Targeting Hypoxic Prostate Tumors Using the Novel Hypoxia-Activated Prodrug OCT1002 Inhibits Expression of Genes Associated with Malignant Progression

Heather Nesbitt1, Niall M. Byrne1,2, S. Nicole Williams3, Louise Ming1, Jenny Worthington1,3, Rachel J. Errington4,5, Laurence H. Patterson6, Paul J. Smith6, Stephanie R. McKeown1,6, and Declan J. McKenna1

Abstract

Purpose: To understand the role of hypoxia in prostate tumor progression and to evaluate the ability of the novel unidirectional hypoxia-activated prodrug OCT1002 to enhance the antitumor effect of bicalutamide.

Experimental Design: The effect of OCT1002 on prostate cancer cells (LNCaP, 22Rv1, and PC3) was measured in normoxia and hypoxia in vitro. In vivo, tumor growth and lung metastases were measured in mice treated with bicalutamide, OCT1002, or a combination. Dorsal skin fold chambers were used to image tumor vasculature in vivo. Longitudinal gene expression changes in tumors were analyzed using PCR.

Results: Reduction of OCT1002 to its active form (OCT1001) decreased prostate cancer cell viability. In LNCaP-luc spheroids, OCT1002 caused increased apoptosis and decreased clonogenicity. In vivo, treatment with OCT1002 alone, or with bicalutamide, showed significantly greater tumor growth control and reduced lung metastases compared with controls. Reestablishment of the tumor microvasculature following bicalutamide-induced vascular collapse is inhibited by OCT1002. Significantly, the upregulation of RUNX2 and its targets caused by bicalutamide alone was blocked by OCT1002.

Conclusions: OCT1002 selectively targets hypoxic tumor cells and enhances the antitumor efficacy of bicalutamide. Furthermore, bicalutamide caused changes in gene expression, which indicated progression to a more malignant genotype; OCT1002 blocked these effects, emphasizing that more attention should be attached to understanding genetic changes that may occur during treatment. Early targeting of hypoxic cells with OCT1002 can provide a means of inhibiting prostate tumor growth and malignant progression. This is of importance for the design and refinement of existing androgen-deprivation regimens in the clinic. Clin Cancer Res. 23(7); 1797–808. ©2016 AACR.

Introduction

Hypoxia occurs in most solid tumors, and it is known to have a major influence on treatment response to both radiotherapy (1) and chemotherapy (2). Untreated prostate tumors are no exception, and they have been found, in several studies, to have very low median oxygen levels (2.4 mm Hg; 0.3% oxygen; refs. 3, 4), which is >12 times lower than oxygen levels found in the normal prostate (30 mm Hg; 3.9% oxygen; ref. 5). Studies have shown that high levels of hypoxia significantly correlate with increasing clinical stage and can predict biochemical failure following radiotherapy (6). Importantly, hypoxia has also been implicated as a causative factor in malignant progression, with many effects mediated through increased expression of HIF-1 (7). In addition, hypoxia has been shown to cause genetic instability (8), gene amplification (9), endothelial-to-mesenchymal transition (EMT; refs. 10, 11) and the selection of cells with diminished apoptotic potential and a greater invasive potential (12–15). Hypoxia is therefore a significant impediment to the successful outcome of many cancer treatments.

One approach to blocking the influence of hypoxic tumor cells is to directly target this subpopulation with a hypoxia-activated prodrug (HAP); these prodrugs are a diverse group of chemicals that can be reduced in cells when oxygen levels are very low (16, 17). The majority of HAPs are reduced in single-electron reduction steps, a process that is reversible when oxygen levels increase and is associated with superoxide formation. In contrast, alkylaminoanthraquinone di N-oxides are irreversibly reduced by the addition of two electrons on each of the two N-oxide side arms of the anthraquinone ring. This results in formation of metabolically stable reduction products; for this reason, it is proposed they should be classified separately as unidirectional HAPs (uHAP). Currently, there are two compounds of therapeutic interest presenting these advantageous properties, namely AQ4N (18) and a recently described deuterated analogue OCT1002.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).  
S.R. McKeown and D.J. McKenna share senior authorship of this article.  
Corresponding Author: Declan J. McKenna, University of Ulster, Cromore Road, Coleraine, Northern Ireland BT52 1SA, United Kingdom. Phone: 44-2870124356; Fax: 44-2870214356; E-mail: dj.mckenna@ulster.ac.uk  
doi: 10.1158/1078-0432.CCR-16-1361  
©2016 American Association for Cancer Research.
Tumor hypoxia is recognized as a major contributing factor in the progression of prostate cancer. This study provides the first evidence that a novel unidirectional hypoxia-activated prodrug, OCT1002, effectively targets hypoxic tumor cells; our study shows that this occurs both in vitro and in vivo. In a xenograft mouse model of prostate cancer, OCT1002 improves the efficacy of the antiandrogen drug bicalutamide in controlling tumor growth. We have shown that initial treatment of androgen-sensitive tumors with bicalutamide causes vascular collapse and increased hypoxia; however, after 21 days, the vasculature recovers. Our evidence shows that OCT1002 causes selective killing of tumor cells with a more malignant genotype. This provides a mechanistic explanation for why patients on hormonal therapy relapse. It also shows that targeting hypoxic tumor cells with OCT1002 offers a means by which malignant progression can be blocked in patients on androgen-deprivation therapy.

(OncoTherics Ltd; ref. 19). Under tumor-relevant hypoxic conditions, the two sequential 2e\(^{-}\) reductions yield the toxic metabolites AQ4 and OCT1001, respectively (5). These stable reduction products have very high affinity for DNA and they also target topoisomerase II (20), which can effect a long-term inhibition of both DNA replication and cell-cycle traverse (21). OCT1002 is distinguished from AQ4N by its highly selective deuterium substitution of the 12 hydrogen atoms contained within the two N-oxide side chains (22). Deuteration can modify the rate of cleavage of covalent bonds to deuterium, creating a deuterium kinetic isotope effect, slowing cytochrome P450 metabolism of the deuterated versions of drug candidates (23). Further, non-covalent interactions between molecules can be modified by deuteration (24), while differences in the polarities of deuterated versus non-deuterated isomers can potentially alter the complex subcellular localization and sequestration properties (25) and contribute to an enhanced intracellular persistence of the activated drug OCT1001 (19).

To test the effectiveness of these uHAPs in vivo, we routinely utilize a murine LNCaP-luc xenograft model of prostate cancer in our laboratory (11, 26). This is an ideal system to investigate tumor hypoxia, because LNCaP/LNCaP-luc tumors have a low oxygen level (~6 mm Hg, 0.8% oxygen) comparable with that observed in patients. Furthermore, we have used this model to demonstrate that this level falls even further to about 0.1% oxygen upon initiation of bicalutamide treatment, due to vascular collapse (26). Bicalutamide is a widely used drug used in androgen deprivation therapy (ADT) and provides for effective control of locally advanced prostate cancer prostate tumors. However, remissions normally last only about 18 to 24 months before tumors recur, often as the more highly metastatic castrate-resistant prostate cancer (CRPC; ref. 27). Our previous results led us to hypothesize that the bicalutamide-induced hypoxia may select for cells with a more aggressive phenotype, a selection which we subsequently demonstrated was characterized by upregulation of several prosurvival genes and a promotion of the EMT phenotype (11). Notably, when a single dose of uHAP (AQ4N) was administered during the bicalutamide-induced hypoxic phase in this model, tumor growth was significantly delayed (26). In the current study, we have investigated if a similar growth delay occurs in the same xenograft model when OCT1002 is used alone and in combination with bicalutamide. We also profile genetic changes in response to both bicalutamide and OCT1002 treatment to determine if bicalutamide-induced alterations in genes associated with metastatic progression were blocked by the use of OCT1002.

Materials and Methods

Cell culture

All cell lines were obtained from ATCC. Cells were frozen at low passage number and used within three to six passages after thawing. Cells were authenticated by in-house genotyping service and routinely tested as mycoplasma-free (InvivoGen). A luciferase-expressing variant of the parental LNCaP cells was developed and confirmed to have similar characteristics to the parental cells (26). These LNCaP-luc cells were cultured in RPMI-1640 culture medium (Life Technologies) supplemented with 10% FBS, n-glucose (10 mmol/L, Sigma) and HEPES (10 mmol/L, Sigma). PC3 and 22Rv1 were cultured in RPMI-1640 supplemented with 10% FBS. For OCT1002 treatment, 5,000 cells were seeded in a 96-well plate and allowed to adhere overnight. Cells were treated with OCT1002 (10\(^{-12}\) to 10\(^{-3}\) mol/l in vehicle) at 37°C. After 72 hours, a cell viability XTT assay (Roche) was carried out. For treatment in hypoxic conditions, 5,000 cells were seeded in a 96-well plate and allowed to adhere overnight. After dosing with OCT1002 (1 μmol/L), cells were placed in normoxia (20% oxygen) or hypoxia (0.1% oxygen) at 37°C in a hypoxia work station (Ruskinn Technology) for up to 48 hours: cell viability was measured using a XTT assay.

Spheroid 3D cell culture

LNCaP-luc cells (5,000) were seeded in nonadherent 24-well plates and spheroids allowed to form upon continued incubation. On day 7, spheroids were treated with vehicle (1% DMSO), bicalutamide (13.8 μmol/L), OCT1002 (1 μmol/L), or bicalutamide + OCT1002. Spheroid growth was measured at days 4, 6, 8, 10, 11 and 12. Images were taken at 100× magnification. For colony-forming assays, spheroids were collected by centrifugation on day 10, resuspended as a single-cell suspension and seeded on adherent 6-well plates. After 7 days, colonies were fixed and stained with crystal violet (2%). Quantification was performed by addition of 0.1% SDS to the wells and absorbance was measured at 595 nm. Spheroids, treated on day 4, were disaggregated and stained 72 hours later with an Annexin/PI apoptosis kit (Life Technologies) and analyzed by flow cytometry on a Gallios Flow Cytometer (Beckman-Coulter).

Quantitative real-time PCR

RNA was extracted from spheroids using TRIzol (Life Technologies) and cDNA prepared using RevertAid reagents (Fermentas). Quantitative real-time PCR (qPCR) was carried out using SYBR green (Fermentas) and gene-specific primers (Supplementary Table S1) on a LC480 Lightcycler (Roche). After normalization to the reference gene(s), relative expression levels of each target gene were calculated using the comparative Ct (ΔΔCt) method. qPCR for miR-210 was performed using the mirCURY LNA microRNA PCR system (Exiqon) using 20-ng template RNA. PCR was performed on the LC480 Lightcycler, normalization was
against U6snRNA and relative expression levels calculated using the comparative C\textsubscript{T} (ΔΔC\textsubscript{T}) method. All PCR graphs represent the combined results from ≥3 independent biological replicates, unless otherwise indicated.

Western blotting
Protein was extracted from spheroids using urea buffer. Primary antibodies used were obtained from Cell Signaling Technology unless otherwise stated and comprised: PARP/Cleaved PARP (#9542, 1:1,000), Bax (#2772S, 1:500), Bcl-2 (#2876S, 1:500), β-actin (#4967, 1:1,000), and GAPDH (Millipore, ABS16, 1:1,000). Membranes were blocked in 5% BSA diluted in 1× TBS-T (0.05%) followed by incubation in the appropriate secondary antibody [goat anti-rabbit IgG-HRP (1:10,000) or goat anti-mouse IgG-HRP (1:10,000)]. Secondary antibodies were purchased from Santa Cruz Biotechnology.

\textbf{In vivo methods}

\textbf{Animal maintenance.} In vivo experiments were conducted in accordance with the Animal (Scientific Procedures) Act 1986 and the UKCCCR guidelines for the welfare of animals in experimental neoplasia (28). Eight- to 10-week-old male BALB/c immune-compromised (SCID) mice weighing 25 to 30 g (Envigo) were housed under standard laboratory conditions in a temperature-controlled (22 ± 2°C; 50% – 55% humidity) pathogen-free environment with a 12-hour light–dark cycle. Food and water were supplied \textit{ad libitum}. All surgical procedures were performed under aseptic conditions, and the body temperature of animals was kept constant using heated pads.

\textbf{Xenograft establishment and tumor growth delay.} LNCaP-luc or 22Rv1 xenografts were established on the dorsum of SCID mice by subcutaneous injection of 2 \times 10^6 cells suspended in 100 μL of Matrigel with a 21-gauge needle (Becton Dickinson). Once the tumor became palpable, dimensions were measured using Vernier calipers.

\textbf{Drug administration.} Bicalutamide was prepared in vehicle (1% DMSO in corn oil; Sigma) and administered orally via gavage as 2 mg/kg daily. OCT1002 (OncoTherics) was prepared in sterile PBS and administered as a single intraperitoneal (i.p.) injection (50 mg/kg). When tumor volume reached between 100 and 150 mm^3, mice were randomly assigned to treatment groups and dosing initiated. Animals were sacrificed at day 28 or when tumor reached the maximum size permitted.
Assessment of metastasis. Upon day 28, animals were injected intraperitoneally with D-Luciferin (150 mg/kg in PBS) 5 minutes prior to imaging. Animals were sacrificed, and the lungs were removed to detect bioluminescence using the IVIS imaging system (Xenogen). Within a region of interest, the total light flux (photons/second) was measured.

Dorsal skin fold model

Dorsal skin fold (DSF) preparation. A bespoke transparent DSF “window chamber” (APJ Trading Co. Ltd) was attached to the dorsum of mice as previously described (26, 29). This consisted of a titanium frame with a viewing port into which a tumor fragment was placed. The DSF preparation was then hydrated with sterile saline and closed with a glass coverslip secured by a spring c-clip.

Imaging of treatment-induced changes in the tumor vasculature.

The tumor fragment was left in place for 1 week to allow vascularization. Bicalutamide treatment was then initiated (day 0). Five minutes prior to imaging (days 0, 7, 14, and 21), mice were given 50 μL 150-kDa fluorescein isothiocyanate-labeled (FITC) dextran (50 mg/mL in saline; Sigma) into the tail vein. Mice were secured in a bespoke restraint and placed laterally onto a modified microscope stage. Tumor microvessels were imaged using an HC PL APO lens (10× magnification) fitted to a TCS SP5 confocal microscope (Leica Microsystems). Three random regions of interest (ROI) were identified at various locations within each tumor, and consecutive images were taken. At each ROI, image stacks were created through the z-plane of the tumor tissue with a final maximum projection created using the Leica application suite advanced fluorescence (LAS AF) software. Images were analyzed offline using ImageJ software (National Institute of Mental Health, Bethesda, MD) and stereological analysis performed using a Merz grid (30). Visualization of OCT1002 was performed 4 hours after drug administration with a helium neon laser (excitation at 633 nm; emission 650 nm–800 nm).

Figure 2.

OCT1002 targets cells in LNCaP-luc spheroids. A, Hypoxic markers HIF1A, IGF-1, and miR-210 in LNCaP spheroids after 10 days of growth. B, Images reveal evidence of hypoxia in LNCaP spheroids as shown by the presence of reduced OCT1002 (i.e., OCT1000). Western blots show increased HIF1α protein after 10 days of growth, compared with day 7. Cycling normoxic LNCaP-luc cells are included as control. C, Graph and representative inset images show spheroid growth is reduced by OCT1002 (1 μmol/L) alone or in combination with bicalutamide compared with vehicle (0.02% DMSO in media) or bicalutamide (13.8 μmol/L) alone. D, Crystal violet staining of colonies formed from cell disaggregates of spheroids, treated as C. E, Annexin V/PI apoptosis assay showing that OCT1002 induces apoptosis. Data, mean ± SE from triplicate experiments (Student t test P values: * P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant).
Images were subsequently merged with FITC–dextran-labeled tumor vasculature.

**PCR custom array gene expression analysis**

Five micrograms of RNA per tumor sample was reverse transcribed using RevertAid (Fermentas), and cDNA from three tumors per treatment on specific days (0, 7, 14, 21, and 28) was pooled. Changes in expression profile were analyzed on Real-Time Ready custom 96-well panels (Roche; Supplementary Fig. S1). PCR was performed on the LC480 lightcycler, and results were normalized to reference genes (HPRT, beta actin, and 18s ribosomal RNA). Fold changes in gene expression of vehicle- and bicalutamide-treated xenografts were compared with pretreatment (day 0) expression levels; genes considered upregulated (≥2) or downregulated (≤0.5) and those which satisfied a P value of <0.05 (Student t test) were deemed statistically significant.

**Immunohistochemistry**

Tumors at the experimental endpoint were excised, fixed in PFA, and wax-embedded. Sections (5 μm) were stained with RUNX2 (27-K, 1:50), ki-67 (SP6, 1:100), and IgG antibodies (all Santa Cruz Biotechnology) using the Superpicture 3rd Gen IHC kit (Life Technologies). Five fields per sections were viewed, scored, and averaged. Immunohistochemical staining was scored using a two-approach method for extent and intensity (31). The extent of staining was defined as the percentage of cells with nuclear immunoreactivity and was scored 0 to 5: negative (0); ≤10% (1); 11%–25% (2); 26%–50% (3); 51%–75% (4); and ≥76% (5). Intensity of staining was scored 0–5: negative (0)–very strong (5). Scores were averaged with three mice per group.

**Statistical analysis**

Data from qPCR analyses and tumor growth delay studies were analyzed using a two-tailed Student t test. All remaining datasets were analyzed using a two-way ANOVA with Bonferroni posttest analysis. All statistical analysis was carried out using the Prism 5.0 software (GraphPad). Differences between points were deemed statistically significant with P < 0.05 (95% confidence interval).
Results

Novel uHAP OCT1002 is reduced to active form OCT1001 in hypoxic conditions

OCT1002 is electrically neutral in aqueous solution, but in hypoxic tumor cells, it can be reduced to the DNA-affinic cytotoxin OCT1001 (Fig. 1A). Imaging 4 hours after OCT1002 administration on day 7 showed extensive distribution of drug far-red fluorescence (19) in the tumor, particularly in the more hypoxic center (Fig. 1B). When the tumors were imaged 7 days later, the bound drug was still clearly visible, particularly away from blood vessels, confirming the longevity of OCT1001 in hypoxic cells. No autofluorescence was seen in untreated tumor fragments (Fig. 1B).

Activated OCT1002 targets hypoxic cells in vitro

Treatment of three prostate cancer cell lines (LNCaP-luc, 22Rv1, and PC3) at a range of OCT1002 concentrations (10⁻¹² to 10⁻³ mol/L) revealed no significant toxicity at concentrations of <1 μmol/L (Fig. 1C). When LNCaP-luc cells were grown in hypoxic conditions (0.1%) for 48 hours, there was an expected decrease in cell viability (Fig. 1D) compared with cells grown in normoxic conditions (20%). However, treatment of these hypoxic cells with 1 μmol/L OCT1002 resulted in a further significant decrease in the viability of the cells (Fig. 1D, gray bars). In contrast, OCT1002 treatment of cells grown in normoxic conditions had no effect (Fig. 1D, white bars), indicating that the drug only targets cells in hypoxic conditions. Similar results were obtained for both 22Rv1 and PC3 cells (Supplementary Fig. S2).

We also utilized a 3D spheroid model of prostate cancer growth to illustrate the effect of OCT1002 on hypoxic LNCaP-luc cells. The increasing presence of hypoxia in growing spheroids was confirmed by the increasing levels of the hypoxic markers IGF1 and miR-210 (Fig. 2A) and HIF1α protein (Fig. 2B). Again, the prevalence and localization of hypoxia-activated OCT1001 in the spheroids could be detected in the far-red spectral window (Fig. 2B, images). The developing hypoxia occurs concurrently with an increase in spheroid size over a 10-day period. Addition of bicalutamide on day 7 significantly attenuated their growth, and an even greater decrease in spheroid growth and reduction in size was found when OCT1002 was added (Fig. 2C). Upon disaggregation, the ability of both bicalutamide- and OCT1002-treated spheroids to form colonies was significantly decreased in comparison with untreated spheroids (Fig. 2D). In spheroids treated with OCT1002, there was a significant increase in the number of...
apoptotic cells (Fig. 2E; Supplementary Fig. S3A). This was consistent with the concomitant decrease in Bcl-2 protein levels and increase in Bax protein levels, as well as evidence of PARP cleavage (Supplementary Fig. S3B). Taken together, we conclude that OCT1002 is indeed activated in hypoxic conditions in vitro, resulting in increased cell death.

OCT1002 in combination with bicalutamide improves tumor growth control and reduces lung metastases

The LNCaP-luc xenograft mouse model of prostate tumorigenesis was used to measure the effect of OCT1002 in vivo (Fig. 3A and B). Tumor growth was measured during treatment with 2 mg/kg bicalutamide; although it initially slowed growth by day 28, the tumor growth delay (1.09 ± 0.55 days) was not significantly different from that found in the vehicle-treated group. OCT1002 was administered as a single low dose (50 mg/kg) on day 7, which we had previously confirmed as the hypoxic nadir following bicalutamide treatment. When given with vehicle, OCT1002 caused a moderate delay in tumor growth (3.88 ± 0.56 days). However, when OCT1002 was combined with bicalutamide, there was a very significant increase in tumor growth delay (22.24 ± 5.17 days). This effect was significantly different from both vehicle-only and bicalutamide-only tumors from day 16 until the end of the experiment (day 28). By day 28, vehicle-treated mice had significant metastatic spread to the lungs (Fig. 3C). Although bicalutamide-treated mice had a slightly reduced level of lung metastasis, this was not significant, whereas OCT1002 in combination with bicalutamide significantly reduced metastatic spread by ~10-fold (Fig. 3D). When a similar experiment was performed in a 22Rv1 xenograft model, the combination of OCT1002 and bicalutamide in this model also resulted in greater tumor growth control (Supplementary Fig. S4).

Effect of bicalutamide on vascular density is abrogated by OCT1002

As previously (26), we used a DSF preparation to evaluate the effect of drug treatments on tumor vasculature (Fig. 4A–D; Supplementary Fig. S5). Vehicle-treated tumors showed little or no change in vasculature over time. Treatment with a single dose of OCT1002 (50 mg/kg) on day 7 caused a reduction in tumor...
microvessels when imaged on days 14 and 21 (Fig. 4A). Treatment with daily bicalutamide reduced the microvessel density (MVD) by half when measured on day 7, with further loss by day 14, yet a marked vascular recovery was evident by day 21 (Fig. 4A), consistent with our previous studies (11, 26). Data from window chamber experiments on 22Rv1 tumors also showed bicalutamide-induced hypoxia and concomitant changes in vasculature similar to that observed for LNCaP tumors (Supplementary Fig. S6). Notably, in LNCaP xenografts, OCT1002 administered on day 7 blocked the reestablishment of microvessels seen after 21 days of bicalutamide treatment (Fig. 4A). Quantification of the vascular coverage at day 21 showed administration of OCT1002, alone or in combination with bicalutamide, resulted in reduced MVD and fewer branch points compared with vehicle or bicalutamide alone (Fig. 4B and C). Conversely, as expected, vessel length was increased in the presence of OCT1002, as the microvascular network was not reestablished unlike that observed in bicalutamide-treated mice (Fig. 4D). Full stereological measurements from day 0 to 21 are shown in Supplementary Fig. S5, demonstrating that the revascularization that occurs at day 21 with bicalutamide treatment is blocked when OCT1002 is present.

**OCT1002 modifies bicalutamide-induced changes in gene expression**

qPCR arrays were used to compare longitudinal changes in expression of 70 genes in tumors during treatment. Expression changes for all genes are shown as scatter plots (Fig. 5A). Genes

---

**Figure 6.**

Upregulation of prosurvival genes induced by bicalutamide is blocked by OCT1002. A, Quantification of immunohistochemical staining of Ki67 and RUNX2 protein expression in LNCaP-luc tumors (data generated from >5 mice per treatment). Insets show RUNX2 staining (brown) present in LNCaP-luc tumors at the experimental endpoint. B, Expression of RUNX2 and downstream targets in LNCaP-luc spheroids. Data, mean ± SE from triplicate experiments. (Student t test P values: *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant). C, Proposed hypothesis for beneficial effect of bicalutamide and OCT1002 combination. Cells that survive the bicalutamide-induced hypoxic stress can re-populate the tumor and progress to CPRC. Addition of OCT1002 targets these cells, prevents reestablishment of tumor microvasculature, and exerts greater tumor growth control.
OCT1002 can improve the antitumor efficacy of bicalutamide treatment is not observed when bicalutamide is combined with OCT1002. More detailed examination of the variations in gene expression on day 21 revealed that the genes highlighted by filtering analysis are involved in promoting cell survival. Notably, the induction of these genes at day 21 in response to bicalutamide was ablated by the addition of OCT1002 (Fig. 5B).

Expression of RUNX2 and prosurvival genes induced by bicalutamide is blocked by OCT1002

Significantly, one of these was RUNX2, which regulates expression of several targets involved in prosurvival pathways and which we had previously noted as important in the hypoxic stress response (32, 33). Immunohistochemistry revealed that the addition of OCT1002 significantly reduced the expression of the RUNX2 protein, as well as Ki67 staining, in LNCaP-luc tumors (Fig. 6A). Furthermore, when we examined the expression of RUNX2 and several of its downstream targets, in our LNCaP-luc spheroid model, we also found that OCT1002 blocked the bicalutamide-induced upregulation of these genes (Fig. 6B).

Discussion

Understanding the physiological and molecular characteristics of the tumor microenvironment before, during, and after treatment is crucial in developing and improving therapies for prostate cancer (34). In particular, the effect of hypoxia upon tumor progression is a key consideration, because it has been consistently associated with an increasingly malignant phenotype (12–15). In our studies, we have used a mouse LNCaP-luc xenograft model of prostate cancer; this provided an ideal method for monitoring genetic and physiological changes longitudinally. It enabled us to examine how targeting hypoxic tumor cells with the novel uHAP, OCT1002, can improve the antitumor efficacy of bicalutamide.

As OCT1002 is a new agent, it was first necessary to confirm that the prodrug was converted to the active form (OCT1001) in hypoxic tumors. We have shown that the drug penetrated throughout tumor fragments grown in window chambers, and that it remained in the reduced form for some considerable time (>7 days; Fig. 1A and B). Because most of the prodrg will have been eliminated after 4 hours (half-life about 45–60 minutes in mice), the fluorescent signal reflects only the highly DNA-affinic reduction product OCT1001, which is bound in hypoxic cells. We then showed that OCT1002 at high concentrations (>10⁻⁶ mol/L) showed cytotoxicity against three prostate cancer cell monolayers and that OCT1002 could target hypoxic prostate cells more effectively in hypoxia (0.1%; Fig. 1C and D; Supplementary Fig. S2). This confirms additional studies we have carried out showing an oxygen-dependent enhancement of cytotoxicity in a range of other cell lines (data not shown). We also used LNCaP-luc spheroids to more closely recapitulate in vivo tumor formation, because hypoxia develops as the spheroid grows (Fig. 2A–C). We show that this spheroid hypoxia reduces OCT1002 and results in significant cell death, evidenced by reduced spheroid size and increased apoptotic markers. Furthermore, spheroids had significantly reduced ability to form colonies after treatment with OCT1002 as compared with controls (Fig. 2D). This suggests that specifically killing hypoxic cells reduces viable clonogens, which may be crucial for reducing cells with metastatic potential in vivo. Flow cytometry suggests that OCT1002 promotes apoptosis, whereas bicalutamide induces more necrosis, presumably due to the differing modes of action of these drugs (Fig. 2E). However, the combination of these agents needed to be explored in vivo to better assess how they would impact upon tumor growth in a more complex microenvironment.

Previously, we have shown that bicalutamide treatment (2–6 mg/kg daily) caused immediate severe hypoxia in LNCaP-luc xenografts over 1 to 14 days due to vascular collapse which slowly recovered over the next 14 days (11, 26). Hence, we have now examined the effect of the novel uHAP, OCT1002, on this effect by administration of a single low dose of OCT1002 (50 mg/kg) at the hypoxic nadir (day 7). In combination with daily bicalutamide, there was a significant slowing of tumor growth; this effect was observed up to the end of the experiment (day 28), indicating that targeting hypoxic cells can enhance the antitumor effect of bicalutamide for some considerable time. Interestingly, treatment with OCT1002 alone also proved to be slightly more effective in controlling tumor growth than bicalutamide treatment alone. Presumably, this is because it is activated by the inherently hypoxic tumor environment (5), even without the extra hypoxia induced by bicalutamide effect on the vasculature. Notably, OCT1002 significantly inhibited metastatic spread to the lungs, suggesting that targeting hypoxic cells also targets cells with increased metastatic potential.

In the absence of another reliable androgen-sensitive xenograft model to validate our results, we investigated 22Rv1 xenografts. However, these tumors are very hematogenous, grow very fast in vivo, and respond only slightly to bicalutamide. Nonetheless, bicalutamide caused marked losses in microvessels and profoundly reduced oxygenation, consistent with the effect in LNCaP tumors (Supplementary Fig. S6). When OCT1002 was combined with bicalutamide, there was improved growth control in 22Rv1 tumors (Supplementary Fig. S4), suggesting that OCT1002 activation was improved when administered during the period of bicalutamide-induced hypoxia. Interestingly, the bicalutamide-treated tumors became less hematogenous and easier to work with, perhaps due to the antivascular effects of the bicalutamide. Even so, 22Rv1 tumors are more challenging to work with than LNCaP tumors and provide, in our opinion, a model that is of limited value for comparison with androgen-sensitive slow-growing human tumors. Even moderate-sized tumors start to deteriorate, and we would urge caution at using 22Rv1 cells as xenografts. Nevertheless, our data show that a single moderate dose of OCT1002 produced significant antitumor effects, providing further preclinical evidence of the potential of OCT1002 in the treatment of prostate cancer.

It is also notable that OCT1002 prevents the revascularization observed during bicalutamide treatment in LNCaP-luc xenograft tumors, suggesting that the cells responsible for driving the vascular recovery are also inhibited or killed by OCT1002 (Fig. 4; Supplementary Fig. S5). These data are particularly pertinent because several chemotherapeutic drugs have also been shown to cause tumor shrinkage and vascular collapse, with a corresponding reduction in oxygen levels, even when if this is not directly linked to the mechanism of action of the drugs (35–37). This hypoxic stress could provide an environment that selects for cells with increased metastatic potential that survive to repopulate...
the tumor, implying that a combination of therapies is required to prevent progression to a more malignant tumor. This was also the conclusion of another recent study that suggested that combination of ADT with inhibitors that block hypoxia-induced chemokine production may prevent emergence of CRPC in a mouse metastatic prostate (TRAMP) model (38). In human, this concept may explain recent results from a large clinical trial that showed that using docetaxel in addition to ADT improved relapse-free survival in patients with high-risk localized prostate cancer (39). Docetaxel would have added cytotoxic effect to the growth-slowing effect of bicalutamide, increasing the total cell kill, and therefore prolonging the time to relapse. Similarly, this may also explain why widespread clinical testing of drugs that directly target tumor angiogenesis has not provided the panacea that was expected, perhaps because they drive hypoxic selection of more malignant cells, which survive to resist further treatment (40). Indeed, the LNCaP xenograft model presented here would be ideal for studying effects of other commonly used prostate cancer drugs, such as enzalutamide and abiraterone, to investigate this phenomenon further and to help inform clinical practice.

We were also interested to see how OCT1002 would impact upon gene expression patterns that we have previously investigated in profiling the response to bicalutamide treatment alone (11). We screened a set of 70 genes at each time point in the treatment groups and filtered the data to identify genes that were consistently and significantly altered (Fig. 5A). We focused on results from day 21, because we expected the hypoxic stress at this time to be reflected in the gene signatures within the tumors. We noted that bicalutamide treatment induced an upregulation in a large number of genes at day 21 in response to the severe hypoxia. However, this was largely absent at this time point when OCT1002 was combined with bicalutamide. Further examination of the genes affected at day 21 (Fig. 5B) showed that they reflected genes involved in promoting cell survival through various mechanisms such as angiogenesis, transcription, adhesion, invasion, apoptosis, and cell-cycle maintenance. Notably, the induction of almost all these genes at day 21 in response to bicalutamide was ablated by the addition of OCT1002 (Fig. 5B). These results support our proposal above that some cells can survive the bicalutamide-induced hypoxia and that this is linked to upregulation of an array of prosurvival genes, which help reestablish the tumor vasculature. Ultimately, this could result in the hypoxia-driven selection of cells that exhibit a more aggressive, malignant phenotype. Worryingly, this suggests that bicalutamide use in patients may in fact increase prostate cancer tumor hypoxia soon after treatment, thereby selecting for cells that have increased metastatic potential. Our data corroborate previous studies of prostate cancer cells, which have shown that hypoxia can indeed select for cells with a more malignant genotype/phenotype (13, 41, 42), with several reports detailing the upregulation of various genes in response to hypoxic insult, including those associated with a proangiogenic phenotype (43). Stem cell maintenance (44), genetic instability (45), protection from apoptosis (46), and malignant progression (15). With this in mind, it is significant that the upregulation of prosurvival genes is not observed at day 21 when the OCT1002 is combined with bicalutamide. This indicates that the cells that survive the hypoxic insult, and subsequently alter gene expression, are targeted and killed, which is evidenced in increased tumor growth control.

We have previously highlighted the link between hypoxic stress and RUNX2 (32, 33), so we were interested to see that induction of RUNX2 expression by bicalutamide treatment was effectively blocked by OCT1002 exposure. This is significant because RUNX2 expression is upregulated in prostate (46), breast (47, 48), and colon cancers (49) and is thought to contribute to a more aggressive, metastatic phenotype by altering expression of many genes involved in migration, invasion, metastasis, apoptosis, and angiogenesis (32, 46, 49). Similarly, in this study, the induction of RUNX2 correlated with induction of several of its downstream targets (Fig. 6A). However, when OCT1002 was added to a bicalutamide treatment, the induction of RUNX2 and the associated targets was not observed, even though a significant hypoxic stress still existed in these tumors given that the vasculature had not been restored. Confirmation of this effect was found when we examined RUNX2 expression and other genes in our spheroid model (Fig. 6B). This corroborates our previous work that proposes that RUNX2 is a key regulator in helping cells survive the hypoxic stress. Our study suggests that the addition of OCT1002 targets those cells, thereby preventing RUNX2 upregulation and subsequently the development of more aggressive tumor cells. Our proposed model is shown in Fig. 6C, wherein the hypoxic-resistant cells within the heterogeneous tumor cell population survive to repopulate the tumor, leading to relapse and CPSC development. However, addition of OCT1002 targets these cells, preventing regrowth of the microvasculature in particular and thereby exerting greater tumor growth control. Future work will further characterize the exact role of RUNX2 and other key molecular changes during this process.

Conclusions

In conclusion, this study shows that the novel uHAP OCT1002 effectively targets hypoxic prostate cancer cells and improves the ability of bicalutamide to control growth of human prostate tumors in mice. We propose that the enhanced efficacy is due to selective killing of cells that would otherwise survive the severe hypoxic conditions induced by vascular collapse soon after initiation of bicalutamide treatment. Because the genes upregulated during bicalutamide treatment are predominantly prosurvival, we hypothesize that these cells survive to establish a tumor with a more malignant phenotype; this may help explain why patients treated solely with ADT exhibit a relatively early relapse. However, our data suggest that inclusion of OCT1002 at the early stage of the ADT regimen to eliminate hypoxic cells could provide a method for eradication of the more malignant hypoxic cell population. Our data provide increased understanding of the physiologic and molecular changes in the prostate cancer microenvironment that occur during treatment and we suggest that more awareness of these changes should be taken into account when deciding on ADT schedules for prostate cancer patients.

Disclosure of Potential Conflicts of Interest

L.H. Patterson holds ownership interest (including patents) in, and is a consultant/advisory board member for Oncotherics Ltd. P. Smith is a co-founder of Biostatus Ltd, and listed as the inventor on a patent, for which Biostatus Ltd is the applicant, for a new series of anticancer drug that can be activated under hypoxia. S.R. McKeown holds ownership interest (including patents) in Biostatus Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H. Nesbitt, R.J. Errington, L.H. Patterson, P.J. Smith, S.R. McKeown, D.J. McKenna
Development of methodology: H. Nesbitt, N.M. Byrne, J. Worthington, P.I. Smith, S.R. McKeown, D.J. McKenna

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Nesbitt, N.M. Byrne, S.N. Williams, L. Ming, J. Worthington, S.R. McKeown, D.J. McKenna

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Nesbitt, N.M. Byrne, S.N. Williams, J. Worthington, S.R. McKeown, D.J. McKenna

Writing, review, and/or revision of the manuscript: H. Nesbitt, N.M. Byrne, L. Ming, R.J. Errington, L.H. Patterson, P.I. Smith, S.R. McKeown, D.J. McKenna

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Nesbitt, N.M. Byrne, J. Worthington

Study supervision: H. Nesbitt, S.R. McKeown, D.J. McKenna

References


Grant Support

This study was supported by a Prostate Cancer UK research grant (PG12-02). Additional support was provided by Department of Employment and Learning, Northern Ireland.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 27, 2016; revised August 11, 2016; accepted August 25, 2016; published OnlineFirst October 3, 2016.
Targeting Hypoxic Prostate Tumors Using the Novel Hypoxia-Activated Prodrug OCT1002 Inhibits Expression of Genes Associated with Malignant Progression

Heather Nesbitt, Niall M. Byrne, S. Nicole Williams, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-1361

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/10/01/1078-0432.CCR-16-1361.DC1

Cited articles
This article cites 46 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/23/7/1797.full#ref-list-1

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://clincancerres.aacrjournals.org/content/23/7/1797.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.