The MCT4 Gene: a Novel, Potential Target for Therapy of Advanced Prostate Cancer

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Management of castration-resistant prostate cancer (CRPC) is a major challenge in the clinic. Although CRPC treatment efficacy has been improved by using more powerful drugs targeting the androgen receptor-signaling axis, patient survival has only marginally increased and further improved versions of such drugs can lead to transdifferentiation of CRPC to currently incurable neuroendocrine prostate cancer. Therefore, new strategies targeting alternative, fundamental cancer properties are critically needed. Increasing evidence indicates that reprogrammed energy metabolism of cancers offers a unique target. For glucose utilization, cancer cells generally prefer glycolysis coupled to lactic acid production, i.e. aerobic glycolysis. This pathway leads to increased lactic acid secretion by cancers facilitating oncogenic processes, including tissue invasion/metastasis and neo-angiogenesis. The lactic acid secretion is predominantly mediated by MCT4, a plasma membrane transporter protein. Here we have shown, both in vitro and in vivo, that MCT4-targeting anti-sense oligonucleotides (ASOs) can inhibit MCT4 expression, lactic acid secretion, aerobic glycolysis and cell proliferation of CRPC models. As such, MCT4-targeting ASOs may provide a novel therapeutic approach for CRPC.
Abstract (250 words)

Purpose: Management of castration-resistant prostate cancer (CRPC) is a major challenge in the clinic. Androgen receptor signaling-directed strategies are not curative in CRPC therapy and new strategies targeting alternative, key cancer properties are needed. Using reprogrammed glucose metabolism (aerobic glycolysis), cancer cells typically secrete excessive amounts of lactic acid into their microenvironment promoting cancer development, survival and progression. Cellular lactic acid secretion is thought to be predominantly mediated by MCT4, a plasma membrane transporter protein. As such, the MCT4 gene provides a unique, potential therapeutic target for cancer.

Experimental Design: A tissue microarray of various Gleason grade human prostate cancers was stained for MCT4 protein. Specific, MCT4-targeting anti-sense oligonucleotides (MCT4 ASOs) were designed and candidate MCT4 ASOs checked for effects on i) MCT4 expression, lactic acid secretion/content, glucose consumption, glycolytic gene expression and proliferation of human CRPC cells and ii) growth of PC-3 tumours in nude mice.

Results: Elevated MCT4 expression was associated with human CRPC and an earlier time to relapse. Treatment of PC-3, DU145 and C4-2 CRPC cultures with candidate MCT4 ASOs led to marked inhibition of MCT4 expression, lactic acid secretion, to increased intracellular lactic acid levels, and markedly reduced aerobic glycolysis and cell proliferation. Treatment of PC-3 tumour-bearing nude mice with the MCT4 ASOs markedly inhibited tumour growth without inducing major host toxicity.

Conclusions: MCT4-targeting ASOs that inhibit lactic acid secretion may be useful for therapy of CRPC and other cancers, as they can interfere with reprogrammed energy metabolism of cancers, an emerging hallmark of cancer.
Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous cancer and a leading cause of cancer death for North American men (1). When the malignancy is confined to the prostate, surgery and radiation therapy can be curative. However, many treated patients experience local cancer recurrence and metastasis (2). While androgen deprivation therapy (ADT), currently the treatment of choice for metastatic prostate cancer, can lead to remissions, tumours frequently return in a form that is highly resistant to ADT and other therapies, i.e. metastatic castration-resistant prostate cancer (mCRPC). Although the efficacy of mCRPC treatment has recently been improved by using more powerful chemotherapeutics targeting the androgen receptor (AR)-signaling axis, such as enzalutamide (3) and abiraterone (4), the overall survival of patients has only marginally increased (5-7). Moreover, it is thought that further improved versions of such drugs can promote transdifferentiation of prostatic adenocarcinoma to neuroendocrine prostate cancer (NEPC), a subtype of the disease that is currently incurable (8-10). There is therefore a critical need for new, more effective strategies for therapy of CRPC that target different key properties of cancer.

There is increasing evidence that targeting reprogrammed energy metabolism of cancers offers a unique approach for effective therapeutic intervention (11). For glucose utilization, cancer cells, as distinct from normal resting cells, in general have a preference for glycolysis coupled to lactic acid production, i.e. a process called aerobic glycolysis (the Warburg effect) (12). This leads to elevated glucose consumption, a near-universal property of primary and metastatic cancers. In addition, aberrant utilization of glutamine, also leading to elevated lactic acid production, has been observed to be highly common for cancers (13). These metabolic energy pathways lead to increased lactic acid secretion by the cancer cells into their microenvironment, facilitating multiple oncogenic, lactate-stimulated processes, including tissue invasion/metastasis, neo-angiogenesis and responses to hypoxia (14-17); furthermore, lactic acid-
induced acidification of the cancer cell microenvironment (to pH 6.0-6.5) can lead to suppression of local host anticancer immunity (14, 18). The phenomenon of enhanced glucose metabolism by cancers is most commonly exploited clinically by $^{18}$F-fluorodeoxyglucose positron emission tomography (FDG-PET). Although this imaging technique is not generally used for prostate cancer (19), there is evidence suggesting that glucose metabolism of prostate cancer cells is increased by AR signaling and progression to treatment resistance (20, 21). As such, targeting the aerobic glycolytic pathway could be effective for treating advanced prostate cancers.

The monocarboxylate transporter (MCT) family consists of plasma membrane transporter proteins involved in the transport of lactic acid and other metabolic monocarboxylates. In particular, the cellular efflux of lactic acid/H$^+$ is thought to be predominantly mediated by MCT4 (SLC16A3) (22). Expression of MCT4 has been associated with highly glycolytic cells (17, 23, 24), and elevated expression of MCT4 in tumours is clinically relevant as it has been associated with poor patient prognosis in multiple types of cancer (25-27), including prostate cancer (28, 29). Furthermore, elevated MCT4 expression may be important in cancer-stroma interactions facilitating prostate cancer progression (30). This information, together with the cancer growth-promoting ability of cancer-generated lactic acid, suggests that inhibition of the expression or function of MCTs provides a promising therapeutic strategy for a wide variety of neoplasms (31). Currently, small molecule inhibitors of MCTs are available that specifically target MCT1 and MCT2 (e.g., AZD3965, AR-C155858) (32, 33), non-specifically inhibit MCTs, including MCT1 and MCT4 (e.g., alpha-cyano-4-hydroxycinnamate) (34, 35), or selectively inhibit lactic acid import via MCT4 (e.g., 7-aminocarboxycoumarins) (36). However, a therapeutic strategy specifically targeting MCT4-mediated efflux of lactic acid is still lacking.

In the present study, we established that development of human CRPC is associated with elevated expression of MCT4. Furthermore, evidence was obtained that $MCT4$-targeting anti-sense
oligonucleotides (ASOs) inhibiting MCT4-mediated lactic acid secretion may be useful for treatment of CRPC.

**Materials and Methods**

**Materials**

Chemicals, solvents and solutions were obtained from Sigma-Aldrich, Oakville, ON, Canada, unless otherwise indicated.

**Cell Cultures**

Human PC-3 and DU145 CRPC cells, human LNCaP prostate cancer cells, and mouse TRAMPC2 prostate cancer cells were purchased from the American Type Culture Collection (ATCC); C4-2 CRPC cells were obtained from Dr. Martin E. Gleave, the Vancouver Prostate Centre, Vancouver, BC, Canada. Human monolayer cultures were maintained in RPMI-1640 (GE Healthcare Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Hyclone, Logan, UT) while TRAMPC2 cells were maintained in DMEM (GE Healthcare Hyclone, Logan, UT) supplemented with 5% FBS. For cell counting, cells were trypsinized to form a single cell suspension and counted using a TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). Cell viability was assessed by trypan blue exclusion.

**Human Prostate Cancer Tissue Microarray (TMA) Construction and Immunohistochemistry**

TMAs were manually constructed, as previously described (37, 38), using various Gleason grades-exhibiting prostate cancer specimens (n=342), obtained from the Vancouver Prostate Centre Tissue Bank with written informed patients’ consent, clinical information and institutional study approval. All specimens were obtained through radical prostatectomy, except CRPC samples that were obtained via transurethral resection of the prostate (TURP). Immunohistochemical staining was conducted using a Ventana autostainer (model Discover XT; Ventana Medical System, Tucson, AZ) with an enzyme-labelled
biotin-streptavidin system and a solvent-resistant DAB Map kit (Ventana). Staining intensity was scored by a trained pathologist on a four-point scale: 0 represents no staining on any tumor cells, 1 represents a faint or focal, questionably present stain, 2 represents a stain of convincing intensity in a minority of cells, and 3 represents a stain of convincing intensity in a majority of cells.

**Antibodies**

The following antibodies and conjugates were used: rabbit anti-MCT4 antibody (Santa Cruz, Santa Cruz, CA; WB 1:4000, IHC 1:100), mouse anti-vinculin antibody (Sigma; WB 1:1000), rabbit anti-cleaved caspase 3 antibody (Cell Signalling Technology, Danvers, MA; IHC 1:50), mouse anti-Ki67 antibody (Dako, Burlington, ON; IHC 1:50), rat anti-CD31 antibody (Dianova, Hamburg, Germany; IHC 1:20), mouse anti-pan-T cell marker CD3 antibody (Dako; IHC 1:50), biotinylated mouse anti-NK1.1 (Cedarlane, Burlington, ON; IHC 1:100), IRDye 800CW goat anti-mouse antibody (Li-Cor Biosciences, Lincoln, NE; WB 1:10,000), IRDye 680RD goat anti-rabbit antibody (Li-Cor Biosciences; WB 1:10,000), biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA; IHC 1:200), biotinylated goat anti-rat antibody (Vector Laboratories; IHC 1:200), and biotinylated goat anti-mouse antibody (Vector Laboratories; IHC 1:200).

**ASO Design and Selection**

First-generation phosphorothioate-modified ASOs against human MCT4 were rationally designed by selecting sequences containing favourable motifs while excluding unfavourable ones (39). Specificity of MCT4-targeting sequences, compared to human and mouse genes (at least 3 of 20 bases mismatched), was evaluated using BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi). Ten sequences (see Table S1) distributed throughout the length of the transcript with perfect complementarity to all human MCT4 transcript variants (NM_001042422.2, NM_001042423.2, NM_001206950.1, NM_001206951.1, NM_001206952.1, and NM_004207.3) were selected and synthesized by Eurofins MWG Operon. The knock-down efficiencies of these ten ASOs were tested by determining target mRNA and protein
expression 48 hours after transfection of cells using qPCR and Western blotting. Two candidate ASOs (#1 and #14) were selected for further studies. Sequences: ASO #1, 5’-TCCCATGGCCAGGAGGGTTG-3’; ASO #14, 5’-AGATGCAGAAGACCACGAGG-3’; a published non-targeting control ASO, 5’-CCTCCCTGAAGGTTCCCTCC-3’ (40, 41).

**ASO and siRNA Transfection**

Cells were transfected in 6-well plates with ASOs at 100 nM for 48 hours (unless otherwise indicated) using Oligofectamine (Invitrogen, Carlsbad, CA) or with MCT4-targeting siRNAs and controls (Dharmacon, Chicago, IL) at 50 nM for 48 hours using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions.

**Quantitative PCR**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Inc., Hilden, Germany) and cDNA synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Primers (see Table S1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR reactions using KAPA SYBR Fast Universal (Kapa Biosystems, Woburn, MA) were performed in triplicate in a ViiA 7 Real-Time PCR system (Applied Biosystems, Foster City, CA). Target genes were normalized to a geometric average of 3 internal reference genes (42).

**Western Blotting**

Cells were harvested and lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Igepal, 0.5% Na-deoxycholate, 0.1% SDS) supplemented with a complete protease inhibitor cocktail (Roche, Nutley, NJ). The protein concentration of the lysate was determined by Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA). The lysate was run on 8% SDS polyacrylamide gel (20 ug of protein per lane) and proteins were transferred onto PVDF membrane (Millipore, Billerica, MA). The blot was blocked with the
Odyssey blocking buffer (Li-cor Biosciences) and probed with anti-MCT4 antibody. Vinculin was used as a loading control. Following overnight incubation at 4°C, the primary antibody was probed with the corresponding secondary antibody and detected using the Odyssey Infrared Imaging System (Li-cor Biosciences) and Image Studio Version 3.1 (Li-cor Biosciences). Densitometry analysis was done using ImageJ (U. S. National Institutes of Health, Bethesda, MD).

**Modified Boyden Chamber Assay**

The migration and invasion potential of PC-3 cells following treatment with MCT4 ASOs was investigated using Matrigel-coated modified boyden chambers (BD Bioscience, San Jose, CA) as previously described (38). Briefly, ASO-treated cells were seeded into the top chamber at 50,000 live cells per well. The cells were then resuspended after 48 hours using dissociation buffer (Trevigen, Gaithersburg, MD) containing calcein AMS (12.5 mM; Trevigen). The number of migrated/invaded cells in the lower chamber was determined by fluorescence measurement (485 nm excitation, 520 nm emission) of the cell suspensions using the Infinite F500 fluorometer (Tecan, Männedorf, Switzerland).

**Lactate and Glucose Determination**

PC-3 cells transfected with ASOs for 48 hours were assessed for lactate and glucose levels. Cells were incubated with fresh media for 4 hours. A sample of the media was then taken and deproteinated with 10K Spin Columns (BioVision, Milpitas, CA) prior to determination of lactate concentration using Lactate Assay Kit (BioVision) and glucose concentration using Glucose Assay Kit (BioVision). Intracellular lactate levels were determined by lysing ASO-transfected cells in MQH2O. Final concentrations were determined by normalizing to the total number of live cells.

**Treatment with MCT4 ASO of PC-3 Tumor-bearing Nude Mice**
PC-3 cells (10^6 cells in 1:1 HBSS:Matrigel) were injected subcutaneously into both flanks of 24 male athymic nude mice (Simonsen Laboratories, Gilroy, CA). Once the mean tumour volume had reached approximately 100 mm³, mice were randomized into four groups and treated with intraperitoneal injections of MCT4 ASO #1, #14, control ASO, or vehicle (PBS) at 10 mg/kg daily for 5 days followed by 2 days off treatment for a total of 15 days. The health of the mice was monitored throughout the study by measuring body weights and checking for abnormal behaviour such as lethargy, lack of hydration, and additional signs of weakness. Tumour size was measured twice weekly and tumour volume calculated using the formula: Volume = Length x width x depth x 0.5236 (mm³). Mice were sacrificed 1 hour after the final dose for tissue harvesting.

**Immunohistochemistry of Tumour Tissue**

Tumour tissue was formalin-fixed and paraffin-embedded for immunohistochemical analysis. Tissues were sectioned, probed, and stained with DAB (Sigma) as previously described (43). For Ki-67 and cleaved caspase 3 staining, images of five random fields at 400x magnification were taken per tumour and cells counted to determine the percentage of positively stained cells. For MCT4, images of five random fields at 200x magnification were taken per tumour and staining intensity was assessed by percentage scoring, using the formula: Intensity = (% area score 3) x 3 + (% area score 2) x 2 + (% area score 1) x 1. The extent of immune cell aggregation was quantified following CD31 staining by imaging the five most prominent regions of aggregates per tumour at 200x magnification and determining the percent area of the field they occupied. The proportions of immune cells were evaluated as the area of positive staining normalized to the area occupied by immune cell aggregates in the same five prominent regions.

**Statistical Analysis**
All pooled results are represented as Mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). The Student t-test was carried out to compare means between two groups. One-way ANOVA followed by the post-hoc Dunnett’s test was used to compare means of more than two groups. Two-way ANOVA followed by post-hoc multiple comparison was applied to compare tumour growth. A contingency test was done to compare staining intensity between patient cohorts on the tissue microarray. A Log-rank test was done to compare patient survival curves. Chi-squared tests were done to correlate MCT4 expression levels with various clinical parameters. Results with a p-value <0.05 were considered statistically significant and are indicated by * for p<0.05, ** for p<0.01, and *** for p<0.001.

Results

**Elevated MCT4 Protein Expression is Associated with CRPC**

A tissue microarray (TMA) composed of tissues from Gleason grade 3, 4 and 5 human prostate cancers was stained for MCT4 protein. As shown in Figures 1A and B, Gleason grade 5 prostate cancers had significantly increased MCT4 protein expression relative to Gleason grade 3 and 4 specimens. Also, elevated MCT4 expression was associated with an earlier time to relapse from primary treatment as measured by increases in serum PSA levels, with the high MCT4-expressing cohort having a median time to relapse of 63.3 months vs. 94.2 months for the low MCT4-expressing cohort (Fig. 1C). Additionally, high MCT4 expression was correlated with other clinical characteristics associated with poor prognosis, such as higher serum PSA levels at diagnosis and clinical T stage (Table S2). Furthermore, elevated MCT4 protein expression was found in tumours from patients subjected to prolonged neo-adjuvant hormone therapy (>6 months) and CRPC patients (Figs 1D, E), indicating that elevated expression of MCT4 protein in prostate cancer is associated with development of CRPC.

**Knockdown of MCT4 Inhibits PC-3 Cell Proliferation**
The potential therapeutic efficacy of inhibiting MCT4 expression was investigated using MCT4-targeting siRNAs and ASOs. Human PC-3 CRPC cells were used as they present a distinct glycolytic metabolic profile, a property associated with MCT4 expression (20). As shown in Figure 2A, treatment of PC-3 cells with MCT4 siRNA led to an inhibition of cell proliferation, suggesting potential therapeutic efficacy of an MCT4 knockdown approach. Accordingly, ASOs specifically targeting human MCT4 were designed. Screening of ten MCT4 ASOs revealed varying capacities of inhibiting PC-3 cell proliferation and MCT4 expression, with ASOs #1 and #14 showing the greatest potency (Figs 2B, 2C). A strong correlation was found between the reduced levels of MCT4 mRNA in PC-3 cells treated with the various ASOs and the resulting cell numbers (Fig. 2D), indicating that the growth inhibition by the ASOs was directly related to MCT4 knockdown.

**Candidate MCT4 ASOs Inhibit PC-3 Cell Proliferation in a Sustained and Dose-dependent Manner**

The growth-inhibitory activities of MCT4 ASOs #1 and #14 were further characterized. As shown in Figure 2E, PC-3 cell proliferation was inhibited by both ASOs in a dose-dependent manner, with ASO #14 being slightly more effective (IC_{50} = 26 nM) than ASO #1 (IC_{50} = 50 nM). The ASOs also reduced MCT4 mRNA levels in a dose-dependent manner, mirroring their inhibition of cell proliferation (IC_{50} ASO #14 = 32 nM, IC_{50} ASO #1 = 50 nM). As shown in Figure 2F, both ASOs maintained the inhibition of cell proliferation even at 96 hours post-transfection. While MCT4 mRNA levels began to increase slightly starting after 48 hours of transfection, MCT4 protein levels remained low even at 96 hours.

**Candidate MCT4 ASOs exert similar growth-inhibitory and MCT4 expression-inhibitory effects in other human prostate cancer cell lines and are specific for human MCT4**

When the MCT4 ASOs #1 and #14 were transfected into human C4-2 CRPC cells they inhibited their proliferation with IC_{50} values comparable to those observed with PC-3 cells, i.e. ASO #1 = 40 nM, ASO #14 = 27 nM. Similarly, they reduced the MCT4 expression with IC_{50} values of 50 nM for ASO #1 and
26nM for ASO #14 (Fig. 3A). Furthermore, when the ASOs were transfected into human DU145 prostate
cancer cells, a similar inhibitory effect on cell proliferation and MCT4 expression was observed with
almost identical IC50 values (Fig. 3B). Transfection of ASOs into LNCaP prostate cancer cells also showed
a similar inhibition of cell proliferation and MCT4 expression (Fig. S1), suggesting that the inhibitory
effect is more associated with a glycolytic phenotype than androgen receptor status. Importantly,
transfection of MCT4 ASOs #1 and #14 into mouse TRAMPC2 prostate cancer cells did not lead to a
significant reduction in cell proliferation or mouse MCT4 expression (Fig. 3C). Taken together, the results
suggest that these ASOs specifically target human MCT4 and that their inhibitory effect on cell
proliferation is a consequence of MCT4 knock-down.

**Candidate MCT4 ASOs are able to inhibit glucose metabolism and tissue invasion/migration of CRPC
cells in vitro**

To further examine the effects of MCT4-targeting ASOs on prostate cancer cells, we measured their
effects on lactic acid secretion, intracellular lactate concentrations, and glucose consumption of PC-3
cells. Transfection of the cells with MCT4 ASOs #1 and #14 led to a marked inhibition of lactic acid
secretion, a corresponding accumulation of intracellular lactate and an extensive decrease in glucose
consumption, measured after 48 hours of transfection (Fig. 4A). Furthermore, as shown in Figure 4B,
treatment with the ASOs resulted in down regulation of various genes involved in glycolysis, i.e. GAPDH,
PGK1, PGAM1 and ENO1. In addition, expression of lactate dehydrogenase A (LDHA) was found to be
depressed, indicative of a decrease in the conversion of pyruvate to lactic acid. Moreover, decreased
expression was found for pyruvate dehydrogenase kinase-1 (PDK1), an enzyme that shunts pyruvate
away from the TCA cycle and promotes its conversion to lactic acid. Thus the treatment with the MCT4
ASOs led to inhibition of aerobic glycolysis.
Treatment with MCT4 ASOs also inhibited the migration and tissue invasion of PC-3 cells in modified boyden chambers (Fig. S2), suggesting that lactic acid secretion as facilitated by MCT4 could also play an important role in the metastatic process.

**Growth of PC-3 Xenografts in Nude Mice Inhibited by Treatment with MCT4 ASOs**

Male athymic nude mice bearing subcutaneous PC-3 tumours were treated with MCT4 ASOs #1 and #14 for a total of 15 days. Both ASOs markedly inhibited the growth of the tumours (Fig. 5A) without inducing major host toxicity as assessed by monitoring animal weights (Fig. S3) and behaviour. Immunohistochemical analysis revealed that the ASO-induced inhibition of tumour growth was associated with an increase in cell apoptosis, as measured by cleaved-caspase 3 staining, and a decrease in cell proliferation, as measured by Ki-67 staining (Fig. 5B). The decrease in tumour growth was associated with a decrease in MCT4 protein expression (Fig. 5C), consistent with an anti-proliferative effect generated by MCT4 knockdown.

**Effects of MCT4 ASOs on Immune Cell Aggregates in Nude Mice**

As lactic acid-induced acidification of tumours has been linked to suppression of local host anticancer immunity (14), it was of interest to determine whether the treatment of the PC-3 tumour-bearing nude mice with MCT4 ASOs had caused changes in the local host immune response of these mice even though their immune reactivity was very limited. To that end, we quantified immune cell aggregates that had extravasated from CD31-positive blood vessels, particularly in the tumour periphery. As shown in Fig. 6A, xenografts treated with the two MCT4 ASOs had significantly larger immune cell aggregates compared to control tumours. Quantification of the natural killer (NK) cell population, the predominant cytotoxic immune cell subtype in nude mice (44), revealed that the treatment with the MCT4 ASOs markedly increased the proportion of tumour-associated NK cells (Fig. 6B). Furthermore, activation of NK cells is facilitated by CD3 (45), a molecule commonly regarded as a T-cell marker for its association...
with the T-cell receptor complex. Its expression in NK cells is detectable by immunohistochemistry (46), and in view of the absence of T cells in nude mice, can be used as an indicator of NK cell activation (47). As shown in Figure 6C, CD3 staining revealed that the proportion of activated NK cells associated with the ASO-treated tumours had also increased.

Discussion

Cancers have a common preference for reprogrammed energy metabolism leading to upregulated glycolysis and excessive lactic acid secretion, an emerging hallmark of cancer (48); the lactic acid secretion is thought to be predominantly mediated by the MCT4 plasma membrane transporter protein (22). The present study indicates that progression of prostate cancer to CRPC, a major challenge in the management of the disease (1), is coupled to an increase in MCT4 expression. Elevated MCT4 protein expression in clinical prostate cancer specimens was associated with increases in Gleason grade, prolonged treatment of patients with neo-adjuvant hormone therapy, CRPC and early disease relapse (Figs 1A-E), as well as other clinicopathological characteristics indicative of poor prognosis (Table S2). These observations are consistent with reports by others (28, 29), indicating that elevated MCT4 expression is clinically relevant and may play an important role in the late and more aggressive stages of the disease. Thus, stimulation of cancer progression by elevated expression of MCT4 leading to increased lactic acid secretion is consistent with the notion that lactic acid generated by cancers can aid their progression by promoting a variety of oncogenic processes.

Support for potential use of MCT4-targeting ASOs for therapy of CRPC comes from our findings that treatment with MCT4 ASOs #1 and #14 markedly reduced MCT4 mRNA expression (Fig. 2C), lactic acid secretion and glucose consumption by PC-3 cells (Fig. 4A) and inhibited in vitro proliferation of CRPC cell lines in a species-specific manner (Figs 2A,B,D,E,F and 3A,B,C). Furthermore, treatment of PC-3 tumour-bearing nude mice with the MCT4-targeting ASOs led to marked inhibition of the growth of the tumours
(Fig. 5A), which was associated with increased apoptosis (Fig. 5B) and a reduction in MCT4 protein levels (Fig. 5C). As well, the treatment with the ASOs did not induce major toxicity to the hosts (Fig. S3).

The MCT4 ASOs produced in the present study appear to be highly specific for human MCT4, as the specificities of their sequences were checked via BLAST analysis (see Materials and Methods) and as they had highly similar inhibitory effects on the MCT4 expression and proliferation of human PC-3, DU145, C4-2, and LNCaP prostate cancer cells (Figs 2A-F, 3A,B, and S1) as distinct from mouse TRAMPC2 prostate cancer cells which were not significantly affected by the ASOs (Fig. 3C). The marked MCT4 ASO-induced inhibition of lactic acid secretion and corresponding increase in intracellular lactic acid concentration are therefore likely a direct result of MCT4 knockdown (Fig. 4A). The ASO-induced inhibition of glucose consumption (Fig. 4A) and down-regulation of enzymes involved in aerobic glycolysis (Fig. 4B) appear to be secondary effects, probably reflecting a response of the cancer cells to reduce the potentially fatal increase in intracellular lactic acid content by shutting off the lactic acid supply. Furthermore, decreasing MCT4 expression also resulted in the reduction of Matrigel invasion and in vitro migration of PC-3 cells (Fig. S2), lending preliminary confirmation that lactic acid secretion may play an important role in local tissue invasion and metastasis (15, 16). Elucidation of the precise mechanisms by which down regulation of MCT4 inhibits proliferation, tissue invasion and migration of prostate cancer cells, and other aspects of cancer biology such as tumorigenesis, requires further studies. In particular, FDG-PET could be used as a metabolic tracer to determine whether glucose uptake by prostate cancer cells in vivo is affected by treatment with MCT4 ASOs. Alternatively, metabolomic approaches using stable isotope-labelled metabolites such as 13C-glucose (49, 50) may be used to more comprehensively assess alterations in glucose metabolism following MCT4 knockdown.

Of interest are the findings that the treatment of the PC-3 tumour-bearing nude mice with the MCT4 ASOs led to increases in i) the number of extravasated immune cell aggregates in the tumour cell micro-
environment (Fig. 6A), ii) the proportions of host NK cells (Fig. 6B) and iii) the proportions of host NK cells that had been activated (Fig. 6C). They indicate that MCT4 knockdown might lead to restoration of cancer-suppressed host immunity, a possibility that should be examined using immuno-competent hosts.

Elevated MCT4 expression has also been reported as clinically relevant and functionally significant in other types of cancer (25, 27). For example, MCT4-targeting siRNA has been reported to be effective at inhibiting renal cell carcinoma growth (51), and MCT4 expression in small cell lung cancer was found to confer resistance to specific MCT1 inhibitors (32). The candidate MCT4 ASOs developed in our laboratory could therefore be useful in multiple clinical settings, in particular if their efficacies can be improved by incorporating second-generation 2’-methoxyethyl modifications in their backbone (52). Additionally, treatment efficacy could also be increased using a combination strategy with other therapeutics currently used for treating CRPC (such as second-generation hormone therapies or docetaxel). Alternatively, combination with other modulators of glucose metabolism (such as metformin or mitochondrial inhibitors) might result in a more complete blockade of glucose utilization by cancer cells.

Conclusions

Development of CRPC was found to be associated with elevated expression of MCT4, a plasma membrane transporter protein mediating lactic acid secretion by cancers. MCT4-targeting ASOs that inhibit lactic acid secretion may be useful for therapy of CRPC and other cancers characterized by excessive lactic acid secretion resulting from reprogrammed cellular energy (glucose) metabolism, an emerging hallmark of cancer.

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References


Figure Legends

Table S1. List of Antisense Oligonucleotides (ASOs) and qPCR Primer Sequences used.

Table S2. Additional Clinicopathological Characteristics associated with high MCT4 expression in patient samples.

Figure 1. MCT4 Staining of CRPC TMAs reveal elevated MCT4 expression as clinically relevant. TMAs were constructed using patient-derived prostate cancer samples available at the Vancouver Prostate Centre Tissue Bank and stained for MCT4 expression. A) Results confirm that elevated MCT4 expression is associated with high Gleason grade. B) Representative images of MCT4 staining show increased staining intensity in Gleason grade 5 specimens. C) High MCT4 expression is associated with earlier time to relapse as measured by increases in serum PSA. D) Patients undergoing prolonged neoadjuvant hormone therapy (NHT) and those with CRPC also show elevated MCT4 expression. E) Representative images of MCT4 staining show an increase in staining intensity in extended NHT and CRPC patient specimens.

Figure 2. Efficacy screening of MCT4-targeting ASOs using PC-3 cells in vitro reveals candidates markedly inhibiting MCT4 expression and cell proliferation. MCT4 ASOs #1 and #14 exert their in vitro effects in a dose dependent manner, and the effects persist past 96 hours after transfection. A) siRNA-silencing of MCT4 in PC-3 cells showed significant inhibition of cell proliferation, indicating that inhibition of MCT4 expression could have potential therapeutic efficacy. B) Screening of ten MCT4-targeting ASOs revealed varying inhibitory effects on cell proliferation, with sequences #1 and #14 showing the most profound inhibitions. C) The MCT4 ASOs induced various levels of MCT4 knockdown as measured by qPCR and Western blot, with ASOs #1 and #14 being the most effective. D) A strong correlation (p<0.001) was found between the MCT4 mRNA expression and the resulting cell numbers, indicating that the inhibitory effects on cell proliferation are strongly related to decreased MCT4 expression.
expression (dotted lines represent the 95% confidence interval). E) Transfection of 5 nM to 200 nM of MCT4 ASOs showed that the inhibition of cell proliferation and expression of MCT4 is similarly dose dependent (IC$_{50}$ = 32 nM for ASO #14, IC$_{50}$ = 50 nM for ASO #1). F) A time course experiment demonstrates that the inhibition of cell proliferation and MCT4 expression following MCT4 ASO transfections persists up to at least 96 hours post-transfection.

Figure 3. MCT4 ASOs #1 and #14 are effective in vitro against human prostate cancer cells other than PC-3, but not against mouse prostate cancer cells. A) Candidate MCT4 ASOs are also able to inhibit C4-2 human prostate cancer cell proliferation and expression of MCT4 in a dose-dependent manner with IC$_{50}$ values comparable to those observed with PC-3 cells. B) Candidate MCT4 ASOs also inhibit DU145 human prostate cancer cell proliferation in a dose-dependent manner with IC$_{50}$ values similar to those obtained with the other cell lines. The inhibition of cell proliferation is accompanied by a decrease in MCT4 expression. C) The candidate MCT4 ASOs do not have any appreciable effects on TRAMPC2 mouse prostate cancer cells. They neither affect cell proliferation nor mouse MCT4 expression levels even at the highest tested concentration of 200 nM. This indicates that our candidate MCT4 ASOs are specific for human MCT4 and that the inhibition of cell proliferation is a phenomenon related to MCT4 knock-down.

Figure 4. Transfection of candidate MCT4 ASOs leads to inhibition of glucose metabolism of PC-3 cells. A) MCT4 ASOs significantly inhibited lactic acid secretion. A corresponding accumulation of intracellular lactate and inhibition of glucose consumption is also observed (u.d. = undetectable) 48 hours post-transfection. B) The changes in metabolism caused by MCT4 knock-down were further characterized by qPCR analysis of gene expression levels of various genes involved in glycolysis and lactic acid conversion. Following treatment with MCT4 ASOs, a marked change in glucose metabolism was observed in PC-3 cells. A significant decrease in expression of various genes in glycolysis suggests an overall decrease in
glucose metabolism. Furthermore, the decreased expression of LDHA and PDK1 suggest a redirection of pyruvate away from lactic acid production toward the TCA cycle for oxidative phosphorylation.

**Figure 5.** MCT4 ASO-induced reduction of MCT4 expression in PC-3 tumour cells *in vivo* was associated with inhibition of PC-3 tumour growth, characterized by an increase in apoptosis and inhibition of cell proliferation. Athymic nude mice bearing subcutaneous PC-3 tumours were treated with intraperitoneal injections of MCT4 ASOs #1, #14, control ASO, or vehicle (PBS) at 10 mg/kg daily for 5 days followed by 2 days off treatment for a total of 15 days. A) Treatment with the MCT4 ASOs significantly slowed down tumour growth. B) The decrease in tumour growth rates following treatment with MCT4 ASOs is attributable to an increase in cell apoptosis and a decrease in cell proliferation. C) Treatment with MCT4 ASOs decreased MCT4 expression in the tumour as measured by IHC staining. Representative images of tumours from each group show presence of strong membrane staining in the control tumours that is absent in the MCT4 ASO-treated tumours.

**Figure 6.** *MCT4*-targeting ASOs increase extravasated immune cell aggregation and alter tumour-associated immune cell proportions *in vivo*. The potential immunomodulatory properties of MCT4 ASOs were investigated through IHC staining. A) Immune aggregates are characterized by areas of small, circular, densely packed nuclei that are distinct from the surrounding tumour cells. Staining for CD31 reveals that these immune cells have extravasated and surround the blood vessels in the tumour periphery. The immunomodulatory effects of treatment with MCT4 ASO are partially exerted through a significant increase in the extent of such immune cell aggregations. B) Treatment with MCT4 ASO also significantly altered the composition of the immune cells present in the aggregates. Staining using the NK cell marker NK1.1 revealed that treatment with MCT4 ASO increased the proportion of NK cells associated with the tumour. C) Because nude mice lack T cells, CD3 was used as a marker for activated
NK cells. Staining using CD3 also revealed that treatment with MCT4 ASO also increased the proportion of activated NK cells associated with the tumour, suggesting stimulation of anti-cancer immunity.

**Figure S1. Candidate MCT4-targeting ASOs are able to inhibit cell proliferation and MCT4 expression of LNCaP human prostate cancer cells 48 hours after transfection.** A) MCT4 ASOs are able to inhibit LNCaP cell proliferation to levels comparable to those observed with other human prostate cancer cell lines. B) The decrease in cell proliferation is associated with a decrease in MCT4 expression. Taken together, the data suggest that the inhibitory effect of MCT4 ASOs may be more associated with a glycolytic phenotype than with androgen receptor status.

**Figure S2. Candidate MCT4 ASOs are able to inhibit PC-3 cell migration and tissue invasion in vitro.** A) Treatment of PC3 cells with candidate MCT4 ASOs resulted in an inhibition of cell migration through a transwell, as indicated by a reduction in the number of migrated cells observed. B) Treatment with MCT4 ASO also inhibited the ability of PC-3 cells to invade Matrigel. As such, MCT4-mediated lactic acid secretion could play an important role in cancer metastasis.

**Figure S3. Treatment of nude mice with MCT4 ASO did not cause host toxicity as measured by animal weights.** A) Animal weights were monitored throughout the duration of the *in vivo* study, and treatment with MCT4 ASOs did not significantly affect the average animal weight of each group. B) The individual animal weights also remained stable throughout the treatment period.
Figure 1

A

![Bar chart showing average MCT4 staining intensity by primary Gleason grade.](image)

B

![Images of tissue samples with Gleason grades 3, 4, and 5.](image)

C

![Kaplan-Meier survival curve showing proportion without recurrence by MCT4 expression score.](image)

D

![Bar chart showing average MCT4 staining intensity by hormone status.](image)

E

![Images of tissue samples for hormone naive, NHT 0-6 months, NHT 6+ months, and CRPC.](image)
Figure 4

A

- Lactate Secretion (mM/10^6 cells)
  - Mock Ctrl #1 #14
  - * **

- Intracellular Lactate (mmol/10^6 cells)
  - Mock Ctrl #1 #14
  - ****

- Glucose Consumption (mM/10^6 cells)
  - Mock Ctrl #1 #14
  - u.d. u.d.

B

Affected Genes in the Glycolysis Pathway
(Fold-Change, ASO vs. Mock)

- GLUT1
  - #1 = -3.87
  - #14 = -6.84

- Glucose-6-P

- Fructose-6-P

- Fructose-1,6-P2

- Glyceraldehyde-3P ↔ Glycerone-P

- GAPDH
  - #1 = -2.10
  - #14 = -2.23

- Glycerate-1,3P2

- PGK1
  - #1 = -6.48
  - #14 = -6.38

- Glycerate-3P

- PGAM1
  - #1 = -4.43
  - #14 = -4.61

- Glycerate-2P

- ENO1
  - #1 = -2.82
  - #14 = -2.53

- Phosphoenolpyruvate

- LDHA
  - #1 = -3.88
  - #14 = -4.70

- Pyruvate

- LDHB
  - #1 = -1.32
  - #14 = -1.33

- PDHA1
  - #1 = 1.14
  - #14 = -1.06

- Acetyl-CoA

- MCT1
  - #1 = -1.51
  - #14 = -1.44

- MCT4
  - #1 = -6.02
  - #14 = -7.49

- ENO2
  - #1 = -2.08
  - #14 = -2.51

- PDK1
  - #1 = -3.54
  - #14 = -2.73
Figure 6

A

![Graph showing Area of Immune Aggregates (%) for Control and MCT4 ASO treatments.](image)

B

![Graph showing Proportion of NK Cells (%) for Control and MCT4 ASO treatments.](image)

C

![Graph showing Proportion of CD3 Positive Cells (%) for Control and MCT4 ASO treatments.](image)
Clinical Cancer Research

The MCT4 Gene: a Novel, Potential Target for Therapy of Advanced Prostate Cancer


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