Blocking Lactate Export by Inhibiting the Myc Target MCT1 Disables Glycolysis and Glutathione Synthesis

Joanne R. Doherty1, Chunying Yang1, Kristen E.N. Scott1, Michael D. Cameron4, Mohammad Fallahi1, Weimin Li1, Mark A. Hall1,1, Antonio L. Amelio1, Jitendra K. Mishra2, Fangzheng Li2, Mariola Tortosa3, Heide Marika Genau1,1, Robert J. Rounbehler1, Yunqi Lu6, Chi V. Dang4,3, K. Ganesh Kumar3, Andrew A. Butler3, Thomas D. Bannister2, Andrea T. Hooper8, Keziban Unsai-Kacmaz8, William R. Rouss7, and John L. Cleveland1

Abstract
Myc oncoproteins induce genes driving aerobic glycolysis, including lactate dehydrogenase-A that generates lactate. Here, we report that Myc controls transcription of the lactate transporter SLC16A1/MCT1 and that elevated MCT1 levels are manifest in premalignant and neoplastic Eμ-Myc transgenic B cells and in human malignancies with MYC or MYCN involvement. Notably, disrupting MCT1 function leads to an accumulation of intracellular lactate that rapidly disables tumor cell growth and glycolysis, provoking marked alterations in glycolytic intermediates, reductions in glucose transport, and in levels of ATP, NADPH, and ultimately, glutathione (GSH). Reductions in GSH then lead to increases in hydrogen peroxide, mitochondrial damage, and ultimately, cell death. Finally, forcing glycolysis by metformin treatment augments this response and the efficacy of MCT1 inhibitors, suggesting an attractive combination therapy for MYC/MCT1-expressing malignancies. Cancer Res; 74(3); 908–20. ©2013 AACR.

Introduction
Myc oncoprotein transcription factors are activated in a large cast of human malignancies, and it has been estimated that 100,000 deaths per year are associated with deregulated MYC expression (1). Myc drives continuous cell growth and division, which triggers DNA damage and apoptotic checkpoints that are then bypassed by mutations that lead to frank malignancy. Accordingly, forced expression of Myc is sufficient to provoke tumor formation in mouse models of human cancer (1, 2). Furthermore, Myc is required to sustain the malignant state, as Myc inactivation usually provokes rapid tumor regression (3, 4).

Myc oncoproteins are basic-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors that regulate a large cast of targets to coordinate cell growth, metabolism, and division (5). Myc functions require dimerization with the related bHLH-Zip partner Max, and Myc:Max dimers activate target genes by binding to E-box elements (CACGTG; ref. 6). In addition, Myc represses transcription via inhibitory interactions with the transcriptional activator Miz-1 (7, 8). Finally, Myc oncoproteins have recently been suggested to function as universal amplifiers of active genes (9, 10), which may occur through their ability to recruit histone modifying enzymes (11) and/or by occupying preexisting open chromatin and promoting transcription or pause release at promoters loaded with RNA polymerase II (9, 10).

Proliferant targets induced by Myc include a cast of metabolic enzymes, some of which drive aerobic glycolysis, a hallmark of cancer cells (12, 13). Indeed, in cell culture models, Myc oncoproteins induce many aspects of cancer cell metabolism, where Myc targets include glucose and glutamine transporters, glutaminase and glycolytic enzymes, including lactate dehydrogenase-A (LDH-A; refs. 14–17).

Increased glycolytic flux in cancer cells leads to high levels of lactate that is exported by proton-dependent twelve-membrane pass monocarboxylic acid solute transporters coined MCT1-4 (18). Cell surface expression of MCT1 and MCT4 requires coexpression of the immunoglobulin-like molecule CD147 (19). Although MCT4 transcription is regulated by hypoxia-inducible factor-1α (HIF-1α; ref. 20) and in renal clear cell cancer by promoter methylation (21), much less is known about the control of MCT1 transcription, other than MCT1 expression is elevated in Myc-expressing MCF10 breast epithelial cells and in some tumors (22).
Blocking lactate transport impairs tumor cell growth through several mechanisms. First, blocking lactate export leads to an accumulation of lactic acid and decreases intracellular pH (23). This response seems to contribute to growth arrest of Ras-transformed fibroblasts triggered by MCT1 inhibitors and to the effects of CD147 knockdown on tumor xenografts (24). Second, some tumor cells rely on lactate as a substrate for oxidative phosphorylation, and in this scenario blocking lactate import inhibits tumor cell growth (25, 26). However, this is the exception as most tumor cells express high levels of LDH-A, which drives the production of lactate from pyruvate (27). Finally, lactate uptake in vascular endothelial cells via MCT1 seems to promote tumor angiogenesis; thus blocking this response impairs tumorigenesis (28, 29). Given these effects, a recent AstraZeneca patent application claims the use of MCT1 inhibitors for the treatment of certain cancers (30).

Given their effects on aerobic glycolysis, we reasoned that Myc oncoproteins would control lactate transport. Here, we report that Myc directly and selectively activates MCT1 transcription and that elevated MCT1 levels are a hallmark of human malignancies with MYC or MYCN involvement. Notably, we show that blocking MCT1 function rapidly disables glycolysis, leading to reductions in ATP and glutathione (GSH) levels, and that cotreatment with metformin, which forces the glycolytic phenotype, augments the in vivo efficacy of MCT1 inhibitors against MYC-expressing malignancies.

Materials and Methods

RNA analyses

Total RNA was prepared from B220<sup>+</sup> B cells of the spleens or bone marrow of 4- to 6-week-old wild-type (WT) and premalignant E<sup>i</sup>-Myc littermates. Lymphomas were from individual animals. B cells were purified using magnetic-activated cell sorting and beads conjugated with antibodies to B220 (Miltenyi). RNA was isolated from purified B220<sup>+</sup> B cells or tumors using RNA shredder and RNA easy kits (Qiagen) or the NucleoSpin RNA II kit (Macherey-Nagel). For quantitative RNA analyses, RNA was mixed for 2 minutes followed by analyte measurements as most tumor cells express high levels of LDH-A, which inhibits tumor cell growth (25, 26). However, this is the exception as most tumor cells express high levels of LDH-A, which inhibits tumor cell growth (25, 26). However, this is the exception as most tumor cells express high levels of LDH-A, which inhibits tumor cell growth (25, 26).

siRNA knockdown

MCF7 cells (2.5 × 10<sup>5</sup> cells/well) were transfected with 50 to 100 nmol/L siRNA On-TARGETplus SMARTpool siRNA against hSLC16a1 (L-007402-00), c-Myc (L-003282-00-0005), or control siRNA (D-001810-01-05) from Thermo Scientific together with 3 μL Lipofectamine-2000 (Invitrogen 11668-027) into a 6-well dish. Cells were incubated for 24 hours before plating for growth assays, metabolic assays, and protein and RNA isolation. For growth assays, cells were plated at 10,000 cells/well into 24-well dishes and three wells/day were counted.

Metabolic analysis

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse Bioscience XF96 Analyzer. Before each measurement, wells were mixed for 2 minutes followed by analyte measurements every 22 seconds for 5 minutes. Each data point is the change in analyte concentration over 5 minutes and is reported as ECAR (mph/minute) and OCR (pmol oxygen/minute). Agents were injected into wells through preloaded reagent delivery chambers in the sensor probe. Final concentration of agents used was MCT1 inhibitors 10 nmol/L to 1 μmol/L, oligomycin 1 μmol/L (Calbiochem), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) 300 nmol/L (Sigma), and rotenone 100 nmol/L (Sigma). Raji, Daudi, and Ramos Burkitt lymphoma cells (60,000 cells/well) were adhered to poly-D-lysine–coated 96-well plates by centrifuging. MCF7 cells were plated at 15,000 cells/well the day before analysis. Protein concentration in wells was measured to normalize ECAR and OCR data.

Glutathione measurements and glutathione rescue

Total glutathione [GSH: GSH plus oxidized GSH (GSSG)] was measured according the manufacturer’s protocol (Cayman’s Glutathione Assay Kit) from Raji cells treated with vehicle or SR13800 (1 μmol/L) for 8 hours. Raji cells (2 × 10<sup>5</sup> cells/mL), cultured in RPMI with FBS, were pretreated with GSH-reduced ethyl ester (3–5 mmol/L; Sigma) for 1 hour followed by treatment with SR13800 (1 μmol/L) and metformin (1 mmol/L). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured by H<sub>2</sub>DCFDA. Viability was measured by Trypan blue dye exclusion. Proliferation was measured by MTT assay 3 days after treatment.

Results

Myc coordinates the control of lactate homeostasis

To assess whether Myc controls lactate production and export in vivo, we used the E<i>j</i>-Myc transgenic mouse model of human B lymphoma (1), where a protracted premalignant state allows one to identify targets that play roles in Myc-driven tumorigenesis (31, 32). Expression analyses of B220<sup>+</sup> splenic and bone marrow B cells from WT and premalignant E<i>j</i>-Myc littermates established that E<i>j</i>-Myc B cells express elevated levels of several glycolytic enzymes, including Ldh-A, and that this response was augmented in neoplastic E<i>j</i>-Myc B cells (Fig. 1A and Supplementary Fig. S1A and S1B). In accordance with these analyses, marked increases in Ldh-A protein were manifest in E<i>j</i>-Myc versus WT B cells (Fig. 1B), and the preponderance of Ldh-A versus Ldh-B suggested that pyruvate would be converted to lactate (27). Indeed, intracellular
lactate levels were elevated 2- to 3-fold in Eμ-Myc versus WT B cells (Fig. 1C). Thus, Myc activates glycolysis and drives lactate production during lymphomagenesis.

We reasoned that lactate was exported from Eμ-Myc B cells by SLC16A/MCT transporters. Notably, there were marked increases in Mct1 mRNA levels, but not of Mct2, Mct3, and Mct4 transcripts, in Eμ-Myc B cells (Fig. 1D and Supplementary Fig. S1C). Levels of CD147 transcripts, the obligate MCT1/MCT4 chaperone (19), were similar in WT and Eμ-Myc B cells (Fig. 1D). Immunoblot analyses confirmed elevated MCT1 protein levels in premalignant and neoplastic Eμ-Myc B cells (Fig. 1E). MCT4 protein was also detected at low levels in some
Eμ-Myc lymphomas (Supplementary Fig. S1D), presumably due to hypoxic regions in these tumors (20, 25). Cell surface expression of MCT1 and CD147 are codependent (19); thus, fluorescence-activated cell sorting (FACS) analyses of CD147 serves as a surrogate for cell surface MCT1. Notably, there were marked increases in the cell surface levels of CD147 in pre-malignant and neoplastic Eμ-Myc B cells versus WT B cells (Fig. 1F). Thus, there are marked increases in MCT1 expression during Myc-induced lymphomagenesis.

**Elevated MCT1 expression is a hallmark of MYC-driven malignancies**

To determine whether elevated MCT1 expression is manifest in human malignancies having MYC involvement, we first queried expression datasets of Burkitt lymphoma having MYC/immunoglobulin chromosomal translocations and of MYCN-amplified neuroblastoma (33, 34). Notably, there are concordantly high levels of MCT1 and MYC mRNA in Burkitt lymphoma, and of MCT1 and MYCN in MYCN-amplified neuroblastoma (Fig. 1G and H). In contrast, MCT2, MCT3, and MCT4 are not specifically elevated in tumors with MYC/MYCN involvement; indeed, MCT4 transcript levels are low in MYC-expressing tumors (Fig. 1G and H). Furthermore, the high-MCT1/low-MCT4 profile is a hallmark of MYC-expressing colon cancer (Supplementary Fig. S2A; ref. 35; though normal colon was heterogeneous in MCT1 mRNA levels) and elevated MCT1 levels are manifest in the squamous carcinoma subtype of non-small cell lung cancer (Supplementary Fig. S2C; ref. 36). Lung adenocarcinoma and breast adenocarcinoma that express high levels of MYC also expressed high levels of MCT1, yet some of these also elevated levels of MCT4, possibly due to hypoxic regions in these tumors (Supplementary Fig. S2C and S2D). Finally, a high-MYC-high-MCT1 signature connotes poor outcome in basal-like breast cancer and all lung cancers (Supplementary Fig. S2E–S2G).

**MCT1 is a Myc transcription target**

We evaluated the control of MCT1 expression in human P493-6 B-lymphoma cells, which bear a tetracycline-repressible c-Myc transgene (37). The induction of c-Myc mRNA and protein in P493-6 cells deprived of tetracycline was followed by marked increases in MCT1 mRNA and protein (Fig. 2A and B). These findings agree with RNA-seq and expression profiling analyses of P493-6 cells (Supplementary Fig. S3A; ref. 9).

To test whether MCT1 is also induced by pharmacologic cues that control c-Myc, we assessed whether MCT1 expression is cytokine dependent. Primary mouse bone marrow-derived pre-B cells grown in interleukin (IL)-7 medium were deprived of and then restimulated with ligand. Like c-Myc, Mct1 expression was also IL-7 dependent and its induction followed that of c-Myc (Fig. 2C). Similarly, MCT1 expression in immortal 32D.3 myeloid cells was IL-3 dependent and followed the induction of c-Myc (Fig. 2D).

To test whether Myc binds to the endogenous MCT1 gene via identified E-boxes, we performed chromatin immunoprecipitation (ChIP) analyses in P493-6 cells and 32D.3 myeloid cells. There was a marked and specific enrichment for c-Myc binding to human MCT1 E-box2 and E-box1 in P493-6 cells and to mouse Mct1 E-box1 in IL-3–stimulated 32D.3 cells (Fig. 2E and F). In contrast, c-Myc binding to human E-box3 or E-box4, or to mouse E-box4 was not detected (Fig. 2E and F). Similar findings were evident in querying the genome-wide ChIP-seq datasets of Myc and Max target genes in P493-6 cells (9), which showed that MCT1 was transcriptionally silent until bound by Myc (Supplementary Fig. S3B). In addition, Myc-directed control of MCT1 was specific, as c-Myc knockdown in MCF7 breast cancer cells impaired proliferation and reduced MCT1 expression but not levels of MCT2 or MCT4 (Fig. 2G), which are expressed in these cells (Supplementary Fig. S3C). Finally, Myc knockdown selectively reduced the activity of a MCT1 promoter-reporter harboring E-box1 and E-box2 (Fig. 2H). Thus, MCT1 is a direct Myc transcription target in normal and tumor cells.

**MCT1 is necessary for tumor cell proliferation**

Most tumor cells produce and export excess lactate. We thus reasoned that blocking MCT1 function would impair the growth of MCT1-expressing tumor cells. Potent and specific inhibitors that disable MCT1- and MCT2-directed lactate transport (but not that of MCT4) block growth of activated T cells (38, 39). We synthesized two of these pyrole pyrimidine-based molecules [Supplementary Fig. S4A; AR-C122982 (hereafter SR13800) and AR-C155858 (SR13801)] and assessed their effects on Raji Burkitt lymphoma cells that only express MCT1 (Supplementary Fig. S3C). Both inhibitors blocked Raji cell proliferation (Fig. 3A and B), at doses at least 10-fold lower than those needed to impair growth of primary bone marrow-derived B cells (Fig. 3C). Growth inhibition was due to proliferative arrest, which was followed by protracted cell death (Supplementary Fig. S4B and S4C). These inhibitors also blocked MCF7 breast cancer cell growth (Fig. 3D), and that of Raji Burkitt lymphoma and 70Z3 mouse B-cell lymphoma in methylcellulose (Supplementary Fig. S4D).

Manipulating MCT1 expression was not feasible in Raji B lymphoma. Thus, to confirm that these inhibitors were on target, MCT1 expression was silenced in MCF7 cells. Silencing MCT1 blocked the growth of MCF7 cells similar to treatment with the inhibitor (Fig. 3E). We also tested the effects of MCT1 or MCT4 overexpression in MCF7 cells. As expected (19), overexpression of either transporter increased CD147 levels (Fig. 3F), and MCT4, but not MCT1, overexpression was sufficient to confer SR13800 resistance (Fig. 3G). MCT1 overexpression augmented lactate transport (Supplementary Fig. S4E) and shifted the IC50 for SR13800 in inhibiting transport of [13C]lactate (Fig. 3H). However, forced expression of MCT1 (or MCT4) did not affect MCF7 cell proliferation (Supplementary Fig. S4F). Thus, in tumor cells that express MCT1, this transporter is necessary for proliferation but does not augment cell growth.

**MCT1 inhibition derails lactate homeostasis, glycolysis, and glutathione synthesis**

In Raji cells treated with SR13800 or SR13801, there were immediate increases in the levels of intracellular lactate and a block in lactate export (Fig. 4A and B). However, levels of intracellular lactate reached a maximum within approximately
4 hours (Fig. 4A), suggesting effects on metabolism. Indeed, there were rapid and marked reductions in the levels of intracellular ATP (Fig. 4C) and in the ECAR (Fig. 4D and G).

In contrast, there were little effects of SR13800 on basal OCR (Fig. 4F). Rapid decreases in ECAR provoked by SR13800 were manifest in other Burkitt lymphoma lines that express MCT1.
Raji lymphoma cells by measured metabolic intermediates in Raji lymphoma cells by disables tumor cell metabolism. Thus, MCT1 inhibition rapidly none treatment, which inhibits mitochondrial complex I, electron donor that drives oxidative phosphorylation. Rote-
capacity (Fig. 4F) without reducing levels of NADH (Fig. 4E), an revealed that MCT1 inhibition abolishes reserve mitochondrial 4D). Notably, dissipating the proton gradient with FCCP induced ECAR in vehicle- but not SR13800-treated cells (Fig. 4H). Similar decreases were observed in MCF7 cells following MCT1 knockdown (Fig. 4I). Furthermore, oligomycin treatment, which blocks ATP synthesis and activates glycolysis, mass spectrometry. Again, SR13800 treatment led to rapid increases in the levels of intracellular lactate and corresponding decreases in extracellular lactate (Fig. 5A and B). Furthermore, as MCT family members transport pyruvate (18), SR13800 treatment led to marked reductions in extracellular pyruvate (Fig. 5B). Notably, MCT1 inhibition increased the levels of glucose- and fructose-6-phosphate, fructose-bisphosphate, and glycerol-3-phosphate, and markedly reduced products of the ATP-generating arm of glycolysis, including phosphoglycerate and pyruvate (Fig. 5A, B, and F). Furthermore, there were marked reductions in NADPH levels and glucose transport (Fig. 5C and D). These changes were not associated with alterations in the levels of glycolytic enzymes (Supplementary Fig. S5C), and reduced glucose transport was not due to changes in the cell surface levels of glucose transporters (not shown).
Decreases in NADPH following MCT1 inhibition were not associated with changes in ribulose-5-phosphate or malate, two intermediates in NADPH production (Fig. 5E). NADPH is consumed in the conversion of GSSG to reduced GSH by GSH reductase. The first step of GSH synthesis, the production of γ-glutamylcysteine (γ-GC), requires ATP (Supplementary Fig. S5D). Thus, we reasoned that MCT1 inhibition might compromise GSH synthesis. Indeed, by two measures, MCT1 inhibition led to rapid reductions in γ-GC and GSH, a net total GSH (GSH and GSSG; Fig. 5G). Reductions in GSH did not reflect changes in the levels of the catalytic (GCLC) and modulatory (GCLM) subunits of glutamyl cysteine ligase (GCL) that catalyze γ-GC production (Supplementary Fig. S5E). Thus, the reductions in ATP levels triggered by inhibiting lactate transport (Fig. 4C) are associated with reduced levels of GSH.

Metformin augments the potency and antitumor activity of MCT1 inhibitors

Metformin disables oxidative phosphorylation (OXPHOS) by inhibiting mitochondrial complex I and forces a glycolytic phenotype that we reasoned may augment sensitivity to MCT1 inhibitors. Though metformin alone had little effect, cotreatment of Raji lymphoma with SR13800 and metformin led to higher levels of intracellular lactate (Supplementary Fig. S6A), rapid growth arrest, and cell death (Supplementary Fig. S6B). Synergy of SR13800 and metformin was evident in all MCT1-expressing human and mouse tumor cell lines (Supplementary Fig. S6C and S6D), yet had, as expected, no effects on either MCT4-expressing human tumor cells or upon Eμ-Myc lymphomas in transplant experiments (not shown), likely due to hypoxia-mediated induction of Mct4 (Supplementary Fig. S1D). Thus, metformin augments the anticancer potency of...
MCT1/MCT2 inhibitors in tumor cells expressing these transporters.

The efficacy of SR13800 + metformin was tested in vivo using nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice injected intravenously with Raji lymphoma, or injected subcutaneously with MCT1-expressing human T47D estrogen receptor-positive breast cancer cells. Immunohistochemical analyses confirmed that high levels of MCT1, but not MCT4, were expressed in both tumor models (Supplementary Fig. S7A and S7B). In vivo drug metabolism and pharmacokinetic (DMPK) analyses demonstrated that a daily intraperitoneal injection of 30 mg/kg of SR13800 was sufficient to maintain serum levels above the EC50 of the drug (≈5 nmol/L; Fig. 3B), and mice tolerated long-term daily dosing of SR13800 for >130 days without toxic effects (not shown). There were also no significant effects of SR13800 treatment on numbers of white or red blood cells that express high CD147 levels (not shown).

Notably, SR13800 treatment delayed the onset and penetration of disease relative to vehicle-treated Raji lymphoma recipients (Fig. 6A and Supplementary Fig. S7C). In contrast, metformin treatment (at doses not affecting blood glucose) did not affect lymphoma onset (Fig. 6A). Recipients treated with both SR13800 plus metformin had remarkably delayed disease (Fig. 6A); indeed, some recipients never developed tumors, even after being taken off drug for over 80 days (data not shown). In the T47D model, SR13800 and metformin had comparable activity in impairing tumorigenecity (Fig. 6B).

Figure 5. MCT1 inhibition impairs glucose transport and glycolysis, and reduces GSH pools. A–C, Raji cells were treated for 8 hours with SR13800 (100 nmol/L) or vehicle and levels of glycolytic metabolites were determined by mass spectrometry (n = 6, representative of four experiments; A and B) and levels of NADPH (C) were determined by NADP/NADPH Quantification Kit (BioVision; n = 3). D, uptake of 14C-2-deoxyglucose (14C-2-DG), (0.5 μCi for 1 hour in quadruplicate) in Raji cells pretreated with vehicle or SR13800 (100 nmol/L). *** P < 0.0001, representative of three experiments. E, levels of pentose phosphate pathway and TCA cycle products from Raji cells treated for 8 hours with SR13800 (100 nmol/L) or vehicle were assessed by mass spectrometry (n = 6). F, summary of effects of inhibition of lactate transport on glycolytic intermediates. Green boxes indicate accumulations and red boxes reductions of intermediates.

G, left, mass spectrometry of γ-GC and GSH levels from Raji cells treated with SR13800 (100 nmol/L) for 8 hours (n = 6, representative of three experiments). Right, total GSH (GSH plus GSSG) levels in Raji cells assayed by DNTP (n = 3, representative of three experiments).
However, the SR13800/metformin combination was again superior in blocking tumor growth, without affecting the health of transplant recipients (Fig. 6B and Supplementary Fig. S7D–S7F). Thus, MCT1 inhibitors have in vivo anticancer activity and the MCT1 inhibitor/metformin combination is an attractive therapeutic strategy for treating MCT1-expressing malignancies.

Metformin overcomes resistance to MCT1 inhibitors due to shifts to OXPHOS

To assess whether, in addition to MCT4 expression, there were other tumor cell intrinsic mechanisms of resistance to MCT1 inhibitors, Raji cells were cultured with low doses of SR13800 and surviving cells serially passaged into media having increased levels in SR13800, to generate independent pools of SR13800-resistant Raji (RajiR) cells (n = 6; Supplementary Fig. S8A). The growth rates of RajiR cells were comparable with parental Raji cells in normal media yet they continued to proliferate in media containing SR13800, whereas parental Raji lymphoma cells underwent growth arrest (Fig. 6C).

The resistance of RajiR cells was not associated with MCT4 induction or changes in MCT1 levels (Supplementary Fig. S8B). However, RajiR cells had elevated levels of intracellular lactate and low ECAR compared with parental Raji cells growing in normal media (Fig. 6D and E). Notably, RajiR cells had an increase in OCR compared with parental cells (Fig. 6F). Treatment of RajiR cells with metformin alone led to a rapid collapse in metabolism, with a marked reduction in OCR (Fig. 6F). Although metformin treatment led to reductions in OCR in parental Raji lymphoma cells, it also,
as predicted, increased ECAR, thus providing energetic compensation for inhibiting OXPHOS (Fig. 6F). Finally, metformin alone led to the rapid death of Raji R but not parental Raji cells (Fig. 6G), demonstrating RajiR cells are dependent on OXPHOS. Thus, a metabolic shift to OXPHOS is a mechanism of resistance to MCT1 inhibitors and metformin overcomes this resistance.

Reductions of GSH pools trigger hydrogen peroxide-induced cell death

GSH is a major antioxidant in cells that neutralizes free radicals and reactive oxygen species (ROS; Supplementary Fig. S5D). Indeed, the marked reduction in GSH in SR13800-treated Raji cells was followed by increases of $H_2O_2$ but not of mitochondrial or cytosolic superoxide anions (Fig. 7A and data not shown). Notably, SR13800/metformin treatment led to a profound increase in $H_2O_2$ levels that were associated with the death of Raji Burkitt lymphoma cells.

ROS compromise the function of mitochondria. Analyses with MitoTracker Green, which measures mitochondrial mass, indicated that treatment of Raji Burkitt lymphoma cells with SR13800, metformin, or SR13800/metformin did not change mitochondrial mass (Supplementary Fig. S8C). However, analyses with MitoTracker Red, which measures mitochondrial membrane potential, demonstrated that SR13800 treatment, and especially SR13800/metformin, triggered marked reductions in numbers of Raji Burkitt lymphoma cells having functional mitochondria (Fig. 7B).

These findings suggested that reductions in GSH and subsequent increases in $H_2O_2$ triggered by SR13800 or
SR13800/metformin compromise tumor cell survival. Indeed, pretreatment of Raji Burkitt lymphoma cells with the antioxidants N-acetyl cysteine (NAC) or GSH blocked the deleterious effects of SR13800 and of SR13800/metformin on Raji cell growth and survival (Fig. 7C–E). Finally, GSH treatment impaired $H_2O_2$ production in SR13800- and SR13800/metformin-treated cells (Fig. 7F). Thus, metabolic demise triggered by the inhibition of lactate transport leads to a collapse in ATP pools, reductions in GSH, and increases in $H_2O_2$, that contribute to tumor cell death (Supplementary Fig. S8D).

Discussion

Aerobic glycolysis is a hallmark of most tumor types, and our findings have established that Myc is sufficient to drive glycolysis in vivo, where premalignant Eμ-Myc B cells express elevated levels of several glycolytic enzymes that leads to increased lactate levels, in accordance with Ldh-A induction by Myc in vitro (14, 40). Furthermore, this response is amplified in Eμ-Myc lymphoma. These findings parallel the increased dependence on glycolysis and lactate production manifest during the stepwise transformation of primary human fibroblasts (41). Finally, in rapidly expanding tumors, HIF-1α also contributes to glycolysis via the induction of hypoxia (42).

In cancer cells, glucose transport and glycolytic flux is high, and this drives the production of ATP and intermediates necessary for anabolic pathways (43). A consequence of this shift is the production of toxic levels of lactate. Here, we show this is diverted by expression of high MCT1 levels in Myc-expressing malignancies and that MCT1 is necessary for the proliferation of these tumor cells. Furthermore, exporting lactate into the milieu may play roles in promoting tumor progression, as lactate can trigger an inflammatory response (44) that drives tumor progression (45). Finally, lactate also plays other roles in tumorigenesis, where it can be converted into pyruvate that enters the tricarboxylic acid (TCA) cycle (25, 26) and/or stimulate endothelial cell angiogenesis (28, 29).

Our findings establish that Myc controls lactate homeostasis by inducing MCT1 transcription. Pharmacologic inhibition and knockdown of MCT1 demonstrate the essential role of this transporter in maintaining the metabolism, growth, survival, and tumorigenecity of MCT1-expressing tumor cells. These findings are consistent with studies showing the anticancer effects of nonspecific inhibitors of lactate transporters such as $\alpha$-cyanocinnamate (25) and with the block in metabolism and growth of Ras-transformed fibroblasts ex vivo by MCT1 inhibitors (24). However, the mechanism by which inhibiting lactate transport compromises cancer cell growth has been unclear and we now show is due to a collapse in glycolysis and accompanying reductions in ATP and GSH (Supplementary Fig. S8D).

Changes in metabolic intermediates suggest how inhibiting lactate export disables glycolysis. In particular, our findings are remarkably akin to the changes in glycolytic intermediates observed in the exercised muscle of LDH-A-deficient patients, where reduced levels of NAD$^+$ compromise glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and favor the oxidation of NADH and the production of glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase (GPDH; ref. 46). In MCT1 inhibitor-treated cells, intracellular lactate levels and NADH levels rise, suggesting that the conversion of pyruvate to lactate and the coupled oxidation of NADH to NAD$^+$ are impeded by high intracellular lactate levels. High NADH levels favor GPDH conversion of DHAP to glycerol-3-phosphate, which diverts triose-phosphates from GAPDH and the ATP-generating arm of glycolysis (Supplementary Fig. S8D). As a consequence, this leads to reductions in phosphoglycerate and pyruvate. This chain of events, coupled with feedback inhibition by lactate on PFK1 (47), explains increased levels of more upstream intermediates such as glucose-6-phosphate (G-6-P) and the effects upon glucose transport. Specifically, G-6-P feedback inhibits hexokinase, which results in increased intracellular free glucose that then inhibits glucose transport. Collectively these events converge to markedly impair ATP levels and the synthesis of the major antioxidant of the cell, GSH, which is a key target, as GSH is sufficient to override the acute effects of MCT1 inhibitors on tumor cell survival.

Importantly, these findings suggest that any agent targeting glycolysis will compromise ATP production and GSH levels, resulting in $H_2O_2$-induced death. In accordance with this notion, LDH-A inhibition triggers oxidative stress and blocks tumor cell proliferation (48) and the antioxidants NAC and GSH block the antitumor activity of MCT1 inhibitors. Our findings also suggest that pro-oxidant therapies will augment the potency of lactate transport inhibitors and that other means of disrupting GSH synthesis, for example, by inhibiting cysteine or cystine transporters (49), will be effective therapeautic strategies for tumors with MYC involvement.

Elevated MCT1 expression is a hallmark of human malignancies with MYC involvement. Notably, many MYC-expressing tumors express reduced MCT4 levels, suggesting MCT1 inhibitors will have a therapeutic benefit in such tumors. However, it is clear that tumor cells that express MCT4 will be refractory to MCT1 inhibitors, and that patient tumors should be assessed for MCT1 and MCT4 expression before using MCT1 inhibitors for treatment (25). A second mechanism of resistance identified herein is a shift of tumor cells to OXPHOS, and we show that agents such as metformin that disrupt OXPHOS disable this resistance, leading to a collapse in tumor cell metabolism and rapid death. Notably metformin use in type-II diabetes seems to decrease cancer risk and may provide benefit as a cancer therapeutic (50). Together, these findings suggest the use of MCT1 inhibitors in combination with metformin, or with metformin derivatives, in therapies for MCT1- and MYC-expressing malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.R. Doherty, C. Yang, K.E.N. Scott, Y. Lu, C.V. Dang, T.D. Bannister, W.R. Roush, J.L. Cleveland


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Doherty, C. Yang, K.E.N. Scott, M.D. Cameron
Blocking Lactate Export Disables Cancer Cell Glycolysis


Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): J.R. Doherty, C. Yang, K.E.N. Scott, M.D. Cameron, M. Fallahi, W. Li, M.A. Hall, A.L. Amelio, F. Li, H.M. Genau, Y. Lu, A.A. Butler, A.T. Hooper, J.L. Cleveland

Writing, review, and/or revision of the manuscript: J.R. Doherty, C. Yang, K.E.N. Scott, C.V. Dang, T.D. Bannister, A.T. Hooper, W.R. Roush, J.L. Cleveland

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.R. Doherty, C. Yang, W. Li, J.K. Mishra, W.R. Roush

Study supervision: J.R. Doherty, C. Yang, K. Ussal-Kacmaz, W.R. Roush, J.L. Cleveland

Acknowledgments

The authors thank Nancy Philp (Thomas Jefferson University, Philadelphia, PA) for MCT antibodies, the FACS, Genomics, and ARC Cores of Scripps Florida, members of the Cleveland laboratory and Anke Klippel-Giese (Pfizer Oncology) for discussions, Marika Kernick for editing, Justin Lucas (Pfizer Oncology) for assistance, and Dr. George Hu (Pfizer Oncology) for pathologic analyses.

Grant Support

This work was supported by NIH grants CA076797 and CA169412 (J.L. Cleveland), CA057341 (C.V. Dang), GM026782, GM038436, CA169412, and U54MH074404 (W.R. Roush), F32 CA134121 (A.L. Amelio), the Jane & Leonard Korman Family Foundation Postdoctoral Fellowship (K.E.N. Scott), and by monies from the State of Florida to Scripps Florida.

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 July 22, 2013; revised October 28, 2013; accepted October 28, 2013; published OnlineFirst November 27, 2013.
Blocking Lactate Export by Inhibiting the Myc Target MCT1 Disables Glycolysis and Glutathione Synthesis

Joanne R. Doherty, Chunying Yang, Kristen E.N. Scott, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-2034

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/11/27/0008-5472.CAN-13-2034.DC1

Cited articles
This article cites 48 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/3/908.full#ref-list-1

Citing articles
This article has been cited by 26 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/74/3/908.full#related-urls

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://cancerres.aacrjournals.org/content/74/3/908.
Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.