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Early reduction of glucose consumption is a biomarker of kinase inhibitor efficacy which can be reversed with GLUT1 overexpression in lung cancer cells

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Abstract

Purpose: Small molecule inhibitors that target oncogenic driver kinases are an important class of therapies for non-small cell lung cancer (NSCLC) and other malignancies. However, these therapies are not without their challenges. Each inhibitor works on only a subset of patients, the pharmacokinetics of these inhibitors is variable, and these inhibitors are associated with significant side effects. Many of these inhibitors lack non-invasive biomarkers to confirm pharmacodynamic efficacy, and our understanding of how these inhibitors block cancer cell growth remains incomplete. Limited clinical studies suggest that early (<2 weeks after start of therapy) changes in tumor glucose consumption, measured by [^{18}F]FDG PET imaging, can predict therapeutic efficacy, but the scope of this strategy and functional relevance of this inhibition of glucose consumption remains understudied. Here we demonstrate that early inhibition of glucose consumption as can be measured clinically with [^{18}F]FDG PET is a consistent phenotype of efficacious targeted kinase inhibitors and is necessary for the subsequent inhibition of growth across models of NSCLC.

Methods: We tested nine NSCLC cell lines (A549, H1129, H1734, H1993, H2228, H3122, H460, HCC827, and PC9 cells) and ten targeted therapies (afatinib, buparlisib, ceritinib, cabozantinib, crizotinib, dovitinib, erlotinib, ponatinib, trametinib, and vemurafenib) across concentrations ranging from 1.6 nM to 5 μM to evaluate whether these inhibitors block glucose consumption at 24 hours post-drug treatment and cell growth at 72 hours post-drug treatment.

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Conflict of Interest Statement

Dr. Clark and Dr. Ghezzi are inventors on a pending patent, to be assigned to the Regents of the University of California, that covers the work described in this manuscript.

We overexpressed the facilitative glucose transporter SLC2A1 (GLUT1) to test the functional connection between blocked glucose consumption and cell growth after treatment with a kinase inhibitor. A subset of these inhibitors and cell lines were studied *in vivo*.

Results: Across the nine NSCLC cell lines, ten targeted therapies, and a range of inhibitor concentrations, whether a kinase inhibitor blocked glucose consumption at 24 hours post-drug treatment strongly correlated with whether that inhibitor blocked cell growth at 72 hours post-drug treatment in cell culture. These results were confirmed *in vivo* with [^{18}F]FDG PET imaging. GLUT1 overexpression blocked the kinase inhibitors from limiting glucose consumption and cell growth.

Conclusions: Our results demonstrate that the early inhibition of lung cancer glucose consumption in response to a kinase inhibitor is a strong biomarker of and is often required for the subsequent inhibition of cell growth. Early inhibition of glucose consumption may provide complementary information to other biomarkers in determining whether a drug will effectively limit tumor growth.

Keywords

positron emission tomography; biomarker; [^{18}F]FDG; tumor imaging; lung cancer; glucose consumption; high-throughput screen

Introduction

Targeted kinase inhibitors are first-line therapy for many patients with non-small cell lung cancer (NSCLC) whose tumors possess certain genetic alterations [1, 2]. Examples include EGFR inhibitors for patients with deletions in Exon 19 or L858R mutations in EGFR, and ALK inhibitors for patients with EML4-ALK fusions [3, 4]. While targeted therapies are effective, they can also fail despite the presence of the associated genetic biomarker alteration [2]. Overall response rates for the EGFR inhibitor gefitinib in NSCLC patients with activating EGFR mutations and the ALK inhibitor ceritinib in NSCLC patients with EML4-ALK fusions are only 74% and 73%, respectively [5, 6]. This is likely due to various mechanisms including variability in drug pharmacokinetics, intrinsic tumor resistance to these drugs caused by co-expression of other oncogenic drivers, and increased expression of anti-apoptotic proteins [7–11]. Second-line therapies for these NSCLC patients who progress systemically on targeted therapies are usually immune checkpoint inhibitors or chemotherapies such as docetaxel or pemetrexed [12]. Determining before or early in a treatment course whether an individual patient's tumor will respond to a targeted therapy remains a challenge. An early predictive pharmacodynamic biomarker of kinase inhibitor efficacy could quickly identify patients whose tumors are not responding to the therapy thus limiting their exposure to drug side effects while allowing them to quickly move on to a potentially effective alternatively therapy.

Early (<2 weeks after start of therapy) kinase inhibitor-induced inhibition of tumor glucose consumption, measured non-invasively with [^{18}F]FDG PET, has been shown in certain preclinical models and patients to correlate with therapeutic efficacy [13–19]. Gefitinib blocks glucose consumption as early as 4 hours post-treatment in cell lines in which gefitinib

subsequently blocks cell growth [13]. Similarly, small clinical studies with fewer than 30 patients have shown that decreased tumor [^{18}F]FDG consumption 2 days or 2 weeks after starting treatment with gefitinib or the EGFR inhibitor erlotinib, respectively, can predict which tumors will respond to the treatment [14, 15]. These studies suggest a potential broad role for the early inhibition of glucose consumption as a predictor of a therapeutic response to a kinase inhibitor, but a number of key questions remain including: How universal is this strategy among targeted kinase inhibitors? Are early changes in glucose consumption only correlative or do they contribute to a therapeutic response?

We recently developed a high-throughput assay for measuring cellular glucose consumption and used this assay to screen 3555 bioactive small molecules for their effect on glucose consumption [20]. The results of that study suggested that many kinase inhibitors currently approved to treat NSCLC may block glucose consumption. Here we study the effect of approved kinase inhibitors and those in development on NSCLC glucose consumption and evaluate whether inhibiting glucose consumption with these kinase inhibitors is sufficient to block cell growth.

Materials and Methods

Cell lines.

A-549 (CCL-185), NCI-H1299 (CRL-5803), NCI-H2228 (CRL-5935), NCI-H460 (HTB-177), NCI-H1734 (CRL-5891), NCI-H1993 (CRL-5909), and HCC827 (CRL-2868) cells were from ATCC. PC-9 cells were from Sigma-Aldrich. NCI-H3122 cells were from the National Cancer Institute. All cell lines were transduced with the FU-EBFP2-H2B-W plasmid that expresses a nuclear-localized blue-fluorescent protein. All cell lines were cultured in RPMI1640 supplemented with FBS (10% v/v), glutamine (4 mM), and penicillin/streptomycin (100 U/mL). PC9, H1229, and H3122 cell lines were also cultured in HPLM supplemented with FBS (10% v/v) and penicillin/streptomycin (100 U/mL).

[^{3}H]2-DG accumulation assays.

H1229, PC9, A549, H460, and H2228 cells (0.25×10^6 cells/mL; 0.5 mL) and HCC827, H1734, H1993, and H3122 cells (0.5×10^6 cells/mL; 0.5 mL) were plated in separate poly-L-lysine-coated wells of a 24 well plate and incubated overnight (37 °C, 5% CO_2). The following day, the cells were washed once with 1x PBS and incubated with [^{3}H]2-DG in glucose-free RPMI (0.5 μCi/well; 15, 30, 60, and 120 minutes). Glucose-free media was used here to avoid the significant blocking effect that glucose in media has on [^{3}H]2-DG accumulation. The cells were washed three times with ice-cold 1x PBS containing 25 mM glucose and were lysed in RIPA buffer. 1x PBS containing 25 mM glucose was used for the washes to limit [^{3}H]2-DG efflux from the cells. The lysate was combined with scintillation fluid and accumulated activity was measured on a scintillation counter. Separate wells treated the same except without the addition of [^{3}H]2-DG were used to count cells. For the kinase inhibitor experiments, cells were treated with buparlisib (1 μM) or cabozantinib (40 nM) in RPMI1640 supplemented with FBS (10% v/v), glutamine (4 mM), and penicillin/streptomycin (100 U/mL) for 4, 16, or 24 hours prior to the [^{3}H]2-DG accumulation assays,

which were run as described above except that buparlisib and cabozantinib were added to the glucose-free RPMI and the cells were incubated with [^{3}H]2-DG for 15 minutes.

Immunoblots.

Immunoblots were conducted as previously described [20] except that the following antibodies were used: EGFR (Cell Signaling 2239S, 1:1000 dilution); phospho-EGFR (Tyr1068, Cell Signaling 3777S, 1:1000 dilution); AKT (Cell Signaling 2920S, 1:1000 dilution); phospho-AKT (Ser473; Cell Signaling 9271S, 1:1000 dilution); Erk1/2 (Cell Signaling 4696S, 1:1000 dilution); and phospho-Erk1/2 (Thr202/Tyr204, Cell Signaling 9101S, 1:1000 dilution).

High-throughput glucose consumption and cell growth assay and screen.

Cells were treated with the following kinase inhibitors and concentrations or DMSO: afatinib, buparlisib, crizotinib, dovitinib, and vemurafenib (8 nM, 40 nM, 200 nM 1 μM, 5 μM); cabozantinib, ceritinib, erlotinib, ponatinib, and trametinib (1.6 nM, 8 nM, 40 nM, 200 nM, 1 μM). The range of concentrations over which we tested each inhibitor was based on the reported IC_{50} value of the inhibitor for its known target(s). 24 hours post-kinase inhibitor treatment, glucose consumption was measured in high-throughput as previously described [20]. Briefly, the cells were removed of media, washed three times with 1x PBS containing 0.25% (w/v) BSA, and incubated with 2-DG (1.25 mM) in 1x PBS containing 0.25% (w/v) BSA for 20 min at 37 °C. During the 20 min incubation, cell numbers in each well were measured on an Image Express XL. Stop buffer (0.4 N HCl containing 2% (w/v) dodecyltrimethylammonium bromide) was added followed by detection reagents that convert 2-DG-6-phosphate to light. Light output was measured on a PerkinElmer EnVision plate reader. 72 hours post-kinase inhibitor treatment, cell numbers on a separate plate were measured on an Image Express XL. Glucose consumption and cell growth values for the inhibitor-treated cells were normalized to corresponding values for the DMSO-treated cells. DMSO-treated control cell values in the graphs are shared across drugs.

[^{18}F]FDG PET imaging and xenograft growth measurements.

[^{18}F]FDG PET imaging experiments were conducted as previously described [20] except that PC9 and H460 cell xenografts were treated with vehicle (0.5% carboxymethyl cellulose), erlotinib (PO; 40 mg/kg/day), or dovitinib (PO; 20 mg/kg/day). Xenografts were measured by calipers immediately before the first treatment and at Day 7 post-treatment. All mouse experiments were conducted in accordance with the UCLA Animal Research Committee (ARC) and Institutional Animal Care and Use Committee (IACUC).

GLUT1 overexpression experiments.

H460, HCC827, and H2228 cells were transduced with the pLenti-eGFP vector expressing human SLC2A1 (GLUT1) or the empty vector and sorted for eGFP expression. No additional selection was performed on these clones, and so they represent a polyclonal population.

Statistics.

Statistical comparisons were made using one-way and two-way ANOVA with Dunnett multiple comparison testing to vehicle-treated cells. For glucose consumption and cell growth analyses, plotted p-values represent the p-value between vehicle-treated cells and cells treated at the maximum inhibitor concentration. All statistical analyses were conducted and graphs plotted in Graphpad Prism. Replicates represent independent wells of cells treated and analyzed independently. All data is presented as mean ± standard deviation.

Results

Representative NSCLC cell lines and clinically relevant kinase inhibitors

NSCLC is the most common subtype of lung cancer, accounting for >80% of all cases [12]. We studied nine NSCLC cell lines that collectively possess the major genetic alterations found in NSCLC (Fig. 1a). PC9 and HCC827 cells have mutations in EGFR, which are present in ~14% of patient tumors [21]. A549, H1299, H1734, and H460 cells have mutations in KRAS or NRAS, which are present in ~34% of patient tumors [21, 22]. H2228 and H3122 cells have ALK fusions, which are present in ~7% of patient tumors [23]. H460 cells have an activating E545K mutation in PIK3CA. PIK3CA mutations are present in ~7% of patient tumors [21]. H1993 cells have a MET amplification, and MET is amplified at the genomic level or mutated in a way that increases its stability in ~8% of patient tumors [24]. As expected given the heterogeneity of driver mutations in these cell lines, the signaling pathways downstream of the mutant and activated proteins were also heterogeneously activated across these cell lines. Phospho-EGFR levels were elevated in PC9 and HCC827 cells consistent with the activating EGFR mutations in these cell lines. Phospho-Akt levels were elevated in the cell lines with activating mutations in both PIK3CA and KRAS (H460), in EGFR (PC9 and HCC827), and in KRAS (A549 and H1229). Phospho-ERK levels were elevated in cells with G13C mutation in KRAS (H1734) (Fig. 1b).

As part of our initial characterization of these cell lines, we measured glucose consumption in each cell line using [^{3}H]2-deoxy-D-glucose ([^{3}H]2-DG) (Fig. 1c). All of the cell lines accumulated 2-DG although at very different rates: H460 cells consumed 2-DG at the highest initial rate of 270 fmol 2-DG/(10^6 cells x min), 27-fold faster than the H1993 cells that consumed 2-DG at the slowest initial rate of 10 fmol 2-DG/(10^6 cells x min).

We chose ten targeted therapies to study that are approved for or that could be used to target driver kinases or downstream signaling pathways as monotherapies or combination therapies for the treatment of NSCLC. These included afatinib, buparlisib, cabozantinib, ceritinib, crizotinib, dovitinib, erlotinib, ponatinib, trametinib, and vemurafenib (Fig. 1d) [1, 2, 25–31]. These inhibitors target proteins known to contribute to NSCLC pathogenesis such as EGFR (afatinib and erlotinib) and ALK (crizotinib and ceritinib) as well as additional proteins that could contribute to the mechanism-of-action of each inhibitor. For example, dovitinib inhibits the NSCLC cancer target FGFR1 but also FLT3, FLT4, PDGFRβ, c-Kit, and FGFR3 all with an *in vitro* IC_{50}≤50 nM [32].

A unique set of kinase inhibitors blocked glucose consumption in each cell line

In early studies, we investigated the time course over which kinase inhibitors blocked glucose consumption. We chose a subset of cell lines and kinase inhibitors for which we knew that the kinase inhibitor would block glucose consumption: PC9, H460, and H3122 cells treated with buparlisib and PC9, H460, and HCC827 cells treated with cabozantinib. The kinetics over which these inhibitors decreased glucose consumption depended on the cell line and inhibitor combination (Supplemental Fig. 1). For example, buparlisib induced a strong decrease in PC9 glucose consumption at 4 hours post-drug treatment that reached its lowest level by 16 hours post-drug treatment and that was maintained through 24 hours post-drug treatment. In contrast, cabozantinib induced a steady decline in glucose consumption over the 24 hours of drug treatment that reached its lowest level at 24 hours post-drug treatment. All of the tested cell line and inhibitor combinations reached or maintained their lowest level of glucose consumption at 24 hours post-drug treatment. Thus, for all subsequent experiments, we studied glucose consumption at 24 hours post-drug treatment.

Given the number of different drug and cell line combinations we wanted to test, we employed a high-throughput glucose consumption assay that we recently developed [20]. In this assay, 2-DG-6-phosphate levels are measured in cells treated with 2-DG. Because 2-DG-6-phosphate levels in this assay are regulated by the same enzymes that regulate [^{18}F]FDG accumulation in cells – the GLUT transporters and hexokinase enzymes [33] – this assay is a cell culture equivalent of a [^{18}F]FDG PET assay.

Each targeted kinase inhibitor blocked glucose consumption as a function of concentration in at least one of the nine cell lines (Fig. 2). However, considerable variability existed across cell lines and inhibitors. The targeted kinase inhibitors blocked glucose consumption in cell lines with activating alterations in the signaling pathway(s) targeted by the inhibitor as well as in cell lines without these activating alterations. For example, consistent with the reported role for EGFR in driving glucose consumption in mutant EGFR-containing cells [13], erlotinib and afatinib, which both inhibit EGFR, blocked glucose consumption in the EGFR mutant cell lines HCC827 and PC9. Unexpectedly, both erlotinib and afatinib also blocked glucose consumption in the EGFR wild-type cell line H1734 suggesting a potential role for EGFR in regulating glucose consumption even in certain cell lines without an activating EGFR mutation. Erlotinib had no effect on glucose consumption in other EGFR wild-type cell lines while afatinib, which can target additional EGFR family members [34], also blocked glucose consumption in H3122, H1993, and H1229 cells. Ceritinib and crizotinib, which both target ALK, blocked glucose consumption in H3122 cells, which harbor an EML4-ALK gene fusion, as well as PC9, HCC827, H2228, H1993, and H1229 cells, which have not been reported to harbor EML4-ALK gene fusions. Crizotinib, which beyond ALK targets different kinases than ceritinib [35, 36], also blocked glucose consumption in H460 and H1734 cells. Finally, despite the well-studied role of PI3K in regulating glucose consumption [37], buparlisib had no significant effect on glucose consumption in A549, H1229, and H2228 cells. Like our previous study [20], we identified no correlation between cell growth and glucose consumption 24 hours post-kinase inhibitor treatment (Supplemental Fig. 2), indicating that the effects of these kinase inhibitors on glucose consumption are not a consequence of inhibiting cell growth.

Among the nine cell lines, there was wide variation in whether glucose consumption could be affected by one or more of the ten inhibitors. Only one inhibitor – vemurafenib – blocked glucose consumption in A549 cells. Nine of the ten inhibitors blocked glucose consumption in H3122 and PC9 cells. The number of inhibitors that blocked glucose consumption in a given cell line had no connection to the rate of glucose consumption in that cell line. This may suggest that glucose consumption in H3122 and PC9 cells is regulated by various, interconnected pathways compared to A549 cells. Collectively, these data suggest that kinase inhibitors can block glucose consumption in NSCLC cell lines but that each kinase inhibitor only blocks glucose consumption in a subset of these cell lines. Possibly due to the wide substrate scope of many of these kinase inhibitors, the exact subset of cell lines for which a given inhibitor will block glucose consumption is difficult to predict based on genetics alone.

Early inhibition of glucose consumption strongly correlated with subsequent inhibition of cell growth across kinase inhibitors and cell lines

Previous studies have suggested a correlation between early inhibition of glucose consumption and subsequent inhibition of cell growth in cell lines or tumors treated with kinase inhibitors [13–19]. However, these studies have been limited in scope to a few inhibitors and cell lines or tumors and usually only one drug concentration despite the fact that the pharmacokinetics of many of these inhibitors can be variable leading to variability in plasma drug concentrations [7, 8, 38]. Our data linking kinase inhibitors across a range of concentrations to inhibition of glucose consumption in a set of cell lines allowed us to systematically test this correlation across a larger set of conditions. To accomplish this, we studied cell growth at 72 hours post-drug treatment. As expected given the known efficacy of many of these compounds in NSCLC [1, 2, 25–31], all of the kinase inhibitors significantly decreased cell growth in at least one and usually multiple of the cell lines (Supplemental Fig. 3).

Comparing the effect of each inhibitor and inhibitor concentration on glucose consumption per cell measured 24 hours post-drug treatment and cell growth measured 72 hours post-drug treatment, we identified a strong link between whether and at what concentration an inhibitor blocked glucose consumption, and whether and at what concentration that inhibitor subsequently blocked cell growth (Supplemental Fig. 4; correlation coefficient r: 0.46; $p<0.0001$). This is exemplified with erlotinib, afatinib, and dovitinib in PC9 cells (Fig. 3a). In all three PC9 cell examples, the kinase inhibitor blocked cell growth (measured at 72 hours post-inhibitor treatment) only at those inhibitor concentrations that blocked glucose consumption (measured at 24 hours post-inhibitor treatment) and *vice versa*. Less frequently, we identified kinase inhibitor and cell line combinations that did not show this strong link including for example trametinib in A549 cells, which strongly blocked cell growth without affecting glucose consumption, and dovitinib in HCC827 cells, which strongly blocked glucose consumption without affecting cell growth (Fig. 3b). Combining all this data, in this system, a >21% inhibition of glucose consumption by a kinase inhibitor at 24 hours post-inhibitor treatment was 77% sensitive and 79% selective for predicting that the kinase inhibitor would decrease cell growth by >40% at 72 hours post-inhibitor treatment (Fig. 3c).

The RPMI1640 media in which we conducted the cell culture studies contains supraphysiological concentrations of various metabolites including glucose (11.1 mM in RPMI1640 media versus 5.5 – 7.8 mM in normal human plasma [39]). HPLM media is a recently developed media that contains similar metabolite concentrations to human plasma including glucose (5 mM in HPLM media) [40]. We chose a random subset of cell lines (PC9, H1229, and H3122) and inhibitors (afatinib, buparlisib, crizotinib, erlotinib, and ponatinib) in which to test the correlation between glucose consumption per cell measured 24 hours post-drug treatment and cell growth measured 72 hours post-drug treatment in cells grown in HPLM media. Among this smaller subset of inhibitors and cell lines cultured in HPLM media, we identified similar results to those obtained for the larger set of inhibitors and cell lines cultured in RPMI1640 media. A unique set of inhibitors blocked glucose consumption and growth in each of the cell lines (Supplemental Fig. 5). Comparing glucose consumption measured 24 hours post-drug treatment and cell growth measured 72 hours post-drug treatment for the cells cultured in HPLM media across a range of concentrations yielded a significant correlation (Supplemental Fig. 5; correlation coefficient r: 0.43; p=0.0001) that was similar to that identified for the cells grown in RPMI1640 media.

We tested in a subset of cell lines and inhibitors whether these results translate *in vivo*. We chose cell lines that we had experience growing as xenografts and inhibitors that depending on the chosen cell lines, did and did not block glucose consumption and growth. In culture, erlotinib and dovitinib reduced PC9 glucose consumption and cell growth (Fig. 2, Supplemental Fig. 3). Consistent with these cell culture results, dovitinib and erlotinib also reduced PC9 xenograft [^{18}F]FDG accumulation compared to pre-treatment levels by 21±3.2% and 25±5.3% respectively, and growth compared to vehicle-treatment by 54±3.7% and 31±4.0%, respectively (Fig. 3d). In culture, erlotinib had no effect on H460 glucose consumption or growth while dovitinib reduced both H460 glucose consumption and growth (Fig. 2, Supplemental Fig. 3). Consistent with the cell culture results, erlotinib had no effect on H460 xenograft [^{18}F]FDG accumulation or cell growth *in vivo*. Different from the cell culture results but consistent with the demonstrated link between glucose consumption and cell growth, dovitinib had no effect on both H460 xenograft [^{18}F]FDG accumulation or growth (Fig. 3e). In all of these studies, the drug treatments did not alter [^{18}F]FDG accumulation in healthy tissues such as the brain and heart (Supplemental Fig. 6), suggesting that these inhibitors specifically block glucose consumption in the tumor cells. These *in vivo* results support our cell culture work in suggesting a close link between early changes in glucose consumption and subsequent changes in cell growth.

Kinase inhibitor-induced inhibition of glucose consumption is necessary for subsequent inhibition of cell growth

The strong correlation we identified between early kinase inhibitor-induced inhibition of glucose consumption and subsequent inhibition of cell growth suggested a potential causative relationship in which inhibition of glucose consumption limits cell growth. We studied this in H460, HCC827, and H2228 cells. We chose these cell lines as their responses to the inhibitors with respect to both glucose consumption and growth were representative of the remaining cell lines. GLUT1 overexpression increased basal glucose consumption in each of the three cell lines (Fig. 4a). In addition, for each cell line, most of the

kinase inhibitors that blocked glucose consumption in control cells could no longer do so in the GLUT1 overexpression cells (Fig. 4b). In all but two of these cases, GLUT1 overexpression also blocked the kinase inhibitors from affecting growth (Fig. 4b). For example, buparlisib caused a 51±4.4% decrease in glucose consumption in control H460 cells but only a 14±4.0% decrease in glucose consumption in H460 cells overexpressing GLUT1. Correspondingly, buparlisib decreased cell growth in the control H460 cells by 44±3.4% but failed to decrease cell growth in the H460 cells overexpressing GLUT1. Dovitinib decreased glucose consumption and cell growth in the control H460 cells by 72±2.0% and 80±0.1%, respectively, but only decreased glucose consumption and cell growth in the H460 cells overexpressing GLUT1 by 28±7.3% and 22±7.4%. The two exceptions to this were HCC827 cells treated with dovitinib, and H2228 cells treated with crizotinib. In the case of HCC827 cells, dovitinib treatment had no effect on control HCC827 growth, and GLUT1 overexpression did not change this. In the case of crizotinib treatment of H2228 cells, GLUT1 overexpression blocked crizotinib-induced inhibition of glucose consumption but not cell growth, which was minimally affected by treatment in control cells. These data suggest that kinase inhibitor-induced early inhibition of glucose consumption is not just correlative to but is often causative of the subsequent inhibition of cell growth.

Discussion

Here we show that across a panel of kinase inhibitors and NSCLC cell lines and a range of inhibitor concentrations there exists a significant correlation between whether a kinase inhibitor blocks glucose consumption and whether that kinase inhibitor subsequently blocks cell growth. However, we also identify interesting exceptions to this. We identify cases in which a kinase inhibitor significantly blocks glucose consumption but has no effect on cell growth. These include HCC827 cells treated with dovitinib and PC9 cells treated with ceritinib. In these same cell lines, we also identify various kinase inhibitors that block both glucose consumption and cell growth, and in the case of HCC827 cells, we show that blocking the loss of glucose consumption rescues growth. This would suggest either that these cell lines activate compensatory mechanisms to maintain growth in response to the inhibition of certain kinases but not others or that certain kinase inhibitors block both glucose consumption and compensatory mechanisms while others do not. Identifying these compensatory mechanisms and understanding these differences will be important work for future studies.

We studied glucose consumption at 24 hours post-inhibitor treatment, a much earlier time point than most clinical studies that measure changes in glucose consumption after weeks or months of treatment [13–19, 41–43]. Our results at this early time point suggest that decreased glucose consumption is a direct mechanistic consequence of the kinase inhibitor treatment rather than a downstream consequence of blocked cell growth. This is further supported by the exceptional cases we identify in which a kinase inhibitor blocks growth without affecting glucose consumption (*i.e.,* A549 cells treated with trametinib) as well as our results showing in many cases that inhibition of glucose consumption is necessary for the subsequent inhibition of cell growth. This data is consistent with limited clinical studies that measure changes in glucose consumption after days of treatment [14]

The exact mechanisms through which targeted kinase inhibitors limit glucose consumption are likely to vary depending on the underlying genetics of the cell lines and the different kinases targeted. Many of the kinase inhibitors we study here have multiple targets, and we do not know which target or targets activate glucose consumption in the different cell lines. Nevertheless, PI3K-AKT signaling has been shown to be a major pathway through which at least receptor tyrosine kinases activate glucose consumption in cancer [37]. PI3K-AKT signaling activates glucose consumption through various mechanisms including activating the transcription factor HIF1α that can induce GLUT1 expression, inhibiting the protein thioredoxin-interacting protein (TXNIP) that induces GLUT1 endocytosis, and activating Hexokinase 2 by enhancing its interaction with the mitochondrial membrane. Although it requires further study, we would anticipate that many of the mechanisms through which kinase inhibitors block glucose consumption in these cell lines function through downregulation of PI3K-AKT signaling.

Although speculative, we can envision two clinical scenarios where this correlation between kinase inhibitor induced inhibition of glucose consumption and of cell growth could be used to improve patient care and drug development. First, our data suggests that early inhibition of tumor glucose consumption, as measured with [^{18}F]FDG PET, could be a broadly applicable pharmacodynamic biomarker of kinase inhibitor efficacy and could provide information on kinase inhibitor pharmacodynamics as early as 24 hours post-drug treatment. This would allow for a personalized medicine approach to treating cancer patients with these kinase inhibitors that goes beyond identifying potentially targetable mutations within a tumor to also accounting for patient-to-patient variability in drug pharmacokinetics and additional genetic alterations in the tumor that could make it resistant to the chosen therapy. Second, in the clinical development of new kinase inhibitors designed to target cancer, early changes in tumor [^{18}F]FDG PET accumulation could be used during dose-escalation studies to carefully define the response of tumors to the drug and potentially identify a maximum effective dose that is lower than the maximum tolerated dose, thereby potentially saving patients from unnecessary toxicities brought on by the maximum tolerated dose. Early changes in tumor [^{18}F]FDG accumulation could be used in a similar way during preclinical drug development to quickly and quantitatively define a dose-response curve for new kinase inhibitors.

A recent study suggests that tumor-infiltrating immune cells may accumulate [^{18}F]FDG at higher levels than some tumor cells [44]. Glucose consumption and [^{18}F]FDG accumulation in these immune cells could be unaffected or even increased by kinase inhibitor treatments, either as a direct consequence of the kinase inhibitor treatment or secondary to immune cell activation following tumor cell death (a so-called “flare” effect). If this were the case, it would complicate the use of [^{18}F]FDG PET as an early pharmacodynamic biomarker of kinase inhibitor efficacy. Determining what role infiltrating immune cells have on [^{18}F]FDG accumulation in tumors in response to kinase inhibitor therapies will be the subject of future work. Nevertheless, the clinical studies showing that early inhibition of [^{18}F]FDG accumulation in tumors can predict tumor responses during kinase inhibitor treatments [14, 15] suggest that infiltrating immune cells may not strongly affect these results. Additionally, the fact that we can see significant decreases in glucose consumption within 24 hours of a kinase inhibitor treatment before any induction in cell death that would trigger an

immune response suggests that any type of immune-mediated “flare” in tumor [^{18}F]FDG accumulation would likely occur after the 24 hour post-treatment [^{18}F]FDG PET timepoint that our results would suggest could be used.

The strong correlative and functional connection between early inhibition of glucose consumption and subsequent inhibition of cell growth in our model systems suggests that finding new ways to inhibit cancer cell glucose consumption may prove to be a successful method of finding new ways to limit cell growth. Here we use the high-throughput assay we developed for measuring glucose consumption to study how kinase inhibitors relevant to the treatment of lung cancer block glucose consumption. However, we also recently showed how this assay can be used to screen for new molecules that block cancer cell glucose consumption [20]. In that work, we identified almost 100 small molecules that block glucose consumption in lung cancer and further studied the cyclin-dependent kinase inhibitor Milciclib. Consistent with our current results, we showed that Milciclib blocks cell growth by inhibiting glucose consumption. Additionally, the fact that many kinase inhibitors block cell growth by blocking glucose consumption suggests that compounds that synergize with glucose deprivation may be potential combination therapies to test with these kinase inhibitors.

Our results showing that GLUT1 overexpression can enable cancer cells to resist the growth inhibitory effect of certain kinase inhibitors suggests that elevated glucose consumption may serve as a resistance mechanism to kinase inhibitors in cancer. Consistent with this, elevated expression or activity of Hexokinase 2, a key enzyme in regulating glucose consumption, is associated with resistance to therapies including paclitaxel and tamoxifen in breast cancer, and cisplatin in ovarian cancer [45–47]. Similarly, acquired resistance to kinase inhibitors such as vemurafenib in preclinical melanoma models, and sorafenib and sunitinib in renal cell carcinoma patients is associated with restored or increased [^{18}F]FDG accumulation despite a decrease in [^{18}F]FDG accumulation when the cells were sensitive to the drug [48–50]. Although various mechanisms could explain this phenomenon, one possible explanation that would be consistent with our data is that the cancer cells gain resistance to the kinase inhibitors by upregulating their glucose consumption. Our results also suggest that blocking glucose consumption could be a way to overcome therapeutic resistance to kinase inhibitors in lung cancer. Consistent with this, a recent study showed that GLUT1 inhibition can sensitize an EGFR resistant lung cancer cell line to an EGFR inhibitor [51].

These studies have a number of limitations. Two-dimensional cell culture systems may less reliably model tumor metabolism compared with three-dimensional cell culture systems. Our high-throughput glucose consumption assay would be challenging to implement in a three-dimensional cell culture system as it requires multiple automated wash steps. Additional assays would need to be developed to enable high-throughput measurements of glucose consumption in three-dimensional culture systems. The cell culture and *in vivo* studies were conducted on samples that consisted entirely or largely of pure cancer cells despite the fact that tumors often consist of a milieu of different cell types. These additional cell types may affect the results reported here. Genetically engineered mouse models of cancer could help us in better answering these questions. Additionally, all of the studies were conducted with established cancer cell lines which are more homogenous than real

tumor cells and have adapted to rapid growth in culture. Tumor cell heterogeneity could affect the overall tumor response to the kinase inhibitors. Patient-derived xenografts could model this heterogeneity. We study the short-term effects of the kinase inhibitors on cell growth at 72 hours post-inhibitor treatment in culture and 7 days post-inhibitor treatment *in vivo*. How well the correlation between kinase inhibitor-induced inhibition of glucose consumption and cell growth holds over a longer term and whether elevated glucose consumption would return once the kinase inhibitors stopped blocking growth remains to be determined.

Conclusions

In conclusion, we show that kinase inhibitor-induced inhibition of glucose consumption is strongly predictive of and in many cases required for those inhibitors to subsequently block growth in lung cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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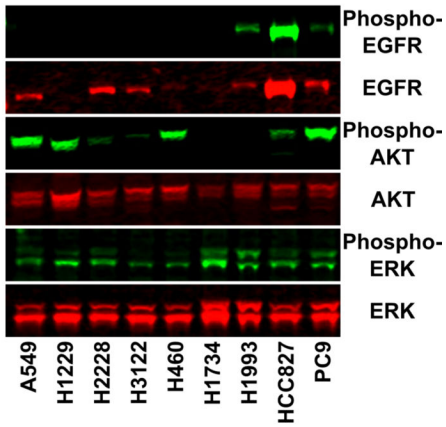
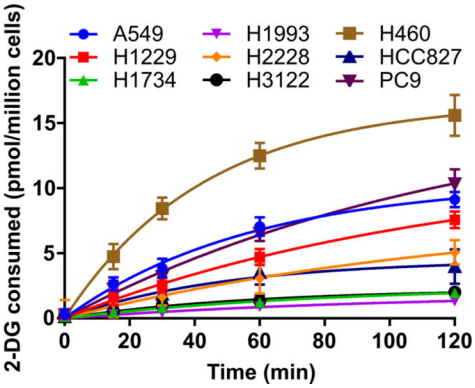
a

Cell line	EGFR	RAS	ALK	PIK3CA	MET
A549	NR	KRAS: G12S	NR	NR	NR
H1299	NR	NRAS: Q61K	NR	NR	NR
H1734	NR	KRAS: G13C	NR	NR	NR
H1993	R309G	NR	NR	NR	Amplified
H2228	NR	NR	EML4-ALK fusion	NR	NR
H3122	NR	NR	EML4-ALK fusion	NR	NR
H460	NR	KRAS: Q61H	NR	E545K	NR
HCC827	Ex19 deletion	NR	NR	NR	NR
PC9	Ex19 deletion	NR	NR	NR	NR

b



c



d

Drugs	Target (IC_{50} < 100 nM)
Afatinib	EGFR, ERBB2
Buparlisib	PIK3CA
Ceritinib	ALK, INSR, IGF1R, FLT3, TSSK1B
Cabozantinib	AXL, FLT3, KIT, MET, RET, TEK, KDR
Crizotinib	MET, ALK
Dovitinib	c-Kit, CSF1R, FGFR1, FGFR3, FLT1, FLT3, FLT4, KDR, PDGFRb
Erlotinib	EGFR
Ponatinib	50 different kinases
Trametinib	MAP2K1, MAP2K2
Vemurafenib	TNK2, BRAF, RAF1, FGR, MAP4K5, SRMS

Fig. 1: Representative NSCLC cell lines and targeted therapies.

(a) Cell lines used in this study and corresponding genetic alterations. NR: Not reported; Ex19: Exon 19. **(b)** Representative immunoblots of phospho- and total- EGFR, AKT, and ERK levels in the studied cell lines. n=2. **(c)** Glucose consumption in the studied cell lines. n=3. **(d)** Kinase inhibitors used in this study and corresponding targets. Data plotted as mean ± standard deviation.

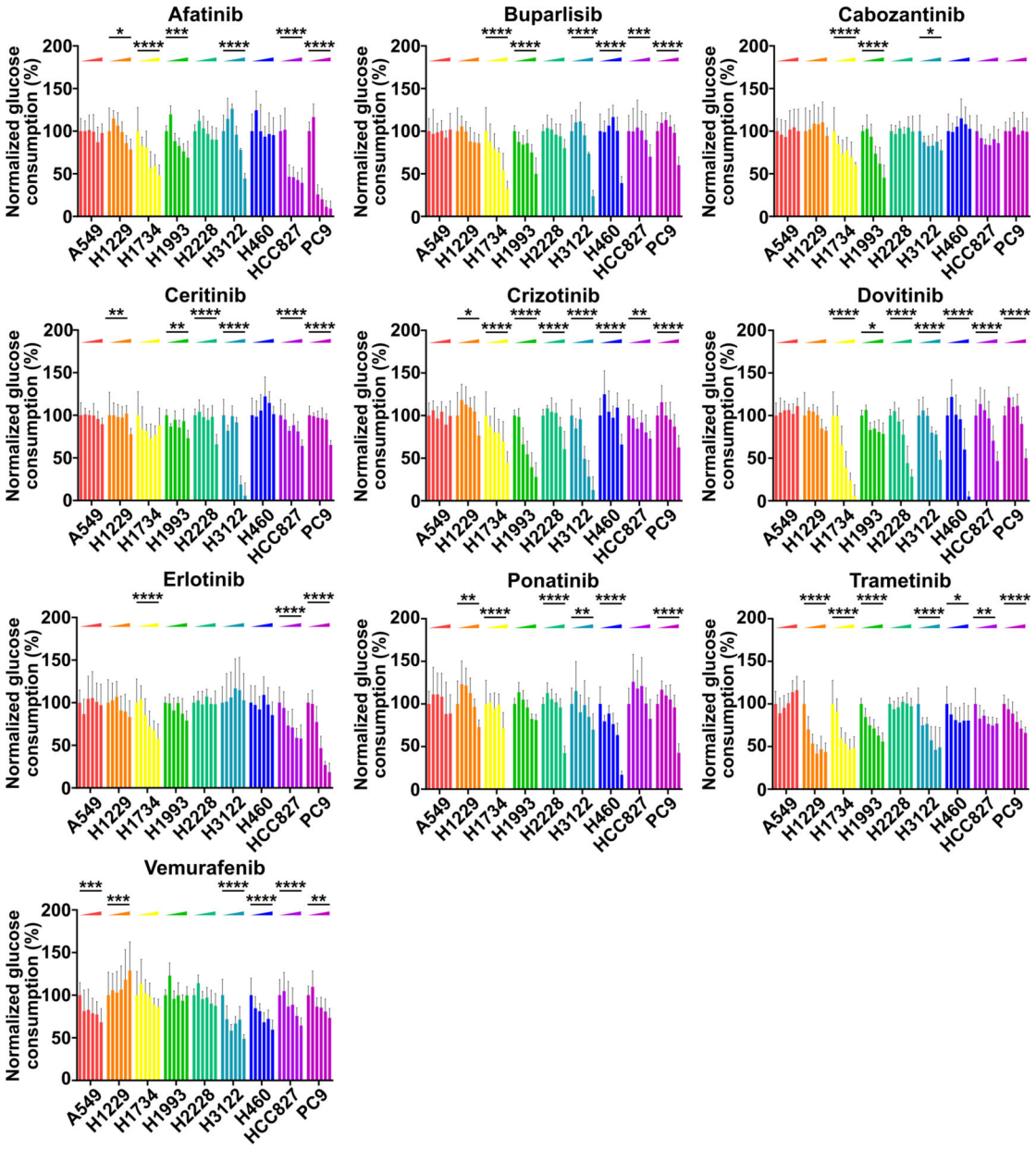


Fig. 2: Each tested kinase inhibitor blocks glucose consumption in a unique subset of cell lines at 24 hours post-inhibitor treatment.

Glucose consumption per cell in the 9 cell lines treated with the 10 kinase inhibitors across five inhibitor concentrations. Glucose consumption normalized to DMSO treatment for each cell line. First bar for each cell line – inhibitor combination represents DMSO-treated cells. Drug concentrations increase from left to right for each treatment graph. n=32–48 for DMSO-treated cells; n=6 for inhibitor-treated cells; statistical comparisons are between control and maximum inhibitor concentration. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001. Data plotted as mean ± standard deviation.

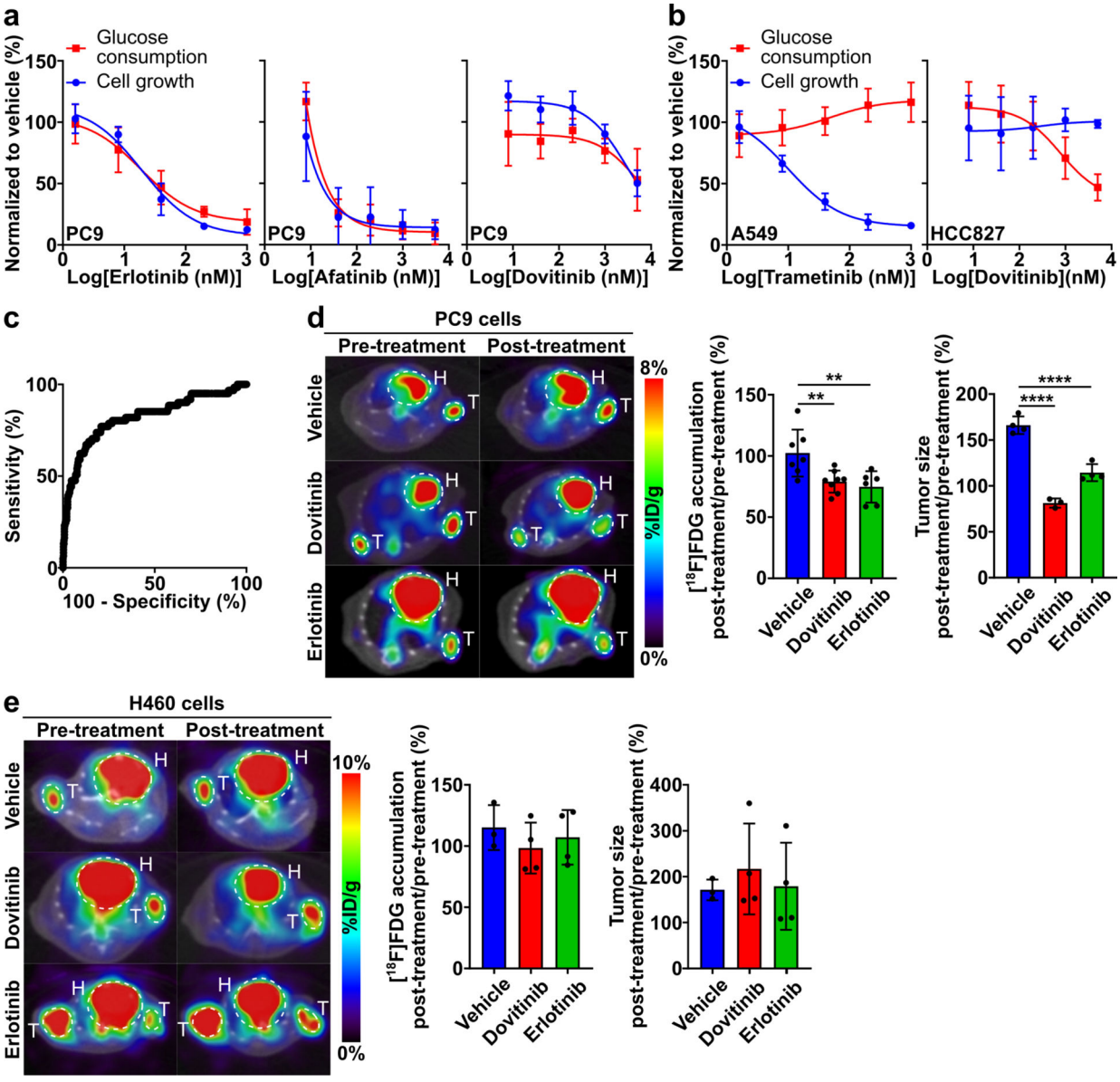


Fig. 3: Early inhibition of glucose consumption is strongly predictive of subsequent inhibition of cell growth across cell lines and kinase inhibitors.

Glucose consumption measured at 24 hours post-inhibitor treatment and cell growth measured at 72 hours post-inhibitors in **(a)** PC9 cells treated with erlotinib (*left*), afatinib (*middle*), and dovitinib (*right*) and **(b)** A549 cells treated with trametinib (*left*) and H2228 cells treated with dovitinib (*right*). n=6. **(c)** Receiver operating characteristic (ROC) curve for predicting inhibition of cell growth based on inhibition of glucose consumption. **(d)** Changes in [^{18}F]FDG accumulation and tumor size in PC9 xenografts following 24 hours and 7 days treatment, respectively, with vehicle, dovitinib, and erlotinib. Representative [^{18}F]FDG PET images (*left*); quantification of [^{18}F]FDG PET and tumor growth (*right*). [^{18}F]FDG accumulation: vehicle: n=7, dovitinib: n=8, erlotinib: n=6. Tumor size: vehicle: n=4, dovitinib: n=3, erlotinib: n=4. **(e)** Changes in [^{18}F]FDG accumulation and tumor size in H460 xenografts following 24 hours and 7 days treatment, respectively, with vehicle,

dovitinib, and erlotinib. Representative [^{18}F]FDG PET images (*left*); quantification of [^{18}F]FDG PET and tumor growth (*right*). [^{18}F]FDG accumulation: vehicle: n=3, dovitinib: n=4, erlotinib: n=4. Tumor size: vehicle: n=3, dovitinib: n=4, erlotinib: n=4. **: P<0.01; ****: P<0.0001. Data plotted as mean ± standard deviation.

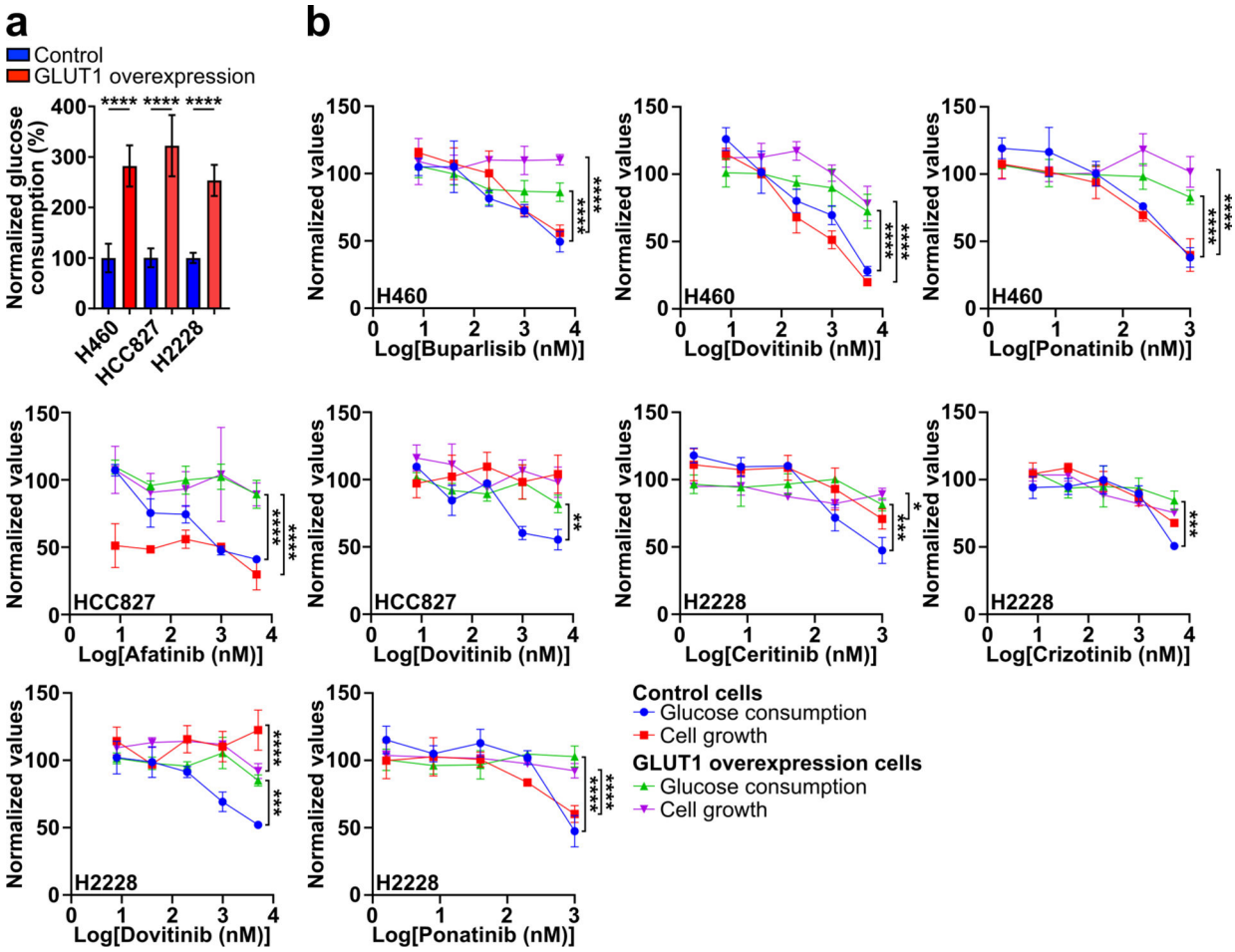


Fig. 4: Inhibitor-induced inhibition of glucose consumption is necessary for therapeutic efficacy in a subset of cell lines and kinase inhibitors.

(a) Glucose consumption in control and GLUT1 overexpressing H460, HCC827, and H2228 cells. Values normalized to control cells for each cell line. n=15. **(b)** Glucose consumption and cell growth in control and GLUT1 overexpression H460, HCC827, and H2228 cells treated with various kinase inhibitors. n=3. *: P<0.05; ***: P<0.001; ****: P<0.0001. Data plotted as mean ± standard deviation.