Targeting Amino Acid Transport in Metastatic Castration-Resistant Prostate Cancer: Effects on Cell Cycle, Cell Growth, and Tumor Development

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Background

L-type amino acid transporters (LATs) uptake neutral amino acids including L-leucine into cells, stimulating mammalian target of rapamycin complex 1 signaling and protein synthesis. LAT1 and LAT3 are overexpressed at different stages of prostate cancer, and they are responsible for increasing nutrients and stimulating cell growth.

Methods

We examined LAT3 protein expression in human prostate cancer tissue microarrays. LAT function was inhibited using a leucine analog (BCH) in androgen-dependent and -independent environments, with gene expression analyzed by microarray. A PC-3 xenograft mouse model was used to study the effects of inhibiting LAT1 and LAT3 expression. Results were analyzed with the Mann-Whitney U or Fisher exact tests. All statistical tests were two-sided.

Results

LAT3 protein was expressed at all stages of prostate cancer, with a statistically significant decrease in expression after 4–7 months of neoadjuvant hormone therapy (4–7 month mean = 1.571; 95% confidence interval = 1.155 to 1.987 vs 0 month = 2.098; 95% confidence interval = 1.962 to 2.235; P = .0187). Inhibition of LAT function led to activating transcription factor 4–mediated upregulation of amino acid transporters including ASCT1, ASCT2, and 4F2hc, all of which were also regulated via the androgen receptor. LAT inhibition suppressed M-phase cell cycle genes regulated by E2F family transcription factors including critical castration-resistant prostate cancer regulatory genes UBE2C, CDC20, and CDK1. In silico analysis of BCH-downregulated genes showed that 90.9% are statistically significantly upregulated in metastatic castration-resistant prostate cancer. Finally, LAT1 or LAT3 knockdown in xenografts inhibited tumor growth, cell cycle progression, and spontaneous metastasis in vivo.

Conclusion

Inhibition of LAT transporters may provide a novel therapeutic target in metastatic castration-resistant prostate cancer, via suppression of mammalian target of rapamycin complex 1 activity and M-phase cell cycle genes.


L-type amino acid transporters (LATs) supply cells with large neutral amino acids, which are not only required for protein synthesis but also contribute to various signaling pathways. Intracellular leucine levels are sensed by the leucyl-transfer RNA synthetase, previously known to catalyze the adenosine triphosphate–dependent ligation of L-leucine to transfer RNA during protein synthesis (1,2). Leucyl-transfer RNA synthetase activates the Rag guanosine triphosphatase–dependent ligation of L-leucine to transfer RNA during protein synthesis (1,2). In this way leucine is not only an essential amino acid but acts as a rate-limiting signaling molecule in the mTORC1 pathway.

In cells deprived of amino acids, there is an accumulation of uncharged transfer RNA, which binds to and activates the general control nonrepressed 2 (GCN2) kinase. In turn, GCN2 phosphorylates the translation initiation factor 2α (eIF2α) on serine 51, triggering translational upregulation of activating transcription factor (ATF) 4 (4). ATF4 itself upregulates the expression of amino acid transporters as a means of restoring intracellular amino acid levels (5). Therefore, understanding how amino acid transporters regulate intracellular leucine levels, and generating novel inhibitors of these transporters, may lead to potent suppressors of mTORC1 signaling.

The two distinct families of LATs are 1) solute carrier 7 (SLC7) members (LAT1/SLC7A5 and LAT2/SLC7A8), which mediate Na+-independent neutral amino acid exchange as heterodimers with the 4F2 cell-surface antigen heavy chain (4F2hc/SLC3A2/C798) glycoprotein (6,7); and 2) SLC43 proteins (LAT3/SLC43A1 and LAT4/SLC43A2) that mediate Na+-independent uniport of neutral amino acids (8,9). Although the expression of each LAT member varies dramatically in different tissues, these transporters are commonly upregulated in cancer. Increased LAT1 expression
has been detected in lung cancer, colon cancer, breast cancer, head and neck cancer, genital cancers, and soft tissue sarcomas (10–12). We and others have shown that LAT1 and LAT3 are overexpressed in prostate cancer (11–14), with LAT1 expression increased in metastasis compared with primary cancer (10,12).

We hypothesized that inhibition of LAT1 and LAT3 may offer an effective therapeutic approach for prostate cancer.

**Materials and Methods**

**Patients**

Prostate cancer specimens (n = 194) were obtained from the Vancouver Prostate Centre Tissue Bank (http://www.prostatecentre.com/our-research/core-facilities/biorepository). This project was approved by the institutional review boards at the University of British Columbia (Vancouver, Canada) and the CHUQ Research Centre (Québec, Canada). Written informed consent was obtained from all participants. The hematoxylin and eosin slides were reviewed, and desired areas were identified on paraffin blocks. Tissue microarrays were manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm for each sample, with quantitative analysis calculated from individual cores (individual Gleason score) or the average of duplicate cores (neoadjuvant hormone therapy tissue microarray analysis). All specimens were from radical prostatectomy except 12 castration-resistant prostate cancer (CRPC) samples that were obtained from transurethral resection of prostate. Supplementary Table 1 outlines the details of the pathology, number of patient samples, and treatment.

**Cell Culture**

Human prostate cancer cell lines LNCaP-FGC and PC-3 were purchased from ATCC (Rockville, MD, USA). LNCaP cells have been passaged directly from original low-passage stocks (2009), and we confirmed PC-3 cell identity by short tandem repeat profiling in 2010 (CellBank, Australia). Cells were cultured in RPMI 1640 medium (Invitrogen, Australia) containing 10% (v/v) fetal bovine serum, penicillin-streptomycin solution (Sigma-Aldrich, Australia), and 1 mM sodium pyruvate (Invitrogen, Australia). Cells were maintained at 37°C in a fully humidified atmosphere containing 5% carbon dioxide.

**PC-3-luc Xenografts and Bioluminescence Imaging**

Athymic (nu/nu) male nude mice (Animal Resource Center, Perth, Australia) 6–8 weeks of age were housed in a specific pathogen-free facility in accordance with the University of Sydney animal ethics committee guidelines. Mice were anesthetized via 2% isoflurane inhalation and received subcutaneous injections of 1 × 10⁶ PC-3-luc cells resuspended in 100 µl of Hank's Balanced Salt Solution. Xenografts were transplanted in both the right and left side dorsal flanks of mice as detailed previously, using five mice per group from three independent experiments (15). Tumor growth was monitored via bioluminescence imaging performed 24 hours following cell implantation and biweekly thereafter for 4 weeks. During the experiments, one shCont and two shLAT1 and shLAT3 mice were euthanized prior to day 29 due to weight loss beyond our ethical guidelines. They were not included in the analyses. Anesthetized mice received intraperitoneal injections of D-luciferin substrate (150 mg/kg in DPBS, Gold Biotechnology, MO, USA), and images were acquired after a 15-minute interval, using the Xenogen in vivo imaging system 100 (Caliper Life Science, Hopkinton, MA, USA). Regions of interest were determined using Living Image Software (Caliper Life Science) and quantified in photons per second. After 29 days, mice were euthanized following the final imaging time point, with tumors collected either in Trizol for RNA analysis or fixed for sectioning and immunostaining. Livers, inguinal lymph nodes, and lungs were removed for in vivo imaging system 100 analysis to detect spontaneous metastases.

**Microarray Gene Expression Analysis**

RNA was isolated using miRVana RNA Isolation Kit (Ambion, USA) from LNCaP cells treated for 48 hours with or without 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, 10 mM; Sigma Aldrich) in either charcoal-stripped serum (CSS) with the synthetic androgen R1881 (1 mM), or CSS with ethanol as a vehicle control. cRNA generated by Agilent’s QuickAmp labeling kit was hybridized to the Agilent 4×44K Human Gene Expression Microarrays (v:014850) using the single-channel gene expression standard protocol (Agilent Technologies, USA). The resulting data were analyzed using the LIMMA R package to determine differential gene expression between the treatment groups. Microarray annotation was downloaded from GEO (platform: GPL6480; September 2012). Gene set enrichment analysis (GSEA; http://www.broad.mit.edu/gsea) was used to identify gene clusters. Depending on sample size, phenotype or gene set permutation analysis with ratio-of-classes or signal-to-noise gene ranking was carried out. Ingenuity Pathway Analysis (Ingenuity Systems, USA) was used to explore the microarray data for differential regulation of pathways. Venn diagrams were drawn using R package software at the gene level. The microarray data were overlaid with published microarray data sets using Oncomine (v.4.4.3).

**Statistical Analysis**

GSEA analyses (http://www.broad.mit.edu/gsea) generate a nominal P value, reflecting the statistical significance, estimated using an empirical phenotype-based permutation test. Microarray data were processed using the LIMMA linear model adjusted using the Benjamini Hochberg multiple test correction. Oncomine analysis uses the Student t test. Statistical analyses of our data were performed using the Mann-Whitney U test or the Fisher exact test in GraphPad Prism v.6. Data are expressed as means with 95% confidence intervals (CIs). All statistical tests were two-sided.

**Results**

**LAT3 Protein Expression in Prostate Cancer Stages**

We and others have shown statistically significantly increased levels of LAT3 messenger RNA (mRNA) in prostate cancer tissue (16) and that LAT3 is an androgen-responsive gene (12). To determine the level of LAT3 protein present in prostate cancer, and whether its expression level was associated with disease state, we performed LAT3 immunohistochemical staining using a prostate cancer tissue microarray (Figure 1 and Supplementary Figure 1). Our tissue microarray contained samples from patients
LAT3 expression was detected at all stages of prostate cancer, with no differences observed between Gleason grades (Figure 1, A and B). This is in contrast to LAT1, which has previously been shown to be associated with high Gleason score (11). Comparison of untreated patients (n = 123 cores) with patients undergoing neoadjuvant hormone therapy showed a decrease in LAT3 expression after 2–3 months of treatment (21.7% decrease; mean score of 2–3 month = 1.643; 95% CI = 1.128 to 2.157 vs mean score of 0 month = 2.098; 95% CI = 1.962 to 2.235; P = .0510), with a statistically significant decrease after 4–7 months (25.1% decrease; mean score of 4–7 month = 1.571, 95% CI = 1.155 to 1.987 vs mean score of 0 month = 2.098; 95% CI = 1.962 to 2.235; P = .0187) (Figure 1, A and C). This is in agreement with our previous data showing that LAT3 mRNA levels statistically significantly decrease after hormone therapy (12). Interestingly, the expression of LAT3 increased 8–12 months posttreatment and returned to untreated levels in recurrent disease (Figure 1, C).

Amino Acid Response Pathway Regulation of Amino Acid Transporter Expression

Because both LAT1 and LAT3 proteins are present in prostate cancer patients, we next set out to determine the potential global effects of LAT inhibition on prostate cancer cells. LNCaP cells were treated with the LAT inhibitor BCH (6,8,17,18), and/or the synthetic androgen R1881 for 48 hours, followed by mRNA microarray analysis. To separate the effects due to androgenic stimulation from those due to amino acid deprivation, four treatment groups were analyzed: control (CSS), BCH (BCH in CSS), R1881 (R1881 in CSS), and BCH and R1881 (BCH and R1881 in CSS). This allowed us to examine BCH-specific effects, R1881-specific effects, as well as effects arising from the combination of BCH and/or R1881.

We first analyzed amino acid transporters that showed a statistically significant change (moderated t statistic, false discovery rate adjusted P value < .05) in expression in either BCH vs control or BCH and R1881 vs R1881 (Figure 2, A). Consistent with previous data, BCH reduced leucine uptake by approximately 60% in LNCaP cells (Figure 2, B) leading to rapid translation of ATF4 protein and subsequent transcriptional increases in LAT1 (SLC7A5) and system xCT (SLC7A11) expression (Figure 2, A and C) (12). This was confirmed both in the presence and absence of R1881 (Figure 2, A).

Interestingly, a group of amino acid transporters appeared to be regulated by both R1881 and BCH (Figure 2, A). These transporters included SLC1A4 (alanine-serine-cysteine-threonine transporter 1 [ASCT1]), SLC1A5 (alanine-serine-cysteine-threonine transporter 2 [ASCT2]), and SLC3A2 (4F2hc; LAT1 heterodimeric partner). Analysis of protein levels for ASCT1, ASCT2, and 4F2hc showed increased expression after leucine deprivation (PC-3 cells; 

Figure 1. Immunohistochemical staining of L-type amino acid transporter 3 (LAT3) in neoadjuvant hormone therapy patient cohort. A) Representative images of LAT3 expression in patient samples with Gleason grade 3, Gleason grade 4, Gleason grade 5, and after neoadjuvant hormone therapy (NHT) treatment following an interval of 1–1.5 months, 4–7 months, 8–12 months, or recurrent cancer (Rec.).

Scale bar is 100 μm. B) Scoring of LAT3 expression in patient cohort with different Gleason grades (n = 88). C) Scoring of LAT3 expression in tissue samples from NHT patient cohort versus untreated (n = 72). Two-sided Mann-Whitney U test was used to determine statistical significance. Error bars represent 95% confidence intervals.
To determine whether ASCT1, ASCT2, and 4F2hc are also regulated by ATF4, we searched for conserved enhancer-like elements in promoters, untranslated regions (UTRs), and downstream exonic and intronic regions. These analyses revealed a conserved amino acid response element (AARE) consensus (nTTnCATCA) for each gene, which may be bound by ATF4 (Supplementary Figure 2). To confirm that ATF4 binds directly to these AAREs, we next performed ATF4 chromatin immunoprecipitation (ChIP) in PC-3 cells in the presence or absence of leucine. Analysis of ChIP samples using quantitative polymerase chain reaction showed a statistically significant enrichment of binding to both intronic regions of 4F2hc and ASCT2 in the absence of leucine (Figure 2, E). Previously known ATF4 regulatory regions from xCT and...
LAT1, which were both statistically significantly altered in the BCH microarray data (Figure 2, A), were used as positive controls. Analysis of the putative AARE in the 5’ untranslated region of ASCT1 showed constitutive binding of ATF4 (above immunoglobulin G control background), which was not enhanced by leucine deprivation (Figure 2, E).

To further validate these results, we constructed luciferase reporter plasmids containing a single copy of a wild-type or mutant 4F2hc, ASCT1, or ASCT2 enhancer region (Supplementary Table 2) and transfected them into PC-3 cells grown in leucine-containing (Control) or leucine-free (Leu free) medium for 18 hours. Luciferase expression from PC-3 cells in leucine-free medium was higher than basal levels in normal medium from the wild-type 4F2hc-containing constructs (3.1-fold; mean fold increase over empty vector of leucine free = 5.904; 95% CI = 4.859 to 6.950 vs mean fold increase over empty vector of control = 1.888; 95% CI = 1.317 to 2.459; *P < .0001), ASCT1-containing (1.4-fold; mean fold increase over empty vector of leucine free = 1.993, 95% CI = 1.732 to 2.248 vs mean fold increase over empty vector of control = 1.372; 95% CI = 1.235 to 1.509; *P < .0001) or ASCT2-containing (1.8-fold; mean fold increase over empty vector of leucine free = 2.193; 95% CI = 1.988 to 2.398 vs mean fold increase over empty vector of control = 1.191; 95% CI = 0.9997 to 1.382; *P < .0001) constructs, respectively (Figure 2, F). In the mutant constructs, basal expression was reduced, and luciferase expression induced by leucine deprivation was completely abolished (Figure 2, F).

A number of amino acid transporters were also increased by R1881, suggesting they are regulated by AR. In silico analysis of prostate cancer patient samples showed that ASCT1, 4F2hc, and LAT3 were statistically significantly reduced in CRPC (Supplementary Figure 3, A), with ASCT1 and ASCT2 (and LAT3 [12]) also being reduced after androgen-deprivation therapy (Supplementary Figure 3, B). We have previously confirmed that LAT3 is androgen regulated and contains an androgen response element in the second intron (12). Analysis of publicly available AR ChIP-on-chip and AR ChIP-seq data confirmed putative binding sites for AR in ASCT1, ASCT2, and 4F2hc (Supplementary Figure 3, C).

**Amino Acid Transporter Expression in Metastatic CRPC**

We next examined the gene expression of amino acid transporters in a published prostate cancer patient cohort (GSE35988) using Oncomine (19). This data set contains gene expression profiling on a matched cohort of benign prostate tissue (28 samples), primary prostate cancer (59 samples), and metastatic CRPC (35 samples). Of the transporters regulated by BCH or R1881 (Figure 2, A), we found that LAT3, xCT, and SLC1A1 exhibit low expression in normal prostate tissue but show a statistically significant increase in primary prostate cancer samples (Figure 2, G). For each of these transporters, the expression levels were reduced in metastatic prostate cancer (Figure 2, G).

Interestingly, ATF4 gene expression is upregulated in metastasis (Figure 2, G), suggesting that ATF4-mediated regulation of genes containing an AARE may be important for the development of metastatic CRPC. ATF4-regulated genes, such as LAT1, ASCT1, 4F2hc, and SLC38A42 (SNAT2), exhibit low expression in normal prostate tissue and primary prostate cancer, but they are all statistically significantly increased in metastasis (Figure 2, G), suggesting that these transporters are involved in nutrient supply required for metastatic prostate cancer.

**Effects of Leucine Deprivation on M-Phase Cell Cycle Genes and E2F Transcription Factors**

To determine which gene sets and pathways were being altered by BCH treatment, we examined our microarray data using GSEA. We performed Gene Ontology analysis for BCH (including BCH and BCH+R1881) versus control (including control and R1881) with false discovery rate q value lower than 25% and normalized P value lower than .05 (Supplementary Table 3). Substantial enrichment was shown for gene sets involved in cell cycle regulation including spindle formation, microtubule cytoskeleton, M phase, mitosis, cell cycle process, and cell cycle phase in the control group (Figure 3, A, Supplementary Figure 4, A, and Supplementary Table 3, A).

We also examined the transcription factor motif gene sets in the BCH versus control group. Interestingly, there was statistically significant enrichment in E2F transcription factor binding sites in the control group (Figure 3, B, Supplementary Figure 4, B, and Supplementary Table 3, B). E2F family members regulate cell cycle and metabolism in cancers (20).

We next examined the genes showing statistically significant changes (fold change >1.5 and false discovery rate adjusted *P value < .05) after BCH treatment, with 122 genes downregulated and 12 genes upregulated (Figure 3, C–F, and Supplementary Table 4). Of the 122 downregulated genes, which included transcription factors E2F1 and E2F2, 72 (59%) were related to cell cycle and proliferation. Using Ingenuity Pathway Analysis, we generated a network of downregulated genes from the E2F transcription factors, showing that approximately half of these downregulated genes are part of a common cell cycle regulatory pathway (Figure 3, G). This analysis indicates that E2F transcription factors have a central role in the regulation of cell cycle gene expression in prostate cancer. Three of these cell cycle genes, *CDK1* (CDC2), *CDC20*, and *UBE2C* (ubiquitin-conjugating enzyme E2C), are upregulated and control cell cycle progression in androgen-independent prostate cancer cells (21). Western blots showed that E2F1, E2F2, CDK1, UBE2C, and CDC20 protein levels are slightly decreased in both PC-3 and LNCaP cells after BCH treatment, with all proteins exhibiting a dramatic decrease after leucine deprivation (Figure 3, H). Some BCH-downregulated genes encode proteins that form the centromere (centromere protein A, E, and F; CENPA, CENPE, CENPF) and kinetochores (baculoviral IAP repeat containing 5, BIRC5; NDC80 kinetochore complex component homologs, NDC80, NUF2, SPC24, and SPC25; spindle and kinetochore associated complex subunit 3 [SKA3]), which may delay the formation of these structures and the segregation of sister chromosomes.

The BCH-upregulated genes include transcription factors ID1, ID3, and ID4 (inhibitor of DNA-binding 1, 3, and 4) and amino acid transporters xCT and SLC9A9 (Supplementary Table 4). ID1–4 are helix-loop-helix proteins that heterodimerize with members of the basic helix-loop-helix family of transcription factors to inhibit transcription activation (22). Interestingly, UBE2E1 (the ubiquitin-conjugating enzyme E2E1) was also upregulated
This protein may function to compensate for loss of UBE2C to mediate the selective degradation of short-lived and abnormal proteins.

Expression of BCH-Downregulated Genes in Metastatic CRPC

We next used GSEA to interrogate metastatic patient samples (GSE35988) (19). GSEA of metastatic prostate cancer versus primary prostate cancer using gene ontology showed a highly similar set of enriched genes in metastasis to that inhibited by BCH treatment (Supplementary Table 5, A). These included M phase, mitosis, cell cycle process, and cell cycle phase, with 7 of 30 of the most statistically significantly enriched gene ontology terms common for the two groups (Figure 4, A). Furthermore, analysis of transcription factor motif gene sets showed enrichment for genes with E2F binding sites, with 17 of 30 of the statistically significantly enriched transcription factor motif gene sets common for metastasis-upregulated and BCH-downregulated genes (Figure 4, B, and Supplementary Table 5, B). This analysis suggested that E2F-regulated cell cycle genes are also crucial for metastatic CRPC.

We then used Oncomine to determine the expression of the BCH-downregulated genes in metastatic prostate cancer (GSE35988), showing that 90.9% of BCH-downregulated genes (111 of 122) are statistically significantly upregulated in metastatic patient samples including E2F1, E2F2, CDK1, CDC20, and UBE2C (Figure 4, C).
Analysis of the statistically significantly upregulated genes following BCH treatment showed that 83.3% of upregulated genes (10 of 12) were statistically significantly downregulated in metastatic patient samples (Figure 4, D). BCH-downregulated genes were also overexpressed in metastasis using Oncomine gene expression signatures (Table 1) (19,23–28). CENPA, UBE2C, BIRC5, CDC20, and CDK1 are statistically significantly overexpressed in most data sets (Supplementary Table 6, A). Also BCH-downregulated genes were overexpressed in patients with recurrent cancer (1–5 years) and poor survival (Table 1) (25,29,30) including BIRC5, CDK1, E2F1, CDC20, and UBE2C (Supplementary Table 6, B).

Effect of Knockdown of LAT1 or LAT3 on PC-3-luc Xenografts

We next used a bioluminescent xenograft model to establish the role of LAT1 and LAT3 in CRPC tumor formation in vivo. Luciferase-positive PC-3 cells (PC-3-luc) were stably transduced with lentiviral shRNA constructs against either LAT1 or LAT3, resulting in decreased transporter expression (Figure 5, A) as well as decreased expression of cell cycle proteins including UBE2C, CDK1, and CDC20 (Supplementary Figure 5, A). PC-3-luc cells expressing shCont, shLAT1, or shLAT3 were transplanted into the dorsal flanks of male nude mice, and tumor formation and growth was monitored using bioluminescent imaging (Figure 5, B). Knockdown of both LAT1 and LAT3 caused a reduction in the tumor take rate: 70% for shLAT1 and 61% for shLAT3 cells compared with 83% for shCont cells (Supplementary Figure 5, B). In addition, PC-3-luc xenografts expressing either shLAT1 or shLAT3 showed statistically significantly decreased tumor growth compared with control cells at day 29 (mean total flux of shLAT1 = 0.8854; 95% CI = −0.1725 to 1.945 vs mean total flux of shCont = 3.304, 95% CI = 1.767 to 4.840, P = .0003; mean total flux of shLAT3 = 0.1850; 95% CI = −0.03710 to 0.4072 vs mean total flux of shCont = 3.304; 95% CI = 1.767 to 4.840; P < .0001, respectively; Figure 5, B and C). Analysis of Ki-67 expression in the tumors showed a statistically significant decrease in proliferation for both the shLAT1 and shLAT3 tumors compared with the shCont group (Figure 5, D and E). Furthermore, we examined the expression of CDK1, UBE2C, and CDC20 in these xenografts, showing decreased expression of CDK1 and UBE2C in shLAT1 and shLAT3 tumors (Figure 5, F) while CDC20 expression also decreased in shLAT3 tumors (Figure 5, F).

To investigate the occurrence of metastases, multiple organs were harvested from mice at the final imaging time point (day 29), and bioluminescence was examined using the in vivo imaging system 100. Of 14 mice with shCont tumors, 8 exhibited metastases to the liver, lymph nodes, and/or lungs (Supplementary Figure 5, C). In contrast, the numbers of metastases in mice harboring either shLAT1 (3 of 13 mice; P = .1201) or shLAT3 (1 of 13 mice; P = .012) tumors were decreased (Figure 5, G; two-sided Fisher exact test). These data suggest that depletion of LAT1 or LAT3 expression in prostate cancer cells may also suppress their metastatic potential.

Discussion

We show that targeting the LAT family inhibits nutrient signaling pathways, in vivo prostate cancer growth, and over 100 genes
overexpressed in metastasis including E2F transcription factors and cell cycle regulators UBE2C, CDC20, and CDK1. We found that inhibition of leucine uptake regulates gene expression at the transcriptional level. Our microarray data showed that a number of amino acid transporters are regulated by two important pathways in prostate cancer, namely AR signaling and ATF4-mediated amino acid response pathways. AR signaling is crucial for prostate cancer survival. Androgen-deprivation therapy inhibits AR signaling not only leading to cell apoptosis, but also alters transporter gene expression, thereby regulating nutrient uptake.

Amino acid deprivation activates ATF4 signaling to induce the expression of a set of transporters that may compensate and restore intracellular amino acid levels. ATF4 expression is commonly increased in prostate cancer metastasis (Figure 2, G), and we have shown that the ASCT1, ASCT2, and 4F2hc transporters are all regulated by ATF4 expression in prostate cancer cells. These glutamine transporters work together with the LAT1/4F2hc heterodimer by providing the abundant amino acid glutamine for LAT1 to exchange with the essential amino acid leucine. They are commonly upregulated together in cancers (31), and although ASCT2 does not appear to be co-regulated in metastasis (potentially due to regulation by other pathways such as AR or extracellular signal–related kinases 1/2 (ERK1/2) signaling (32)), ASCT1, LAT1, and 4F2hc were all statistically significantly increased in our in silico analysis (Figure 2, G). This coordinate response may provide leucine for activation of the mTORC1 pathway in metastasis. This leucine/mTORC1 pathway appears critical in metastatic prostate cancer because we have previously shown that BCH treatment directly inhibits mTORC1 activity (p-mTOR and ribosomal protein S6 kinase, 70 kDa, poly-peptide 1; p-p70S6K) in both LNCaP and PC-3 cells, decreasing cell growth and clonogenicity in vitro (31).

Overexpression of E2F1 and E2F2 in metastasis (33). E2F1 can also interact and cooperate with AR to regulate gene expression in the presence of androgen (34). In metastatic CRPC patient samples, E2F-regulated genes are statistically significantly increased over primary cancer, indicating that E2F family members are crucial for cell cycle gene expression in metastatic CRPC. Among the cell cycle pathway genes that were also inhibited by BCH were CDK1, CDC20, and UBE2C, which have been shown to be critical M-phase regulators of cell cycle in metastasis (21,35). In the presence of high CDK1 activity, CDC20 is phosphorylated, and activated anaphase-promoting complex/cyclosome (APC/C) is ubiquitinated and degraded, allowing cyclin B to promote the metaphase-anaphase transition (36,37). UBE2C, an APC/C-specific E2 ubiquitin-conjugating enzyme, inactivates the M phase checkpoint and drives M-phase cell-cycle progression (38,39). Silencing or attenuation of UBE2C in CRPC arrests cell cycle in G2/M phase and inhibits cell proliferation in both AR-negative and -positive CRPC cells (21,40,41). BIRC5, encoding survivin, which is a regulator of apoptosis and the mitotic spindle checkpoint (42), was also decreased after BCH treatment (Supplementary Table 4).

Survivin interacts with X-linked inhibitor of apoptosis (XIAP) to stimulate tumor cell invasion and metastasis (43). This suggests that inhibition of leucine uptake in metastasis may provide an effective block for M-phase cell cycle progression through E2F transcriptional pathways. Although some studies have shown that LAT inhibitors induce apoptosis in epidermoid carcinoma cells and melanoma cells (44,45), we have not observed apoptosis in prostate cancer cells (12); nor was there enrichment for gene sets related to apoptosis in the GO enrichment list (Supplementary Table 3, A).

Although our data did not directly identify how E2F transcription factors are regulated, recent studies have suggested that E2F signaling may regulate UBE2C mRNA expression through AR coactivators, such as forkhead box A1 (FOXA1), GATA binding protein 2 (GATA2), and mediator complex subunit 1 (MED1) (40). The amino acid stress response can also induce the expression of jun proto-oncogenes (c-JUN and JUN-B), FBJ murine osteosarcoma viral oncogene homologs (c-FOS and FOS-B) through ATF4 or ATF2 (46,47). MicroRNAs may also be involved in this process, such as mir-203, which has been shown to decrease the expression of E2F1 and survivin in metastatic prostate cancer (48).

Table 1. Overexpressed BCH-downregulated genes in Oncomine metastatic/clinical prostate cancer data sets

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Figure 5. Effect of reduced expression of LAT1 and LAT3 in PC-3-luc xenografts. PC-3-luc cells stably expressing shCont, shLAT1, or shLAT3 were tested for L-type amino acid transporter 1 (LAT1) and L-type amino acid transporter 3 (LAT3) levels by Western blot ([A]; representative from n = 3), with α-tubulin used as a loading control. ([B]) PC-3-luc cells were injected subcutaneously into the right and left side dorsal flanks of male nude mice. Bioluminescent images are shown at day 1 and day 29. ([C]) Tumor growth curves of bioluminescence (total flux measured in photons/second) from PC-3-luc cells expressing shCont, shLAT1, and shLAT3. Data are the mean with 95% confidence intervals from three independent experiments using five mice per group. Statistical significance was assessed at day 29 using the two-sided Mann-Whitney U test. ([D] and [E]) Quantitation (n = 6 tumors, means with 95% confidence intervals) and representative images of Ki-67 expression in shCont, shLAT1, and shLAT3 tumor sections from two separate experiments. Scale bar is 50 µm. ([F]) Representative images of cell division cycle associated 2 (CDK1), ubiquitin-conjugating enzyme E2C (UBE2C), and cell division cycle 20 homolog (CDC20) expression in shCont, shLAT1, and shLAT3 tumor sections from two separate experiments. Scale bar is 50 µm. ([G]) Spontaneous metastasis was decreased in mice developing shLAT1 and shLAT3 tumors. The number of spontaneous metastases at day 29 in shCont (n = 14), shLAT1 (n = 13), and shLAT3 (n = 13) mice are shown with statistical significance assessed using the Fisher exact test. All statistical tests were two-sided.
Further investigation is needed to study the mechanism regulating the long-term effects of leucine deprivation.

In addition to transcriptional level regulation of metastatic cell proliferation, other mechanisms may also suppress CRPC metastasis. LAT1 protein is highly expressed in high Gleason score prostate cancer samples (11), and its expression is associated with metastatic cancer (10,12). LAT1 has also been shown to bind to β1-integrin through the heterodimeric association with 4F2hc (49). Therefore, knockdown of LAT1 may affect β1-integrin distribution on the cell membrane and further inhibit cell migration. We have previously shown that BCH inhibition or short hairpin RNA knockdown of LAT1 or LAT3 suppresses mTORC1 signaling pathway, cell cycle, and cell growth in both LNCaP and PC-3 cells (12). Furthermore, we observed that inhibition of LAT activity by BCH and inhibition of mTORC1 by rapamycin exhibit similar effects on p70S6K activation (Supplementary Figure 6, A) and prostate cancer cell growth (Supplementary Figure 6, B and C). This suggests that the E2F cell cycle effects we have observed may be linked to mTORC1 activation. Interestingly, recent studies have shown that mTORC1 regulates mRNA translation of genes involved in cell invasion and metastasis, including YB1 (Y-box binding protein 1), vimentin, MTA1 (metastasis-associated 1), and CD44 (50). Other studies have also shown that mTORC1 and mTORC2 regulate metastasis via ras homolog family member A (RhoA) and ras-related C3 botulinum toxin substrate 1 (Rac 1) signaling in colorectal cancer (51) or via signal transducer and activator of transcription 3 (STAT3) in glioblastoma (52). Thereby inhibition of LATs, leading to reduced mTORC1 activity, may also affect these metastatic pathways.

LAT1 expression is increased in a variety of cancers, in particular in metastasis (10–12). This is likely due to the critical requirement of nutrients for cell growth pathways such as mTORC1, and it suggests that targeting LAT transporters may be an effective and generalizable therapy across multiple cancers including breast and colon cancer. This study also had some limitations, most notably that although downregulation of LAT1 and LAT3 in tumor cells suppressed cell growth, it remains to be determined which other cell types may also be inhibited by targeting LAT1 or what resistance mechanisms (such as ATF4 activation) may be triggered. In particular, LAT1 was recently shown to be critical in effector T-cell differentiation and pathogen response, suggesting there may be immune side effects of targeting LAT1 (53) that needs to be tested in preclinical models.

In conclusion, we have shown that prostate cancer cells use AR and ATF4 transcriptional pathways to maintain amino acid transporter levels during primary and metastatic prostate cancer. Targeting LAT transporters, thereby inhibiting leucine uptake, may offer a new therapeutic opportunity for metastatic CRPC, affecting tumor growth and metastasis through inhibition of M-phase cell cycle and mTORC1 signaling pathways.

References


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Notes

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