Patient-derived Models of Abiraterone- and Enzalutamide-resistant Prostate Cancer Reveal Sensitivity to Ribosome-directed Therapy


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1. Introduction

Over the last decade, new systemic treatments for advanced prostate cancer have extended patient survival, but raised new clinical challenges [1]. Metastatic castration-resistant prostate cancer (CRPC) often maintains androgen receptor (AR) signalling due to resistance mechanisms including intracrine androgen synthesis, genomic alterations to the AR gene, and a neuroendocrine-like AR-null phenotype. Despite their heterogeneity, all models were sensitive to the combination of ribosome-targeting agents CX-5461 and CX-6258.

In this study, we developed new patient-derived xenografts (PDXs) of contemporary CRPC from patients resistant to conventional androgen deprivation therapies, second-generation AR-directed inhibitors, and chemotherapy, with a broad spectrum of resistance mechanisms. We used these PDXs, as well as explants and organoids derived from them, to test candidate therapies. This strategy revealed the effectiveness of targeting ribosomes with the combination of a ribosome-targeting agent and a PIM kinase inhibitor. Together, this work identifies a promising therapeutic strategy to target a range of CRPC subtypes and also provides new patient-derived models to prioritise treatment strategies for clinical translation.

2. Patients and methods

See the Supplementary material for further details of all experiments.

2.1. Patient-derived xenografts

PDXs were established by grafting CRPC tissue under the renal capsule of NOD-SCID or NSG mice according to human and animal ethics approvals [8]. For castration experiments, PDXs were regrafted into...
testosterone-supplemented mice before half the mice were castrated and their testosterone implants removed [9,10]. For the combination therapy, mice received four doses of vehicle or 50 mg/kg CX-5461 and 100 mg/kg CX-6258 (Cylene Pharmaceuticals, San Diego, USA) via oral gavage over 9 d [11].

2.2. DNA and RNA analysis

Low-coverage whole genome sequencing was run on an Illumina Nextseq platform (paired-end 75 bp). Similarity between samples was calculated using Spearman’s correlation. Targeted DNA sequencing was performed for a pan-cancer panel and the AR [12]. RNA sequencing with Illumina TruSeq V2 libraries was performed on a HiSeq 2500 (single-end 50 bp reads). Single sample gene set enrichment analysis was used to calculate the enrichment of MSigDB50 pathways. Xenomapper was used to remove mouse reads. Genomic aberrations were validated using Sanger sequencing, digital droplet polymerase chain reaction (PCR), or fluorescence in situ hybridisation. AR mutations were examined using transactivation assays with PC3 cells [13]. Quantitative reverse transcriptase PCR (RT-PCR) for AR variants (ARVs) was performed as described [14].

2.3. Preclinical drug testing

Explants were performed as described [15], with minor modifications. Tissues were treated with drugs for 48 h. Immunohistochemistry on explants was performed using antibodies listed in Supplementary Table 1 and scored using Aperio ImageScope software (Leica Biosystems, Melbourne, Australia). Organsoids were treated for 4 h with vehicle or M CX-5461, 5 μM CX-6258, or both compounds. Protein lysates from organsoids were analysed with Western blotting using antibodies listed in Supplementary Table 1.

2.4. Statistics

Statistical analyses were performed using Graphpad Prism 7.0. All tests were two tailed and p values <0.05 were regarded as significant.

3. Results

3.1. Abiraterone- and enzalutamide-resistant tumours exhibit genomic diversity

To establish new preclinical models of prostate cancer, we collected 109 tumour samples from 29 patients via the Melbourne Urological Research Alliance (MURAL) platform (Supplementary Fig. 1A). This yielded 10 new serially transplantable PDXs (Supplementary Fig. 1B). We focused on four PDXs derived from rapid autopsy samples from two patients (27 and 201) with CRPC who had exhausted multiple treatments, including enzalutamide and/or abiraterone (Fig. 1A–C), surmising that these aggressive tumours would set a high threshold for preclinical testing of drug responses. These PDXs were from metastases to dura (27.1 and 201.1), an inguinal lymph node (27.2), and lung (201.2), and all grew for multiple generations (Fig. 1C).

Genomic profiling with low-coverage whole-genome sequencing and targeted deep sequencing of 623 cancer-related genes showed that the features of the autopsy specimens were maintained by corresponding PDXs at early (G1–3) and late (G6–8) generations (Fig. 2A and 2B, Supplementary Fig. 2A, and Supplementary Tables 2 and 3). Indeed, there was strong overall concordance between the original autopsy samples and PDXs with respect to genome-wide copy number profiles (Fig. 2A), candidate genomic alterations (Fig. 2B and Supplementary Fig. 2B–F), and histopathology (Supplementary Fig. 3A and 3B).

After establishing that the PDXs faithfully recapitulated the original samples, we compared the four CRPC metastases in more detail. The genome–wide copy number profiles of patients 27 and 201 were dissimilar (Spearman rho = 0.401–0.522 between samples; Fig. 2A). Within each patient, the autopsy samples from patient 27 were highly correlated (rho = 0.923), but those from patient 201 were more distinct (rho = 0.557; Fig. 2A). Differences between the metastases from patient 201 include putative pathogenic alterations in two mismatch repair genes, MLH1 and MSH6 (Supplementary Fig. 2D) present only in 201.1, and loss of heterozygosity of TP53, with a pathogenic TP53 mutation that was present only in 201.2 (Fig. 2B and Supplementary Fig. 2B). Therefore, these new PDXs are typical of the heterogeneity that is observed in CRPC [16].

3.2. Abiraterone- and enzalutamide-resistant tumours exhibit diverse AR-associated mechanisms of resistance

CRPC usually remains reliant on AR signalling for growth, so resistance mechanisms associated with this pathway are common [12]. We evaluated androgen responsiveness of the PDXs by comparing their growth in castrated or testosterone-supplemented mice (Fig. 3A and Supplementary Fig. 4A–C). All PDXs grew in castrate hosts, as expected for tumours derived from CRPC. Three PDXs (27.1, 27.2, and 201.1) were also androgen responsive, with significantly greater growth in testosterone-supplemented mice (Fig. 3A and Supplementary Fig. 4A–C). In contrast, PDX 201.2 grew at the same rate in castrated and testosterone-supplemented mice. Supporting these findings, transcriptomic data demonstrated robust expression of AR-responsive genes in all PDXs except for 201.2 (Supplementary Fig. 4D).

To further investigate potential AR-related resistance mechanisms, we examined the architecture of the AR gene using deep, targeted sequencing. This approach revealed AR genomic structural rearrangements (AR-GSRs) in PDXs 27.1 and 27.2, resulting in the loss of a region encompassing AR exons 5–7 (Fig. 3B and 3C, and Supplementary Table 4). PDX 27.2 had at least two clones, with only one harbouring AR-GSRs. PDXs 201.1 and 201.2 did not have demonstrable AR-GSRs (Supplementary Table 4). AR-GSRs can promote the expression of ARVs with truncated carboxy (C) termini, some of which are constitutively active, androgen-independent transcription factors that mediate castration resistance [2,12,16,17]. Therefore, we examined ARV expression using quantitative RT-PCR and immunohistochemistry with antibodies recognising different regions of the AR or two clinically relevant variants, AR-V7 and ARv567es. We found varying full-length AR and ARV expression among the PDXs, with AR-GSRs linked to ARv567es expression as a possible mechanism of castration resistance in tumours from patient 27, but not from patient 201 (Fig. 3D and Supplementary Fig. 4E).
Fig. 1 – Establishing PDXs of CRPC from rapid autopsy specimens. (A) Sites that tumour material was collected from for two patients (patients 27 and 201) who consented to the CASCADE rapid autopsy programme. The metastases where there was sufficient fresh tissue for xenografting are marked. A subset of tumours formed successful PDXs. The four tumours that produced serially transplantable PDXs (27.1, 27.2, 201.1, and 201.2) are shown in colour, while other sites that were initially grafted, but did not establish long-term PDXs that are shown in white. (B) Serum PSA measurements from diagnosis to death for patient 27 and patient 201 and the systemic treatments they received. (C) Time course of the first 400 d of PDX growth for the four PDXs from patients 27 and 201. Each step represents a new generation (27.1—blue; 27.2—green; 201.1—red; 201.2—yellow). AR = androgen receptor; CRPC = castration-resistant prostate cancer; LN = lymph node; PDX = patient-derived xenograft; PSA = prostate-specific antigen.
Gain-of-function mutations of the AR are another common mechanism of resistance to AR-directed therapies. PDX 201.1 had two mutations, T878A and C687Y, each occurring with at least 94% frequency, indicating that this tumour predominantly expresses a double mutant form of the AR (Supplementary Table 4). The common T878A AR mutation is known to increase promiscuity in ligand binding by the AR\(^2,16\), while the C687Y mutation has not been reported previously. Transcriptional activation assays with an AR-responsive probasin luciferase construct showed that the C687Y mutation conferred agonist properties to hydroxyflutamide, nilutamide, and bicalutamide (Fig. 3E). Notably, compared with wild-type AR or single mutants, the C687Y/T878A double mutant exhibited increased responsiveness to flutamide, hydroxyflutamide, and dexamethasone (Fig. 3E). The double mutant also conferred partial resistance to enzalutamide compared with wild-type AR and single mutants (Supplementary Fig. 5A), despite all proteins being expressed at equivalent levels (Supplementary Fig. 5B). The only genomic alteration of the AR gene in this tumour was the T878A mutation (Supplementary Table 4), implying that loss of AR expression was likely due to epigenetic changes, as previously shown \(^{24}\). Collectively, these features place tumour 201.2 on the spectrum of AR-null or NE prostate cancers.

The transition of CRPC to an AR-null/AR-independent phenotype is another important mechanism of resistance to AR-directed therapies \(^4\). Since tumour 201.2 lacked the expression of AR and AR-regulated genes and was unaffected by androgen levels in vivo, we examined whether it had other features of AR-null and NE CRPC. PDX 201.2 neither had classical small cell pathology, nor expressed the NE markers synaptophysin and chromogranin A (Fig. 3F and Supplementary Fig. 6A). Nevertheless, it had several characteristics of AR-null and NE prostate cancer including: (1) focal staining of CD56; (2) concurrent genomic loss of TP53, PTEN, and RB1 \(^{18–20}\); (3) increased SOX2, BRN2, and FOXA2 expression \(^{19,21,22}\) and decreased FOXA1 expression \(^{23}\); (4) FGF pathway activation \(^4\); and (5) enrichment of an NE gene signature \(^3\) (Fig. 3F and 3G, and Supplementary Fig. 6A–D). The only genomic alteration of the AR gene in this tumour was the T878A mutation (Supplementary Table 4), implying that loss of AR expression was likely due to epigenetic changes, as previously shown \(^{24}\). Collectively, these features place tumour 201.2 on the spectrum of AR-null or NE prostate cancers. Therefore, the four PDXs harbour diverse mechanisms of resistance to AR-directed therapies, including ARV expression, AR mutations, and AR loss, highlighting the need to target additional pathways in CRPC.

### 3.3. Rapid therapeutic testing with integrated preclinical models

To expedite preclinical drug testing, we established explants and organoids from the PDXs (Fig. 4A). Explants maintained high levels of proliferation and low levels of apoptosis (Fig. 4B and 4C). Organoids grew efficiently with 5–14% of individual cells forming colonies from each PDX sample. The organoids also re-formed tumours in mice, confirming that they were still tumourigenic (Fig. 4A, 4D, and 4E).
Fig. 3 – PDXs exhibit diverse mechanisms of resistance to AR-directed therapies. (A) Average weight of grafts from each PDX grown in control mice bearing testosterone implants (+T) or established in mice bearing testosterone implants and then castrated for 3–28 d. Fast growing PDXs from patient 201 were harvested early due to animal ethics guidelines (unpaired T tests for +T versus castrate at each time point, n = 4–8 grafts/group, ± SEM). (B) Schematic of the AR gene showing the locations of two genomic inversions in PDXs 27.1 and 27.2. (C) Plots showing sequencing coverage across coding and noncoding regions of the AR gene in PDXs 27.1 and 27.2. (D) Representative images of immunohistochemistry for the AR N terminal (AR-N), AR C
showed that the gene set for MYC targets had the highest enrichment score, consistent with MYC gains amongst the tumours (Supplementary Fig. 7). Pathways associated with androgen response, cell cycle, and DNA repair were also enriched. Therefore, we selected inhibitors targeting AR signalling (current standard of care, enzalutamide, and a novel AR antagonist galeterone), DNA repair (talazoparib and cisplatin), and cell cycle progression (CDK4/6 inhibitor and ribociclib). To target MYC, we selected inhibitors of BET proteins (iBET151 and JQ1), which decrease c-MYC levels and also target the AR [25]. To block the central downstream function of c-MYC in elevating protein synthesis, we also selected inhibitors of ribosome biogenesis (RNA polymerase I transcription inhibitor: CX-5461) and ribosome function (pan-PIM kinase inhibitor: CX-6258) [11,26,27], drugs that we recently demonstrated to be effective in genetically engineered mouse models, prostate cancer cell lines, and a single xenograft [11].

3.4. Abiraterone- and enzalutamide-resistant tumours are sensitive to dual inhibition of ribosome biogenesis and function

In our explant system, only PDX 27.1 had decreased proliferation (Ki67 staining) and/or increased apoptosis (cleaved caspase 3 staining) in response to standard doses (10 μM) of AR-directed therapies (Fig. 5A). There was no change in AR localisation or prostate-specific antigen (PSA) expression after enzalutamide treatment in any of the tumours, including 27.1, consistent with abundant ARv567es expression in this tumour (Supplementary Fig. 8A and 8B). All other drugs were tested at 1 μM concentration. Only 27.1 was weakly responsive to the PARP inhibitor talazoparib, and none of the four tumours was responsive to cisplatin (Fig. 5B). This is consistent with the lack of homozygous defects in genes involved in homologous recombination in any of the tumours (Supplementary Fig. 2D). None of the PDXs responded to the CDK4-6 inhibitor ribociclib, likely due to homozygous loss of RB1 in all tumours, a known mechanism of resistance to this therapy (Fig. 5B).

Compounds that indirectly target MYC were, in general, more effective than the other classes of drugs in all PDXs. More specifically, BET inhibitors decreased proliferation and increased apoptosis in the three androgen-responsive tumours (27.1, 27.2, and 201.1), although there was no significant response in the AR-null 201.2 tumour (Fig. 5C).

Notably, the two compounds targeting ribosome biogenesis and function, CX-5461 and CX-6258, elicited significant changes in all four tumours, either as a single agent or in combination (Fig. 5D).

We examined mechanisms underlying the activity of CX-5461 and CX-6258 using short-term treatments of organoids from 201.1 and 201.2. The combination therapy significantly decreased the volume of 201.2 PDXs compared to the vehicle control after just 2 wk (Fig. 5F). To evaluate the ribosome-directed inhibitors in vivo, we selected model 201.2 since there is an urgent unmet need to develop therapies for AR-null/NE CRPC. The combination therapy significantly decreased the volume of 201.2 PDXs compared to the vehicle control after just 2 wk (Fig. 5F).

Fig. 4 – Integrated models for rapid preclinical testing. (A) Tumour 201.1 as a PDX, an explant, organoids, and regrafted organoids (Organoid-PDX) from the same generation (scale bars: upper panel—2, 2, 0.1, and 2 mm from left; lower panel—50 μm). Average percentage of (B) Ki67 and (C) cleaved caspase 3 (Casp3)-positive cells in explants after 48 h. Each point represents average data for three explants per PDX generation (n = 3–6 generations; SEM). (D) The average percentage of individual cells from each PDX that form organoids (% organoid-forming efficiency, OFE). Each point represents a different PDX generation. (E) Average diameter (μm) of organoids for each PDX over time. PDX = patient-derived xenograft; SEM = standard error of the mean.

(A) Examples of tumour tissues embedded in Matrigel (PDX) or explanted (Organoid-PDX) are shown for each tumour model. Images were acquired using a 20X objective. Scale bar = 50 μm. (B) Average transcriptional activation assays with an AR-responsive probasin luciferase construct comparing wild-type AR (AR-wt) with single and double mutant transfections. Transfected PC3 cells were treated for 24 h with vehicle or DHT alone or in combination with dexamethasone (Dex), enzalutamide (Enz), bicalutamide (Bic), flutamide (Flut), hydroxyflutamide (OH-Flut), or nilutamide (Nil). Data represent the average luciferase signal relative to Renilla (±SEM) for a representative experiment with n = 6 wells per treatment. (F) Heatmap showing the relative expression of genes associated with AR-null and neuroendocrine prostate cancer. Data represent log, fold changes in transcript abundance relative to the median for each gene. (G) GSEA of up- and downregulated genes in the Beltran signature in tumour 201.2 versus tumour 201.1. AR = androgen receptor; AR-FL = AR full length; AR-GSR = AR genomic structural rearrangement; CRPC = castration-resistant prostate cancer; FDR = false discovery rate; GSEA = gene-set enrichment analysis; NE = neuroendocrine; NGS = normalised enrichment score; PDX = patient-derived xenograft; PDX E = early-generation PDX; PDX L = late-generation PDX; SEM = standard error of the mean. * p < .05. ** p < .01.
Fig. 5 – Diverse tumours are sensitive to dual inhibition of ribosome biogenesis and function. The results of explants treated for 48 h with (A) AR-directed inhibitors (Gal—10 μM galeterone; Enz—10 μM enzalutamide), (B) cell cycle or DNA repair inhibitors (PARPi—1 μM talazoparib; CDDP—1 μM cisplatin; CDKi—1 μM ribociclib), (C) BET inhibitors (I-BET—1 μM I-BET 151; 1 μM JQ1), or (D) ribosome-targeted inhibitors (1 μM CX-5461; 1 μM CX-6258; 1 μM CX-5461 + 1 μM CX-6258). Graphs summarise the combined data from multiple experiments and represent average changes in the percentage of Casp3- and Ki67-positive cells versus the vehicle control (n = 3, ±SEM, * p < 0.05, **p < 0.01, ***p < 0.001, T test of absolute Ki67 and EUROC.
extend this observation, we selected another AR-null PDX (PDX 305; Supplementary Fig. 1B). This large-cell NE tumour lacked AR, PSA, and prostate-specific membrane antigen, but expressed chromogranin A, synaptophysin, neuron-specific enolase, CD56, TTF-1, and c-MYC. Notably, the combination of CX-5461 and CX-6258 significantly decreased PDX 305 tumour volume in vivo compared to the vehicle control (Fig. 5G).

In summary, with our new patient-derived models, we demonstrated that combined targeting of RNA Pol I and PIM kinases represents a promising therapeutic strategy for diverse CRPC subtypes, including NE-like AR-null disease.

4. Discussion

Despite radical changes in the treatment of CRPC, including the approval of new AR-targeted therapies (abiraterone and enzalutamide), chemotherapies, immunotherapy, and bone-targeted agents, this disease remains incurable and a major cause of cancer mortality [31]. In this study, we developed new PDXs of CRPC as models to test novel therapeutics. A limitation of establishing serially transplantable PDXs of prostate cancer is the low take rate compared to other tumour types [32]. We generated 10 new PDXs of CRPC with an overall take rate of 9% of samples from 29% of patients. Most PDXs were from soft tissue metastases, which often provide more fresh tissue for xenografting than bone metastases. Notably, several PDXs were established from CRPC patients who failed enzalutamide and/or abiraterone in the clinic. These PDXs are a valuable resource, since most models of prostate cancer were established prior to the routine use of these agents. Four CRPC PDXs were from two patients who consented to the CASCADE rapid autopsy programme, which enabled extensive sampling of metastases, including sites that are often inaccessible prior to death, such as dura [33]. Importantly, these PDXs exemplify the heterogeneity of CRPC, exhibiting differing genomic features and distinct mechanisms of AR-driven resistance, including AR-GSRs, mutations, and AR loss. Indeed, the divergence between tumours 201.1 and 201.2 was particularly striking. This fascinating case of a patient with both androgen-responsive (201.1) and AR-null (201.2) metastases highlights the need for new treatments against diverse subtypes of CRPC.

We used explants to screen candidate drugs targeting core oncogenic pathways that were enriched in the four PDXs, as determined by transcriptomic analyses. These experiments identified consistent sensitivity to treatment with CX-5461 plus CX-6258, extending our previous work with these compounds to diverse castration-resistant tumours [11]. More broadly, our approach highlights the utility of different types of patient-derived models, including explants and PDXs, in prostate cancer research [5,34–36].

A limitation of this study is the use of only four PDXs from soft tissue metastases from heavily pretreated patients. However, soft tissue metastases are becoming more common in patients who fail multiple lines of treatment as diverse mechanisms of therapy resistance evolve. Our data using ribosome-targeted inhibitors hold promise for future studies that will test the drug combination in models from bone metastases and earlier phases of patient treatment. Additionally, we will assess potential predictive biomarkers of responsiveness. Since CX-5461 also elicits DNA strand breaks, specifically at the ribosomal DNA locus [28], these biomarkers could include DNA repair defects, MYC, PIM substrates such as p27, and measurements of RNA Pol I activity [37–39].

The efficacy of the combination therapy emphasises that high rates of protein synthesis are critical for the unchecked proliferation of cancer cells and are an Achilles' heel of cancer compared to normal tissues [27,40,41]. In prostate cancer, several upstream pro-oncogenic signalling events converge on the protein synthesis machinery, including increased expression of PIM kinases [42], loss of PTEN [43], and frequent amplification or overexpression of MYC, which act cooperatively [40]. The resulting increase in RNA Pol I activity was recently shown in patient prostate cancer tissue by measuring the levels of ribosomal RNA expression [37]. Therefore, prostate cancer cells may be sensitive to inhibitors of ribosome biogenesis (RNA polymerase I inhibitors) and ribosome function (PIM kinase inhibitors) due to the elevated activity of these processes. Importantly, our results indicate that combining these therapies to simultaneously inhibit different parts of the ribosome pathway improves efficacy. This strategy may also curtail the resistance of tumour cells to treatment, as shown in other tumour types [11,44].

5. Conclusions

We established new PDXs of CRPC from men who failed abiraterone and/or enzalutamide and chemotherapy, including an AR-null tumour with NE features. Importantly, we showed that all these CRPC models are sensitive to dual inhibition of ribosome production and function. Since ribosome targeting agents are in clinical trials for haematological malignancies and breast cancer [38,39,45,46], our findings provide a strong rationale to pursue clinical trials of these drugs in CRPC.

Author contributions: Gail P. Risbridger had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Appendix A. Supplementary data

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References


