



Glucose-dependent growth arrest of leukemia cells by MCT1 inhibition: Feeding Warburg's sweet tooth and blocking acid export as an anticancer strategy



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ABSTRACT

This study aims to investigate the utilization of The Warburg Effect, cancer's "sweet tooth" and natural greed for glucose to enhance the effect of monocarboxylate transporter inhibition on cellular acidification. By simulating hyperglycemia with high glucose we may increase the effectiveness of inhibition of lactate and proton export on the dysregulation of cell pH homeostasis causing cell death or disruption of growth in cancer cells. MCT1 and MCT4 expression was determined in MCF7 and K562 cell lines using RT-PCR. Cell viability, growth, intracellular pH and cell cycle analysis was measured in the cell lines grown in 5 mM and 25 mM glucose containing media in the presence and absence of the MCT1 inhibitor AR-C155858 (1 μ M) and the NHE1 inhibitor cariporide (10 μ M). The MCT1 inhibitor, AR-C155858 had minimal effect on the viability, growth and intracellular pH of MCT4 expressing MCF7 cells. AR-C155858 had no effect on the viability of the MCT1 expressing K562 cells, but decreased intracellular pH and cell proliferation, by a glucose-dependent mechanism. Inhibition of NHE1 on its own had a no effect on cell growth, but together with AR-C155858 showed an additive effect on inhibition of cell growth. In cancer cells that only express MCT1, increased glucose concentrations in the presence of an MCT1 inhibitor decreased intracellular pH and reduced cell growth by G1 phase cell-cycle arrest. Thus we propose a transient hyperglycemic-clamp in combination with proton export inhibitors be evaluated as an adjunct to cancer treatment in clinical studies.

1. Introduction

The German physiologist, Otto Warburg observed that cancer cells utilize a higher amount of glucose compared to noncancerous cells [1]. Not only do cancer cells consume more glucose, but the metabolic pathway they employ to completely metabolize glucose also differs from the pathway used by normal cells. Cancerous tissue and cells rely on pyruvate reduction to lactate for energy production even in the presence of oxygen [2]. This phenomenon was first described by Otto Warburg, and is referred to as "the Warburg effect" [3]. To avoid a decrease in intracellular pH due to increased metabolic activity, lactate molecules and protons are quickly and efficiently exported out of the cell [4,5]. This function is performed by proton-linked monocarboxylate transporters, although there are multiple proton export pathways in the plasma membrane including the sodium-hydrogen exchanger (NHE), sodium bicarbonate transporters (NBC), chloride bicarbonate exchangers (AE) and others (Fig. 1).

The monocarboxylate transporter (MCT) family includes 14

members, from which only a few have been described as proton-linked short-chain monocarboxylic acid transporters [6,7]. They are MCT1-MCT4, and the concentration gradient of protons and monocarboxylates such as pyruvate, lactate, and ketone bodies dictates the direction in which that substrate will be transported [8]. Due to the importance of MCTs in monocarboxylate and proton transport and their high expression levels in cancer cells [9], MCTs are now being considered in cancer prognosis [10]. The levels of MCT1 and MCT4 have been noticeably upregulated in breast, cervical, colorectal, and gastric cancers, along with some glioblastomas [11]. Consequently these two representatives of the MCT family have become important novel targets for cancer therapy [12–15].

AR-C155858 is a potent MCT1 inhibitor and was first created by AstraZeneca to function as an immunosuppressant to block the proliferation of T-lymphocytes [16], but eventually the drug found its application in cancer treatment [17]. AR-C155858 binds to and inhibits MCT1, but has no effect on MCT4 function up to a concentration as high as 10 μ M [16,17]. The viability of cells expressing MCT4 should not be

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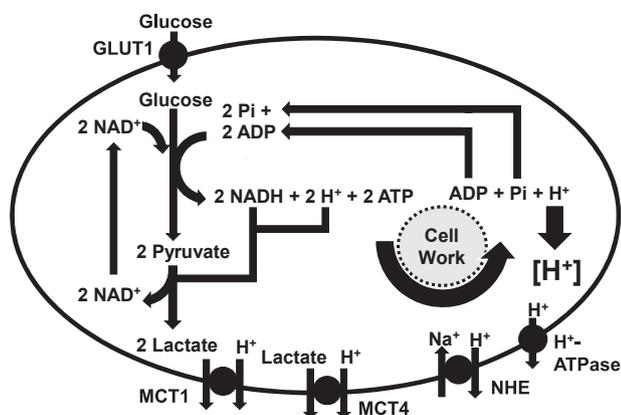


Fig. 1. Schematic diagram of Warburg metabolism and proton export pathways in a cancer cell. Cancer cells rely heavily on glycolysis for energy production, even in the presence of oxygen and produce lactate as a byproduct, this was first discovered by Otto Warburg and is known as The Warburg Effect. In the case of Warburg metabolism, metabolic acidosis results when the rate of ATP hydrolysis from cell work, exceeds the rate at which ATP is produced via glycolysis. Hence, the biochemical mechanism of proton accumulation is not lactate production, but ATP hydrolysis. As the rate of ATP hydrolysis exceeds all other reactions, the rate of proton release eventually exceeds metabolic proton buffering and protons will accumulate inside the cell and cytosolic acidification results. Tumor cells prevent intracellular acidification by having multiple proton export pathways including the Na⁺/H⁺ exchanger 1 (NHE1), the vacuolar H⁺-ATPase, and the proton coupled monocarboxylate transporters (MCT1 and MCT4). We will focus on the monocarboxylate transporters MCT1 and MCT4 due to their high expression and upregulation in many cancers, and potential as an anti-cancer pharmaceutical target. The concept for this figure was unashamedly taken from Robergs et al. 2004 [38].

affected by the MCT1 inhibitor AR-C155858, due to the presence of the additional lactate and proton coupled transporter, MCT4. However cells that only express MCT1 (such as K562 cells) would fail to export protons in the presence of AR-C155858 leading to an intracellular acidification.

The current theories on targeting the Warburg Effect of cancer usually refer to exploiting their reliance on glycolysis [18] and targeting one of the several enzymes involved glucose metabolism. Our theory is the exact opposite, we are proposing to let cancer's sweet tooth and greed for sugar become its Achilles' heel. By increasing the amount of available glucose, we are promoting its uptake and utilization by cancer cells (due to reliance on the Warburg effect), causing an increase in their metabolic rate and a rise in generated lactate and protons. These protons need to be extruded from the cell to maintain a physiological intracellular pH. We propose to supplement glucose to hyperglycemic levels followed by inhibition of lactate and proton transport with the MCT1 inhibitor AR-C155858. We hypothesize these cells will show an increased intracellular acidification with subsequent cell distress and deleterious effects such as inhibition of growth or death.

2. Materials and methods

2.1. Cell culture

The MCF7 (GFP) human breast cancer cell lines was obtained from Cell Biolabs, INC (San Diego, CA). The non-fluorescent MCF7 and K562 cells, the human myelogenous leukemia cell line were obtained from American Type Culture Collection, Manassas, VA, and were a kind gift of Dr. Eric Mendenhall (University of Alabama in Huntsville). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing no phenol red, no glucose and no pyruvate. DMEM was supplemented with 10% fetal calf serum, 100 Units/ml of penicillin and 100 µg/mL of streptomycin solution, glutamine (2 mM), non-essential amino acids (1x) and the appropriate amount of glucose to give either 5 mM or 25 mM final glucose concentrations. All cell culture reagents

were purchased from (Life Technologies™, Carlsbad, CA). Cells were passaged every 3–5 days by trypsinization and grown at 37 °C in a humidified 5% CO₂ incubator. K562 cells were cultured in a similar manner, except they were a suspension culture and did not need to be trypsinized. Cells were passaged with a 1:3 to 1:5 split every 3–5 days. For experiments, cells were used directly from the T-25 flasks or plated on 24 well plates. The MCT1 inhibitor AR-C155858 and the NHE1 inhibitor cariporide (obtained from Tocris Biosciences, Minneapolis, MN) were dissolved as a stock solutions of either 1 mM or 10 mM in DMSO (ATCC, Manassas, VA) and stored at –20 °C. The AR-C155858 and cariporide were made up at a working concentrations of 1 µM and 10 µM respectively which would expose the cells to a final DMSO concentration of 0.1 or 0.2% DMSO.

2.2. RNA isolation and cDNA generation and RT-PCR analysis

An appropriate number of cells, usually 5×10^6 – 1×10^7 cells were taken from suspension culture or trypsinized off a T-25 flask, pelleted by centrifugation at $1000 \times g$ for 5 min and the supernatant discarded. Essentially the RNA isolation protocol was similar to the Qiagen RNeasy® kit instructions (catalog No. 74104). To make cDNA, about 1 µg of total cellular RNA was thawed on ice, and cDNA according to the QuantiTect Reverse Transcription Kit protocol (Qiagen Catalogue No. 205311). The cDNA was then quantified using the Nanodrop spectrophotometer and frozen at –20 °C until use for real-time PCR analysis. Real-time PCR was performed using a 7500 Fast Real-time PCR system (Life technologies™, Carlsbad, CA). RT-PCR was set up in a 96-well plate in a reaction volume of 25 µL per well 2x Fast SYBR® Green DNA polymerase (Catalog number 4385617). All MCT oligos were purchased from Qiagen Inc. (Valencia, CA). SDHA oligos were synthesized by Eurofins MWG Operon LLC (Huntsville, AL), SDHA_F: TCTGCACTCTG GGAAGAAG and SDHA_R: CAAGAATGAAGCAAGGGACA. The PCR efficiency was determined for the target genes MCT1, MCT4 and the reference gene SDHA by PCR analysis of serial dilutions of cDNA. The threshold crossing point values (C_T) were linearly correlated with the logarithmic value of the DNA amount. The slope of this line provided the PCR efficiency number for the gene under the given parameters (primers used and PCR-protocol).

To determine the fold change of MCT1 and MCT4 expression over the reference gene SDHA we used the quantification method and guidelines of Pfaffl 2001 [19]. The efficiencies of all three genes examined in this study were close to the ideal efficiency of two, and we simplified our analysis and assigned E_{target} a value of two. The threshold crossing RT-PCR cycle (C_t) for reference and target genes were obtained from the RT-PCR cyclers software. The reference gene SDHA showed constant expression over different glucose concentrations, therefore no normalization was needed and a simplified equation could be used, Fold change over SDHA = $(E_{target})^{\Delta C_t(\text{ref} - \text{target})}$.

2.3. K562 and MCF7 (GFP) cell counting, viability assay and cell cycle assay

MCF7 (GFP) and K562 cells were cultured in media containing different concentrations of glucose with or without MCT1 inhibitors. Cells were then isolated for counting, viability and cell cycle assays using a Tali® cell counter (Life technologies™, Carlsbad, CA). Cell cycle progression was measured by quantification of cellular DNA content. As cells progress through the cell cycle, the amount of DNA doubles. This doubling can be tracked and used to determine the cell cycle phase (G1, S, and G2/M). This procedure was performed using manufacturer instructions (Tali® Cell Cycle Kit - Catalog no. A10798). The cell number/fluorescence data from the Tali® was analyzed with FCS Version 5 (De Novo, Glendale, CA) and Multicycle AV (Phoenix Flow Systems, Inc., San Diego, CA) using the mathematical fitting models of Kallioniemi et al. [20]. The proportion of cells in the G1, S and G2 phases was determined and plotted.

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