

A Non-destructive, Time-resolved, Multi-analyte Technology for Sensing Metabolic Changes in Cancer Cells

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Abstract

Cancer cells exhibit a unique dependence on glycolysis as a possible means to enhance their survival in a hypoxic tumor environment. We have developed a novel microplate-based instrument for profiling the metabolic changes in adherent cells in response to chemical or biological agents. Measurement of extracellular flux (XF) rates, including cellular oxygen consumption rate and extracellular acidification rate demonstrates a correlation between the metastatic potentials of cancer cell lines, and also shows the unique responses of certain cell lines to the combination of a glycolysis inhibitor and an anti-cancer drug.

A near-universal property of primary and metastatic cancers is the increased requirement for glucose and glycolysis, as Warburg observed more than 70 years ago.^{1,2} Tumors that produce large amounts of lactate have been shown to correlate with a poor clinical outcome.^{3,4} It was recently reported that Novartis' Imatinib reverses the "Warburg effect" in BCR-ABL positive cancer cells by switching their metabolism from glycolysis to mitochondrial respiration.^{5,6}

The Seahorse Bioscience XF24 instrument profiles the metabolic rates of cells in microplates by measuring the extracellular flux (XF) of analytes. Oxygen consumption rate (OCR) primarily quantifies mitochondrial respiration, while extracellular acidification rate (ECAR) which is caused by proton release, reflects primarily lactate excretion.

Using the Seahorse XF assay technology, we have observed a correlation between the metabolic profile and metastatic potential of non-small-cell lung carcinoma (NSCLC) cancer cell lines. Within minutes of exposure to the following glycolysis inhibitors: oxamate, 3-bromopyruvic acid (3BrPA), iodoacetate (IAA) and fluoride, both cell lines showed a dose-dependent decrease in ECAR and a concomitant increase in OCR, suggesting a pathway switch for respiration from glycolysis to mitochondrial oxidation. Lactate dehydrogenase (LDH) inhibitor, oxamate, yielded much lower IC₅₀ values in H460 than in A549 cells, suggesting the presence of higher affinity glycolytic enzymes in H460 cells. Interestingly, the IC₅₀ values of hexokinase inhibitor 3BrPA, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) inhibitor, iodoacetate, are very similar for both cell lines. Addition of phloretin, the glucose transporter GLUT1 inhibitor, reduced the ECAR of H460 cells to a greater extent than that of A549 cells implying an increased expression of GLUT1 in H460 cells.

Thus, it appears that a set of high affinity glycolytic enzymes and glucose transporters are expressed in the highly metastatic and glycolytic NSCLC H460 cells, perhaps conferring on them a growth and metastatic advantage within a low glucose tumor environment *in vivo*.

Glycolysis inhibitors have also been reported to sensitize cancer cells to chemotherapeutic drugs such as doxorubicin.⁷ Seahorse XF assays confirmed that the glycolysis inhibitor, 2-deoxyglucose (2-DG), sensitized H460 cells to doxorubicin mediated cytotoxicity.

Seahorse XF assays are a powerful new tool for investigating compounds targeting the metabolic pathways in cancer cells. The label free, non-destructive, time resolved hallmark of these assays will allow new insight into drug action mechanisms and will facilitate validating novel cancer targets and screening for novel anti-cancer compounds.

Materials and Methods

Cell Culture and Chemical Reagents

H460 and A549 cell lines were obtained from the DCTD Tumor/Cell Line Repository at the National Cancer Institute. The cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 100 mg/ml penicillin-Streptomycin (Invitrogen). Sodium oxamate, 3-bromopyruvic acid (3BrPA), sodium iodoacetate (IAA), sodium fluoride, and phloretin were obtained from Sigma (St. Louis, MO). Doxorubicin was purchased from EMD/Calbiochem (La Jolla, CA). All compounds were prepared according to the manufacturers' instructions.

ATP Assay

Cells were seeded in 96-well tissue culture microplates at a density of 10⁴ cells 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).

IC₅₀ Value Determination

Data was analyzed with Prism 4 (GraphPad Software, San Diego, CA).

XF Metabolic Assay

Measurements were made with a prototype Seahorse XF instrument. Cells were seeded in 24-well Seahorse tissue culture microplates at density of 3 x 10⁴ cells per chamber in each well. Approximately 45 minutes prior to the assay, the culture medium was exchanged with a low-buffered RPMI assay medium (Molecular Device, Sunnyvale, CA) to ensure accurate ECAR readings. For detection of acute drug responses, OCR and ECAR were measured for 5 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.

Seahorse XF Instrument Overview

The Seahorse instrument measures the rate of change of analytes in the media surrounding living cells. 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.

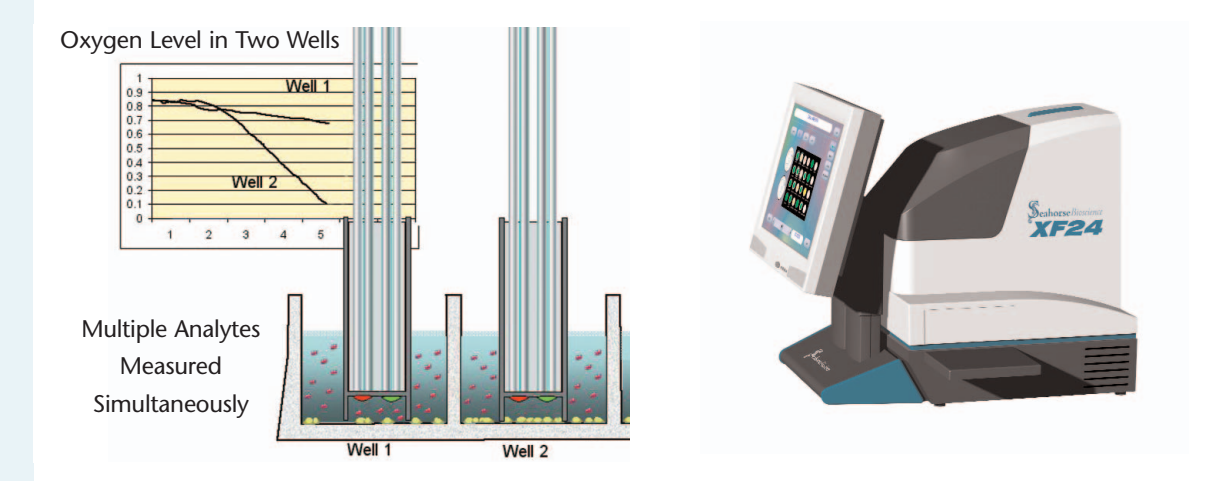
A unique feature of the XF technology is its ability to make accurate and repeatable measurements in as little as five minutes. This is accomplished by isolating an extremely small volume (less than 10 µL) of media above the cell monolayer. Cellular metabolism causes rapid, easily measured changes to 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 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 totally non-invasive, cells can be profiled over a period of minutes, hours or days.

Figure 1. XF Technology Measurement Method and Instrument



Results

Figure 2. Acute Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) Changes in H460 and A549 Cells in Response to Inhibitors of Glycolysis and Glucose Transporters

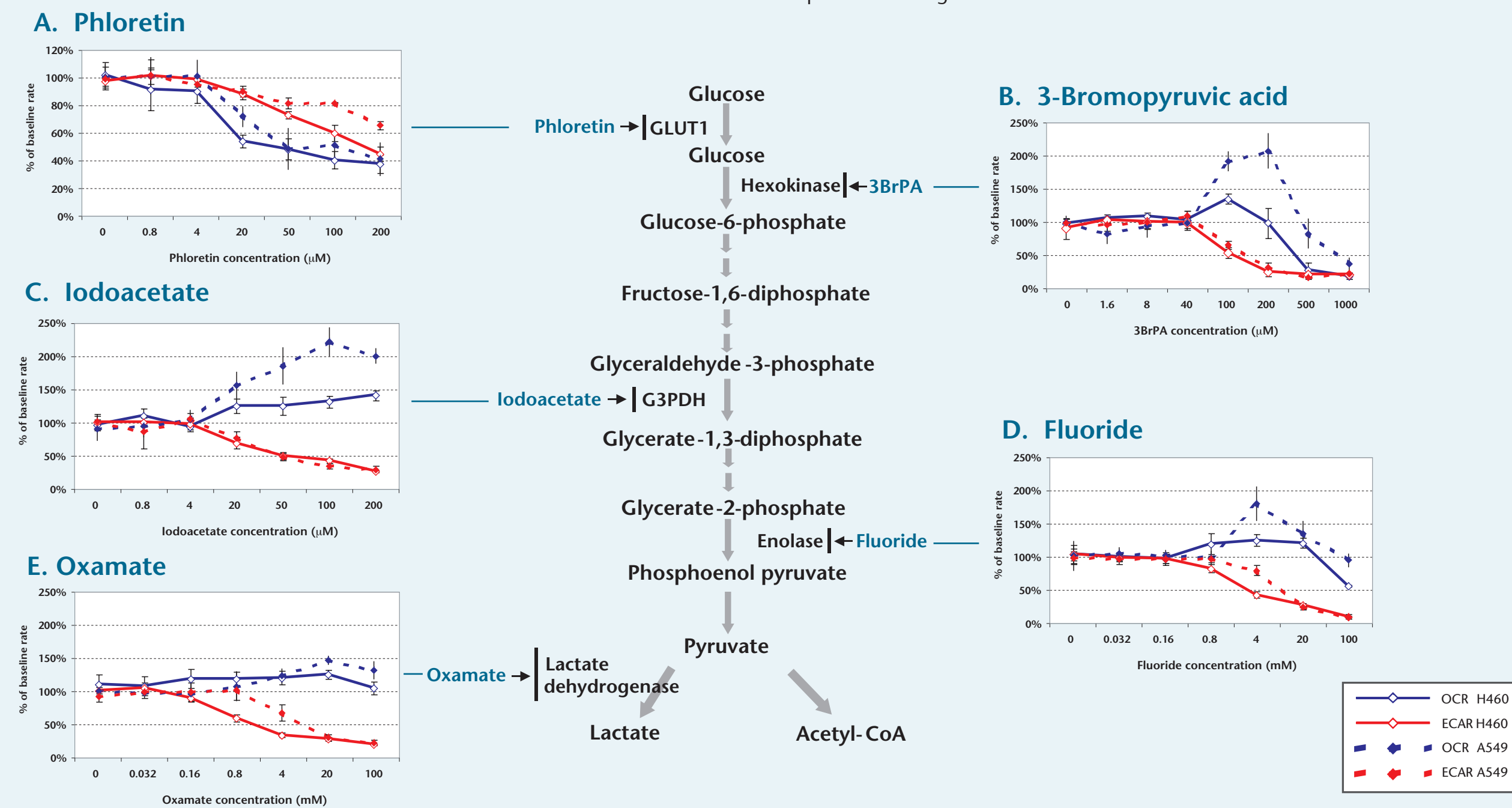
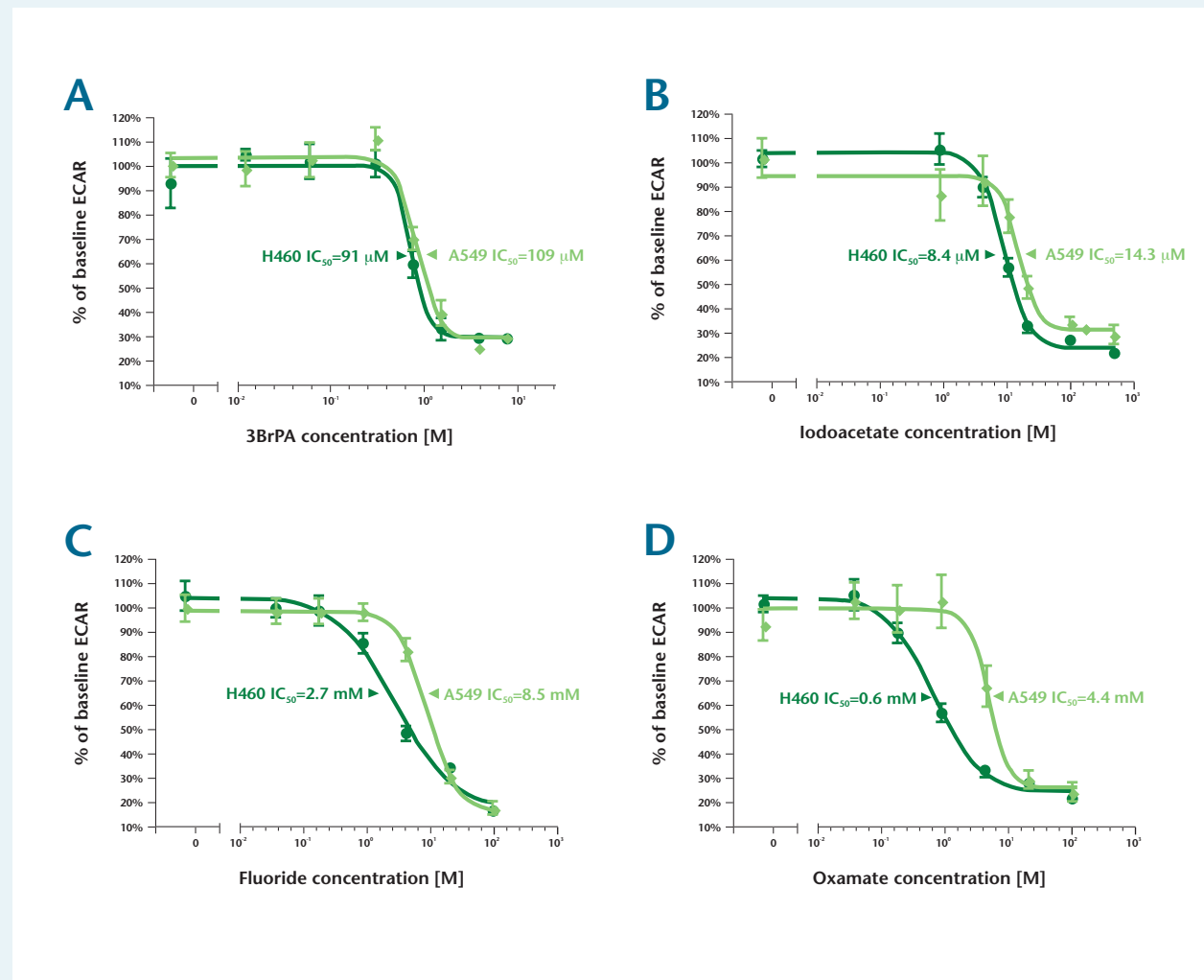
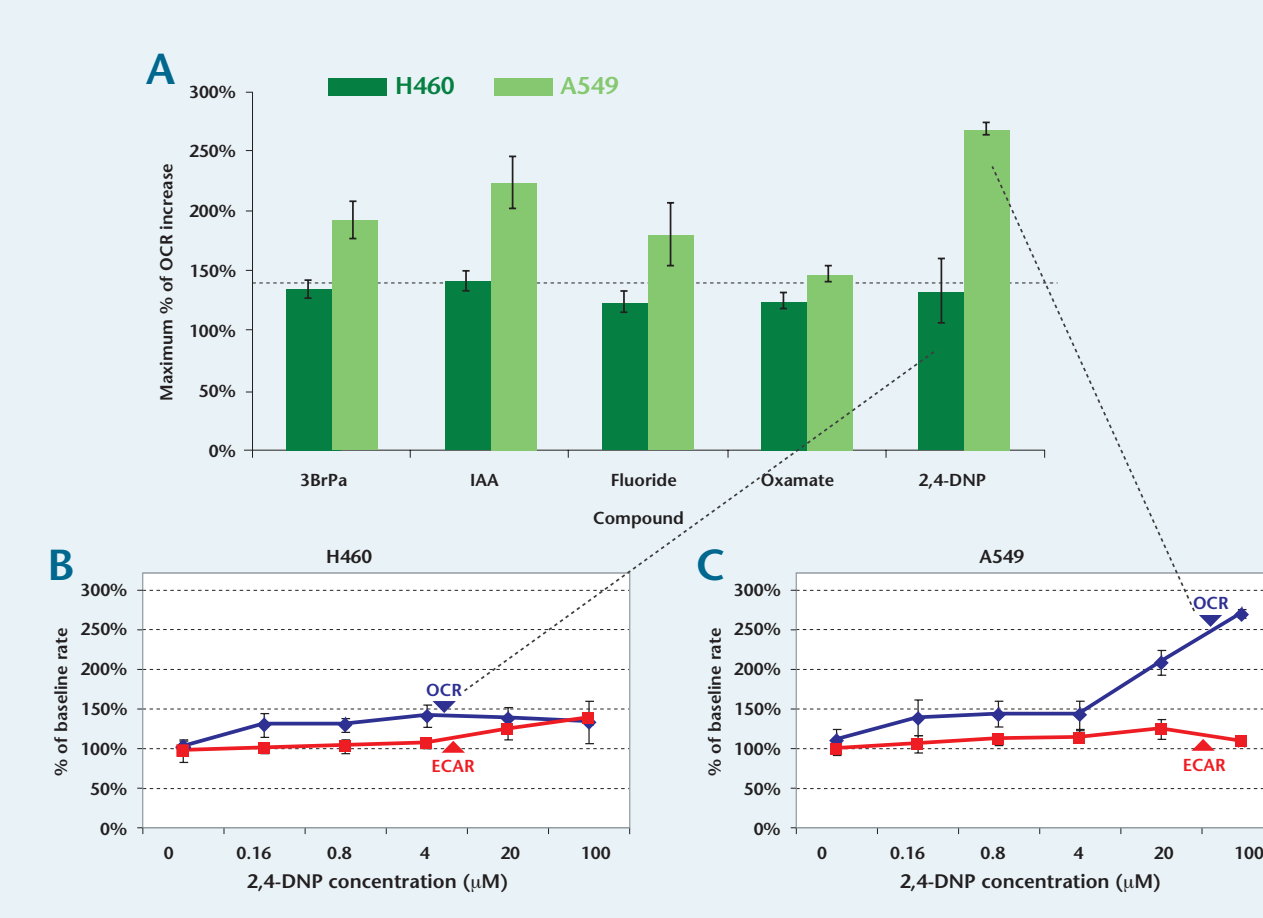


Figure 3. Comparison of Glycolysis Inhibitor IC₅₀ Values, as Measured by ECAR, in H460 and A549 Cells Which Differ in Metastatic Potential



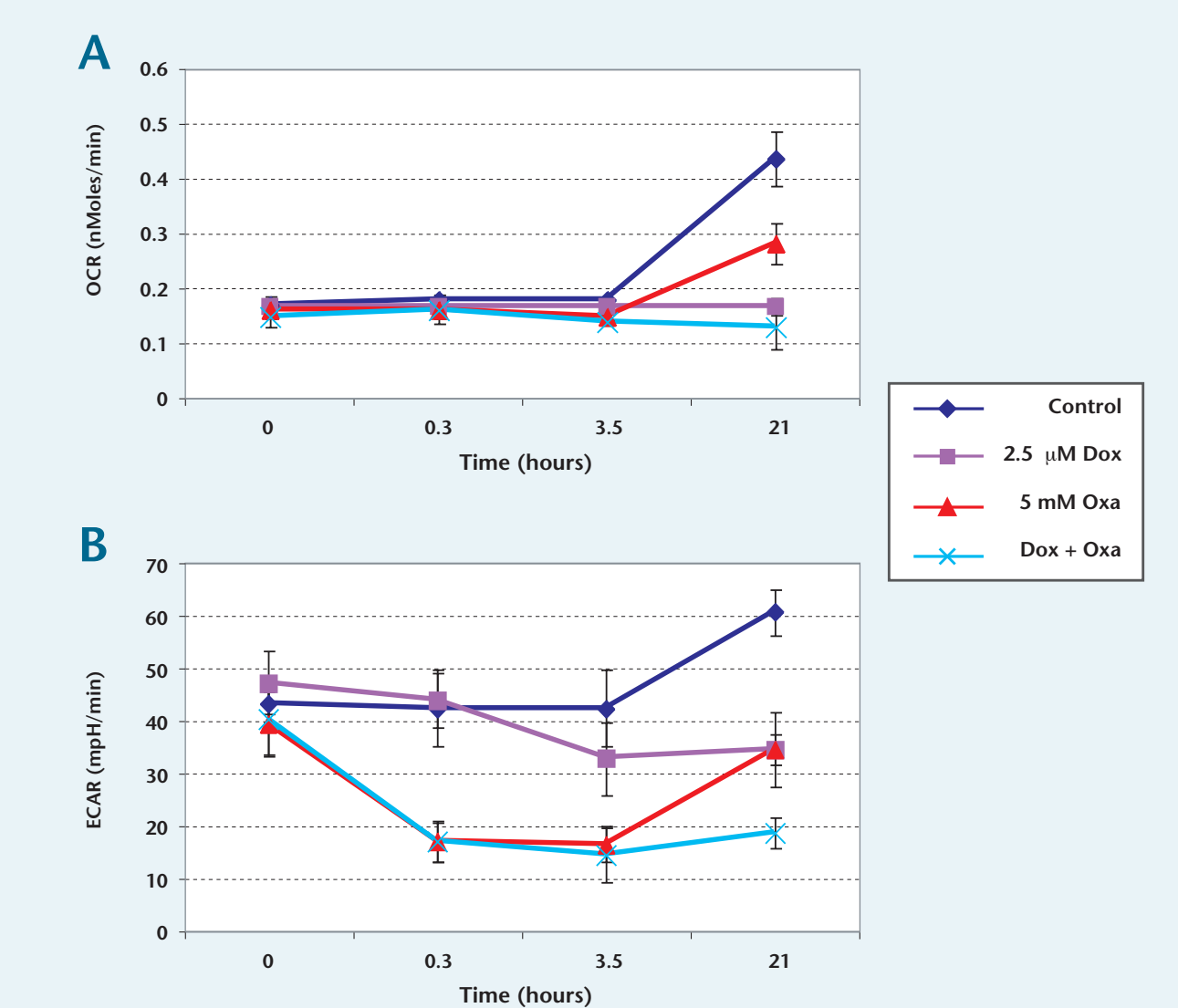
The IC₅₀ value for oxamate (D) is much lower in H460 than in A549 cells. However, IC₅₀ values for 3-bromopyruvic acid (A) and iodoacetate (B) are similar in both cell lines. It has been reported that high affinity lactate dehydrogenase (LDH) is expressed in many cancer cell lines. It is possible that the expression of high affinity LDH is associated with increased metastatic potential in H460 cells.

Figure 4. H460 Cells Exhibit Attenuated Mitochondrial Respiratory Capacity as Compared to A549 Cells



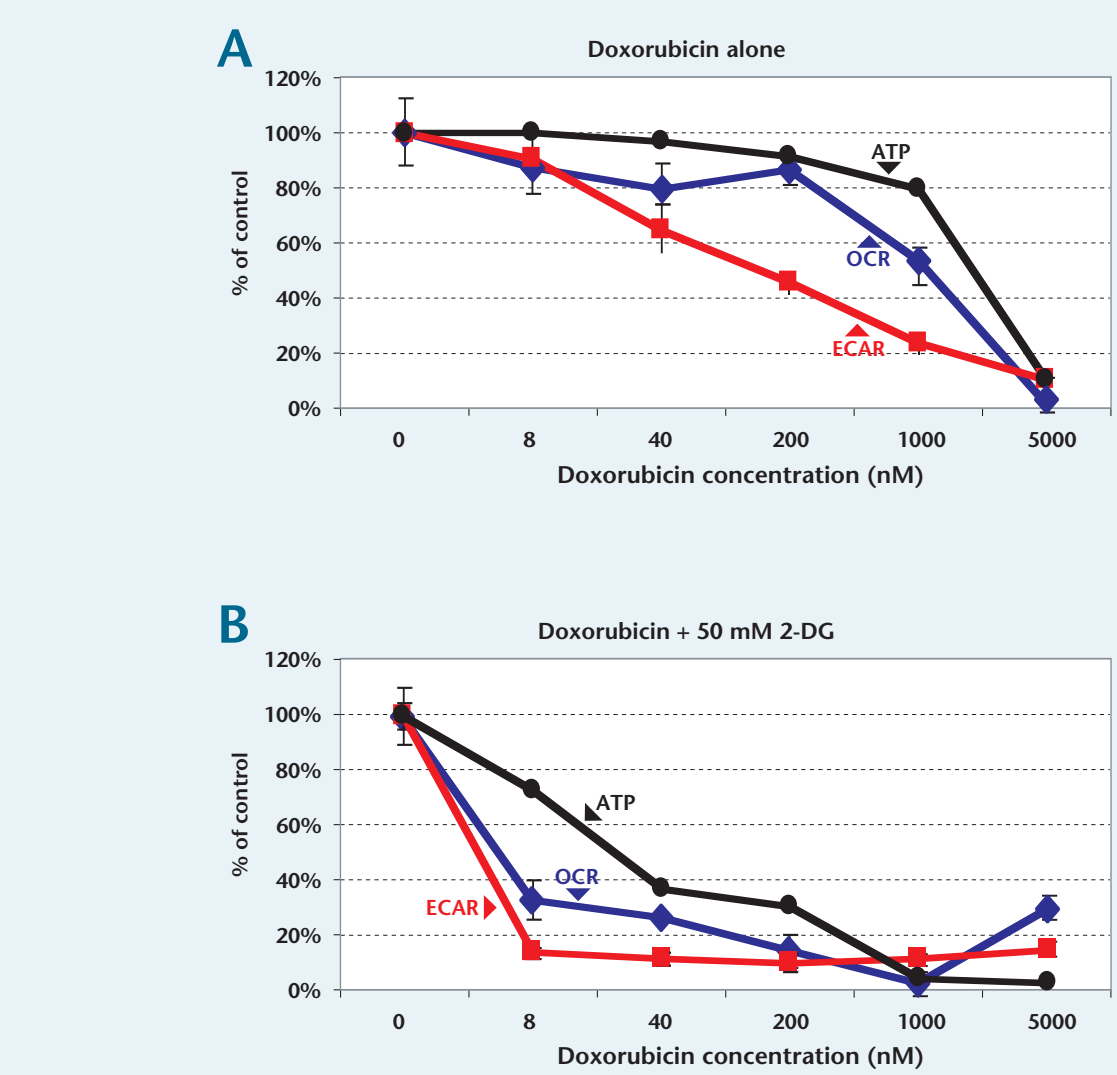
A maximal increase of 150% in OCR over baseline was observed in H460 cells in response to glycolysis inhibitors (A), oxamate, 3-bromopyruvic acid, iodoacetate and fluoride. Since the maximum OCR increase following treatment with the mitochondrial uncoupler 2,4-DNP was also 150%, this suggests that lower mitochondrial respiratory capacity is an intrinsic characteristic of H460 cells (B). In comparison, maximal 225% and 250% responses were observed in A549 cells following glycolysis inhibitors or 2,4-DNP respectively.

Figure 6. Time-resolved Measurement of Oxamate Sensitization of H460 Cells to Doxorubicin Mediated Cytotoxicity



OCR (A) and ECAR (B) of H460 cells treated with either doxorubicin or oxamate alone and combination of both compounds over a 21 hour period. OCR and ECAR were measured at 0, 0.3, 3.5 and 21 hours post treatment.

Figure 5. 2-Deoxyglucose (2-DG) Sensitizes H460 Cells to Doxorubicin Mediated Cytotoxicity



Shown is a doxorubicin dose response in H460 cells after 72 hours treatment (A). Shown is a dose response of H460 cells to doxorubicin in the presence of 50 mM 2-Deoxyglucose (2-DG) after 72 hours combination treatment (B).

Summary

The unique, highly glycolytic phenotype of cancer cells represents both an informative biomarker and a potential therapeutic target. Seahorse's new XF technology provides a non-invasive, time resolved, high content method to profile this metabolic phenotype.

Using the XF technology, we have shown a difference between the glucose transporter and glycolytic enzymes in two NSCLC cancer cell lines that correlates with their metastatic potential. In addition, we have shown glycolysis inhibitors sensitize H460 cells to cytotoxic drugs.

Acknowledgements

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