzalutamide: 26.3 ± 6.9 μM, CCG-1423: 9.9 ± 2.2 μM, CCG-257081: 3 ± 1.1 μM, lestaurtinib 0.1 ± 0.1 μM) (Figure 1, panel A, and Table 1).

Combinations of enzalutamide with either CCG-257081 (Figure 1, panel B) or lestaurtinib (Figure 1, panel C) showed decreased IC_{50} values in both cell lines when treated with both combinations, which was in line with what we have previously shown for CCG-1423 in combination with enzalutamide [13].

To further understand whether this decrease in IC_{50} values in the combination treatments was due to an additive or synergistic effect, we analyzed the combination index (CI) values using the Compusyn Inc Software [22]. This analysis showed a synergistic effect (with some additive effect for certain concentrations) for all three combinations in LNCaP parental cells (Figure 2, panel A). No synergy was observed in LNCaP Abl cells for CCG-1423 and CCG-257081, while synergy between enzalutamide and lestaurtinib was also shown in this cell line (Figure 2, panel B).

3.2 SRF inhibition, singly or combined with enzalutamide, alters the expression of G1/S-phase cell cycle proteins

Next, we investigated the possible molecular mechanisms that are affected following inhibition of SRF and AR. SRF and androgens play an important role in the G1/S phase transition of the cell cycle [23–25]. For example, it was previously shown that knockdown of SRF in hepatocellular carcinoma resulted in lowered E2F1, cyclinD2 and CyclinE2 at the mRNA and protein level [26]. Furthermore, SRF downregulation in the esophageal squamous cell line Eca-109 resulted in cell cycle arrest in the G1 phase with lowered CyclinD1 levels [27]. In addition to this, CCG-1423 treatment in PC3 cell line led to reduced E2F1 and CyclinE2 protein levels [24], suggesting that G1/S-phase cell cycle proteins' expression is affected by SRF inhibition. Moreover, the mitogenic action of androgens is partially due to their ability to induce the expression of cyclin D1 which, in complex with CDK4/6, phosphorylates retinoblastoma (Rb), a protein involved in the G1/S phase transition of the cell cycle, of which AR is a master regulator [25].

With these studies in mind, we analyzed the expression of key proteins involved in the G1/S phase transition of the cell cycle (summarized in Figure 3, panel A). Following 48-h treatment of the cells with IC_{50} values of CCG-1423, CCG-257081 and lestaurtinib, singly and in combination with enzalutamide, Western Blotting (WB) analysis of the following proteins was performed: retinoblastoma (Rb) and phosphorylated Rb (pRB), cyclin D3/E1(cycD, cycE1), cyclin-dependent kinases 2, 4 and 6 (CDK2, CDK4 and CDK6), p21 and p27.

In general, we observed more changes in these cell cycle proteins, in response to treatments, in the LNCaP parental cells in comparison with the Abl cells. Among the three SRF inhibitors, CCG-1423 seemed to have a more pronounced effect on the expression of cell cycle proteins, which was similar in the two cell lines (Figure 3, panels B-Figure 3C), whereas CCG-257081 showed only a modest effect in the parental cells (Figure 3, panel D) and virtually no effect in the Abl cells (Figure 3, panel E). Lestaurtinib had a modest effect in both cell lines in altering the expression of cell cycle proteins involved in the G1/S transition (Figure 3, panels F-G). In both cell lines, while no change in the phosphorylation of Rb was detected, a significant decrease in Rb expression was observed following treatment with all three inhibitors, singly and in combination with enzalutamide, with the exception of CCG-257081 in the Abl cells (Figure 3 panels B-G). While a modest increase in the expression of p21 (a cyclin-dependent kinase inhibitor, which inhibits cell

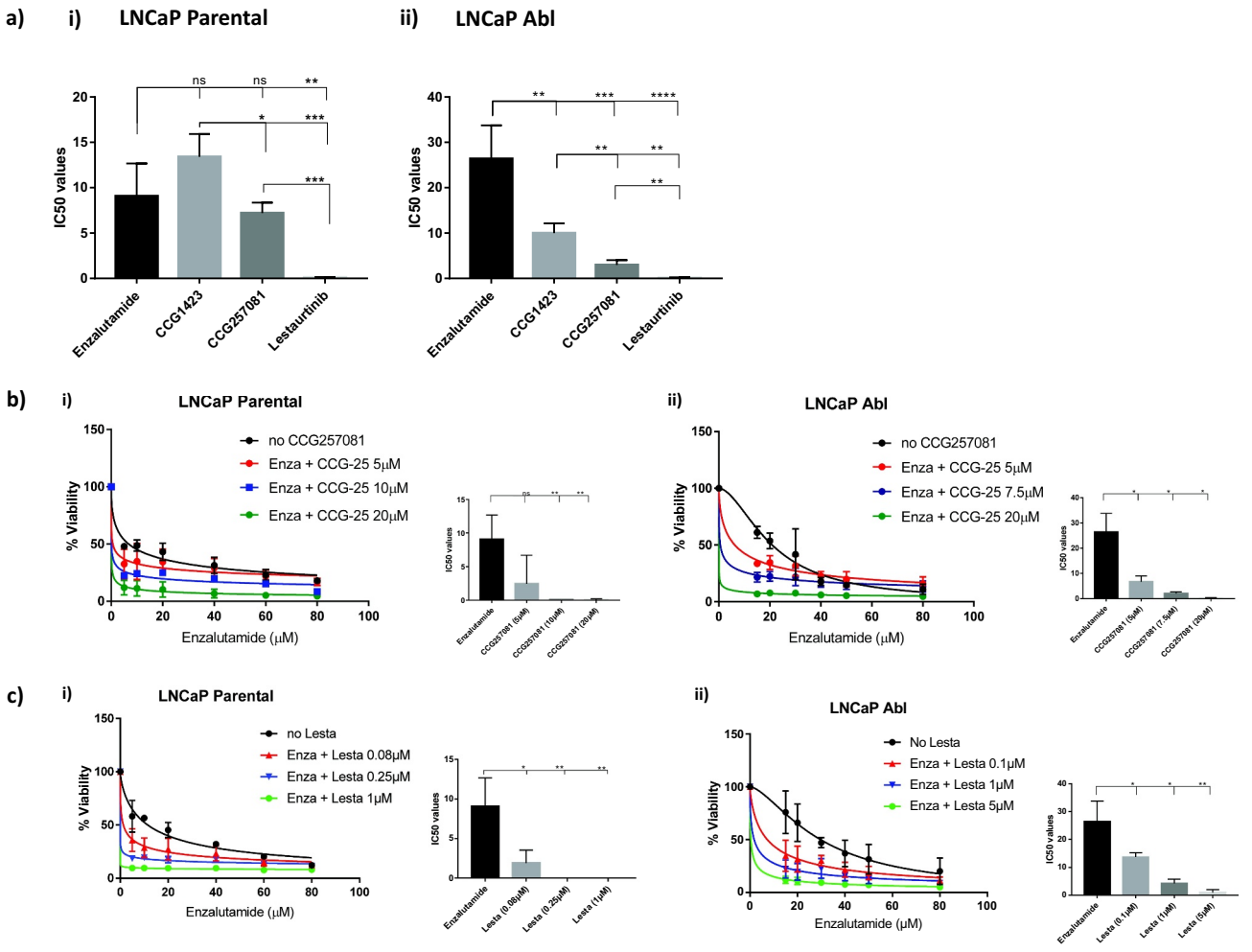


Figure 1. Inhibition of SRF with CCG-1423, CCG-257081 and lestaurtinib, singly and in combination with enzalutamide, results in decreased cell viability in the isogenic cell line pair LNCaP parental and Abl. Cells were seeded in 96 well plates at a cell density of 3 x 10^6 per well. Cells were treated the next day with either the vehicle (DMSO) or increasing concentrations of either enzalutamide, CCG-1423, CCG-257081 or lestaurtinib, singly or in combination, and incubated for 5 d. Cell viability was measured using MTT assay and the IC_{50} values of the inhibitors were calculated using the GraphPad Prism 7 program. Bar charts represent the average of IC_{50} values obtained from at least three independent experiments in triplicate ± standard deviation (SD). Only three concentrations are shown in panel B and C for better visualisation of results. Panel A: i): LNCaP parental and ii): LNCaP Abl. IC_{50} values of single treatments with enzalutamide, CCG-1423 and CCG-257081 and lestaurtinib. Panel B: Combination treatments of CCG-257081 and enzalutamide. i) LNCaP parental: Enzalutamide concentrations (μM): 5, 10, 20, 40, 60, 80. CCG-257081 concentrations (μM): 1, 5, 7.5, 10, 20, 40. ii) LNCaP Abl: Enzalutamide concentrations (μM): 15, 20, 30, 40, 60, 80. CCG-257081 concentrations (μM): 1, 5, 7.5, 10, 20, 40. *P* values were calculated using a one-way ANOVA analysis, followed by Dunnett's test to compare the mean IC50 value of enzalutamide with the IC50 values of the combined concentrations. *p<0.05, **p<0.01, NS = not significant. Panel C: Combination treatments of lestaurtinib and enzalutamide. i) LNCaP parental: Enzalutamide concentrations (μM): 5, 10, 20, 40, 60, 80. Lestaurtinib concentrations (μM): 0.06, 0.08, 0.1, 0.5, 1, 5. ii) LNCaP Abl: Enzalutamide concentrations (μM): 15, 20, 30, 40, 60, 80. Lestaurtinib concentrations (μM): 0.025, 0.08, 0.25, 0.5, 1, 2. *P* values were calculated using a one-way Anova analysis, followed by Dunnett's test to compare the mean IC_{50} value of enzalutamide with the IC_{50} values of the combined concentrations.
*p<0.05, **p<0.01.

cycle progression) and p27 (an inducer of cellular senescence) was observed in both LNCaP parental and Abl cell lines, following CCG-1423 treatment, this was not statistically significant when analyzed by densitometry. CCG-1423 treatment resulted in a significant decrease in the expression of CDK2 (0.37 ± 0.08 fold change (FC) as single treatment, $p < 0.001$ and 0.31 ± 0.07 FC in combination with enzalutamide, $p < 0.001$), CDK6 (0.04 ± 0.03 FC as single treatment, $p < 0.001$ and 0.1 ± 0.09 FC in combination, $p < 0.01$), cyclin D3 (0.23 ± 0.42 FC as single treatment, $p < 0.01$ and 0.21 ± 0.37 FC in

Table 1. IC_{50} values of enzalutamide, CCG-1423, CCG-257081 and lestaurtinib in LNCaP isogenic cell lines.

	IC50 @ 5 d ± SD (μM)			
Cell line	Enzalutamide	CCG-1423	CCG-257081	Lestaurtinib
LNCaP Parental	8.8 ± 3.4	13.4 ± 2.5	7.2 ± 1.1	0.1 ± 0.02
LNCaP Abl	26.3 ± 6.9	9.9 ± 2.2	3 ± 1.1	0.1 ± 0.1

a) LNCaP Parental

	Enzalutamide (μM)					
CCG1423 (μM)	0.2	2	20	30	60	80
10	0.5 ±0.34	0.8 ± 0.43	0.8 ± 0.34	0.6 ± 0.04	0.6 ± 0.18	0.7 ± 0.21
12	0.6 ±0.22	0.6 ± 0.21	0.7 ± 0.07	0.7 ± 0.02	0.7 ± 0.12	0.7 ± 0.15
14	0.7 ±0.22	0.7 ± 0.12	0.8 ± 0.05	0.8 ± 0.01	0.7 ± 0.07	0.8 ± 0.11
16	0.8 ±0.17	0.8 ± 0.15	0.8 ± 0.06	0.8 ± 0.01	0.8 ± 0.02	0.9 ± 0.05
18	0.9 ±0.17	0.9 ± 0.14	0.8 ± 0.11	0.9 ± 0.05	0.9 ± 0.03	0.9 ± 0.03
20	1.0 ±0.18	1.0 ± 0.18	0.9 ± 0.12	1.0 ± 0.09	1.0 ± 0.11	0.9 ± 0.11

	Enzalutamide (μM)					
CCG257081(μM)	5	10	20	40	60	80
1	0.6 ± 0.4	1.0 ± 0.9	1.8 ± 1.4	1.4 ± 1.6	1.1 ± 1.1	1.0 ± 0.88
5	0.8 ± 0.5	1.3 ± 1.2	1.6 ± 1.4	1.8 ± 1.4	1.3 ± 0.9	1.0 ± 0.58
7.5	0.7 ± 0.2	0.9 ± 0.4	1.1 ± 0.5	1.1 ± 0.5	1.0 ± 0.4	0.6 ± 0.43
10	0.8 ± 0.1	0.9 ± 0.3	1.2 ± 0.5	1.1 ± 0.6	1.0 ± 0.5	0.5 ± 0.36
20	0.9 ± 0.2	0.9 ± 0.3	0.8 ± 0.3	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.02
40	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.13

	Enzalutamide (μM)					
Lesta (μM)	5	10	20	40	60	80
0.025	0.6 ± 0.4	0.7 ± 0.5	1.6 ± 1.4	1.4 ± 1.0	0.7 ± 0.2	0.7 ± 0.3
0.08	0.6 ± 0.4	0.5 ± 0.4	0.8 ± 0.5	1.0 ± 0.5	0.7 ± 0.3	0.6 ± 0.2
0.25	0.4 ± 0.3	0.5 ± 0.3	0.6 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.2
0.5	0.5 ± 0.3	0.6 ± 0.4	0.7 ± 0.4	0.7 ± 0.3	0.6 ± 0.3	0.7 ± 0.1
1	0.6 ± 0.5	0.6 ± 0.4	0.7 ± 0.5	0.7 ± 0.3	0.7 ± 0.4	0.8 ± 0.2
2	0.6 ± 0.5	0.6 ± 0.5	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.3

b) LNCa PAbl

	Enzalutamide (μM)					
CCG1423 (μM)	5	10	20	40/50	60	80
2.5	1.9 ± 1.0	1.1 ± 0.4	1.0 ± 0.1	1.3 ± 0.1	1.2 ± 0.04	1.3 ± 0.2
5	2.0 ± 0.5	1.9 ± 1.2	1.4 ± 0.4	0.9 ± 0.4	1.3 ± 0.05	1.3 ± 0.2
10	1.3 ± 0.5	1.4 ± 0.3	1.4 ± 0.3	1.1 ± 0.4	1.4 ± 0.1	1.5 ± 0.2
12	0.9 ±0.01	1.0 ±0.04	1.1 ± 0.2	1.2 ± 0.1	1.4 ± 0.2	1.5 ± 0.2
14/15	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.3	1.4 ± 0.1	1.6 ± 0.3
20	1.0 ± 0.3	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.4	1.6 ± 0.2	1.8 ± 0.3

	Enzalutamide (μM)					
CCG257081 (μM)	15	20	30	40	50	80
1	3.2 ± 0.8	2.1 ± 0.3	1.5 ± 0.5	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.3
5	1.4 ± 0.4	1.5 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.3
7.5	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.3
10	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.3
20	1.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2
40	1.4 ± 0.2	1.4 ± 0.2	1.5 ± 0.3	1.6 ± 0.2	1.6 ± 0.1	1.9 ± 0.3

	Enzalutamide (μM)					
Lesta (μM)	15	20	30	40	50	80
0.06	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
0.08	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
0.1	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.2
0.5	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1
1	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
5	1.1 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.3	0.8 ± 0.2

CI<0.7	0.7<CI<0.9	0.9<CI<1.1	1.1<CI<1.1	CI>1.45
Synergy	Moderate Synergy	Additive	Moderate Antagonism	Antagonism

Figure 2. Synergy tables of enzalutamide combined with CCG-1423, CCG-257081 and lestaurtinib. A) LNCaP parental and B) LNCaP Abl. The reciprocal of the percentage cell viability of single treatments and combination treatments were obtained and inputted into the ComPuSyn software to calculate the CI value. The CI values represent the average of at least three independent experiments in triplicate ± SD. CI from 0.7–0.9 represents synergy in green. CI from 0.9–1.1 represents additive effect in yellow. CI from 1.1–1.45 represents moderate to strong antagonism in orange and red respectively.

combination, $p < 0.01$) and Rb (0.3 ± 0.44 FC as single treatment, $p < 0.05$ and 0.15 ± 0.23 FC in combination, $p < 0.01$) in the LNCaP parental cells (Figure 3, panel B). Similarly, in the Abl cells, CCG-1423 caused a significant decrease in the protein expression of CDK2 (0.45 ± 0.48 FC only in combination with enzalutamide, $p < 0.05$), CDK4 (0.81 ± 0.29 FC as single treatment, $p < 0.05$ and 0.64 ± 0.28 FC in combination, $p < 0.01$), cyclin D3 (statistically significant only for enzalutamide vs. combination, FC 0.48 ± 0.34, $p < 0.05$) and Rb (0.32 ± 0.28 FC as single treatment, $p < 0.0001$ and 0.30 ± 0.43 FC in combination, $p < 0.0001$ (Figure 3, panel C). CCG-257081 treatment had no significant effect on the expression of cell cycle proteins in the Abl cells (Figure 3, panel E). In the parental cells, CCG-257981 caused a significant increase in the expression of p21 (only in combination with enzalutamide, FC 2.40 ± 0.88, $p < 0.05$) and decrease in CDK6 (0.38 ± 0.27 FC as single treatment, $p < 0.05$; 0.21 ± 0.06 FC in combination with enzalutamide, $p < 0.01$) and Rb (0.26 ± 0.23 FC, single treatment, $p < 0.001$ and 0.14 ± 0.09 FC in combination, $p < 0.001$) (Figure 3, panel D). Treatment with lestaurtinib had little effect on the expression of the G1/S phase transition checkpoint proteins. In the LNCaP parental cells, it caused a significant decrease in Rb expression (0.33 ± 0.21 FC as single treatment, $p < 0.01$ and 0.19 ± 0.20 FC in combination $p < 0.001$) (Figure 3, panel F). In the Abl cells, treatment with lestaurtinib caused a significant decrease in CDK2 expression (0.27 ± 0.18 FC only in combination with enzalutamide $p < 0.01$)

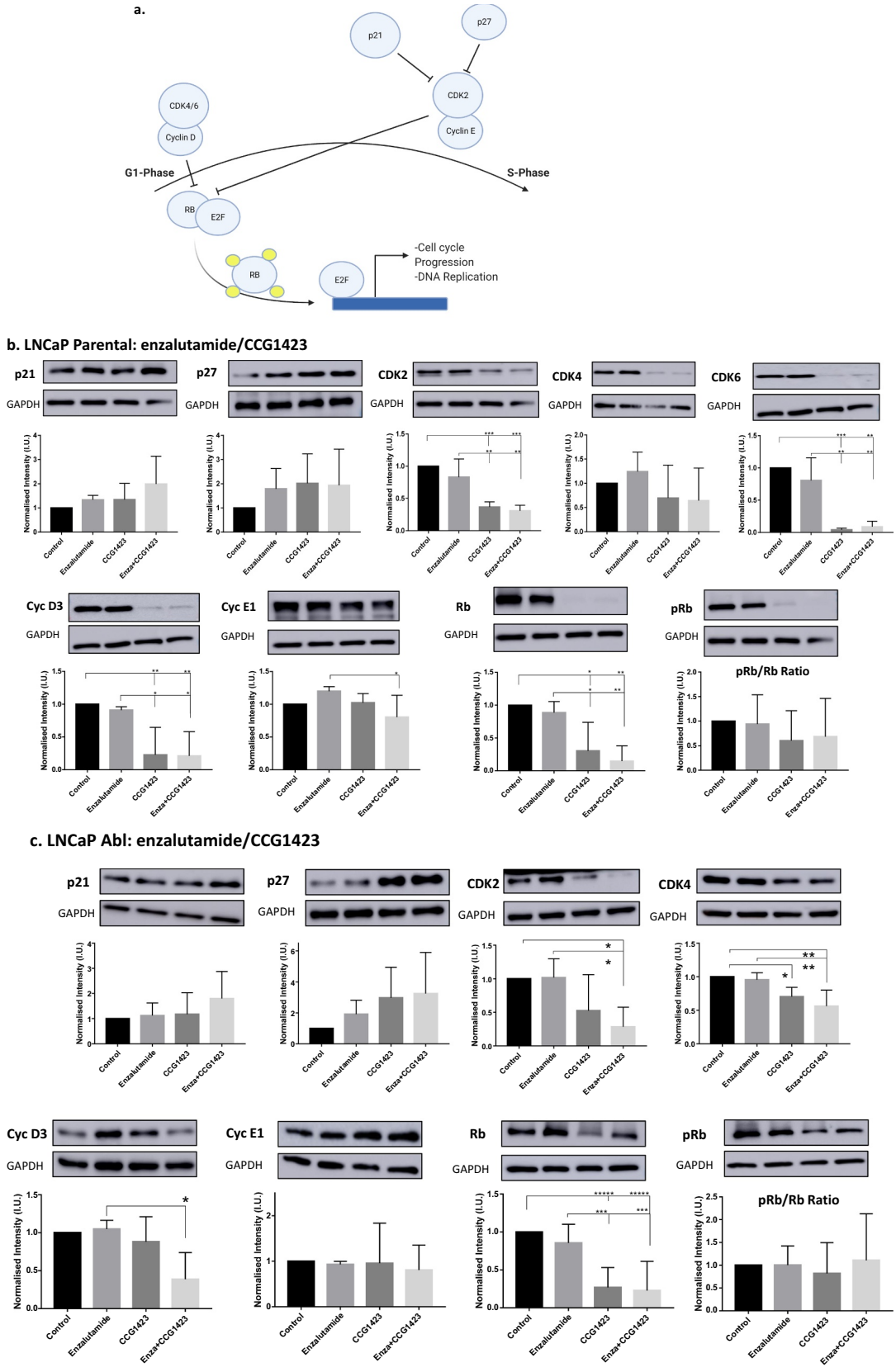


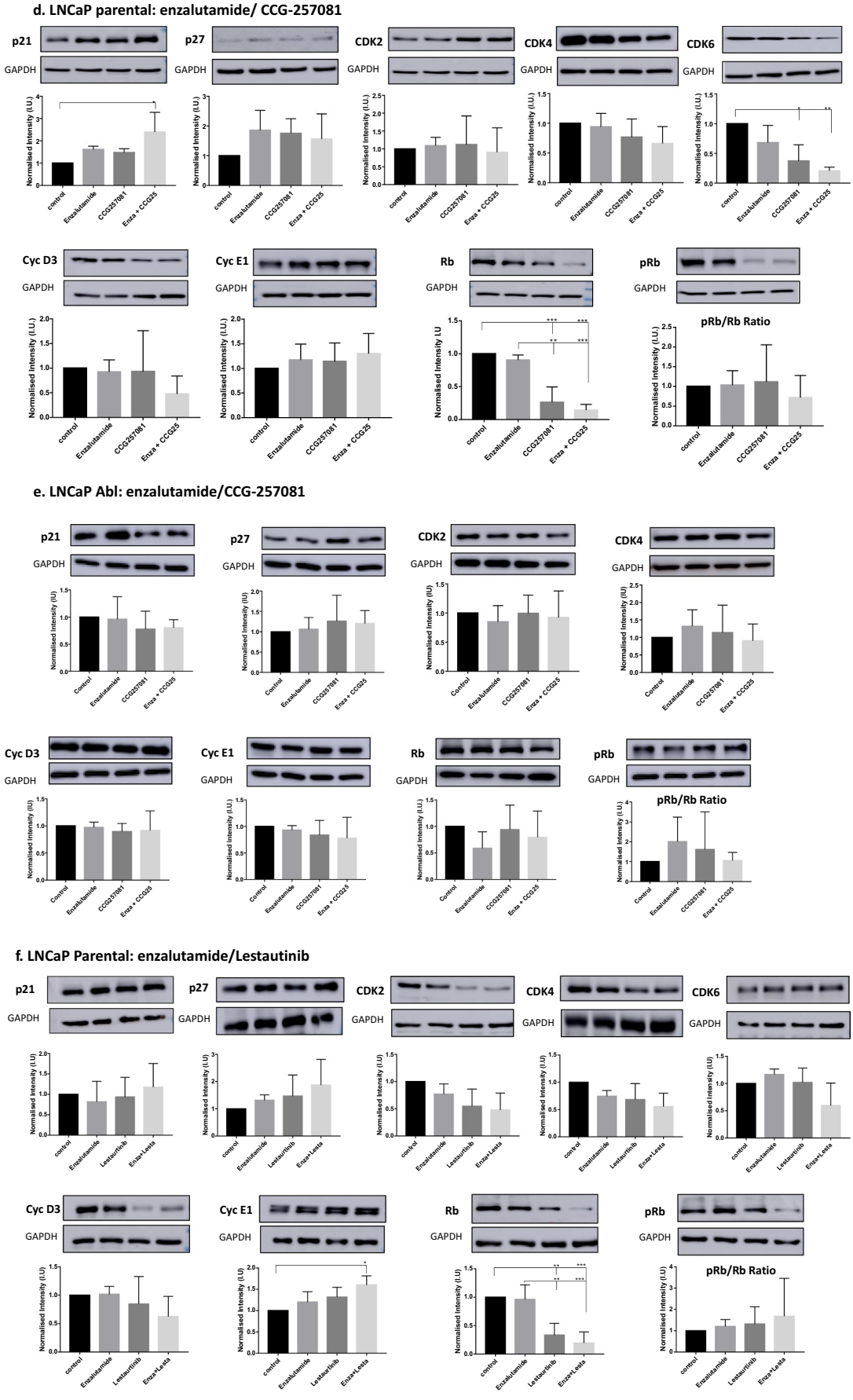
Figure 3. WB analysis of G1/S phase proteins, following single and combination treatments with enzalutamide, CCG-1423 and CCG-257081 and lestaurtinib. Cells were seeded at a cell density of 3.5 x 10^5 per well in 6-well plates and treated with either the vehicle (DMSO), enzalutamide (Parental: 5μM, Abl: 24μM), CCG-1423 (Parental 13μM, Abl: 10μM), CCG-257081 (Parental: 7μM, Abl: 3μM) and lestaurtinib (Parental: 0.1μM, Abl: 0.1μM) singly or in combination for 48 h before whole cell lysate collection. Bar charts represent

(Figure 3, panel G). Cyclin E1 was the only protein which was not affected by any treatment in either cell line, apart from a moderate increase in the parental cells in response to lestaurtinib treatment (1.60 ± 0.21 FC only in the combination treatment, $p < 0.05$) (Figure 3, panel F).

3.3 Inhibition of SRF alone or in combination with enzalutamide results in cell cycle arrest and decrease in cell proliferation

To further assess the effect of SRF inhibition on cell cycle and cell proliferation, flow cytometry experiments post ethynyl-labeled deoxyuridine (EdU) incorporation and DAPI staining were performed. The S phase (indicative of DNA replication) of the cell cycle was taken as a proxy for cell proliferation. For these experiments, we tested only one member of the CCG family (CCG-1423, because it had shown a more marked effect on cell cycle protein expression) and lestaurtinib, which inhibits SRF through a different mechanism from the CCG inhibitors. Both cell lines showed a significant response to both SRF inhibitors in terms of a decrease in the percentage of cells in the S phase of the cell cycle, indicative of reduced proliferation (Figure 4, panels A-E). In the LNCaP parental cells the S phase was decreased from 35.3%±5% in the control group to 25%±6.1% post enzalutamide treatment ($p < 0.01$), 12.8% ±8.7% post CCG-1423 ($p < 0.0001$), 16.3%±1.1% post lestaurtinib ($p < 0.0001$), 10.7%±6.8% post combination of enzalutamide and CCG-1423 ($p < 0.0001$) and 10.6%±11% post combination of enzalutamide and lestaurtinib ($p < 0.0001$) (Figure 4, panels A, B, E). Similarly with the LNCaP Abl cell line, there was a decrease in the percentage of cells in the S phase, from 36.2% ±6.5% in the control group to 30%±5% post enzalutamide treatment (NS), 20.4%±7.9% post CCG-1423 ($p < 0.0001$), 13.3%±4.4% post lestaurtinib ($p < 0.0001$), 6.6%±3.2% post combination of enzalutamide and CCG-1423 ($p < 0.0001$) and 13.4%±4.7% post combination of enzalutamide and lestaurtinib ($p < 0.0001$). As expected, there was no significant decrease in S phase percentage in the cells treated with enzalutamide in the Abl, because these cells are resistant to ADT (Figure 4, panels C, D, F). Both cell lines showed significant cell cycle arrest at the G0/G1 phase for almost all treated conditions. In the LNCaP parental cells, there was an increase in the G0/G1 phase from 54.3%±4.4% in the control group to 67%±7.7% post enzalutamide treatment ($p < 0.01$), 77.8% ±9.5% post CCG-1423 ($p < 0.0001$), 74.6%±1.9% ($p < 0.0001$) post lestaurtinib, 81.8%±8.3% post combination of enzalutamide and CCG-1423 ($p < 0.0001$) and 81%±11.2 post combination of enzalutamide and lestaurtinib ($p < 0.0001$). Similarly, a G0/G1 increase was observed in the LNCaP Abl cells from 52.4%±6.6% in the control group to 59.9%±6.2% post enzalutamide treatment (NS), 70%±8.6% post CCG-1423 ($p < 0.001$), 69%±6.2% post lestaurtinib ($p < 0.001$), 87%±8.6% post combination of enzalutamide and CCG-1423 (<0.0001), 58.4%±12% post combination of enzalutamide and lestaurtinib (NS). Again, there was no significant increase in the percentage of cells in G0/G1 phase, following enzalutamide treatment, in the LNCaP Abl cells, which are resistant to ADT. There was no significant change in G2/M phase of the cell cycle in both cell lines, except for LNCaP Abl cells treated with a combination of enzalutamide and lestaurtinib. Specifically, an increase in the percentage of cells in G2/M phase from 9.6%±0.9% in the control group to 22.4% ±10.5% post combination of enzalutamide and

the average band densitometry, calculated with Image J, ± standard deviation (SD). Full-length WB blots are shown in Supplementary Figure S1. Bar charts represent protein expression following normalisation with GAPDH. Statistical analysis was performed using one-way Anova followed by Tukey's test. *p<0.05, **p<0.01, ***p<0.001. Panel A: Schematic summary of the G1/S cell cycle proteins analysed. WB show representative images from at least three independent experiments. Panel B: LNCaP parental: enzalutamide/CCG-1423 combination; Panel C: LNCaP Abl: enzalutamide/CCG-1423 combination; Panel D: LNCaP parental: enzalutamide/CCG-257081 combination; Panel E: LNCaP Abl: enzalutamide/CCG-257081; Panel F: LNCaP parental: enzalutamide/lestaurtinib; Panel G: LNCaP Abl: enzalutamide/lestaurtinib.



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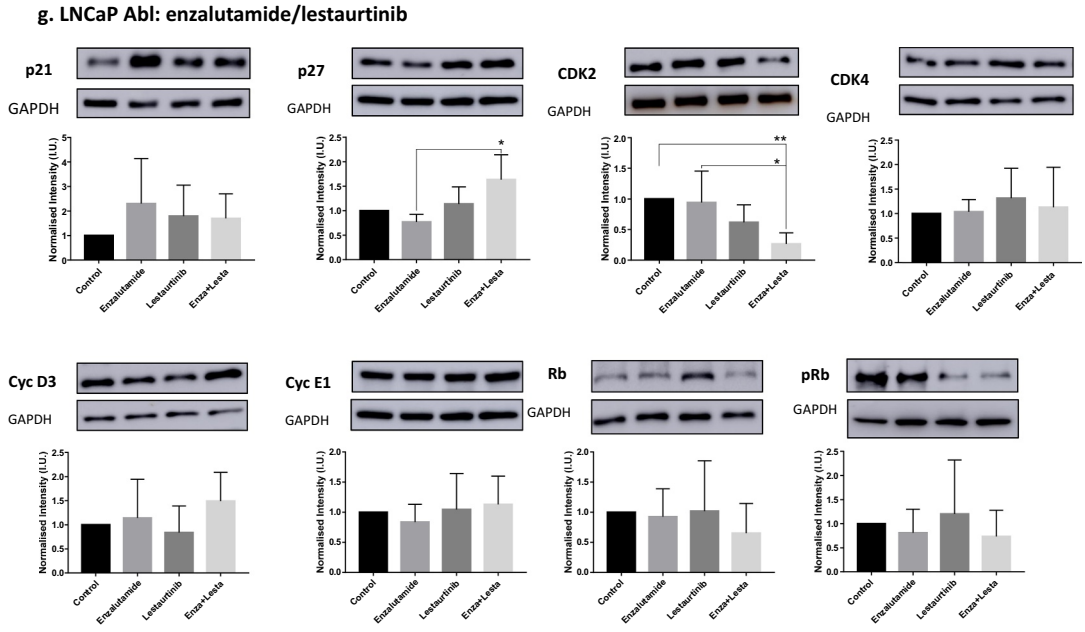

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lestaurtinib ($p < 0.0001$) was demonstrated. Additionally, there was a modest but significant ($p < 0.001$) increase in the percentage of polyploid cells from 1.7%±0.9% in the control group to 5.9% ±4.7% in the enzalutamide/lestaurtinib combination group. A summary of the statistical results can be found in Tables 2 and 3.

3.4 Lestaurtinib and CCG257081, singly and in combination with enzalutamide, increase senescence-associated β-galactosidase activity in LNCaP Abl cells

Cell cycle analysis, through WB and flow cytometry, showed only a modest effect of CCG-257081 and lestaurtinib on cell cycle, especially in the LNCaP Abl cells. Moreover, cells treated with lestaurtinib appeared to be flattened and enlarged, a cell morphology suggestive of cellular senescence. To test whether SRF inhibitors induced senescence, LNCaP parental and Abl cells were stained with X-galactosidase solution, following treatment with CCG-1423, CCG-257081 and lestaurtinib, singly and in combination with enzalutamide. LNCaP parental cells showed no significant changes in β-galactosidase activity, following treatment with each SRF-inhibitor, singly and in combination with enzalutamide (Figure 5, panel A). However, in the LNCaP Abl cells, there was a significant increase in senescence-associated β-galactosidase activity, following treatment with lestaurtinib (1.6 ± 0.06 FC, $p < 0.01$) and CCG-257081 (1.6 ± 0.1 FC $p < 0.01$), singly and in combination with enzalutamide (1.8 ± 0.2 FC, $p < 0.001$ and 1.8 ± 0.2 FC, $p < 0.0001$), when compared to the control (Table 4, Figure 5, panel B). Furthermore, lestaurtinib and CCG-257081 in combination with enzalutamide also showed a significant increase in senescence-associated β-galactosidase activity when compared to enzalutamide alone (from 1.2 ± 0.04 FC for enzalutamide to 1.8 to ±0.2 FC for both combinations, $p < 0.01$), as well as CCG-1423 alone (from 1.2 ± 0.2 FC for CCG-1423 to 1.8 to ±0.2 FC for both combinations, $p < 0.01$) and CCG-1423/enzalutamide combination (from 1.2 ± 0.09 FC for CCG-1423/enzalutamide combination to 1.8 to ±0.2 FC for both combinations, $p < 0.01$) (Table 4, Figure 5, panel B).

4. Discussion

Although anti-androgens such as enzalutamide and abiraterone have improved the overall survival of patients with PCa, development of resistance to

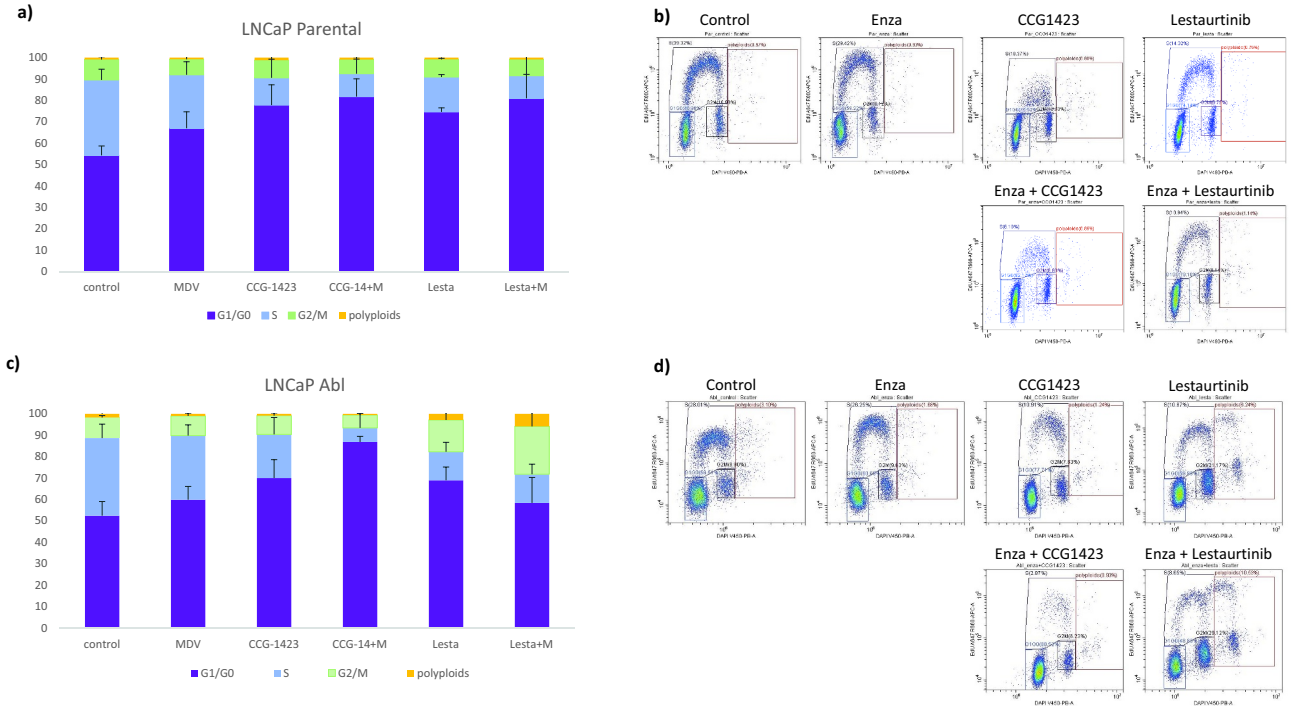

Figure 4. Effects of single and combination treatments with enzalutamide, CCG-1423 and lestaurtinib on the cell cycle. LNCaP cells were seeded at a density of 3 x 10^5 in a six-well plate and treated with either the vehicle (DMSO), enzalutamide (Parental: 5μM, Abl: 24μM), CCG-1423 (Parental 13μM, Abl: 10μM) or lestaurtinib (Parental 0.1μM, Abl: 0.1μM), singly or in combination for 48 h. Cell cycle distribution was analysed using flow cytometry. Bar charts show the average percentage of cells in different phases of the cell cycle from at least three independent experiments in triplicate ±SD for LNCaP parental (Panel A) and LNCaP Abl (Panel C). Panels C and D show representative images from flow cytometry for LNCaP parental and Abl respectively. MDV: enzalutamide. CCG-14+M: CCG-1423 + enzalutamide. Lesta+M; lestaurtinib + enzalutamide.

these drugs is still a major challenge. Emergence of AR splice variants and the involvement of cofactors of AR, such as SRF, are common determinants of ADT resistance [5,28,29]. Regarding aberrant activation of AR cofactors, we believe that targeting AR transcriptional activity, through these co-factors, without directly targeting the AR protein, may avoid triggering certain resistance mechanisms. We have previously shown that high SRF expression in patients with CRPC correlated with resistance to enzalutamide and that SRF inhibition with CCG-1423 retained AR in the cytoplasm [13], which suggests a possible role for SRF in enzalutamide resistance. Here, we show that inhibition of SRF, using the small molecule inhibitors CCG-1423 and CCG-257081 and the multikinase inhibitor lestaurtinib, decreases cell viability through cell cycle arrest in the LNCaP parental and Abl isogenic model of CRPC. These results are particularly promising in the LNCaP Abl cells, which are ADT-resistant and show relatively high IC_{50} values for enzalutamide (26.3 ± 6.9 μM). In these cells, using the SRF-inhibitors seems to be more effective than using enzalutamide, even as single drugs, with significantly lower IC_{50} values compared to enzalutamide and a significant bigger effect on cell cycle arrest, reduction in proliferation and induction of cellular senescence. When it comes to combination treatments, a synergistic effect between the three SRF inhibitors and enzalutamide was shown in the parental cells, where the majority of the combination doses showed synergy, as indicated by CI values of less than 1. In the LNCaP Abl cells, combination treatment of enzalutamide with either CCG-1423 or CCG-257081 are mostly antagonistic with a CI value of over 1.2, while combination of enzalutamide with lestaurtinib are synergistic. As this antagonism between enzalutamide and CCG-1423/CCG25–7081 only occurs in the LNCaP Abl cells, in contrast with the LNCaP parental line, it suggests that androgens influence the synergy between these drugs. This is in line with our previous studies showing that

Table 2. One-way ANOVA analysis of flow cytometry results for LNCaP parental.

	Enza	CCG1423	Enza + CCG1423	Lesta	Enza + Lesta
% G0/G1 Cells					
Control	0.001	<0.0001	<0.0001	<0.0001	<0.0001
Enza	-	0.014	NS	NS	0.0004
CCG1423	-	-	NS	NS	NS
Enza + CCG1423	-	-	-	NS	NS
Lesta	-	-	-	-	NS
% S Cells					
Control	0.0046	<0.0001	<0.0001	<0.0001	<0.0001
Enza	-	0.0028	0.0004	0.0391	0.0002
CCG1423	-	-	NS	NS	NS
Enza + CCG1423	-	-	-	NS	NS
Lesta	-	-	-	-	NS
% G2/M Cells					
Control	NS	NS	NS	NS	NS
Enza	-	NS	NS	NS	NS
CCG1423	-	-	NS	NS	NS
Enza + CCG1423	-	-	-	NS	NS
Lesta	-	-	-	-	NS

Statistical significance was calculated using One-Way ANOVA (Tukey's multiple comparisons test). P-value < 0.05 was considered significant. Enza, Enzalutamide; Lesta, Lestaurtinib; NS, non-significant.

Table 3. One-way ANOVA analysis of flow cytometry results for LNCaP Abl.

	Enza	CCG1423	Enza + CCG1423	Lesta	Enza + Lesta
% G0/G1 Cells					
Control	NS	0.0006	<0.0001	0.0004	NS
Enza	-	NS	<0.0001	NS	NS
CCG1423	-	-	0.0043	NS	0.0359
Enza + CCG1423	-	-	-	0.0005	<0.0001
Lesta	-	-	-	-	0.036
% S Cells					
Control	NA	<0.0001	<0.0001	<0.0001	<0.0001
Enza	-	0.0234	<0.0001	<0.0001	<0.0001
CCG1423	-	-	0.0005	NS	NS
Enza + CCG1423	-	-	-	NS	NS
Lesta	-	-	-	-	NS
% G2/M Cells					
Control	NS	NS	NS	NS	<0.0001
Enza	-	NS	NS	NS	<0.0001
CCG1423	-	-	NS	NS	0.0001
Enza + CCG1423	-	-	-	0.0275	<0.0001
Lesta	-	-	-	-	NS

Statistical significance was calculated using One-Way ANOVA (Tukey's multiple comparisons test). P-value < 0.05 was considered significant. Enza, Enzalutamide; Lesta, Lestaurtinib; NS, non-significant.

transient knockdown of SRF in DHT stimulated cells led to increased AR activity in the LNCaP Abl cells, but not in the LNCaP parental line [10]. This suggests that androgens affect transcriptional activity of the SRF/AR axis in CRPC, which may explain the different behavior in the synergistic effect between LNCaP parental and Abl cell lines. While there is a difference between LNCaP parental and Abl cells in the synergistic effect in response to combination of enzalutamide with the CCG compounds, combination with lestaurtinib was synergistic in both cell lines. This may be due to the different mechanisms of action of these compounds, which target different proteins. Specifically, the CCG family of small molecule compounds target SRF activity by inhibiting Pirin, which affects myocardin-related transcription factor A (MRTF-A) nuclear translocation [30]. MRTF-A partners with SRF to induce transcription of genes involved in the cytoskeleton organization (important for AR translocation to the nucleus) and cell migration [31–33]. Lestaurtinib disrupts SRF transcriptional activity by inhibiting its co-factor PKN1, which has been

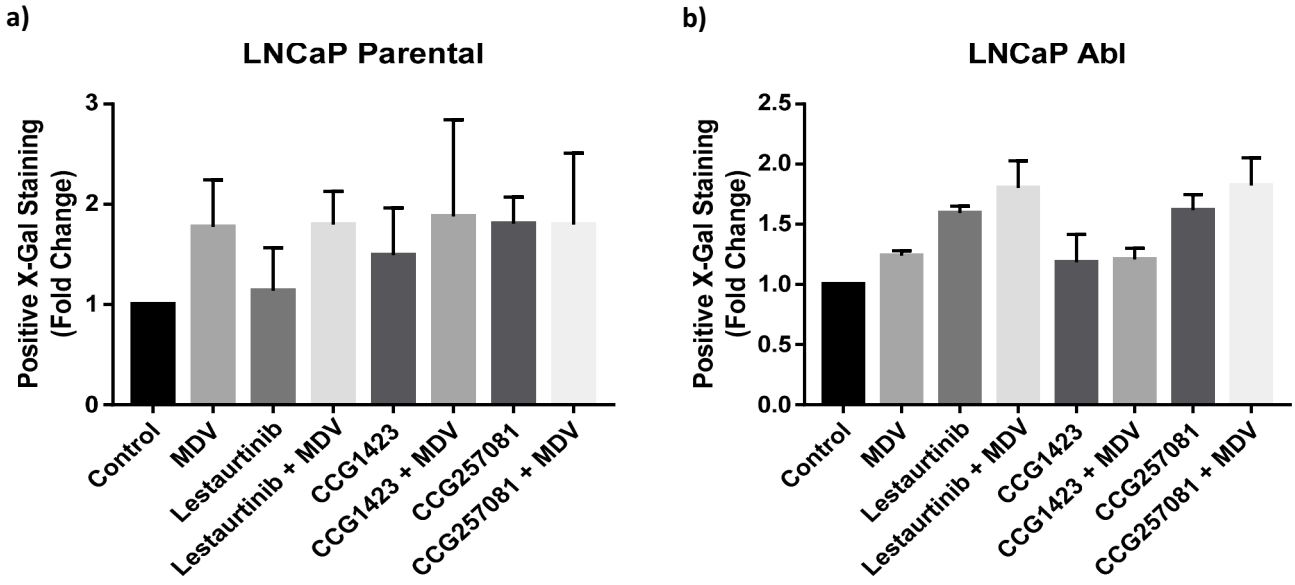

Figure 5. Lestaurtinib and CCG257081, singly and in combination with Enzalutamide, increase senescence-associated β-galactosidase activity in LNCaP Abl cells. LNCaP Parental and LNCaP Abl cells were seeded in 6-well plates at a density of 50×10^3 cells per well and treated with CCG1423 (Parental: 13 μM, Abl:10 μM), CCG257081 (Parental: 7 μM; Abl: 3 μM) and lestaurtinib (Parental: 0.1 μM, Abl: 0.1 μM), singly and in combination with enzalutamide, or left untreated. Following treatment for 48 hours, the cells were stained with X-Gal solution and imaged. Bar charts show the fold change of β-gal positive cells from three independent experiments ± SD, relative to the control condition. MDV; Enzalutamide (Parental: 5 μM, Abl: 24 μM). (A) LNCaP parental. (B) LNCaP Abl. (C) Representative images of β-gal staining. i. Negative control (no β-gal), ii and iii positive control (H_2O_2 10 μM), iv-xi different conditions as indicated in the image. Black arrows indicate senescent cells.

shown to modulate SRF activity in response to androgens and is part of the same immunocomplex as SRF [12]. In fact, PKN1 also co-precipitates with AR both *in vitro* and in PCa patients' tissues [34]. This interaction in patient tissues is enhanced in the presence of synthetic androgen R1881 [34]. Such interaction may indicate why our results show synergy between enzalutamide and lestaurtinib concentrations also in the LNCaP Abl cells as the AR pathway is targeted directly by enzalutamide and indirectly with lestaurtinib. Importantly, our findings align with a previous study where combined treatments of lestaurtinib and leuprolide, a drug that decreases testosterone levels via inhibition of gonadotropin, resulted in significantly reduced tumor weight and recurrence in the Dunning *R*-3327 H rat model of CRPC [34]. This study and our results bolster the fact that lestaurtinib combined with ADT may prove beneficial in the treatment of advanced PCa. The efficiency of combination strategies is an important area of ongoing research, as exemplified by the use of the antiangiogenetics on the treatment of PCa [35].

Both SRF and AR signaling are implicated in the progression of the cell cycle. In fact, using reporter assays, Lim and colleagues showed that CDK6 upregulates AR activity in the presence of DHT. Furthermore, this occurs via CDK6 directly binding to AR and modulating transcriptional activity of AR [36]. Additionally, cyclin E1 also coactivates AR by enhancing AR's transactivation activity [37], thereby highlighting a role for AR interaction with cell cycle regulators in the G1 phase of the cell cycle. In the LNCaP parental cells, we see a significant decrease in CDK6 expression following inhibition with CCG-1423 and CCG-257081, singly and in combination with enzalutamide. However, CDK6 expression was not detected in the LNCaP Abl cells (data not shown). While CCG-257081 treatment did not show any effect on the Abl cells, there is significant decrease in CDK2 expression following inhibition with CCG-1423 and lestaurtinib singly

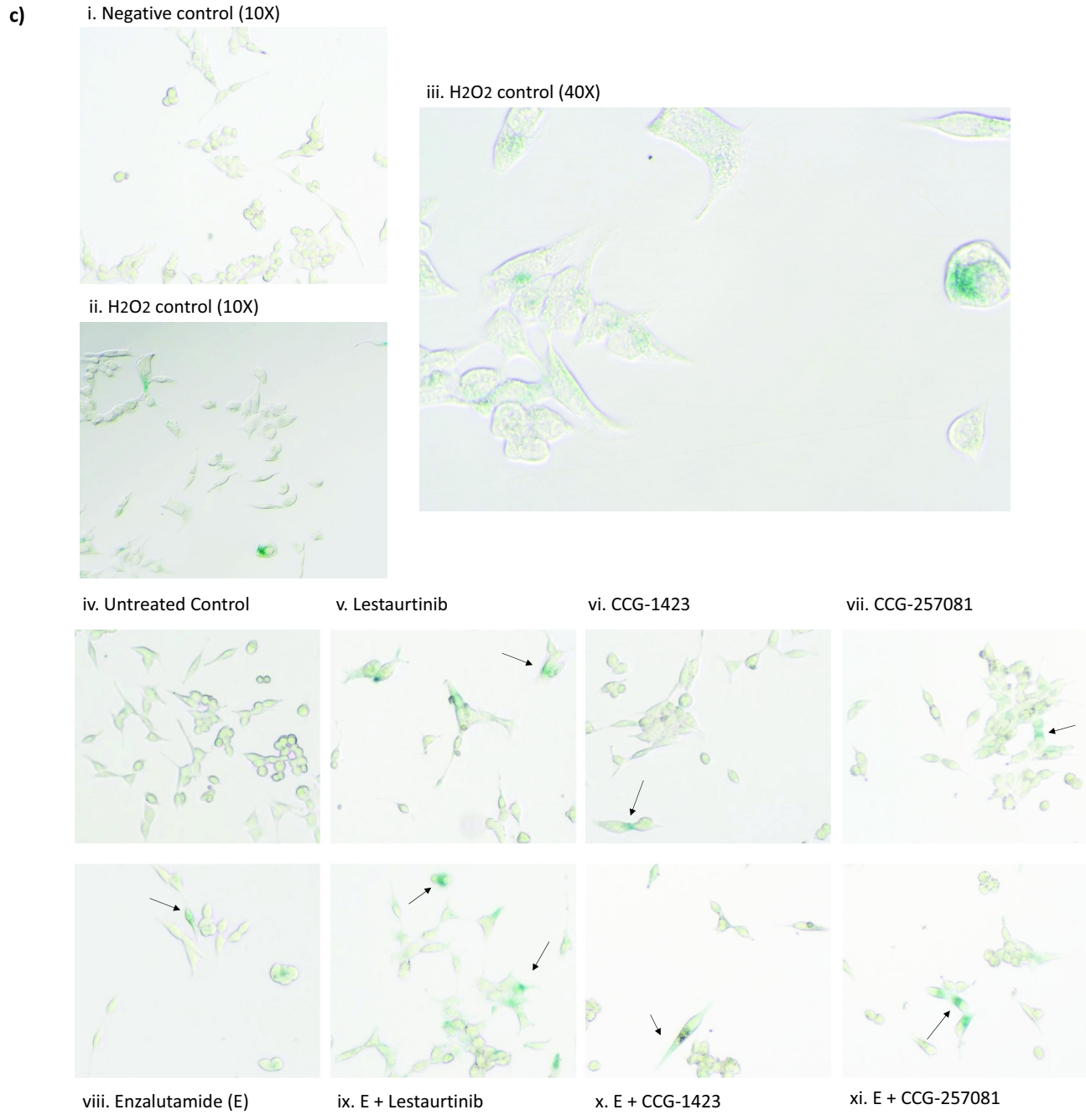

Figure 5. (Continued).

and combination with enzalutamide. These data are in agreement with a previous study, which showed that CCG-1423 inhibition suppressed markers of the G1/S phase transition [24] and that transient knockdown of SRF in hepatocellular carcinoma cells induced G1 phase cell cycle arrest [26]. Similarly, we observed that CCG-1423 alone or in combination with enzalutamide significantly reduced the number of cells in the S phase and increased the number of cells in G1 phase in both LNCaP parental and Abl cells. Interestingly, in the Abl cells, there is a significant increase in G2/M cells after combination with enzalutamide and lestaurtinib whereas this effect resulted in G1 arrest in the parental cells. This difference in the mechanism of action may be due to the presence of androgens in the LNCaP parental media but not in the Abl media, since androgens influence the

Table 4. Statistical significance of comparison between treatment conditions for LNCaP Abl using One-Way ANOVA.

Conditions	Adjusted P-Value
Control ***vs.*** Lestaurtinib	0.0042
Control ***vs.*** Lestaurtinib + MDV	0.0002
Control ***vs.*** CCG257081	0.0029
Control ***vs.*** CCG257081 + MDV	0.0001
MDV ***vs.*** Lestaurtinib + MDV	0.0068
MDV ***vs.*** CCG257081 + MDV	0.0049
Lestaurtinib + MDV ***vs.*** CCG1423	0.003
Lestaurtinib + MDV ***vs.*** CCG1423 + MDV	0.0042
CCG1423 ***vs.*** CCG257081 + MDV	0.0022
CCG1423 + MDV ***vs.*** CCG257081 + MDV	0.0031

Statistical significance was calculated using One-Way ANOVA (Tukey's multiple comparisons test). P-value < 0.05 was considered significant. Comparisons of treatment conditions not shown were not considered significant. Vs, versus; MDV, Enzalutamide.

transcriptional activity of AR. Another difference between LNCaP parental and Abl cells was the induction of cellular senescence, which resulted statistically significant only in the Abl cells and only for CCG-257081 and lestaurtinib, which had shown less changes in cell cycle checkpoint proteins. This is intriguing from a mechanistic point of view, suggesting that the decrease in cell viability and proliferation following treatment with these inhibitors follows different pathways depending on the specific inhibitor. Additionally, distinct subgroups of PCa exist which show different responses to treatment [38]. Interestingly, despite CCG-257081 was reported to have enhanced pharmacokinetic properties compared to CCG-1423, CCG-1423 induced a more pronounced effects on the expression of cell cycle proteins. This apparent discrepancy can be explained considering that the improved pharmacokinetic properties of CCG-257081 refer to its enhanced solubility and metabolic stability [39], which are not reflected in the drug effects on cell cycle and the other cellular fates studied here.

The induction of senescence following cancer therapy has been the subject of intense debate for the past few years. In fact, while the proliferation arrest resulting from senescence has been regarded as a positive outcome, the senescence-associated secretory phenotype, which can potentially induce cellular transformation and epithelial–mesenchymal transition in the neighboring cells, is undesirable [40]. However, the emergence of senolytic therapy opens a wide range of sequential treatments to ensure clearance of the senescent cells when needed [41]. Since prostate cancer is usually slow growing and development of CRPC occurs late in the management of the disease, the induction of senescence, especially in older patients, may be seen as a desired outcome.

Lestaurtinib was previously investigated in a phase II trial in 2007 in patients with CRPC [18] before its known inhibition of PKN1. Lestaurtinib was given as an oral drug to men with CRPC to determine an optimum dosage for treatment [19] and was generally well tolerated as a single agent. Despite this, all patients presented >50% serum PSA levels following treatment, which declined once treatment was stopped. For this reason, the trial had ended. Notably, while lestaurtinib increases PSA levels, it also decreases SRF transcriptional targets, such as Four and a half LIM domains protein 2 and Chemokine (C–C motif) ligand 8 that are involved in cell proliferation and cell migration [12]. This highlights the possibility of using the AR/SRF crosstalk for more appropriate companion biomarkers, which would be better suited than PSA to track response, when treating patients with lestaurtinib. Furthermore, there is growing evidence to suggest that PSA, as a biomarker, is unreliable in the context of CRPC [42]. Our pre-clinical findings also present enhanced synergy when lestaurtinib is combined with enzalutamide, something that would be worth testing in clinical trials.

Conclusions

This study presents pre-clinical data on three SRF inhibitors, which are effective in reducing cell proliferation, through cell cycle arrest and induction of cellular senescence, in an isogenic model of CRPC. Inhibition of AR's co-factors, such as SRF, provides a promising example to overcoming resistance to AR inhibitors currently used in the clinic.

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Disclosure statement

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Author contributions

HA: investigation, formal analysis, writing (original draft); **SM**: investigation, formal analysis, writing (review & editing), **SC**: investigation, formal analysis, writing (review & editing); **WMG**: validation, writing (review & editing); **MP**: conceptualization, data curation, validation, writing (review & editing), visualization, supervision, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

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