Effective combinatorial immunotherapy for castration-resistant prostate cancer

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A significant fraction of patients with advanced prostate cancer treated with androgen deprivation therapy experience relapse with relentless progression to lethal metastatic castration-resistant prostate cancer (mCRPC)1. Immune checkpoint blockade using antibodies against cytotoxic-T-lymphocyte-associated protein 4 (CTLA4) or programmed cell death 1 (PD1/PD-L1) generates durable therapeutic responses in a significant subset of patients across a variety of cancer types2. However, mCRPC showed overwhelming de novo resistance to immune checkpoint blockade3−5, motivating a search for targeted therapies that overcome this resistance. Myeloid-derived suppressor cells (MDSCs) are known to play important roles in tumour immune evasion6. The abundance of circulating MDSCs correlates with prostate-specific antigen levels and metastasis in patients with prostate cancer7−9. Mouse models of prostate cancer show that MDSCs (CD11b+Gr1+10) promote tumour initiation10 and progression11. These observations prompted us to hypothesize that robust immunotherapy responses in mCRPC may be elicited by the combined actions of immune checkpoint blockade agents together with targeted agents that neutralize MDSCs yet preserve T-cell function. Here we develop a novel chimaeric mouse model of mCRPC to efficiently test combination therapies in an autochthonous setting. Combination of anti-CTLA4 and anti-PD1 engendered only modest efficacy. Targeted therapy against mCRPC-infiltrating MDSCs, using multikinase inhibitors such as cabozantinib and BEZ235, also showed minimal anti-tumour activities. Strikingly, primary and metastatic CRPC showed robust synergistic responses when immune checkpoint blockade was combined with MDSC-targeted therapy. Mechanistically, combination therapy efficacy stemmed from the upregulation of interleukin-1 receptor antagonist and suppression of MDSC-promoting cytokines secreted by prostate cancer cells. These observations illuminate a clinical path hypothesis for combining immune checkpoint blockade with MDSC-targeted therapies in the treatment of mCRPC.

Mouse models of prostate cancer (PCa) engineered with signature mutations of human PCa exhibit autochthonous tumour evolution in an intact immune system12−14. However, traditional germline genetic modelling has limited capacity in generating the cohort sizes needed to conduct multi-arm drug testing. This issue is particularly pressing for PCa models based on PB-Cre15, with optimal intercrosses producing 12.5% PCa-prone males (Extended Data Fig. 1a). Here, we employed a

Figure 1 | Strong combination synergy by ICB with cabozantinib or BEZ235 in mCRPC. a, Spontaneous prostate tumour development in the CPPSML model with tumours detected by fluorescence, bioluminescence, and MRI. Scale bar, 5 mm. b, c, Procedures and representative MRI images for testing prostate tumour response to castration and enzalutamide diet in PB-Cre+ Pten12 mice and CPPSML mice (n = 5, biological replicates). d−f, Preclinical trial results of prostate tumour mass, lymph node metastasis score, and lung micrometastasis number (n = 11, 6, 7, 4, 9, 6, and 7, respectively, biological replicates). Red bar, mean. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, #P > 0.05, Mann–Whitney U-test.

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novel non-germline mCRPC model in a C57BL/6 background through first establishing HJ61 and HJ58 mouse embryonic stem cell (mES cell) clones (Extended Data Fig. 1b–d) derived from the following genotypes: PB-Cre<sup>+</sup> Pten<sup>fl/fl</sup> p53<sup>−/−</sup> mTmG<sup>LT</sup>/<sup>L</sup> LUC<sup>LT</sup>/<sup>L</sup> (CPPSML), which exhibited age-dependent green fluorescent protein (GFP<sup>+</sup>) LUC<sup>+</sup> PCa development (Fig. 1a). In high-percentage chimaeras derived from HJ61 or HJ58 mES cells (Extended Data Table 1a), 50% of mice (4 out of 8 necropsied) developed GFP<sup>+</sup> cancer cells at 3 months of age and showed dissemination of cancer cells to draining lymph nodes and lung (Extended Data Fig. 1e, f). In prostate, GFP<sup>+</sup> areas corresponded to CK<sup>+</sup>/CK5<sup>5</sup> adenocarcinoma (Extended Data Fig. 1g, h). Importantly, we observed a fourfold increase in the rate by which PCa-bearing mice can be generated using chimaeric modelling (Extended Data Fig. 1a).

To study combination therapy targeting mCRPC, we first employed the PB-Cre<sup>+</sup> Pten<sup>fl/fl</sup> p53<sup>−/−</sup> mTmG<sup>LT</sup>/<sup>L</sup> LUC<sup>LT</sup>/<sup>L</sup> germinel model and demonstrated that an androgen deprivation therapy protocol (castration followed by enzalutamide-admixed diet) generated a significant, albeit transient, survival benefit (Extended Data Fig. 2a). Next, CPPSML chimaeras were subjected to the same androgen deprivation therapy to induce CRPC. To ensure consistency, MRI was used to assign chimaeras with prostate tumour volumes over 150 mm<sup>3</sup> before 18 weeks of age: 86 out of 107 (80.4%) chimaeras met this criterion (Fig. 1b and Extended Data Fig. 2b). We validated emergence of CRPC in CPPSML chimaeras by comparing the response of size-matched primary prostate tumours with androgen deprivation therapy in three cohorts: the chimaeras, CPPSML mice through breeding, and castration-sensitive PB-Cre<sup>+</sup> Pten<sup>fl/fl</sup> mice (Fig. 1c). All treated chimaeras succumbed to primary CRPC, with metastases in lymph nodes and micrometastases in lungs (Extended Data Fig. 2c). Thus, the CPPSML chimaera models provide a speedy platform to test multiple therapies on mCRPC.

Next, mCRPC-bearing chimaera mice were assigned to therapeutic trials. The targeted agents were selected on the basis of (1) strong activity in preclinical PCA models, (2) initial activity and safety in early phase trials but failure to improve overall survival in phase III trials of mCRPC, and/or (3) purported immunomodulatory activities and thus the potential to enhance or negate immune checkpoint blockade (ICB). The agents selected were the tyrosine kinase inhibitors dasatinib (Dasa)<sup>15</sup> and cabozantinib (Cabo)<sup>16</sup>, and the phosphoinositide 3-kinase (PI3K) mTOR dual inhibitor BEZ235 (BEZ)<sup>17,18</sup>. Of relevance to this study, previous evidence suggests that PI3K pathway activation in both cancer cells and cancer-associated myeloid cells can mediate immunosuppression<sup>19–23</sup> and that BEZ exhibits minimal inhibitory activity on mouse T cells<sup>24</sup>. For ICB, we used a cocktail of anti-CTLA4 and anti-PD1 antibodies to maximize the blockade of checkpoint pathways, a regimen in line with a clinical treatment protocol currently being tested in a phase II trial for mCRPC (NCT02601014). CPPSML chimaeras (generated from HJ61) with induced and MRI-documented mCRPC were randomized to receive single or combination treatments for 4 weeks before endpoint analysis (Extended Data Fig. 2d). While all targeted agent monotherapies or dual ICB cocktails had minimal impact on prostate tumour mass, the combination of Cabo + ICB or BEZ + ICB showed potent synergistic efficacy in targeting primary and metastatic PCA growth (Fig. 1d–f and Extended Data Fig. 2e). In contrast, ICB alone or Dasa + ICB showed minimal impact on primary or metastatic disease burden, although ICB alone resulted in significant reduction of lymph node metastasis and lung micrometastasis (Fig. 1d–f and Extended Data Fig. 2e). In a corroborating study with chimaeras derived from HJ58, Cabo + ICB also generated significant efficacy in the mCRPC setting (Extended Data Fig. 3a–c). At necropsy, Cabo + ICB- and BEZ + ICB-treated CRPC mice showed minimal residual tumour cells in the prostate (Extended Data Fig. 2e), reduced proliferation, and pronounced apoptosis (Extended Data Fig. 3d–g).

We catalogued the constellation of intratumoural immunocytes by time of flight mass cytometry (CyTOF)<sup>21</sup> in the various treatment arms as part of end-point analyses. Continued Dasa, but not Cabo or BEZ, treatment was associated with a significant reduction of...
Cabo (1 μM), or BEZ (1 μM), measured with cytokine array (n = 2, biological replicates). h, RNA expression levels of indicated genes by intratumoral MDSCs when cultured in conditioned medium by CPPSML PCa cell lines, measured by quantitative polymerase chain reaction with reverse transcription (qRT–PCR) (n = 3, biological replicates). i, Effect on gene expression by pre-treating CPPSML PCa cells with Cabo and BEZ before conditioned medium was collected to culture MDSCs (n = 3, biological replicates). j, Quantification of cytokine levels in conditioned medium from CPPSML PCa cell lines pre-treated with vehicle, Cabo (1 μM), or BEZ (1 μM), measured with cytokine array (n = 2, biological replicates). In a and d–j, data represent mean ± s.d. **P < 0.01, ***P < 0.001, Student’s t-test. For gel source data, see Supplementary Fig. 1.

To explore the impact of these agents on cells in the tumour microenvironment, in vitro viability assays were performed to audit Cabo, BEZ, and Dasa activity on MDSCs, CD8+ T cells, and GFP+ PCa cells collected from CRPC in CPPSML. Relative to CD8+ T cells and GFP+ PCa cells, MDSCs displayed a significantly higher sensitivity to Cabo and BEZ, but not to Dasa (Fig. 2c, d and Extended Data Fig. 5a). Similar half-maximum inhibitory concentration (IC50) results were obtained when MDSCs were assayed in medium supplemented with 10 ng ml−1 granulocyte–macrophage colony-stimulating factor (GM-CSF) (Extended Data Fig. 5b) or in medium supplemented with GM-CSF and pre-conditioned for 12 h by PCa cell lines established from the CPPSML model (Extended Data Fig. 5c). Moreover, Cabo or BEZ treatment alleviated the suppressive activity of intratumoural MDSCs on CD4+ and CD8+ T-cell proliferation (Fig. 2e and Extended Data Fig. 5d). On the other hand, CD8+ and CD4+ T-cell in vitro proliferation was only moderately suppressed by Cabo or BEZ, yet completely blocked by Dasa (Fig. 2f, g and Extended Data Fig. 5e). Equivalent drug effects on interferon (IFN)–γ and interleukin-2 (IL-2) production by T cells were observed (Fig. 2h, i). In summary, Cabo and BEZ elicited a pronounced effect on the infiltration and activity of MDSCs.

Next, phospho-receptor tyrosine kinase (phospho-RTK) antibody arrays were used to assess the effect of Cabo and BEZ on the phospho-RTK signalling in treated CPPSML tumours (Extended Data Fig. 6a), revealing Cabo-induced downregulation of pEGFR, pErkB2, pErkB3, pAxl, and pPDGFRα, and partial downregulation of pEGFR, pErkB3, and pAxl through indirect effect by BEZ (Fig. 3a). We further observed that Cabo or BEZ also reduced phosphorylated MET (pMET) and phosphorylated vascular endothelial growth factor receptor 2 (pVEGFR2) levels, and significantly suppressed PI3K–Akt–mTOR signalling in CPPSML tumours (Fig. 3b). Correspondingly, Cabo or BEZ decreased pS6 signalling in intratumoural Gr1+ MDSCs in the CPPSML tumour microenvironment (Extended Data Fig. 6b, c). These findings raised the possibility that Cabo and BEZ compromise MDSCs through inhibition of PI3K signalling. To test this hypothesis, we sought to rescue the viability of Cabo- or BEZ-treated MDSCs with...
enforced downstream activation of signalling surrogates. Specifically, MDSCs were isolated from the induced CRPC in CPPSML and co-transfected with recombinant active ERK2 and p70S6K proteins (Fig. 3c). Transfected MDSCs showed improved survival under Cabo or BEZ treatment (Fig. 3d, e). Similar results were obtained when the assay was performed in enhanced medium (Extended Data Fig. 6d, e). These results reinforce the view that Cabo and BEZ exert an impact on the PCa tumour microenvironment in part via selective depletion of MDSCs.

As cytokine signalling plays a pivotal role in the recruitment and activation of MDSCs, we further explored the impact of combination treatment on cytokine production in primary CRPC. Cytokine arrays revealed that several key cytokines involved in regulating recruitment and activity of immunosuppressive myeloid cells, including CCL5, CCL12, CD40, and HGF, were reduced by Cabo + ICB or BEZ + ICB treatment. These treatments were also associated with increased IL-1α, CD142, and VEGF (Fig. 3f and Extended Data Fig. 7a). IL-1α may contribute to reduced MDSC infiltration. We confirmed that recombinant IL-1α inhibited IL-1β-induced chemoattraction of MDSCs, and this effect was blocked by IL-1α-neutralizing antibody (Fig. 3g). Notably, the cytokine changes were significantly less pronounced in Dasa + ICB treatment (Extended Data Fig. 7b). Noting that cytokine production by cancer cells may influence the functional status of myeloid cells, we cultured MDSCs isolated from CRPC with conditioned medium from the CPPSML PCa cell lines (Extended Data Fig. 7c) and showed upregulated expression of genes responsible for MDSC-induced immune suppression, including Arg1, Cybb, Ncf1, and Ncf4 (refs 6, 11) (Fig. 3h). Critically, the expression induction was largely abolished if the PCa cells were pre-treated with Cabo or BEZ before conditioned medium was collected (Fig. 3i), whereas direct treatment of MDSCs with Cabo or BEZ caused insignificant expression changes (data not shown), suggesting that certain cytokines in the conditioned medium may drive the gene upregulation. Through cytokine array (Extended Data Fig. 7d), we identified ten cytokines significantly downregulated in the conditioned medium of PCa cells pre-treated with Cabo or BEZ (Fig. 3j), among which CCL5 was also identified as a downregulated cytokine in Cabo + ICB- and BEZ + ICB-treated CRPC (Fig. 3f). When MDSCs isolated from CRPC tumours were treated with each of the ten cytokines in the presence of Cabo or BEZ, significant upregulation of Arg1, Cybb, Ncf1, and Ncf4 was observed with most of the cytokines tested (Extended Data Fig. 7e). Therefore, PCa cells are capable of driving immunosuppression-related gene expression in MDSCs through secretion of multiple cytokines, and this paracrine signalling is impaired by Cabo or BEZ treatment.

BEZ targets multiple p110 isoforms. To test whether isoform-specific PI3K inhibitors would generate a comparable level of synergistic advantage when combined with ICB, we tested PI-3065 (p110β-selective inhibitor27) and GSK2636771 (p110δ-selective inhibitor28). Moreover, given the critical role of Cxcr2 in MDSC recruitment10,11 and the downregulation of Cxcr2 ligands Cxcl1 and Cxcl2 in the conditioned medium of PCa cells treated with Cabo and BEZ (Fig. 3j), we also tested a novel clinical-stage Cxcr1/2 inhibitor SX-682 (Extended Data Fig. 7f, g) as monotherapy or in combination with ICB in the CPPSML model. Mild to moderate effects of PI-3065, GSK2636771, or SX-682 as single agents on CRPC progression were observed, yet combination with ICB produced strong efficacy (Fig. 4a, b). To give further credential to our model and assess the pattern of MDSCs in human PCa, a 32-antibody CyTOF panel was developed (Extended Data Table 1b) and used to analyse 12 fresh fine-needle biopsy samples from ten treatment-naive PCa tumours (Extended Data Table 1c). Analysis with SPADE software displayed the heterogeneous immune cell populations (Extended Data Fig. 8). Results showed the prominence of Gr-MDSCs relative to Mo-MDSCs, with the latter occupying no nodes in the SPADE tree (Fig. 4c). Total T-cell load across...
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Fig. 9). The differential sensitivity of MDSCs and CD8+ T cells to Cabo, BEZ, and possibly other phosphokinase inhibitors should provide an avenue for optimizing the dose and schedule for effective silencing of MDSCs while simultaneously sparing cytotoxic T lymphocytes to attack cancer cells. As with all preclinical model systems and human clinical pathological correlations, prospective clinical trials will be needed to substantiate the hypotheses of our work. Future studies should explore the combination therapy in the context of both established mCRPC and newly diagnosed PCa together with selective anti-androgens to achieve durable clinical response in this major cancer of men.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions X.L., R.A.D., Y.A.W., and J.W.H. conceived the project and discussed experiments; J.W.H. designed the methodology for, and oversaw, the chimeric modelling; X.L., E.P., and X.S. provided study drug SX-682 and technical assistance for its use; J.A.Z. suggested MDSC experiments with SX-682 in the model; P.S. provided key suggestions on experiments; Y.A.W., and R.A.D. supervised the research; X.L., R.A.D., Y.A.W., and D.J.S. wrote the manuscript.

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Specifically, cell lines were cultured to roughly 80% confluence on inactivated mouse embryonic fibroblasts in wells of a 24-well plate in mES cell culture media described above. Cells cultured for infection were split 11–16 h before preparation for microinjection. Cells were removed from incubation, washed with sterile PBS, and trypsinized with 0.25% trypsin-EDTA. Trypsin was quenched with mES cell culture media containing FBS and the cell culture plate was returned to incubation for 45 min to allow the larger mouse embryonic fibroblasts to re-attach to the bottom of the cell culture well. After that incubation, the desired mES cells were lifted up using a 15 ml conical tube. The suspension was brought up to 5 ml with mES cell culture media, gently pipetted up and down to mix, and then spun down at 4°C and 800 rpm for 4 min. All except 100–200 μl of medium was aspirated from the tube. The bottom of the conical tip was tapped externally to break up the cell pellet and the tube was placed on ice for microinjection use. C57BL/6N Tac-Tyr<sup>Im4Art</sup> female mice that had achieved successful strain-matched mating after superovulation via timed gonadotropin administration were used as donors for 3.5 day blastocysts. Blastocysts were collected by uterine flush and cultured in M16 medium (Millipore, MR-016-D) overlaid with embryo-tested oil in a 35 mm culture dish at 37°C with 5% CO<sub>2</sub>. Blastocysts were each micro-manipulated to insert roughly 12 individual mES cells into its blastocoele. Injected blastocysts were then implanted into the uteri of pseudo-pregnant females. Each pseudo-pregnant female received up to 14 micromanipulated blastocysts (up to seven blastocysts per uterine horn). Chimaeras were verified with prostate tumour formation by MRI and assigned into preclinical studies through randomization which maximized the chance that male mice housed in the same cage received different drugs. Blinding was not applied, because allocation required investigators to assess mouse prostate tumour size with MRI before randomization. Pups were excluded from tumour analysis if they died without significant sign of tumour formation as judged by MRI imaging.

**Single-nucleotide polymorphism analysis.** Single-nucleotide polymorphism analysis was performed in the Genetic Services core at MD Anderson Cancer Center. For background characterization of the JH61 ES cell line, 80 microsatellite (simple sequence length polymorphism, SSLP) markers, polymorphic between FVB/N and 129S6 inbred strains, and evenly distributed along the genome (that is, genome scan), were used as described<sup>13</sup>. To analyse the MHC region on mouse chromosome 17, an extra 15 polymorphic SSLPs flanking the H2 complex were added. The results (allelotype) obtained for the SSLP in the H2 region were confirmed by means of H2 polypeptide chain reaction–restriction fragment length polymorphism (PCR–RFLP) genotype as described<sup>13</sup>. Non-invasive mouse and *ex vivo* imaging. For MRI imaging with Bruker ICON, the animals were anaesthetized with 1–3% isoflurane and placed on the ICON animal bed. The MRI coil was secured into position over the animal and the entire bed assembly was placed into the Bruker ICON 1T MRI bore. Rapid acquisition with relaxation enhancement (RARE) T2-weighted images were acquired in both the coronal and axial planes. After the imaging was completed, the animals were allowed to recover under a heating lamp until fully conscious. MRI images were loaded into ImageJ to manually demarcate the contour of the prostate and calculate the total volume. Bioluminescence imaging with IVIS Spectrum was performed as previously described<sup>14</sup>. Fluorescent tissue sections of dissected prostate tumours, the pair of draining lymph nodes, and lung was performed with a Leica M165FC fluorescence stereomicroscope. Lymph node metastasis score was assigned as 0 (no GFP<sup>+</sup> tumour cells), 1 (sparse GFP<sup>+</sup> tumour cells on one lymph node), 2 (sparse GFP<sup>+</sup> tumour cell clusters on both lymph nodes), or 4 (strong GFP<sup>+</sup> tumour cell patches on the other lymph node), or 3 (strong GFP<sup>+</sup> tumour cell patches on the two lymph nodes). Spontaneous lung micrometastasis was quantified by counting tumour cell clusters with more than ten GFP<sup>+</sup> tumour cells in each cluster. Immunohistochemistry, immunofluorescence, and western blot. Tissues were fixed in 10% formalin overnight and embedded in paraffin. Immunohistochemical and immunofluorescence staining was performed as previously described<sup>11,14</sup>. Immunohistochemical slides were scanned with a Panoramic Digital Slide Scanner (3DHISTECH) and images were cropped from virtual slides in Panoramic Viewer. Immunofluorescence slides were imaged with a Nikon A1R Confocal Laser Microscope and quantified with ImageJ. Primary antibodies used included CK5 (Covance, PB-160P), CK8 (Covance, MM-162P), K67 (Fisher, RM-9106-S1), cleaved caspase 3 (Cell Signaling Technology, 9661), Gr-1 (BioLegend, 108401), phospho-S6 (Cell Signaling Technology, 4858). For immunofluorescence, cell sections were blocked in 1% BSA in PBS containing 0.5% Tween-20 for 1 h and incubated overnight at 4°C with primary antibodies. Slides were washed in PBS, and then incubated in Alexa Fluor 488 or 568 (Invitrogen) or Cy3 (Jackson Laboratory) labeled secondary antibodies for 1 h. Images were acquired on a Zeiss AxioObserver microscope using AxiosVision 3.0 software.
phospho-mTOR (Cell Signaling Technology, 5536), phospho-p70 S6K (Cell Signaling Technology, 9234), phospho-S6 (Cell Signaling Technology, 4856), and vinculin (Millipore, 05-386).

**Targeted pathway inhibitors.** Enzalutamide (MedKo Biosciences, 201812) was admixed with Purina 5053 Chow at 50 mg of drug per kilogram of diet (Research Diets), which, when fed to mice, could reach approximately 10 mg per kg per day dose42 (calculation based on average body mass of 25 g and daily food intake of 5 g per day, with online calculator at www.researchdiets.com/resource-center-page/diet-dosage). To induce castration-resistant prostate tumour confirmed by MRI were surgically castrated and started on an Enza-mixed diet for 3 weeks before being moved back to regular chow and treated with therapeutic drugs. For in vivo pharmacological inhibition, BEZ235 (Selleck Chemicals, S1009), cabetinib (Selleck Chemicals, S1119), dasatinib (Selleck Chemicals, S1012), PI-3065 (MedKo, 407192), and GSK2636771 (MedKo, 205844) were orally administered at daily doses of 45 mg per kg, 30 mg per kg, 30 mg per kg, 30 mg per kg, and 30 mg per kg, respectively, daily on a Monday–Friday schedule. SX-682 (Syntrox Biosystems) was orally administered twice a day at 50 mg per kg actual dose on a Monday–Friday schedule. Similar drug dosing methods were described previously8,9,37,38. The doses we used for cabetinib and BEZ235 are clinically relevant: for cabetinib, to convert the mouse dose into human dose, we calculated 30 mg per kg × (1/1.23) = 24.2 mg per kg daily in human (the conversion factor 12.3 can be found in FDA guidance at http://www.fda.gov/downloads/Drugs/.../Guidances/UCM078932 and in ref. 39). Given a typical human weight of 60 kg, the clinical dose range of 60 mg daily (PCa, renal cell carcinoma) to 140 mg daily (medulillary thyroid cancer) converts to a human dose range of 1–2.3 mg per kg daily. Therefore, our cabetinib dose in mice approximates the approved dose for treating medulillary thyroid cancer, and is 1.4 times higher than used in COMET-1 and COMET-2 phase III trials of advanced PCa. For BEZ235, previous studies showed that while 300 or 400 mg twice daily65,66 showed significant toxicity in patients, 200 mg twice daily displayed higher tolerability19,40. The dose we used in the mice study, 45 mg per kg daily, is approximately equivalent to 225 mg daily in humans, and thus is within range of tolerable clinical doses.

For TCR and GR1-neutralizing antibody treatment, anti-PD-1 (clone RMPI-14, BioXcell, BE0146), anti-CTLA4 (clone 9H10, BioXcell, BE0131), or anti-Gr1 (clone RB6-8C5, BioXcell, BE0075) antibodies (or their respective isotype IgG controls) were intraperitoneally administered at 200 μg per injection three times per week. The duration of drug treatment was typically 4 weeks before endpoint analysis.

**CyTOF of human specimens and mouse tumours.** CyTOF analysis of mouse prostate tumours with data analysed in Flowwo (Tree Star) followed our previous methods41. Human prostate fine-needle aspirate specimens were acquired under approved institutional review board Protocol PA14-0420 at MD Anderson Cancer Center. Informed consent was obtained from all subjects. Prostate tumour single cells were isolated using a Human Tumour Dissociation Kit (Miltenyi Biotec, 130-094-538) and plated in RPMI-1640 supplemented with 10% FBS and antibiotics. From the cell line established and grown in complete DMEM supplemented with 10% FBS and antibiotics. Treatment of MDSCs with PCa-cell conditioned medium. Reagent WST-1 (Sigma-Aldrich, 11644807001).

**Preparation of SX-682.** SX-682 was synthesised by the Conditional Chemical Biology Group at the University of Texas at Austin. The dose we used in the mice study, 45 mg per kg daily, is approximately equivalent to 225 mg daily in humans, and thus is within range of tolerable clinical doses.

**Pharmacokinetics.** The dose we used in the mice study, 45 mg per kg daily, is approximately equivalent to 225 mg daily in humans, and thus is within range of tolerable clinical doses. This conversion factor is based on the conversion factor for weight (1.23), which is derived from the FDA guidance at http://www.fda.gov/downloads/Drugs/.../Guidances/UCM078932 and is supported by the study of Enzalutamide (MedKo Biosciences, 201812).

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General chemicals, reagents, and precursors for synthesis were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA), Boron Molecular (Research Triangle Park, North Carolina, USA), and Frontier Scientific (Logan, Utah, USA). Solvents were purchased from either VWR International (West Chester, Pennsylvania, USA) or Sigma-Aldrich (Milwaukee, Wisconsin, USA) and used without further purification. The synthesis steps are described below as illustrated in Extended Data Fig. 7g.

2-Chloro-pyrimidine-5-carboxylic acid (1) (3.16 g, 20 mmol, Frontier Scientific, Logan, Utah, USA) was suspended in dichloromethane (40 ml), and oxalyl chloride (3.30 g, 26 mmol) was added, followed by DMF (three drops) as catalyst. The reaction started to vigorously evolve gas. The reaction was heated to reflux for 1 h, and then allowed to cool to room temperature. 4-Fluoroaniline (2.44 g, 22 mmol) was added, vigorous bubbling was seen again, and the reaction mixture warmed up considerably. Triethylamine (4.05 g, 40 mmol) was added, and a flocculent precipitate immediately formed. The reaction mixture was heated to reflux once again for another hour, removed from heat, and stirred at room temperature for 18 h under nitrogen. The reaction was diluted with EtOAc (100 ml), and the organic layer washed with H2O, saturated NaHCO3, H2O, 1 N HCl, H2O, brine, then dried over Na2SO4. The liquid was filtered, and evaporated to yield 3.44 g (68%) of the intermediate. The intermediate was carried forward without further purification.

In a round-bottomed flask, the intermediate 2-chloro-pyrimidine-5-carboxylic acid (4-fluoro-phenyl)-amide (2.52 g, 10.0 mmol) and anhydrous sodium hydroxide solid. Electrospray ionization–mass spectrometry (ESI–MS) ([M]+: 325.2). The NMR spectrum also contained peaks consistent with the presence of residual DMF. The intermediate was carried forward without further purification.

The pinacol boronate ester was deprotected via hydrolysis of the intermediate trifluoroborate43. Compound 3 (5.66 g, 10.3 mmol, 1 eq.) was dissolved in methanol (100 ml). The reaction vessel was charged with 4.5 M aqueous potassium hydrogen fluoride (11.5 ml, 5 eq.) and the resulting solution was stirred for 1 h. The methanol was removed by rotary evaporation at room temperature and the resulting mixture of yellow and off-white solids was suspended in acetone. The suspension was gravity filtered to remove the insoluble salts, and the resulting clear yellow solution was added via pipette to a flask of H2O (2 l) and placed in the refrigerator. After cooling for about 1.5 h, the resulting off-white precipitate was collected by vacuum filtration, rinsing with water. The funnel was dried overnight in a vacuum desiccator to afford 3.87 g (80%) of 2-(2-boronic acid-5-trifluoromethoxy-benzylsulanyl)-pyrimidine-5-carboxylic acid (4-fluoro-phenyl)-amide (4) (ESI-MS) ([M]+: 362.1). Melting point = 211–214°C. 1H NMR (300 MHz, DMSO-d6) δ 10.49 (s, 1H), 9.09 (s, 2H), 8.33 (bs, 2H), 7.78–7.75 (m, 2H), 7.66 (d, J = 8.5 Hz, 1H), 7.46 (s, 1H), 7.25–7.19 (m, 3H), 4.70 (s, 2H); 13C NMR (100.6 MHz, DMSO-d6) δ 173.7, 161.9, 159.8, 157.4, 156.8, 148.8, 144.7, 136.0, 135.7, 135.0, 123.8, 122.3, 122.2, 121.7, 121.4, 118.8, 118.3, 115.5, 115.3, 34.3; 19F NMR (300 MHz, DMSO-d6) δ = −56.5 (3F), −118.2 (1F); 11B NMR (118.4 MHz, DMSO-d6) δ 21.7; HRMS (m/z): [M]+ calculated for C19H15BF4N3O4S, 468.0807; found, 468.0803; analysis (calculated, found for C19H15BF4N3O4S): (C: 48.34, 48.91), H (3.02, 3.20), N (8.99, 8.99), S (6.86, 6.73).

Statistical analysis. Data are presented as mean ± s.d. unless indicated otherwise. Sample size was chosen to ensure 80% power to detect significant effect size on the basis of our recent publications using the transgenic PCA mouse models and thereof derived primary tumour and myeloid cells11,13,14. A Student’s t-test assuming two-tailed distributions or a non-parametric Mann–Whitney U-test was used to calculate statistical significance between groups (no assumption was made that variance was similar between the groups being statistically compared). P < 0.05 was considered statistically significant.

Data availability statement. Source Data for the main and Extended Data Figures are provided in the online version of this paper. All other data are available from the corresponding authors upon reasonable request.

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Extended Data Figure 1 | Chimaeric modelling as an efficient approach to generating spontaneous metastatic PCa. a, Comparison of probability of obtaining PCa-bearing males with CPPSML genotype in a litter, through breeding or chimaeric modelling. In chimaeric modelling, >75% coat colour contributed by injected mES cells (mESC in the figure) is defined as positivity for chimaera. b, c, Predicted and experimental results for PCR–RFLP genotyping of the H2 locus from several mouse strains and two mES cell lines (JM8 is a standard mES cell line derived from C57BL/6 strain as control; Ep61 is also known as JH61). Red asterisk in b and yellow highlight wells in c indicate that the H2 haplotype for JH61 is H2b, the same as the C57BL/6 strain. d, SSLP marker analysis of the region on chromosome 17 flanking the H2 complex locus (34–46 Mb), showing that JH61 has 100% C57BL/6 background in the H2 locus, identical to the standard C57BL/6 mES cell line JM8. e, Experimental steps for generating the CPPSML chimaeras. f, Fluorescence images of prostate, draining lymph node (LN), and lung from a representative chimaera at 3 months old. GFP+ signals indicate the presence of metastasis to lymph nodes and disseminated tumour cells and micrometastasis in lung. Scale bars: prostate, 5 mm; lymph node and lung, 1 mm. g, Fluorescence microscopy and H&E image of snap-frozen prostate tumour from chimaera showing that the GFP+ area corresponds to adenocarcinoma and the GFP− area corresponds to normal host cells. Scale bar, 500 μm. h, Immunohistochemical staining showing the expansion of both CK8+ luminal lineage and CD5+ basal lineage in the prostate tumour formed in CPPSML chimaera. Scale bar, 50 μm.
Extended Data Figure 2 | Experimental design for preclinical therapy of mCRPC in CPPSML chimaeras. a, Significant yet transient survival benefit by castration followed by diet admixed with enzalutamide (50 mg per kg diet) in PB-Cre\(^+\) Pten\(^{-/-}\) p53\(^{-/-}\) Smad4\(^{-/-}\) mice (n = 40 and 18, respectively). ****P < 0.0001, log-rank test. b, Record of assignment for drug trials showing the time range of prostate tumour formation in the CPPSML chimaera. c, Representative CPPSML chimaera with primary CRPC, lymph node metastasis, and micrometastasis in lung. d, Experimental flow for creating mCRPC cohorts and preclinical testing of monotherapy and combination therapy, followed by tumour characterization. e, Representative images of prostate tumours with H&E staining, and GFP\(^+\) tumour cells in the lymph node and lung. Scale bars: prostate, 5 mm; lymph node and lung, 1 mm. AP, DLP, and VP denote anterior, dorsolateral, and ventral prostate lobes, respectively.
Extended Data Figure 3 | Significant combination efficacy by cabozantinib and ICB observed in chimaeras generated from JH58.

a, Experimental design for JH58 chimaeras, similar to the JH61 chimaera experiments. b, Longitudinal MRI images from representative chimaeras in control or combination cohorts. Red contour denotes area of prostate tumour. c, Strong anti-tumour effect by combination therapy in JH58 chimaeras shown by prostate tumour mass, lymph node metastasis scores and lung micrometastasis number (n = 3, biological replicates).

For panels d and e, data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control using Student’s t-test.

f, g, Quantification of tumour cell proliferation by Ki67 immunohistochemistry (n = 4, biological replicates) with representative images. Anterior prostate (AP) and dorsolateral prostate (DLP) were quantified separately. Scale bar, 100μm.
Extended Data Figure 4 | Combination efficacy by Gr1 neutralizing antibody with ICB. 

**a**, Dasatinib, but not cabozantinib or BEZ235, significantly reduced the frequency of infiltrating T cells in CRPC of CPPSML mice (n = 4, biological replicates). Data represent mean ± s.e.m. *P < 0.05, Mann–Whitney U-test. 

**b**, Frequency of Gr-MDSCs and Mo-MDSCs in CPPSML prostate tumours (n = 13, biological replicates). In **b**, data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, #P > 0.05, Mann–Whitney U-test.

**c**, Mass and representative whole-organ and H&E images of prostate tumours from CPPSML chimaeras induced to develop CRPC and treated with 1 month of control IgG, ICB (anti-CTLA4 plus anti-PD1 antibodies), anti-Gr1 neutralizing antibody, or combination of ICB and anti-Gr1 (n = 4, biological replicates). Scale bars: 3 mm for organ images, 200 μm for H&E images. In **b** and **c**, data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, #P > 0.05, Mann–Whitney U-test.
Extended Data Figure 5 | Characterization of the effect of drugs on MDSCs. a, Comparison of in vitro sensitivity to Dasa by MDSCs, CD8+ T cells, and GFP+ cancer cells isolated from CRPC of CPPSML mice. Cell viability was measured 24 h after the start of drug treatment using the WST-1 assay. IC50 values are indicated. b, Comparison of in vitro sensitivity to BEZ, Cabo, and Dasa by MDSCs isolated from CRPC of CPPSML mice. The assay was performed in RPMI1640 supplemented with 10% FBS and 10 ng ml⁻¹ GM-CSF (n = 2, biological replicates). c, Comparison of in vitro sensitivity to BEZ, Cabo, and Dasa by MDSCs isolated from CRPC of CPPSML mice. The assay was performed in RPMI1640 supplemented with 10% FBS, 10 ng ml⁻¹ GM-CSF, and pre-conditioned for 12 h by PCa cell lines established from the CPPSML model (n = 2, biological replicates). d, Representative CFSE flow cytometry histograms showing the effect on in vitro T-cell proliferation by MDSCs isolated from CRPC of CPPSML mice treated with the indicated drugs. Position of CFSE peaks can be used to denote the T-cell division times. e, Representative CFSE flow cytometry histograms showing the effect of Cabo, BEZ, and Dasa on in vitro T-cell proliferation.
Extended Data Figure 6 | Cabozantinib and BEZ235 inhibit PI3K signalling in prostate tumour and intratumoural MDSCs. a, Mouse phospho-RTK array measuring phospho-RTK activity in prostate tumours with indicated treatments. Numerals 1–5 represent pEGFR, pErbB2, pErbB3, pAxl, and pPDGFRα, respectively (n = 2, biological replicates). b, c, Reduced pS6 signal in intratumoural MDSCs by Cabo and BEZ treatment, revealed by immunofluorescent co-staining of pS6 and Gr-1 (n = 3, biological replicates). Scale bar, 100 μm. d, WST-1 assay showing that co-transfection of active ERK2 and p70S6K proteins mediated the resistance of MDSCs isolated from CPPSML tumours to the cytotoxicity by Cabo (1.5 μM) or BEZ (0.15 μM). The assay was performed in RPMI1640 supplemented with 10% FBS and 10 ng ml⁻¹ GM-CSF (n = 3, biological replicates). e, WST-1 assay similar to d, but performed in RPMI1640 supplemented with 10% FBS, 10 ng ml⁻¹ GM-CSF, and pre-conditioned for 12 h by PCa cell lines established from the CPPSML model (n = 3, biological replicates). In c–e, data represent mean ± s.d. ***P < 0.001, Student’s t-test.
Cabozantinib or BEZ235 suppress secretion by PCa cells of several cytokines that promote MDSC activity.

**Extended Data Figure 7**

**a**, Quantification of intratumoural cytokine levels in CRPC chimaera tumours with indicated treatment using cytokine array (n = 2, biological replicates). Numerals 1–9 represent CCL5, CCL12, CCL21, CD40, CD142, HGF, IGFBP-6, IL-1ra, and VEGF, respectively.

**b**, Quantification of intratumoural cytokine levels in Dasa + ICB combination-treated CPPSML chimaera CRPC with mouse cytokine assay, with image and relative intensity of the numbered cytokines shown (n = 2, biological replicates).

**c**, Experimental design for MDSC culture in the presence of PCa conditioned medium.

**d**, Cytokine array results for conditioned medium from CPPSML PCa cell lines treated with vehicle, Cabo (1 μM), or BEZ (1 μM) for 12 h (n = 2, biological replicates). Boxed cytokine is CCL5.

**e**, Effect of supplementation of individual cytokines to the conditioned medium from PCa cell lines treated with Cabo (1 μM) or BEZ (1 μM) on Arg1, Cybb, Ncf1, and Ncf4 from cultured MDSCs (n = 3, biological replicates).

**f, g**, Chemical structure and synthesis of allosteric CXCR1/2 antagonist SX-682 (Syntrix Biosystems). For details, please refer to the corresponding section in Methods. In **b**, **e**, and **f**, data represent mean ± s.d.
Extended Data Figure 8 | Detailed cell population annotation in SPADE tree. **a**, SPADE tree coloured by the median intensity of individual markers (indicated above colour bar) to facilitate the assignment of tree branches to individual cell populations (shown on the top of each plot) \((n = 12\), biological replicates). **b**, Surface markers of different immune subpopulations representing small branches of the SPADE tree.
Extended Data Figure 9 | Model depicting the combination therapy strategy in treating mCRPC. As demonstrated in the CPPSML chimaera model, targeted therapy with agents that inhibit MDSC infiltration frequency and immunosuppressive activity can synergize with ICB to invigorate T-cell immunity in the prostate tumour microenvironment and thus impair CRPC progression.
Extended Data Table 1 | Additional information on mouse model, clinical samples, and methodology

| JH61 chimeras | # chimera | Percent |
|---------------|-----------|---------|
| 90-100%       | 56        | 48.7    |
| 75-90%        | 35        | 30.4    |
| 50-75%        | 14        | 12.2    |
| under 50%     | 10        | 8.7     |
| **TOTAL**     | **115**   | **100** |

### a

- **a**, Coat colour chimaerism for chimaeras derived from JH61.
- **b**, CyTOF antibody panel for human prostate tumour samples.
- **c**, Clinical information of the fresh fine-needle biopsy specimens.

### b

| Marker | Clone | Label | Vendor | Cat# |
|--------|-------|-------|--------|------|
| CD19   | HiB19 | 142Nd | DVS-Sunnyvale | 3142001B |
| CD357, GITR | 621 | 143Nd | BioLegend | 311602 |
| CD11b  | ICRF44 | 144Nd | DVS-Sunnyvale | 3144001B |
| CD4    | RPA-T4 | 145Nd | DVS-Sunnyvale | 3145001B |
| CD8a   | RPA-T8 | 146Nd | DVS-Sunnyvale | 3146001B |
| CD278, ICOS | C398.4A | 147Sm | BioLegend | 313502 |
| CD134, OX40 | Ber-ACT35 | 148Nd | BioLegend | 350002 |
| CD223, LAG-3 | Poly | 149Sm | R&D | AF2319 |
| CD202b (Tie2/Tek) | 33.1 (Ab33) | 150Nd | BioLegend | 334202 |
| CD123  | 6H6   | 151Eu | DVS-Sunnyvale | 3151001B |
| CD137  | 4-1BB | 152Sm | BD | 555955 |
| CD133/2 | 293C3 | 153Eu | Miltenyi | 130-090-851 |
| CD45   | HI30  | 154Sm | DVS-Sunnyvale | 3154001B |
| TIM-3  | F38-2E2 | 156Gd | BioLegend | 345002 |
| CD33   | WM53  | 158Gd | DVS-Sunnyvale | 3158001B |
| CD11c  | Bu15  | 159Tb | DVS-Sunnyvale | 3159001B |
| CD161  | 191B8 | 161Dy | Miltenyi | 191B8 custom |
| CD69   | FN50  | 162Dy | DVS-Sunnyvale | 3162001B |
| CD45RA | HI100 | 163Dy | BioLegend | 304102 |
| CD15   | WBD3  | 164Dy | DVS-Sunnyvale | 3164001B |
| CD16   | 3G8   | 165Ho | DVS-Sunnyvale | 3165001B |
| CD44   | BJ18  | 166Er | DVS-Sunnyvale | 3166001B |
| CD27   | O223  | 167Er | DVS-Sunnyvale | 3167002B |
| CD38   | HIT2  | 168Er | BioLegend | 303502 |
| CD25   | 2A3   | 169Tm | DVS-Sunnyvale | 3169003B |
| CD3    | UCHT1 | 170Er | DVS-Sunnyvale | 3170001B |
| CD62L  | DREG-56 | 171Yb | BioLegend | 304802 |
| CD274, PD-L1 | 29E.2A3 | 172Yb | BioLegend | 329702 |
| CD14   | HCD14 | 173Yb | BioLegend | 325602 |
| HLA-DR | L243  | 174Yb | DVS-Sunnyvale | 3174001B |
| CD279, PD-1 | J105 | 175Lu | MBL | D133-3 |
| CD56   | HCD56 | 176Yb | DVS-Sunnyvale | 3176001B |

### c

| Patient ID | Gleason Score (Grades) | pStage | pN Stage | Race |
|------------|------------------------|--------|----------|------|
| 880709     | 9(4+5)                 | pT3b   | N0       | H    |
| 880503     | 7(3+4)                 | pT2    | N0       | W    |
| 882696     | 7(3+4)                 | pT2    | N0       | W    |
| 883678     | 7(4+3)                 | pT2    | N0       | W    |
| 883121     | 7(4+3)                 | pT3a   | N0       | W    |
| 882002     | 9(4+5)                 | pT3b   | N1       | W    |
| 883318     | 9(4+5)                 | pT3b   | N0       | W    |
| 885177     | 7(4+3)                 | pT2    | N0       | A    |
| 885567     | 7(3+4)                 | pT2    | N0       | W    |
| 885592     | 7(4+3)                 | pT2    | N0       | W    |

a. Coat colour chimaerism for chimaeras derived from JH61. b. CyTOF antibody panel for human prostate tumour samples. c. Clinical information of the fresh fine-needle biopsy specimens.
Erratum: Effective combinatorial immunotherapy for castration-resistant prostate cancer

Xin Lu, James W. Horner, Erin Paul, XiaoYing Shang, Patricia Troncoso, Pingna Deng, Shan Jiang, Qing Chang, Denise J. Spring, Padmanee Sharma, John A. Zebala, Dean Y. Maeda, Y. Alan Wang & Ronald A. DePinho

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In this Letter, owing to an error during the production process, the x-axis label 'BEZ + ICB' appeared twice in each of the graphs in Fig. 1d–f, and the 'Cabo + ICB' label was missing. In each graph, the first 'BEZ + ICB' label should have read 'Cabo + ICB'. The original Letter has been corrected online.