A Novel Assay for Profiling Metabolic Changes Mediated by HIF-1α in Cancer Cells

Min Wu, Amy Swift, Diane Parslow, Suzanne Armitstead, Kristie Lemire, David Ferrick

Seahorse Bioscience
16 Esquire Road
North Billerica, MA 01862

Abstract

Aerobic glycolysis is a near universal property of tumor cells, as Warburg observed more than 70 years ago.[1,2] This is also the basis of the widely used clinical imaging tool FDG-PET (2-[^{18}F] Fluoro-2-deoxyglucose positron emission) which is used for cancer diagnosis and for monitoring therapeutic responses.[3] Seahorse Bioscience has developed a novel microplate based instrument for rapidly measuring cellular metabolic effects from genetic or chemical perturbations. The Seahorse instrument measures the extracellular flux (XF) of molecular oxygen and protons within each well. This platform provides for the first time direct demonstration of the Warburg effect and its reversal by simultaneously measuring glycolysis and oxidative phosphorylation in response to agents that modulate oncogenic signaling pathways in cancer cells.

To gain a better understanding of the link between metabolism and cancer as well as test the utility of this technology in drug discovery, we performed two types of experiments. One type of experiment measured oxygen consumption rate (OCR), which primarily reflects mitochondrial respiration, and extracellular acidification rate (ECAR), which primarily reflects lactic acid production (glycolysis). We profiled the metabolic sensitivities and degree of inhibition/stimulation of a number of cancer cell lines to modulators of anaerobic and aerobic energy metabolism (phloretin, 3-bromopyruvic acid, iodoacetate, flouride and oxamate). Analysis of the sensitivities of the cell lines to these modulators provides insights into their bioenergetic states and their global physiological responses to the modulators. Another type of experiment was performed to investigate changes in cancer cellular energetics triggered by cancer drugs or agents that modulate oncogenic signaling pathways. We determined the effects on aerobic (OCR) and anaerobic (ECAR) metabolism of two compounds that either increase (dimethyloxalylglycine) or decrease (topotecan, a cancer drug) HIF-1α expression. We found that exposure of the non-metastatic prostate LNCaP cells to dimethyloxalylglycine for 24 hours reproduced the Warburg effect, that is, increased rate of glycolysis and concomitant attenuation of mitochondrial respiration. Conversely, treatment of C4-2 cells, a metastatic variant derived from parental LNCaP, with topotecan for 24 hours reduced the glycolysis rate and concomitantly elevated the mitochondrial oxygen consumption rate reversing the Warburg effect. Thus the Seahorse XF technology provides a powerful new tool for discovering and qualifying novel compounds that target metabolic pathways in cancer cells.

Introduction

Dysregulation of energy homeostasis is a fundamental process that is associated with cellular transformation.[4,5] Hypoxia-inducible factor 1 (HIF-1) is a master regulator of oxygen homeostasis by mediating a wide range of cellular adaptive physiological responses to reduced oxygen availability, including switching to glycolytic energy production.[6,7] In addition, growth factors such as EGF and oncogenes such *src*, *ras* and *myc* have been shown to increase HIF-1α expression and subsequently its adaptive physiological responses independent of hypoxia.[8-10] Both the hypoxic and genetic activation of HIF-1 involve the phosphatidylinositol- 3-kinase/Akt signaling pathways.[9,10] Many cancer drugs including topotecan perturb these signaling pathways.[3,11] Seahorse has developed a technology that enables rapid profiling of bioenergetic pathways in cancer cells as they respond to anti-cancer drugs. This allows one to identify if the tumor has become dependant on a particular metabolic pathway for their growth and malignancy. Using Seahorse XF technology. we have shown that imatinib reduced the rate of glycolysis in human BCR-ABL-positive leukemia cells,[12] consistent with previously reported effects of imatinib on glucose metabolism as assessed by MRS and GC-MS.[13,14] In this poster we demonstrate that increased expression of HIF-1α, achieved by adding the stabilizer, dimethyloxalylglycine (DMOG), stimulated glycolysis and suppressed mitochondrial respiration. Conversely, treatment with the HIF-1a inhibitor, topotecan, had the anticipated opposite effect; it inhibited glycolysis and promoted mitochondrial respiration.

Materials and Methods

Cell Culture and Chemical Reagents

Non-small cell lung carcinoma cell lines H460 and A549 were obtained from the DCTD Tumor/Cell Line Repository at the National Cancer Institute. Prostate cell line LNCaP was obtained from ATCC (Manassas, VA). C4-2 cell line (derived from parental LNCaP cell line) was obtained from ViroMed laboratories (Minneapolis, MN). LNCaP cells were maintained in Modified RPMI 1640 (ATCC) supplemented with 10% FBS (Hyclone Logan, UT) and 100 µg/ml penicillin-Streptomycin (Invitrogen). C4-2 cells were maintained in T medium (Invitrogen). For experiments involving dimethyloxalylglycine and topotecan, cells were exposed to these compounds for 24 hours prior to XF assay. The number of viable cells was determined by using a ViCell automated trypan blue counter (Beckman-Coulter, Fullerton, CA). Sodium oxamate, sodium iodoacetate (IAA), sodium fluoride, 2-deoxyglucose, phloretin, 2,4-dinitrophenol and topotecan were obtained from Sigma (St. Louis, MO). Dimethyloxyloglycine was purchased from Frontier Scientific (Logan, UT). Calcein AM was obtained from Invitrogen. All compounds were prepared according to the manufacturers' instructions.

ATP assay

Cells were seeded in white 96-well tissue culture microplates at indicated cell density per well 24 hours prior to compound treatment. Cell Titer-Glo luminescent ATP assays (Promega, Madison, WI) were performed at the indicated treatment time using a FLUOstar Optima plate reader (BMG Labtech, Durham, NC).

Calcein AM stain

Cell were seeded in black 96-well tissue culture microplates at the indicated cell density per well. Calcein AM (2 µM) staining was performed in cells treated with the indicated compounds and at the indicated times.

XF Metabolic Assay

Measurements were made with a prototype Seahorse XF instrument. Adherent cells were seeded in 24 well Seahorse cell culture microplates at various cell densities per chamber per well. Approximately 45 minutes prior to the assay, the culture medium was exchanged with a low-buffered RPMI assay medium (Molecular Devices, Sunnyvale, CA) to ensure accurate ECAR readings. For detection of acute drug responses, OCR and ECAR were measured for five minutes in each well to establish a baseline. Compound solution was then added and followed by measurement of OCR and ECAR. For time resolved measurements, test measurements were made at time points as indicated.

Slowly adhering LNCaP and C4-2 cells were seeded onto poly-D-lysine (PDL)-coated Seahorse cell culture microplates to facilitate complete and rapid adhesion. PDL treatment of plates was performed according to manufacturer's instruction. At the end of each assay, cell number in each well was determined after the cells were incubated with Trypsin.

Seahorse XF Instrument Overview

The Seahorse instrument measures the rate of change of analytes (currently oxygen and pH) in the media immediately surrounding living cells cultured in a microplate. Changes in the extracellular media are caused by the consumption or production of analytes by the cells. Therefore, a sensitive measurement of the media can be used to determine rates of cellular metabolism with great precision and in a totally non-invasive, label-free manner.

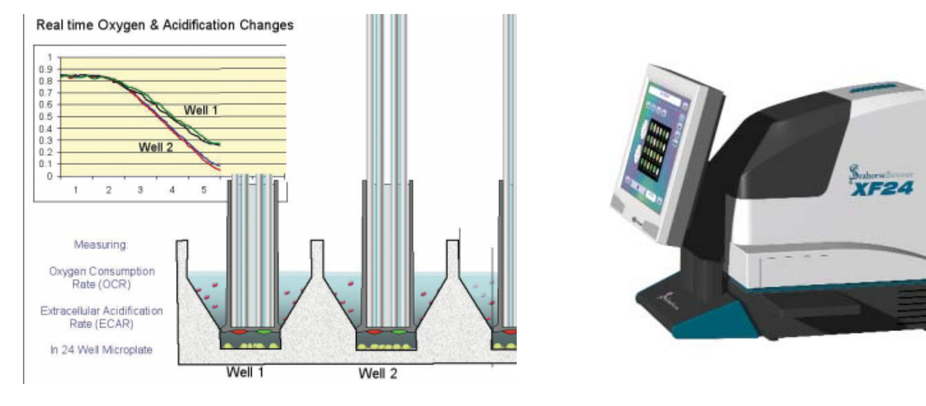
A unique feature of the XF technology is its ability to make accurate and repeatable measurements in as little as five minutes. This is accomplished by isolating an extremely small volume (less than 10 µL) of media above the cell monolayer. Cellular metabolism causes rapid, easily measured changes to the "microenvironment" in this small volume.

Typically, a measurement cycle is performed for 2-10 minutes. During this time, analyte levels are measured every 8 seconds until oxygen concentration drops approximately 10% and media pH declines approximately 0.1 unit. The measurement is performed using fluorescent biosensors embedded in a sterile disposable cartridge that is placed above a 24 well tissue culture microplate.

Baseline metabolic rates are typically measured twice, and are reported in nmol/min for OCR and mpH/min for ECAR. Drug is then added to the media and mixed for 5 minutes, and then the post-treatment OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR changes.

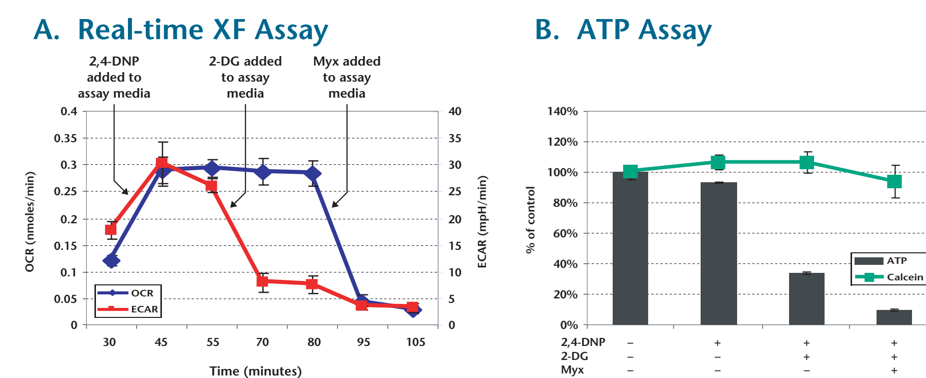
Because XF measurements are non-destructive, cells can be profiled over a period of minutes, hours or days.

Figure 1. XF Technology Measurement Method and Instrument



Results

Figure 2. Real-time Measurement of Changes in Bioenergetic Pathways in LNCaP cells in Response to Metabolic Modulators.



A. Mitochondria uncoupler 2,4-DNP (20 µM), glycolysis inhibitor 2-deoxyglucose (100 mM) and mitochondria complex III inhibitor, myxothiazol (0.1 µM) were injected sequentially into wells containing LNCaP cells. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured before any compound injection and after each compound injection. Mitochondria uncoupler, 2,4-dinitrophenol (2,4- DNP), stimulated the oxygen-consumption and extracellular acidification rates within ten minutes after its administration. The elevated ECAR was brought down by hexokinase inhibitor, 2-deoxyglucose (2-DG), that was injected 25 minutes after 2,4-DNP administration while OCR remained high. The final injection of myxothiazol at 25 minutes after 2-DG administration completely diminished cellular oxygen consumption rate.

B. Cellular ATP level (bars) in response to 2,4-DNP (20 µM), 2-DG (100 mM) and myxothiazol (0.1 µM) treatment (30 minutes) individually or in combinations. Cells remain viable under all conditions as shown by Calcein AM stain (green line). 15,000 cells per well were seeded 24 hours prior to the assay.

Figure 3. Purtubational Profiling of Bioenergetics of H460 and A549 Cells using Glycolysis Inhibitors

Percent change in OCR and ECAR relative to basal rate, in response to compound treatments. The various compounds modulate specific steps of the metabolic pathway as shown. Note: The GLUT1 inhibitor phloretin also inhibited mitochondrial respiration resulting in decreased OCR and ECAR.

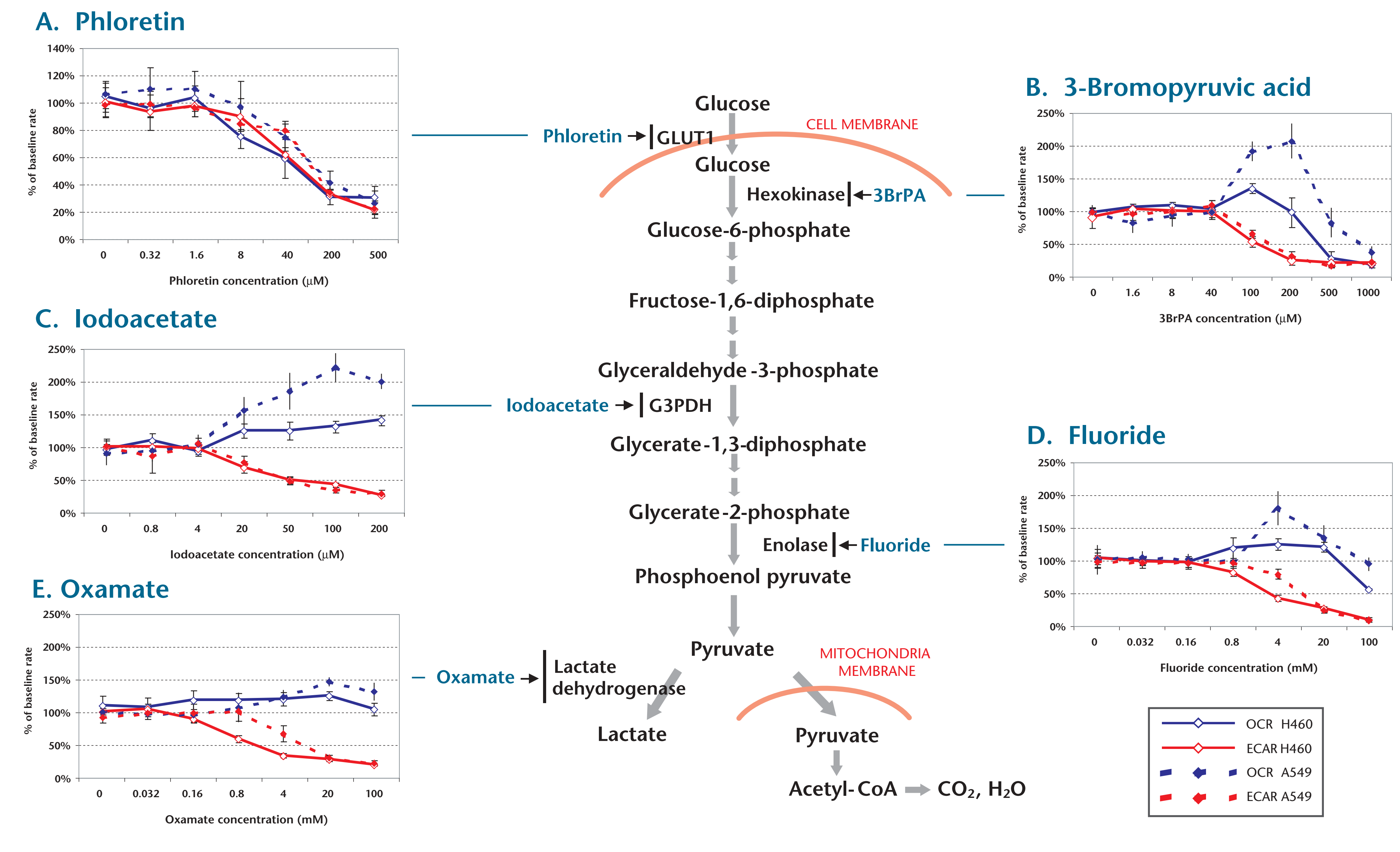
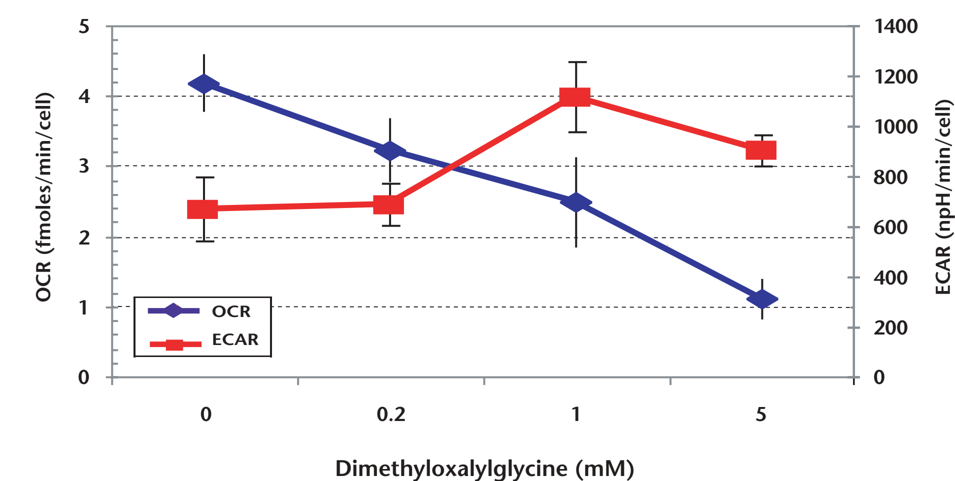
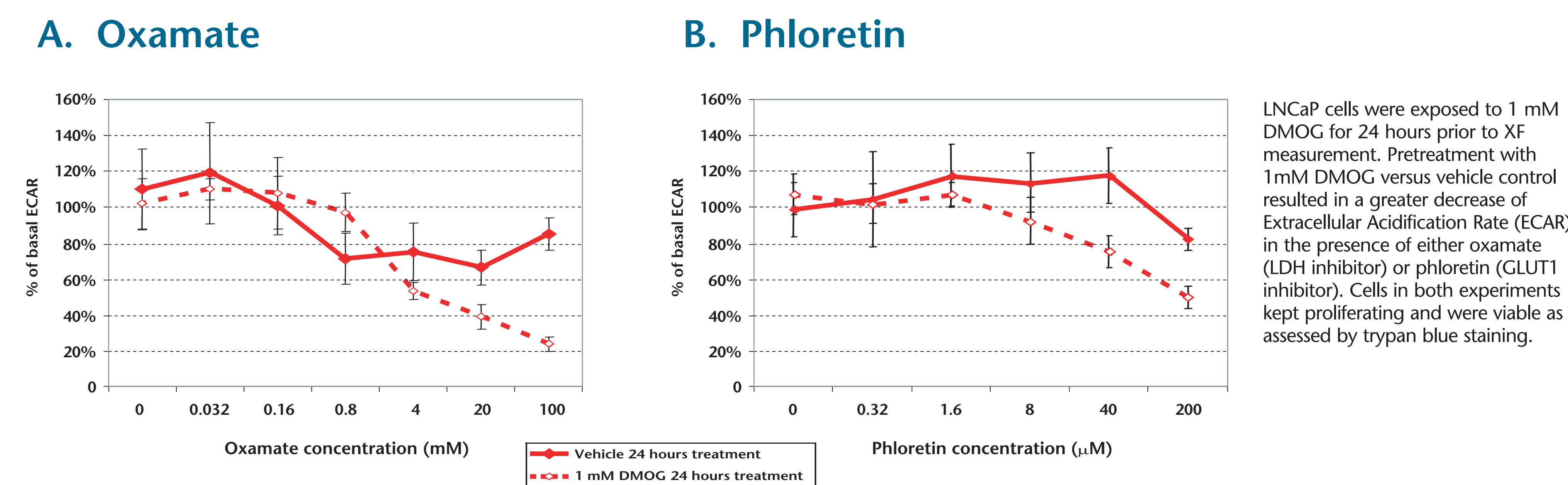


Figure 4. HIF-1α Mediated Recapitulation of the Warburg Effect in LNCaP Cells.



LNCaP cells were exposed to increasing concentrations of dimethyloxalylglycine (DMOG) for 24 hours. DMOG, a prolyl hydroxylase inhibitor (which increases the half life of HIF-1α by preventing VHL-dependent HIF-1α destabilization) stimulated the cellular rate of glycolysis and simultaneously suppressed the cellular rate of mitochondrial respiration. This suggests that HIF-1α down regulated expression of mitochondrial respiration genes in addition to its reported activity of upregulating glycolytic enzyme expression. OCR and ECAR were normalized to rate per cell.

Figure 5. Altered Bioenergetic Profile in LNCaP Cells Treated with Dimethyloxalylglycine.



LNCaP cells were exposed to 1 mM DMOG for 24 hours prior to XF measurement. Pretreatment with 1mM DMOG versus vehicle control resulted in a greater decrease of Extracellular Acidification Rate (ECAR) in the presence of either oxamate (LDH inhibitor) or phloretin (GLUT1 inhibitor). Cells in both experiments kept proliferating and were viable as assessed by trypan blue staining.

Figure 6. Topotecan Reversed the Warburg Effect in C4-2 Cells

C4-2 cells derived from the LNCaP cell line were exposed to increasing doses of topotecan for 24 hours before XF measurement. Topotecan reduced the cellular rate of glycolysis and concomitantly stimulated the cellular rate of mitochondrial respiration. Cells slowed proliferation but remained viable as assessed by trypan blue staining.

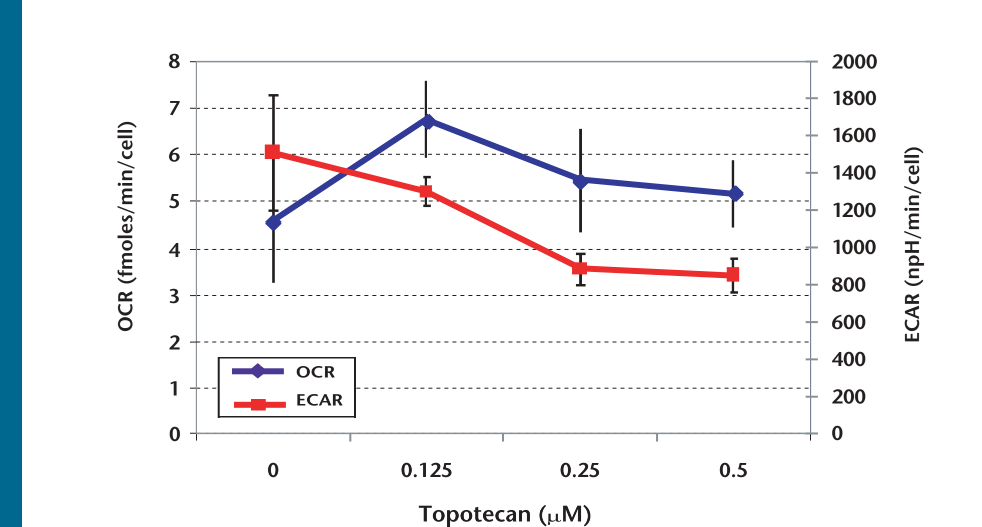
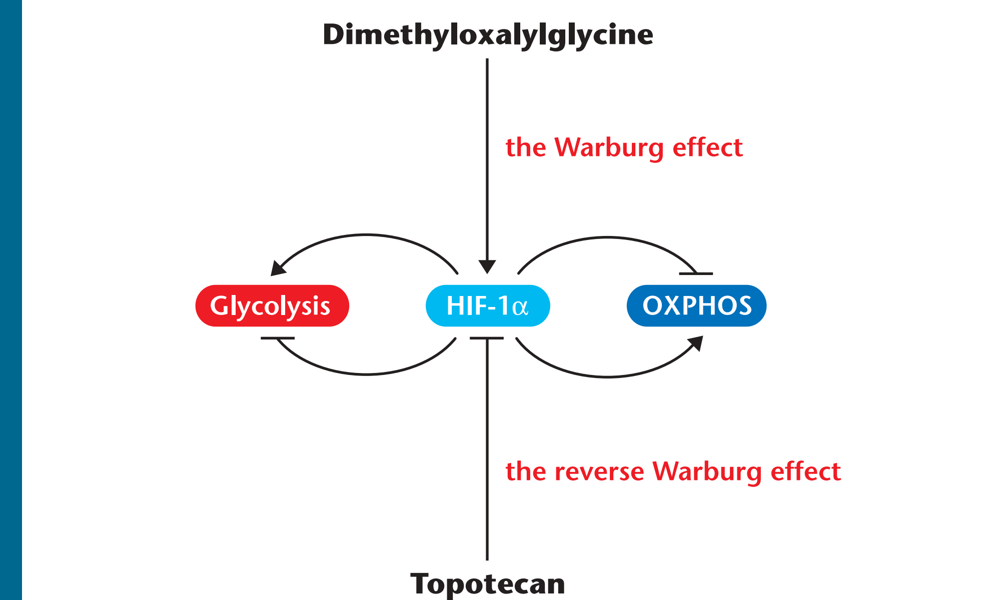


Figure 7. Schematic Illustration of HIF-1α Mediated Warburg Effect and its Reversal.



Summary

1. Cancer cells exhibit an altered bioenergetic dependency which can be exploited for discovering novel anti-cancer drugs.
2. We have validated the novel Seahorse technology approach by rapidly measuring the expected metabolic responses to compounds that are known to modulate these pathways essential for cancer cell growth and survival.
3. Using XF technology, we have not only demonstrated increased glycolysis due to HIF-1α stabilization, as reported in the literature, but also found for the first time that HIF-1α stabilization simultaneously repressed mitochondrial respiration in the prostate cancer cell line LNCaP.
4. We conclude that metabolic profiling using the Seahorse platform combined with conventional screening approaches will be useful in discovering and validating novel anticancer drugs.

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