Fatty acid oxidation is an adaptive survival pathway induced in prostate tumors by heat shock protein 90 inhibition

Zeyad D. Nassar\textsuperscript{1,2,3}, Chui Yan Mah\textsuperscript{1,2,3}, Margaret M. Centenera\textsuperscript{1,2,3}, Swati Irani\textsuperscript{1,3}, Martin C. Sadowski\textsuperscript{4}, Julia S. Scott\textsuperscript{1,2,3}, Elizabeth V. Nguyen\textsuperscript{5}, Shilpa R. Nagarajan\textsuperscript{6}, Max Moldovan\textsuperscript{3}, David J. Lynn\textsuperscript{3,7}, Roger J. Daly\textsuperscript{5}, Andrew J. Hoy\textsuperscript{6}, Lisa M. Butler\textsuperscript{1,2,3*}

\textsuperscript{1} Adelaide Medical School, University of Adelaide, Adelaide, Australia
\textsuperscript{2} Freemasons Foundation Centre for Men’s Health, Adelaide, Australia
\textsuperscript{3} South Australian Health and Medical Research Institute, Adelaide, Australia
\textsuperscript{4} Australian Prostate Cancer Research Centre-Queensland, Institute of Health and Biomedical Innovation, School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Princess Alexandra Hospital, Translational Research Institute, Brisbane, QLD, Australia
\textsuperscript{5} Cancer Program, Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia
\textsuperscript{6} Discipline of Physiology, School of Medical Sciences, Charles Perkins Centre, Faculty of Medicine and Health, The University of Sydney, Sydney, Australia.
\textsuperscript{7} College of Medicine and Public Health, Flinders University, Bedford Park, Australia.

* Corresponding Author

Prof Lisa Butler, Adelaide Medical School, University of Adelaide,
c/o Cancer Theme, Level 5N, SAHMRI, PO Box 11060, Adelaide, SA 5001, Australia.

Phone: (+61) 8 8128 4360; Email: lisa.butler@adelaide.edu.au

Running title: Targeting FAO sensitizes PCa cells to AUY922 treatment
Keywords: Fatty acid oxidation, AUY922, HSP90, drug combinations, patient-derived explants

Disclosure of Potential Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this article.

Word count: 4618

Number of figures & tables: 5 figures & 0 tables

Supplementary materials: 8 figures & 4 tables
Abstract

HSP90 is a molecular chaperone required for stabilisation and activation of hundreds of client proteins, including many known oncoproteins. AUY922 (luminespib), a new generation HSP90 inhibitor, exhibits potent preclinical efficacy against several cancer types including prostate cancer (PCa). However, clinical use of HSP90 inhibitors for PCa has been limited by toxicity and treatment resistance. Here, we aimed to design an effective combinatorial therapeutic regimen that utilizes subtoxic doses of AUY922, by identifying potential survival pathways induced by AUY922 in clinical prostate tumors. We conducted a proteomic analysis of 30 patient-derived explants (PDEs) cultured in the absence and presence of AUY922, using quantitative mass spectrometry. AUY922 significantly increased the abundance of proteins involved in oxidative phosphorylation and fatty acid metabolism in the PDEs. Consistent with these findings, AUY922-treated PCa cell lines exhibited increased mitochondrial mass and activated fatty acid metabolism processes. We hypothesized that activation of fatty acid oxidation is a potential adaptive response to AUY922 treatment and that co-targeting this process will sensitize PCa cells to HSP90 inhibition. Combination treatment of AUY922 with a clinical inhibitor of fatty acid oxidation, perhexiline, synergistically decreased viability of several PCa cell lines, and had significant efficacy in PDEs. The novel drug combination treatment induced cell cycle arrest and apoptosis, and attenuated the heat shock response, a known mediator of HSP90 treatment resistance. This combination warrants further preclinical and clinical investigation as a novel strategy to overcome resistance to HSP90 inhibition.

Implications: Metabolic pathways induced in tumor cells by therapeutic agents may be critical but targetable mediators of treatment resistance.
Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer deaths in men in developed countries [1]. In the last decade, second generation anti-androgen drugs have been clinically approved, and significant advances in disease detection and imaging techniques have been introduced. Nevertheless, men continue to succumb to this disease as all tumors develop resistance to current available drugs, and ~360,000 men worldwide died of PCa in 2018 [1]. Suggested strategies to overcome the emergence of drug resistance include targeting several oncogenic pathways simultaneously by inhibition of a single upstream central regulatory protein [2]. Another strategy is to use combinations of drugs in which one targets the adaptive survival pathways elicited by the other/s [3]. In this study, we applied both strategies; the former by targeting molecular chaperone heat shock protein 90 (HSP90) using AUY922 (luminespib, structure published in [8]), and the latter by identifying and targeting possible AUY922 treatment adaptive pathways.

HSP90 is a chaperone protein that plays an important role in stabilisation, activation and post-translational maturation of more than 400 client proteins (https://www.picard.ch/downloads/hsp90interactors.pdf). Many HSP90 clients are oncoproteins, cell cycle regulators, steroid hormone receptors and transcription factors. HSP90 is overexpressed in many types of cancer including PCa [4], and is thought to address the high level of cellular stress induced by hypoxia, production of reactive oxygen species, increased DNA damage, and accumulation of misfolded and aggregated proteins [5]. Inhibition of HSP90 activity stimulates proteasomal degradation of its client proteins, and consequently induces cell death. Among its many client proteins, HSP90 is essential for the late stage maturation, stability and function of the androgen receptor (AR), the main driver of prostate tumorigenesis and diseases progression, and HSP90 inhibitors are efficient in degrading the AR [6]. Since the discovery of geldanamycin in early 1990s, numerous HSP90
inhibitors were developed and more than 18 distinct inhibitors have entered Phase I and II clinical trials [7]; however, most clinical trials failed due to pharmacokinetic and toxicity complications. A second generation of HSP90 inhibitors with improved toxicity and solubility profiles was developed to enhance clinical efficacy. One of the most potent second generation HSP90 inhibitors AUY922, a synthetic resorrinyl isoxazole amide (structure published in [8]), showed modest efficacy against myeloma, lung, gastrointestinal stromal tumors and breast cancers [9-12]. Preclinical studies demonstrated that AUY922 was a potent antitumor agent in PCa [13, 14] and more effective than the first generation HSP90 inhibitor, 17AAG [13]. We reported that AUY922 perturbs PCa cell invasion in vitro [15] and inhibits cell proliferation in patient derived explants (PDEs) [13].

Despite this promising preclinical efficacy, AUY922 along with other HSP90 inhibitors induces heat shock response (HSR) genes via activation of the transcription factor, heat shock factor 1 (HSF1). Measurement of HSR gene induction is one of the most consistent pharmacodynamic markers of HSP90 inhibition used clinically [7]. HSR genes encode a diverse set of chaperone proteins such as HSP27 and HSP70 that demonstrate cytoprotective activities [16], and their induction is a key mechanism of resistance to HSP90 inhibitors [7]. Although AUY922 was considered well-tolerated in many clinical trials at dose of 70 mg/m² [9, 10, 12], using maximally tolerated doses concomitantly increases the HSR and consequently treatment resistance. Using HSP90 inhibitors as a component in a combination regime might enable the use of lower doses of AUY922, lead to reduced activation of HSR, and result in better clinical outcomes. Only a few studies have investigated co-targeting HSP90 in PCa [14, 17, 18], and little is known about effective combination therapy involving new generation agents such as AUY922 in PCa.

In our previous study, using cultured human prostate tumors, referred to as patient-derived explants (PDEs), we identified response protein biomarkers correlating with the anti-
proliferative response to AUY922 [19]. In this study, we used this model system to identify possible survival pathways activated in response to AUY922 treatment and identified fatty acid oxidation as one of the prominent activated pathways. We then evaluated the efficacy of co-targeting HSP90 and fatty acid oxidation using PCa cell lines in vitro and ex vivo PDEs. Our results suggest that targeting fatty acid oxidation sensitizes PCa cells to HSP90 inhibition.

**Materials and Methods**

**Cell lines and reagents**

The human non-malignant prostate epithelial cell line PNT1 was from the European Collection of Authenticated Cell Cultures (ECACC). The prostate carcinoma cell lines LNCaP, C4-2B and 22RV1 were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell lines were authenticated by short tandem repeat profiling in March 2018 (Cell Bank Australia), and were regularly tested for Mycoplasma using PCR-based detection methods. The cells were propagated in RPMI-1640 medium containing 10% fetal bovine serum (FBS) purchased from Sigma-Aldrich (NSW, Australia). Cells were cultured in 5% CO₂ in a humidified atmosphere at 37°C. New batches of cells were thawed after approximately 15 passages. All chemicals were purchased from Sigma-Aldrich (NSW, Australia), unless otherwise mentioned. AUY922 (Novartis), perhexiline (PEX), etomoxir, 2-bromopalmitate and metformin were dissolved in dimethyl sulfoxide (DMSO). The sources and experimental conditions for the stains and primary antibodies are listed in Supplementary Table 1. Primers were obtained from Sigma-Aldrich and their sequences are detailed in Supplementary Table 2.

**Ex vivo culture of human prostate tumors**
Patient derived-explant culture was carried out according to techniques established in our laboratory and as described previously [20]. In brief, 6-mm biopsy cores were collected from men undergoing robotic radical prostatectomy at St. Andrew's Hospital (Adelaide, South Australia). The study was conducted in accordance with the Declaration of Helsinki and a written informed consent by each patient was obtained through the Australian Prostate Cancer BioResource, with ethical approval from the Human Research Ethics Committees of St Andrew’s Hospital and the University of Adelaide (approval H-2012-016). Clinicopathological details of the deidentified patients are included in Supplementary Table 3. In order to determine the tissue tumor content, a longitudinal section of the entire tissue core was collected for hematoxylin and eosin (H&E) analysis. The tissue then was dissected into small pieces (1 mm³) and cultured on gelatin sponges (Gelfoam, Pfizer, USA) pre-soaked in 500 ul of the cultural medium (RPMI-1640 with 10% FBS, antibiotic/antimycotic solution, 0.01 mg/ml hydrocortisone and 0.01 mg/ml insulin) in 24-well plates. Treatments were then added into each well with another 500 ul of the culture medium, and the plates were kept in 5% CO₂ in a humidified atmosphere at 37°C. After 48 hours, tissues were snap frozen in liquid nitrogen and stored at −80 °C, or formalin-fixed and paraffin-embedded until further analysis.

**Protein preparation and mass spectrometry analysis**

Protein was extracted and prepared for mass spectrometry analysis from PDEs cultured in the absence or presence of AUY922 (500nM) as described previously [19]. Samples were analysed on an UltiMate 3000 RSLC nano LC system (Thermo Scientific) coupled to an LTQ-Orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Scientific).

**Pathway over-representation analysis**
Pathway over-representation analysis was conducted using annotations from Molecular Signatures Database v6.1 [21]. To assess statistical significance, a hypergeometric test was implemented using the fisher.test function in the R v3.5.0 “stats” package. Pathways/gene-sets returning an FDR adjusted P value <5% were accepted as statistically enriched, and are listed in Supplementary Table 4. Heatmaps and enrichment plots were generated using the heatmap.2 and ggplot functions from gplots and ggplot2 R packages, respectively.

**Immunohistochemistry**

Immunostaining for paraffin-embedded tissues for Ki67 and cleaved caspase-3 were conducted as described previously [20]. A manual counting of at least 400 malignant cells from 10-20 randomly selected fields of view at 40× magnification was used to determine the percent of Ki67 and CC-3 positively stained nuclei.

**Measurement of cell viability**

Cell viability was determined using two methods, the CyQUANT cell proliferation assay (Thermo Scientific) according to the supplier’s protocol, or live imaging microscopy with the IncuCyte FLR and Zoom system (Essen BioScience, Ann Arbor, Michigan, USA). Synergism scores were determined using the SynergyFinder web application [22].

**Fatty acid oxidation assay**

PCa cells seeded in 6 well-plates were pre-treated with AUY922 for 24 hours then washed with PBS to remove AUY922 and serum, and then incubated with freshly prepared low glucose DMEM containing [1-14C] olate (0.5 µCi/mL; PerkinElmer, Boston, MA), 0.5 mM olate (Sigma-Aldrich, NSW, Australia), 1 mM L-carnitine, and 2% w/v fatty acid free BSA (Bovagen) for 4 hours. Afterwards, 14CO2 was liberated by transferring the media into glass vials containing 1 M perchloric acid and trapped in microcentrifuge tubes containing 1 M NaOH for 2 hours. NaOH was transferred to scintillation vials with 3 ml of scintillation
cocktail (Ultima Gold, Perkin Elmer). Aliquots of media were transferred into scintillation vials to measure the specific activity. The radioactivity was measured using a Tri-Carb 2810TR liquid scintillation counter. Readings were normalised to total cellular protein content. Results were reported as mean pmol/min/mg of protein ± standard error of the mean.

**Flow cytometric analysis of cell cycle, apoptosis, mitochondrial mass, and cellular ROS**

PCa cells were cultured at 6-well plates at a density of $3.5 \times 10^5$ per well. Cells were allowed to attach overnight, then the medium was replaced with fresh one containing treatments. Floating and adherent cells were collected and washed with ice-cold PBS. For mitochondria mass determination, cells were incubated with MitoTracker Green FM (for 30 min at 37 °C before cell collection). For cell cycle analysis, cells were fixed with 70% ethanol for 30 min at 4°C and then re-suspended in 1 mL of PBS containing 100 μg/mL ribonuclease A and propidium iodide (PI). Cells were incubated in the dark for 30 minutes at room temperature before analysis. For apoptosis analysis, after washing with cold PBS, cells were resuspended in 200 μl of binding buffer (Hanks’ Balanced Salt solution, 1% HEPES, 5 Mm CaCl$_2$), AnnexinV-PE and the viability stain 7AAD. Cellular ROS was analysed using the CellROX® Deep Red assay kit (Thermo Scientific) according to the manufacturer’s instructions. Cells were analysed using a LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA). Data were evaluated using FlowJo version 10.

**RNA isolation and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was isolated, and cDNA was synthesised as described previously [23]. RT-qPCR was performed with a 1:10 dilution of cDNA using SYBR green (Bio-Rad) on a CFX384 Real-Time System (Bio-Rad). Data were analysed using the comparative Ct method and was normalized by geometric averaging of internal control genes GUSB and L19.
Immunoblotting

The expression of cellular proteins of interest was measured using Western blotting as described previously [20]. Protein level was normalized to the matching densitometric value of the internal control HSP90.

Statistical Analysis

Results are reported as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism (V7.0 for Windows). Differences between treatment groups were compared by one-way ANOVA followed by Tukey or Dunnett post hoc test. P values <0.05 were considered significant.

Results

AUY922 induces fatty acid oxidation and mitochondrial respiration pathways in clinical prostate tumors

Hyper reaction monitoring (HRM), a novel data-independent analysis (DIA) mass spectrometry (MS) workflow, was previously performed on a cohort of 30 individual PDEs to identify novel protein markers of AUY922 activity in clinical tumors (Figure 1a) [19]. Here, we performed pathway and Gene Ontology (GO) over-representation analysis to identify AUY922-upregulated pathways that may contribute to treatment resistance. Oxidative phosphorylation and fatty acid metabolism pathways were the top upregulated pathways in AUY922 treated explants (Figure 1b). Gene Ontology over-representation analysis confirmed the overexpression of key fatty acid pathways (Figure S1a). Notwithstanding the predicted high inter-individual heterogeneity inherent to PDEs, heatmaps of each pathway revealed consistent activation of members of these pathways at an individual patient level (Figure 1c).
We validated our results by analysing the effect of AUY922 treatment on the expression of candidate genes involved in fatty acid metabolism in three human PCa cell lines, LNCaP, C4-2B and 22RV1. AUY922 treatment significantly induced the expression of CPT1A, DECR1, ECH1 and ACADM genes (P<0.05) (Figure S1b). We measured fatty acid β-oxidation in the PCa cell lines using radiolabelled oleate. Basal levels of fatty acid oxidation in LNCaP and C4-2B cells were 3-fold higher than in PNT1 non-malignant prostate cells (Figure 2a). Consistent with the observed induction of fatty acid oxidation gene expression, treatment of the cancer cells with AUY922 significantly increased fatty acid oxidation as indicated by the increased CO₂ generation from extracellular oleate. However, AUY922 treatment did not significantly increase fatty acid oxidation in non-malignant PNT1 cells (Figure 2b).

We next measured mitochondrial mass as a surrogate marker for increased mitochondrial biogenesis, which is associated with increased cellular oxidation capacity [24]. MitoTracker Green FM was used as its accumulation in mitochondria is independent of mitochondrial membrane potential. AUY922-treated LNCaP, C4-2B and 22RV1 cells exhibited a significant increase in mitochondrial mass compared to vehicle control, suggesting an increase in mitochondrial biogenesis (Figure 2c, S1c). Furthermore, AUY922 treated cells also exhibited an increase in reactive oxygen species (ROS), which is linked to increased mitochondrial mass [25, 26] (Figure 2d, S1d).

**Fatty acid oxidation inhibition sensitizes PCa cells to AUY922 treatment**

We next tested whether pharmacological inhibition of fatty acid oxidation could sensitize PCa cells to AUY922 treatment. Cells were co-treated with fatty acid oxidation inhibitors (Etomoxir, Perhexiline or 2-Bromopalmitate), or a mitochondrial respiratory chain complex I inhibitor (Metformin). All of these tested agents sensitised cells to AUY922 treatment (Figure S2). We selected Perhexiline (PEX) for further evaluation, as an approved clinical agent. The combination of AUY922 and PEX significantly reduced LNCaP C4-2B, and
22RV1 PCa cell viability (Figure 3a, S3). Synergy testing using the Bliss, Zero Interaction Potency (ZIP) and Highest Single Agent (HSA) methodologies revealed synergistic activity for the AUY922 and PEX at multiple concentrations and time points (Figure 3b &c, S3). Although the non-malignant prostate PNT1 cell line was less affected by the combination treatment after 24 hours of treatment, the combination acted synergistically and significantly inhibited cell viability by 48 and 72 hours (Figure S4). For subsequent mechanistic evaluations of the combination treatment, concentrations of 10 nM for AUY922 and 10 µM PEX were selected, as these doses showed strong synergy and robustly inhibited fatty acid β-oxidation (Figure S5).

PEX and AUY922 combination treatment induces cell cycle arrest and apoptosis

AUY922 co-treatment with PEX decreased mRNA gene expression of many cell cycle proteins to a greater extent than each individual drug treatment. In LNCaP cells, the combinational treatment decreased CDK4, CDK6, AURKB, CCD20, CCND1, CCNE2 and E2F1 mRNA expression significantly compared to the control and to each individual drug treatment (Figure 4a). In C4-2B cells, the combination treatment decreased CDK4, CDK6, CCND1, and E2F1 and increased mRNA expression of the cyclin/CDK complex inhibitor CDKN1A (p21) significantly compared to the control and to each individual drug treatment (Figure 4a). CDK4 was selected to prove that the changes at the mRNA level translated to protein levels. The expression of CDK4 protein level decreased markedly in both cancer cell lines when treated with the combination treatment compared to the control and to each individual drug treatment (Figure 4a). Investigation of key cellular proliferation and survival signalling pathways showed modest downregulation of pERK and pAKT (Ser473) in C4-2B cells compared with the control (Fig S6). The marked cell death induced by the combination treatment was characteristic of apoptosis, as evidenced by increased levels of cleaved PARP compared to all other treatment groups (Figure 4a).
Consistent with the changes observed in cell cycle gene expression, the combination treatment arrested the cells in G1 phase to a greater extent than each individual drug treatment. Specifically, combination-treated LNCaP cells exhibited a significant increase of cells in the G0/G1 phase from 55.6% to 85% when compared to control treatment (P<0.001), compared to AUY922 (58.7%, P<0.001) or PEX (78.1%, P<0.05), individually (Figure 4b). Similarly, the co-treatment increased the C4-2B G0/G1 phase population significantly from 40.7% to 78.6% (P<0.001), significantly more than single treatment with AUY922 (40.1%, P<0.001) or PEX (58.4%, P<0.001) (Figure 4b).

Levels of AnnexinV/7AAD in LNCaP cells were increased from 20.2% to 48.0% when cells were exposed to the co-treatment (P<0.05), compared to AUY922 (32.5%, P<0.001) or PEX (25.2%, P<0.001) single-drug treatment (Figure 4c). Likewise, the co-treatment significantly increased apoptotic C4-2B cells, as evidenced by increased levels of AnnexinV/7AAD, to 41.8% compared to 7% in control treated cells (P<0.001), and this was higher than single-drug treatment with AUY922 (14.7% P<0.001) or PEX (30.7%, P<0.001) (Figure 4c). We further validated the combined AUY922 and PEX-induced apoptotic cell death on the 22RV1 cell line (Figure S7a) and the non-malignant prostate PNT1 cell line (Figure S7b). Collectively, this demonstrates that the combined targeting of HSP90 and fatty acid oxidation inhibition activates apoptosis.

**PEX and AUY922 combination treatment minimizes AUY922-induced resistance mechanisms**

Clinical resistance to HSP90 inhibitors (including AUY922) is commonly attributed to the induction of the heat shock response (HSR) in tumor cells. Reducing the HSR may prevent or delay resistance to AUY922 and thereby enhance its clinical efficacy. Consistent with previous studies [18, 20], AUY922 increased mRNA expression levels of the HSR elements CLU and HSPB1 by approximately 7-fold in LNCaP cells and by 2.5-fold in C4-2B cells.
Combining AUY922 with PEX markedly attenuated HSR gene expression; CLU and HSPB1 gene expression decreased significantly from 6.9-fold and 6.3-fold in AUY922 treated cells to 2.1-Fold and 3.74-fold in combination treated cells, respectively (P<0.05) (Figure 4a). Similarly, CLU and HSPB1 gene expression in C4-2B cells decreased significantly from 2.6-fold and 2.5-fold in AUY922 treated cells to 1.9-fold and 1.7-fold in combination treated cells, respectively (P<0.01) (Figure 4a).

**PEX and AUY922 combination treatment exhibits pro-apoptotic and anti-proliferative effects in clinical prostate tumors cultured *ex vivo***

We previously reported the pronounced anti-proliferative properties of AUY922 (500 nM) in *ex vivo* cultured human prostate tumors [13]. In order to investigate whether PEX could enhance the efficacy of AUY922, we opted to use a lower concentration of AUY922 (i.e. 250 nM) than in our previous report (Figure 5a). The clinicopathologic features of PCa patients who had undergone radical prostatectomy are provided in Supplementary Table 4. Individual Ki67 proliferative index (percent) values for each individual patient and treatment are depicted in Figure 5b, and the mean inhibition of cell proliferation for the 9 patients is shown in Figure 5c. AUY922 treatment inhibited cell proliferation by 64.0% ±27 in 8 of 9 prostate tissue explants derived from 9 different patients (P<0.01). In contrast, PEX (10 µM) showed no efficacy in 6 out of 9 explants and only weak efficacy in 3 out of the 9 explants. The mean inhibition of cell proliferation by PEX alone was 17.9% ±9.3. Notably, the combination treatment was effective in all tested explants (9/9), significantly inhibiting cell proliferation (82.4% ±15.5; P<0.0001). This demonstrates that the combination treatment reduced cell viability more than the individual drug treatments in 8/9 of the PDEs and, in 6 of these, there was more than 10% enhancement in cell proliferation inhibition. We also investigated the effect of the combinational treatment on the induction of apoptosis in human PCa tissues (Figure 5a &c, S8). PEX failed to induce apoptosis compared to vehicle control. In contrast,
AUY922 single treatment and the combination treatment significantly increased the percentage of CC-3 positive cells by 283% (P < 0.05) and 335% (P<0.01), respectively.
Discussion

The results of this study emphasize the important role of lipid metabolic pathways in PCa progression, and reinforce the emerging preclinical evidence of the therapeutic benefit of lipid metabolism inhibitors in combinatorial regimens with other clinically available drugs [27-29]. In addition, it proposes a new strategy to maximize the clinical benefit of HSP90 inhibitors, which showed promising preclinical activities but modest clinical efficacy.

Despite the development and regulatory approval of potent anti-androgen drugs over the past decade, advanced PCa is still not curable. HSP90 inhibitors are rational therapeutics for advanced PCa as they cause AR destabilization and degradation, a distinct mechanism of action against AR than current clinically available AR antagonists. In addition, HSP90 inhibitors maintain their efficacy in cells expressing AR variants that mediate advanced, castration-resistant PCa, and are capable of degrading glucocorticoid receptor [30], which confer resistance against AR antagonists [31]. However, the very promising preclinical activities of HSP90 inhibitors have not translated well into clinical activity due to drug resistance and unfavourable pharmacokinetic and toxicity profiles. Although AUY922 showed improved solubility and tolerated toxicity, it induces the HSR which can mediate drug resistance. We hypothesized that AUY922 clinical use would be more successful if a lower drug concentration could be achieved within a therapeutic regimen by co-applying an agent that prevents AUY922-induced survival pathways and thus elicits less activation of the HSR and sensitises cancer cells to AUY922 treatment.

In an attempt to improve clinical performance of AUY922, here we identified novel survival pathways induced by AUY922 in cultured clinical prostate tumors using quantitative proteomic mass spectrometry. In contrast to previous resistance studies utilizing cell line-based models of PCa, we opted to include PDEs alongside cell lines as PDEs recapitulates disease heterogeneity and complexity of the clinical tissue microenvironment, in which
cancer cells co-exist with fibroblasts, immune cells, adipocytes and endothelial cells. The tumor stroma plays essential roles in cancer development, progression and therapeutic response via cell-cell interactions mediated by autocrine and paracrine signals, and can mediate drug resistance by hindering drug delivery into the cancer cells, modifying tumor cell metabolism and by secreting cytokines, hormones, growth factors and apoptosis inhibitors [32, 33]. Using PDEs is therefore a powerful approach to not only evaluate drug efficacy in cancer cells, but to anticipate and target potential stromal-promoting resistance pathways. This may in turn allow selection of the most promising treatments and combinations for subsequent clinical evaluation.

While the culture times in this study were only 2 days, short-term adaptive changes in response to ADT have been proven to be significant mediators of CRPC and antiandrogen resistance. For instance, overexpression of the glucocorticoid receptor, an established mechanism of CRPC and ADT resistance, takes place within days of antiandrogen treatment in vivo or after mouse castration [31, 34]. In addition, the activation of HSR genes in response to HSP90 inhibition takes place rapidly and is suggested to mediate drug resistance against these agents. In this study, pathway over-representation analysis of proteomics data generated from 30 PDEs revealed a striking overexpression of lipid metabolism and mitochondrial respiration pathways in response to AUY922 treatment (Figure 1). Consistent with this, in vitro cell treatment with AUY922 significantly increased PCa cell fatty acid oxidation, cytosolic ROS production and mitochondrial mass (Figure 2). Mitochondrial fatty acid β-oxidation is associated with the production of ROS, and previous reports have shown an increase in ROS to be linked to an increase in mitochondrial mass [25, 26], as a potential adaptive mechanism to facilitate maximum oxygen uptake and a higher production of energy within the cell [35, 36]. Increased mitochondrial mass has been linked to drug resistance and shown to be overcome by inhibition of oxidative phosphorylation [37, 38]. These
observations led us to hypothesize that these activated pathways serve to provide the cells with more energy to help the cell survive the considerable stress of HSP90 inhibition, and that targeting these pathways will sensitize cells to AUY922 treatment.

It has become established that altered metabolic pathways are a hallmark of cancer cells and that a rewired metabolism is crucial for cancer growth and progression. An accumulating body of evidence demonstrates the impact of metabolism rewiring on cancer cell responses to established first-line chemotherapy in various types of cancer, suggesting that the alteration of metabolic pathways mediates cancer cells drug resistance and these could be therapeutically targetable [39-41]. PCa cells have a distinct metabolic phenotype as they depend on fatty acid oxidation as a predominant source of energy [42]. The overexpression of lipid metabolism enzymes is characteristic of both early and late stages of PCa [43]. Proteomic analyses of primary and bone metastatic prostate tumors have revealed fatty acid oxidation as a significant mediator of PCa initiation and progression [44, 45], suggesting that targeting lipid metabolism pathways may be particularly relevant in PCa compared to other type of tumors. Targeting fatty acid synthesis has been a major focus of research attention, but with limited clinical success to date due to serious adverse effects. Recently, targeting fatty acid oxidation and uptake has started to gain more attention [27, 46, 47]. An emerging body of evidence shows that targeting lipid or cholesterol metabolism enzymes such as CPT1A or HMG-CoA reductase could be effective in CRPC or enzalutamide-resistant PCa cells [27-29]. Notably, numerous inhibitors of lipid metabolism pathways have been developed for liver and heart diseases, and several are approved for clinical use with established toxicity profiles, which facilitates drug-repurposing and rapid clinical integration of these drugs into PCa management.

In this study we used the fatty acid oxidation inhibitor and clinically approved antianginal agent in Australia and New Zealand PEX (Pexsig®). The clinical use of PEX as an
antianginal agent is based on its ability to shift myocardial metabolism from fatty acid oxidation to glucose consumption. Although its selectivity for CPT1 is questionable [48], PEX inhibits oxidative phosphorylation and oxidation of short, medium and long-chain fatty acids *in vitro* and *in vivo* [49]. Importantly, when we tested other inhibitors of fatty acid oxidation (Etomoxir and 2-bromopalmitate), both drugs acted very similarly to perhexiline and significantly sensitized PCa cells to the AUY922 treatment, strengthening the case that fatty acid oxidation is the key target for this combination. We further showed that the PEX and AUY922 combination effect on cell viability is mediated by induction of apoptosis and cell cycle arrest pathways. Although the cell death mechanism by the other 2 inhibitors (Etomoxir and 2-bromopalmitate) and the oxidative phosphorylation inhibitor (Metformin) in combination with AUY922 are expected to be similar to PEX, further characterization of cell death mediated pathways induced by these combinations is warranted.

PEX has previously demonstrated preclinical activity against several types of cancer [50, 51], while in PCa, PEX suppressed cancer cells growth *in vitro* and *in vivo*, and sensitized PCa cells to enzalutamide treatment [27]. Although the co-treatment was less toxic on the non-malignant PNT1 prostate cells than malignant cells after 24 hours, the co-treatment also inhibited PNT1 cell viability. However, as in vitro viability is not an indication of toxicity/safety of the combination, further *in vivo* experiments are needed to evaluate its potential therapeutic safety. Collectively with previous studies, these findings suggest that targeting fatty acid oxidation might be beneficial with other established first and second-line androgen receptor targeting strategies. While the use of PEX has been associated with adverse effects such as peripheral neuropathy, papilloedema, hypoglycaemia and weight loss, PEX is tolerated with regular monitoring of liver function, and glucose and drug plasma concentrations [52].
In addition to its ability to prevent fatty acid oxidation induced by AUY922 treatment, we found that PEX surprisingly reduced the AUY922-induced heat shock response (Figure 4a). In considering potential mechanisms for this observation, it is likely that production of ROS is a key factor. Mitochondrial respiration is the major source of intracellular ROS [53], and the cell responds to increased levels of ROS by activation of the HSR [54]. PEX has been reported to inhibit ROS production [55] and, presumably by this mechanism, minimises HSR activation whilst inhibiting fatty acid oxidation (Figure 5d).

In summary, we provide evidence that fatty acid oxidation is not only a primary source of energy for PCa cells but can also be enhanced as an adaptive survival pathway in response to drug treatment. In addition, our findings reinforce the use of patient-derived preclinical models to discover adaptive survival pathways that may contribute to clinical drug resistance, and to design efficacious combinational therapeutic regimens that overcome these adaptive responses.

**Acknowledgements**

ZDN is supported by an Early Career Fellowship from the National Health and Medical Research Council of Australia (1138648), a John Mills Young Investigator Award from the Prostate Cancer Foundation of Australia (YI 1417) and the Cure Cancer Australia Priority-driven Collaborative Cancer Research Scheme (1164798). CYM is supported by a Master of Philosophy International Scholarship and a Top-Up Scholarship from the Freemason’s Foundation Centre for Men’s Health. JSS is supported by University of Adelaide George Fraser Scholarship, and a Top-Up Scholarship from the Freemason’s Foundation Centre for Men’s Health. LMB was supported by an Australian Research Council Future Fellowship (130101004), and a Cancer Council SA Beat Cancer Project Principal Cancer Research Fellowship (PRF1117). AJH is supported by a Robinson Fellowship and funding from the
University of Sydney. DJL is supported by an EMBL Australia Group Leader award. RJD is supported by an NHMRC Principal Research Fellowship (1058540). This research was supported by funding from the Movember Foundation and the Prostate Cancer Foundation of Australia (MRTA3, to LMB, AJH; MRTA1, to MCS), and from Cancer Australia (1085471, to LMB, MMC, RJD).

The authors thank the Monash Biomedical Proteomics Facility, and SAHMRI Flow and Laser Scanning Cytometry Facility.
References


Figure Legends

Figure 1. AUY922 induces the expression of proteins in the fatty acid metabolism and oxidative phosphorylation pathways. a) Hyper reaction monitoring (HRM), a novel data-independent analysis (DIA) mass spectrometry (MS) was performed on 30 patient-derived explants (PDEs) treated with control (DMSO) or AUY922 (500 nM) for 48 hours. b) Pathway and Gene Ontology (GO) over-representation analysis of proteins significantly upregulated in AUY922 treated PDEs. Pathways/GO terms with an FDR < 0.05 were considered to be statistically enriched. c) Heatmaps showing normalised protein abundance in PDEs treated with DMSO or AUY922. Differentially abundant proteins in the “oxidative phosphorylation” and “fatty acid metabolism” Molecular Signatures Database “Hallmarks” gene sets are shown for matched Control and AUY922-treated PDEs (n=30).

Figure 2. AUY922 induces fatty acid metabolism in PCa cells and is associated with increased PCa mitochondrial mass and cellular ROS. a) Comparison of $^{14}$C-oleate oxidation in non-malignant and PCa cells. b) Effect of AUY922 treatment on $^{14}$C-oleate oxidation in non-malignant and PCa cells. c) Flow cytometry analysis of mitochondrial mass using MitoTracker® Green FM. d) Flow cytometry analysis of cellular ROS with CellROX® Deep Red. Cells were treated with control (DMSO) or AUY922 at the indicated concentration for 24 hours. Data is presented as mean±SEM *P<0.05, **P<0.01 and ***P<0.001, (n=3).

Figure 3. AUY922 and PEX act synergistically on PCa cells. a) LNCaP, C4-2B and 22RV1 cells were treated for 72 hours at the indicated concentrations. Cell viability was measured using CyQUANT Cell Proliferation Assay. The percent of cell viability inhibition compared to the control is visualized as a heatmap (n=3). b) The landscapes of the combination responses for AUY922 and PEX based on HSA model. c) Average synergy scores based on Bliss, ZIP and HSA were calculated using the Synergyfinder software. Cells were treated
with AUY922 and PEX for 24h, 48h and 72 hours. Synergy scores > 0 indicate synergism (red regions) and scores < 0 indicate antagonism (green regions).

**Figure 4. AUY922 and PEX combination treatment induces apoptosis and cell cycle arrest.**

*Left* qRT-PCR analysis of key markers of cell cycle and heat shock response and apoptosis in LNCaP and C4-2B PCa cells. *Right* western blot analysis of the cell cycle marker CDK4 and the apoptosis marker c-PARP. Cells were treated with vehicle control (DMSO), AUY922 (10 nM), PEX (10 µM) alone, or in a combination of the two drugs at the same concentration. Densitometry was used to quantify protein expression levels relative to HSP90 loading control. Values are presented as fold change relative to control treatment. Results are presented as mean±SEM *P<0.05, **P<0.01 and ***P<0.001 versus control, P#<0.05, P##<0.01 and P###<0.001 versus combination, and in heat shock response markers $P<0.05, $$P<0.01 and $$$P<0.001 AUY922 versus AUY922 treatment. *b and c* Flow cytometry analysis of *b* cell cycle distribution using propidium iodide (PI) staining, and *c* analysis of apoptosis by Annexin V/7AAD double staining. Cells were treated with vehicle control (DMSO), AUY922 (10 nM), PEX (10 µM) alone, or in a combination of the two drugs at the same concentration for 24 hours. Results are presented as mean±SEM *P<0.05, **P<0.01 and ***P<0.001 versus control. #P<0.05, ##P<0.01 and ###<0.001 versus the combination treatment, (n=3).

**Figure 5. AUY922 and PEX combination treatment induces apoptosis and decreases cell proliferation in cultured primary prostate tumors.**

*a* Representative immunostaining in sections of cultured primary prostate tumors using the proliferation marker Ki67 and the apoptosis marker CC-3. *b* Percentages of Ki67 positive cells are reported for each individual patient included in this study. Quantification of positively stained nuclei of at least 400 cells/sample are shown. *c* Mean percent of Ki67 and CC-3 positive cells of 9 patients are shown. Tumor tissue explants were treated with control (DMSO), AUY922 (250 nM), PEX
(10 µm) alone, or in combination for 48 hours. Results are presented as the mean percentage of positive cells ±SEM, *P<0.05, **P<0.01 and ****P<0.0001. n=9. d) Proposed mechanistic model for fatty acid oxidation (FAO) targeting in sensitizing PCa cells to HSP90 inhibition by AUY922. AUY922 treatment induces HSP90 chaperone complex remodeling, resulting in polyubiquitination and proteasomal degradation of HSP90 client proteins, and ultimately cell death. In clinical prostate tumors, the HSR, FAO, and mitochondrial biogenesis are all activated as survival pathways to minimize the efficacy of AUY922 and mediate drug resistance. Combination treatment with PEX blocks FAO, decreases ROS production and subsequently minimises HSR, thereby leading to cell cycle arrest and induction of apoptosis. * The diagram was created with BioRender.com.
Figure 1

a) Prostate Explants (n=30)
- Control (DMSO)
- AUY922 (500nM)

48hr culture

LC-MS/MS and HRM-DIA analysis

b) Enriched Hallmarks
- Oxidative phosphorylation
- Adipogenesis
- Fatty acid metabolism
- Protein secretion
- UV response up
- Glycolysis
- Peroxisome
- Epithelial–mesenchymal transition
- MTORC1 signalling
- Myogenesis
- Xenobiotic metabolism
- Hypoxia
- DNA repair
- Cholesterol homeostasis
- Apoptosis
- Bile acid metabolism
- Reactive oxygen species pathway
- Androgen response
- P53 pathway
- MYC target V1
- G2M checkpoint

Enriched KEGG Pathways
- Valine leucine isoleucine degradation
- Fatty acid metabolism
- Oxidative phosphorylation
- Propanoate metabolism
- Butanoate metabolism
- Tryptophan metabolism
- Glycolysis gluconeogenesis
- Citrate cycle TCA cycle
- Glutathione metabolism
- Biosynthesis of unsaturated TCA cycle
- Beta alanine metabolism
- Limonene and pinene degradation
- Pyruvate metabolism

(c) Heatmaps
- Oxidative phosphorylation
- Fatty acid metabolism

Color Key

Log2 abundance

Downloaded from mcr.aacrjournals.org on May 14, 2021. © 2020 American Association for Cancer Research.
Figure 2

(a) Basal Oleate Oxidation (pmol/min/mg) in PNT1, LNCaP, and C4-2B cells with varying concentrations of AUY922. 

(b) PNT1 Oleate Oxidation (pmol/min/mg) in Control, AUY 25 nM, and AUY 50 nM conditions. 

(c) Mitochondria Mass Intensity in LNCaP and C4-2B cells with AUY922 25 nM and 50 nM. 

(d) ROS Intensity in LNCaP and C4-2B cells with AUY922 25 nM and 50 nM.
Figure 3

a) Dose-response matrix (inhibition)

b) HSA synergy score: 15.821
   HSA synergy score: 25.449
   HSA synergy score: 10.36

c) LNCaP
   Bliss  | 24 h | 48 h | 72 h
   ZIP   | -2.7 | 2.9  | 8.9
   HSA   | 2.7  | 10.1 | 15.8

   C4-2B
   Bliss  | 5.2  | 4.2  | 16.9
   ZIP    | 5.2  | 3.5  | 16.5
   HSA    | 10.9 | 8.1  | 25.5

   22RV1
   Bliss  | 5.3  | -3.3 | 5.1
   ZIP    | 5.9  | -2.5 | 5.2
   HSA    | 7.0  | 3.4  | 10.4
Figure 5

(a) Immunohistochemical analysis of Ki-67 and CC-3 expression in different treatment groups.

(b) Bar charts showing the percentage of Ki-67 expression in various treatment conditions.

(c) Graphs depicting proliferation and apoptosis rates under different conditions.

(d) Schematic diagram illustrating the mechanisms of HSR, mitochondrial biogenesis, and drug resistance.

Downloaded from mcr.aacrjournals.org on May 14, 2021. © 2020 American Association for Cancer Research.
Molecular Cancer Research

Fatty acid oxidation is an adaptive survival pathway induced in prostate tumors by heat shock protein 90 inhibition

Zeyad D Nassar, Chui Yan Mah, Margaret M. Centenera, et al.


Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-20-0570

Supplementary Material  Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2020/07/15/1541-7786.MCR-20-0570.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/early/2020/07/15/1541-7786.MCR-20-0570. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.