TITLE: Lactic acidosis induces resistance to the pan-Akt inhibitor uprosertib in colon cancer cells

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Abstract

Background
Akt signalling regulates glycolysis and drives the Warburg effect in cancer, thus decreased glucose utilisation is a pharmacodynamic marker of Akt inhibition. However, cancer cells can utilise alternative nutrients to glucose for energy such as lactate, which is often elevated in tumours together with increased acidity. We therefore hypothesised that lactic acidosis may confer resistance to Akt inhibition.

Methods
The effect of the pan-Akt inhibitor uprosertib (GSK2141795), on HCT116 and LS174T colon cancer cells was evaluated in the presence and absence of lactic acid in vitro. Expression of downstream Akt signalling proteins was determined using a phosphokinase array and immunoblotting. Metabolism was assessed using $^1$H nuclear magnetic resonance spectroscopy, stable isotope labelling and gas chromatography-mass spectrometry.

Results
Lactic acid induced resistance to uprosertib was characterised by increased cell survival and reduced apoptosis. Uprosertib treatment reduced Akt signalling and glucose uptake irrespective of lactic acid supplementation. However, incorporation of lactate carbon and enhanced respiration was maintained in the presence of uprosertib and lactic acid. Inhibiting lactate transport or oxidative phosphorylation was sufficient to potentiate apoptosis in the presence of uprosertib.

Conclusions
Lactic acidosis confers resistance to uprosertib, which can be reversed by inhibiting lactate transport or oxidative metabolism.
BACKGROUND

Historically, upregulated glycolysis (also known as the Warburg effect) was considered necessary to provide rapidly proliferating cancer cells with sufficient energy and biosynthetic intermediates\(^1\). However, we now appreciate that cancer cell metabolism is far more flexible and heterogeneous\(^2\). For instance, tumours utilise mitochondrial metabolism and oxidative phosphorylation (OXPHOS)\(^3\) for energy production and anabolism. Furthermore, glucose concentrations within tumours are often low\(^4\), therefore, cancer cells must adapt to survive under severe nutrient stress, often through utilisation of alternative nutrients such as amino acids and lipids\(^5\)\(^–\)\(^9\).

Lactate is the end product of glycolysis and is often present at elevated concentrations within tumours (10 - 20 mM)\(^1\)\(^–\)\(^10\)\(^,\)\(^11\). Lactate transport via monocarboxylate transporters (MCTs) is bi-directional\(^12\)\(^–\)\(^13\), thus cells can import lactate, to be used as a fuel for oxidative metabolism and gluconeogenesis\(^3\)\(^,\)\(^14\)\(^–\)\(^16\).

Moreover, preferential utilisation of lactate as a carbon source over glucose in the tricarboxylic acid (TCA) cycle has been suggested to occur in some human lung tumours\(^15\). It is becoming increasingly apparent that the role of lactate in promoting cancer cell survival and progression has been underestimated.

Akt signalling is implicated in multiple mechanisms that upregulate glucose metabolism, thus, oncogenic Akt activity is considered a key driver of the Warburg effect in cancer\(^17\). As a result, therapeutic targeting of Akt leads to reduced glucose uptake, which is considered a pharmacodynamic marker of Akt inhibition\(^18\).

Uprosertib (GSK2141795) is an ATP-competitive pan-Akt inhibitor in phase II clinical trials\(^19\)\(^–\)\(^21\). Despite this, efforts to identify patients that are most likely to respond have
been relatively unsuccessful, with most responders demonstrating tumour growth inhibition and stable disease, as opposed to disease regression\textsuperscript{22}. Since uprosertib treatment leads to reduced glucose uptake \textit{in vitro} and in patient tumours\textsuperscript{19,23}, in the current study we investigated whether lactate oxidation could induce resistance to uprosertib. We report that lactic acidosis induces resistance to uprosertib in colon cancer cells, which is reversed upon abolition of MCT activity or therapeutic inhibition of OXPHOS.

**METHODS**

**Cell culture**

The human colon cancer cell line, HCT116, was a generous gift from Dr. Volker Arlt at King’s College London (London, UK). The human colon cancer LS174T cells, with either wild-type or MCT4 knockout\textsuperscript{24} were generously provided by Prof. Jacques Pouysségur at the University of Nice (Nice, France). Dulbecco’s Modified Eagle’s Medium (DMEM; Cat #A14430; Gibco, Grand Island, New York, US), which contains a sodium bicarbonate buffer, was used for routine culture and all experiments. For routine culture, DMEM was supplemented with penicillin/ streptomycin (100 µg/ mL), D-glucose (5.6 mM), L-glutamine (2 mM) and 10% foetal bovine serum purchased from Gibco (Grand Island, New York, US). Cells were maintained in a 37 °C, 5% CO\textsubscript{2} humidified incubator. Cell lines were authenticated using short-tandem repeat DNA profiling by Public Health England and mycoplasma tested.

Prior to experiments, cells were gradually adapted to increasing lactate concentrations to reduce the potential for cellular stress and growth inhibition caused by exogenous lactic acid supplementation. To do this, 3 x 10\textsuperscript{6} cells were cultured in
T150 flasks for 3 days in routine culture medium to produce conditioned medium containing lactate. Cells were subsequently plated in conditioned medium for 24 hours before experimental conditions were applied.

**Sulforhodamine B (SRB) assay**

To investigate the cytotoxic effects of uprosertib (Selleckchem, Houston, Texas, US) SRB assays were performed according to manufacturer’s instructions. Cells were plated into 96-well plates at a density of 4 x 10^4 cells per well and incubated for 24 hours. Media were subsequently changed to DMEM (0 hours) supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS, lactic acid (0 to 20 mM; Sigma-Aldrich, St. Louis, Missouri, US) and uprosertib (0 to 15 µM) or vehicle (0.1% DMSO). Test media were replenished every 24 hours to control for changes in extracellular metabolite concentrations. The SRB assay was performed at 0 hours and at 72 hours post initial treatment. Data were presented as the Log2 of the optical density (OD; 565 nm) at 72 hours relative to the OD at 0 hours.

**Cell counts**

Cells were plated at a density of 1 x 10^5 cells per well into 12-well plates and incubated for 24 hours. DMEM supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS, lactic acid (0 to 20 mM) and uprosertib (10 µM) or vehicle (0.1% DMSO) was subsequently added to wells. Media were replenished every 24 hours. TrypLe Express (ThermoFisher Scientific, Massachusetts, US) was used to des-badhere cells from wells. Cells were counted using the Vi-Cell XR Cell Viability
Analyzer (Beckman Coulter, Indianapolis, US) at 0 hours and 72 hours after initial

treatment.

**Caspase 3/7 and ATP measurements**

Cells were plated at 4 x 10^4 cells per well into 96-well plates and incubated for 24

hours before media were replaced with test media (0 hours) supplemented with

glucose (5.6 mM), glutamine (2 mM), 10% FBS, lactic acid (0 to 20 mM) and

uprosertib (5 µM or 10 µM) or vehicle (0.1% DMSO). Apoptosis was determined at

24 or 48 hours post initial treatment using the Caspase-Glo 3/7 assay system

according to the manufacturer’s instructions (Promega, Madison, Wisconsin, US).

ATP was measured after 24 hours of treatment using the CellTitre-Glo® 3-D

luminescent assay (Promega, Madison, Wisconsin, US) according to the

manufacturer's instructions. Luminescence was measured using the CLARIOstar

plate reader (BMG Labtech, Ortenberg, Germany) and readings were normalised

first to cell density determined using an SRB assay performed in a parallel plate and

second to the vehicle controls.

**Culture of 3-D spheroids**

HCT116 cells were plated into 96-well Ultra-Low Attachment plates (Corning, New

York, US) at a density of 1 x 10^3 cells per well in 50 µL of media supplemented with

glucose (5.6 mM), glutamine (2 mM), 10% FBS and lactic acid (0 or 10 mM) and

incubated for 24 hours to allow 3-D spheroids to form. Subsequently, 50 µL of test

media containing uprosertib (0 to 15 µM) was added on top of the original 50 µL and

spheroids were incubated for 72 hours. Brightfield images of spheroids were
obtained using the IN Cell Analyzer 2000 (GE Healthcare, Chicago, Illinois, US) using a 4 x 0.2 NA objective lens. Scale bars represent 100 µm and were added using Image-J software. To quantify spheroid viability after 72 hours of uprosertib treatment, we used the CellTitre-Glo® 3-D luminescent assay (Promega, Madison, Wisconsin, US) according to the manufacturer’s instructions.

**Phospho-kinase array**

LS174T cells were treated with uprosertib (10 µM) in the presence or absence of lactic acid (10 mM) for 1 hour before cells were lysed and protein was examined using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN, US) according to the manufacturer’s protocol. Chemiluminescent signals were exposed using the Supersignal™ West Pico plus chemiluminescent substrate (Thermo Scientific, Rockford, US) onto Amersham Hyperfilm™ ECL films (GE Healthcare, Chicago, Illinois, US) using the Optimax X-ray film processor (Protec Healthcare, Oberstenfeld, Germany). Densitometry was performed using Image-J software and data were normalised to the loading control antibodies on each membrane. The fold change in phosphorylation compared to the 0 mM lactic acid vehicle controls was subsequently calculated.

**Western blotting**

Cells were plated into 6-well plates at a density of 5 x 10^5 cells per well and incubated for 24 hours, before media were replaced with DMEM (0 hours) supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS, lactic acid (0 or 10 mM) and uprosertib (10 µM) or vehicle (0.1% DMSO). After 1 hour, media were aspirated from wells and cells were washed with 1 x PBS. On ice, RIPA lysis buffer
(Sigma-Aldrich, St. Louis, Missouri, US) containing 1 x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, Massachusetts, US) was added to wells, which were scraped to extract whole-cell protein lysates. Protein quantification was determined using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, US) according to the manufacturer’s protocol. Cell lysates containing 15 µg of protein were loaded onto 4-20% Mini-PROTEAN® TGXTM Precast Protein Gels (Bio-Rad, Hercules, California, US) and separated by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis before being transferred to nitrocellulose membranes. Membranes were incubated using selected antibodies and chemiluminescent signals were enhanced using the Supersignal™ West Pico plus chemiluminescent substrates (Thermo Scientific, Rockford, US) and detected on Amersham Hyperfilm™ ECL films (GE Healthcare, Chicago, Illinois, US) using the Optimax X-ray film processor (Protec Healthcare, Oberstenfeld, Germany). Densitometry was performed using Image-J software. Data were normalised to the relevant total protein and subsequent fold change in phosphorylation compared to the 0 mM lactic acid vehicle control was calculated. The following primary antibodies were used: Pan Akt (C67E7, #4691), phosphorylated Akt Serine (S) 473 (D9E, #4060), Phosphorylated PRAS40 Threonine (T) 246 (#2640) and total PRAS40 (#2610) purchased from Cell Signalling Technologies (Danvers, Massachusetts, US). Anti-β-actin (#A5441) was purchased from Sigma-Aldrich (St. Louis, Missouri, US).

1H nuclear magnetic resonance (NMR) spectroscopy

Cells were plated into 12-well plates at a density of 1 x 10^5 cells per well and incubated for 24 hours, before media were replaced with DMEM (0 hours)
supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS, lactic acid (0 or 10 mM) and uprosertib (10 µM) or vehicle (0.1% DMSO). After a further 24 hours, media were collected from wells into microcentrifuge tubes and centrifuged at 150 g for 5 mins. A volume of 550 µL of each media sample was transferred to a clean microcentrifuge tube. Subsequently, 50 µL of the internal calibration standard 4-4-dimethyl-4-silapentane-1-sulfonic acid in deuterium oxide (12 mM) was added before tubes were vortexed and centrifuged at 20 000 g for 1 minute. Samples were transferred into 5 mm diameter NMR economy sample tubes (Wilmad-LabGlass, New Jersey, US).

High-resolution 1-dimensional ¹H NMR spectroscopy was performed using the 14.1 T Bruker AVANCE 400 MHz spectrometer (Bruker BioSpin, Billerica, Massachusetts, US) at 298 K. NMR spectra were acquired using a conventional ZGPR solvent pre-saturation method with a single radiofrequency pulse, a recycle delay (d1) of 4 seconds, spectral width of 6402.049 Hz, 32 free induction decays and 64 000 data points. Data were automatically Fourier-transformed before being processed in MATLAB® software (Mathworks) using in-house scripts developed by J.T. Pearce, H.C. Keun, T.M.D. Ebbels and R. Cavill at Imperial College London (London, UK). Phase correction, baseline correction and normalisation to the internal standard reference peak was automatically done before spectral peaks were identified with reference to the Human Metabolome Database.

The rate of metabolite uptake and release was determined by calculating the difference in metabolite concentration (X) in spent medium compared to the initial medium. These values were subsequently normalised to the cell number obtained
(area under the curve) using the Vi-Cell XR cell viability analyser, to give the rate in fmol/ cell/ hour. Negative values were converted to positive values and referred to as metabolite uptake.

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\text{Metabolite uptake and release} = \frac{[X]_{\text{spent}} - [X]_{\text{initial}}}{\text{Area under the curve}}
\]

**Stable isotope labelling experiments by gas chromatography-mass spectrometry (GC-MS)**

HCT116 and LS174T cells were seeded into 6-well plates at a density of 6 x 10^5 cells per well and incubated for 24 hours. For glucose-labelling experiments media were supplemented with glutamine (2 mM), lactic acid (10 mM) and ^13^C_6-glucose (5.6 mM, Sigma Aldrich, Missouri, US). For lactic acid-labelling experiments media were supplemented with glutamine (2 mM), glucose (5.6 mM) and ^13^C_3-lactic acid (10 mM, Sigma Aldrich, Missouri, US). Media were also supplemented with uprosertib (10 \(\mu\)M) or vehicle (DMSO, 0.1%). Cells were treated for 4 hours before intracellular metabolites were extracted and aqueous fractions were analysed using the Agilent 7890 GC system linked to an Agilent 5975 Mass Selective Detector using methods published previously^26. AMDIS software was used with reference to the NIST mass spectral library^27 to identify metabolites. Peak integration was done using the in-house developed GAVIN^28 scripts for MATLAB® (MathWorks).

**Respiration measurements**

To measure the oxygen consumption rate (OCR), LS174T cells were seeded at a density of 1 x 10^5 cells per well into 96-well plates and incubated for 24 hours. Cells were subsequently dosed in media (90 \(\mu\)L) supplemented with uprosertib (10 \(\mu\)M) and metformin (10 mM) for 2 hours, before 10 \(\mu\)L of the MitoXpress Xtra Oxygen
Consumption reagent (Agilent Technologies, Santa Clara, California, US) was added to each well according to the manufacturer's instructions. Positive controls containing carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP; 1.25 µM) were used. Wells were rapidly sealed with a layer of pre-warmed mineral oil and the plate was immediately read using the CLARIOstar plate reader at 37 °C (BMG Labtech, Ortenberg, Germany) in continuous cycles for 2 hours. Fluorescent lifetime (µs) was calculated using the CLARIOstar MARS data analysis software (BMG Labtech, Ortenberg, Germany). OCR (µs, hour) was determined by calculating the maximum of the slope of the fluorescent lifetime.

Statistical analysis

Statistical significance (*p<0.05; **p<0.01; ***p<0.001 or #p<0.05; ##p<0.01; ###p<0.001) comparing groups with two independent variables was calculated using two-way ANOVA with Bonferroni’s multiple comparisons post hoc test. Statistical significance comparing three or more independent groups was calculated using one-way ANOVA with Bonferroni’s multiple comparisons post hoc test. Statistical significance (*p<0.05; **p<0.01; *** p<0.001) comparing two independent groups was calculated using the Student’s t test. Calculations were performed and graphs were plotted using GraphPad Prism software version 8.10.

RESULTS

Lactic acidosis induces resistance to uprosertib in colon cancer cell lines.

SRB cytotoxicity assays were used to determine the dose-response to uprosertib (1 to 15 µM) in the presence or absence of lactic acid (0, 10 or 20 mM) in HCT116 and LS174T cells after 72 hours of treatment (Fig. 1a). Results were presented as Log2 of
the OD at 72 hours normalised to the 0 hour OD to determine the cytotoxic or
cytostatic effects of uprosertib treatment. Adding 20 mM of exogenous lactic acid
reduced growth of HCT116 cells (Fig. S1), therefore this concentration was not used
for further investigation of this line.

Uprosertib induced dose-dependent cytotoxicity in both cell lines in the absence of
lactic acid. Lactic acid supplementation mediated a significant increase in the relative
cell growth compared to conditions in the absence of lactic acid. Specifically, a
cytostatic response to uprosertib in the presence of lactic acid was observed.
Increasing the concentration of lactic acid increased the resistance to uprosertib,
which was also demonstrated using cell counts (Fig. 1b).

To further investigate the role of lactic acid in protecting cells from the cytotoxic
effects of uprosertib, apoptosis was measured using a Caspase-Glo 3/7 assay after
24 hours of treatment (Fig. 1c). Results confirmed that uprosertib induced a dose-
dependent increase in caspase-3/7 activation in HCT116 and LS174T cell lines,
however, lactic acid mediated a significant reduction in caspase-3/7 activity. In
LS174T cells, the higher the lactic acid concentration, the lower the caspase-3/7
activity; thus lactic acid was associated with reduced apoptosis induction in cells
treated with uprosertib. Uprosertib treatment in the presence of lactic acid was also
associated with a significant increase in ATP levels (Fig. 1d). Overall, these data
indicate that lactic acidosis induces resistance to uprosertib by promoting survival
and a switch from a cytotoxic to a cytostatic response to treatment. Similar results
were also observed in the presence of pyruvic acid (Fig. S2), which is another
monocarboxylate that is transported via MCTs$^{12,13}$. 
Supplementing media with lactic acid was necessary for resistance to occur while supplementing with sodium lactate was insufficient (Fig. S3). Since lactic acid has a pKa of 3.86, it will dissociate into the lactate anion and H⁺ in media, lowering the pH (to 6.8 at 10 mM lactic acid, Fig. S4a). Lowering the extracellular pH to 6.5 alone using hydrochloric acid in the absence of lactate was not sufficient for protection against the cytotoxic effects of uprosertib (Fig. S4b).

Lactic acidosis induces resistance to uprosertib in HCT116 3-D spheroids.

It is well established that 3-D culture models better recapitulate the tumour microenvironment than monolayer culture by promoting the natural formation of pH, oxygen and nutrient gradients, for example. We therefore used 3-D spheroids to investigate the response to uprosertib in the presence or absence of lactic acid (10 mM). Since LS174T cells did not readily form tight spheroids (data not shown), they were not investigated using this method.

A dose-dependent reduction in spheroid viability and size occurred upon treatment with uprosertib (Fig. 1e), however, spheroid viability was elevated in conditions with lactic acid supplementation compared to spheroids treated in the absence of lactic acid (p=0.0145). Visualisation of spheroid morphology showed that spheroid integrity was better maintained at higher doses of uprosertib (12.5 µM and 15 µM) in the presence of lactic acid compared to spheroids treated in the absence of lactic acid. These data support the conclusion that lactic acidosis is associated with resistance and enhanced viability of colon cancer cells treated with uprosertib.
Uprosertib treatment decreases downstream Akt signalling irrespective of the presence or absence of lactic acid.

Studies have demonstrated that uprosertib treatment leads to reduced phosphorylation of multiple proteins downstream of Akt, as well as an increase in feedback phosphorylation of Akt itself\textsuperscript{19,20}. Therefore, we investigated whether downstream inhibition of Akt signalling induced by uprosertib was altered in the presence of lactic acid. We used a human phosphokinase array to examine the expression of multiple phosphoproteins in LS174T cells after 1 hour of uprosertib treatment (10 \( \mu \)M) in the presence or absence of lactic acid (10 mM) (Fig. 2a).

Densitometry was performed and data were expressed as the mean fold change in phosphorylation relative to the 0 mM lactic acid vehicle control (0.1 % DMSO) (Fig. 2b).

Uprosertib treatment in the absence of lactic acid caused a reduction in phosphorylation of several proteins downstream of Akt activation including GSK-3\( \alpha/\beta \) (S21/S9), PRAS40 (T246), CREB (S133) and WNK1 (T60). Further, a moderate reduction in p70 s6 kinase phosphorylation (T421, S424 and T389), no change in TOR phosphorylation (S2446) and increased Akt phosphorylation at both T308 and S473 were observed, which corresponds with previous reports of the consequences of uprosertib treatment\textsuperscript{19}. Lactic acid supplementation was associated with a slight increase in phosphorylation of Akt (T308), as well as several other downstream phosphoproteins including p70 s6 kinase (T421, S424 and T389), eNOS (S1177), WNK1 (T60) and PRAS40 (T246). Despite this, uprosertib treatment produced the same alterations to protein phosphorylation regardless of exogenous lactic acid supplementation. Western blotting subsequently validated that uprosertib treatment
increased phosphorylation of Akt (S473) and decreased PRAS40 (T246) phosphorylation in the presence and absence of lactic acid (Fig. 2c). LC-MS/MS analysis also confirmed that cellular uptake of uprosertib was the same in the presence and absence of exogenous lactic acid (Fig. S5). Overall, these data indicate that uprosertib-induced changes in Akt signalling are unaffected by the presence of lactic acid.

**Uprosertib inhibits glucose uptake and utilisation.**

Reduced glucose uptake is a pharmacodynamic marker of uprosertib treatment\(^2^3\), therefore, we investigated how glucose uptake and utilisation was influenced by uprosertib (10 µM) in the presence and absence of lactic acid (10 mM) using \(^1\)H NMR spectroscopy (Fig. 3).

In the absence of lactic acid supplementation, uprosertib treatment caused a significant reduction in the rate of glucose uptake in both lines (40% and 48% reduction in HCT116 and LS174T cells respectively, Fig. 3a). Uprosertib treatment in the presence of lactic acid reduced the rate of glucose uptake in both lines to that observed with treatment in the absence of lactic acid. The reduction in glucose uptake in the presence of lactic acid and uprosertib was significant in LS174T cells. Uprosertib treatment in the presence of lactic acid also caused reductions in the rate of lactic acid release in LS174T cells (Fig. 3b). Lactic acid supplementation alone did not significantly alter the glucose uptake rate or lactate release rate in either line.

Since previous reports have indicated that lactate utilisation stimulates oxidative glutaminolysis\(^3^0\), we also examined glutamine uptake in cells treated with uprosertib
in the presence or absence of lactic acid (Fig. S6a and Fig. S6b). Uprosertib and
lactic acid caused a modest reduction in the glutamine uptake rate in both lines (12-
46\% in HCT116 and 8-17\% in LS174T cells), although these differences were not
statistically significant.

To further investigate how glucose utilisation was influenced by uprosertib and
exogenous lactic acid, stable isotope labelling using $^{13}$C$_6$-glucose was used to trace
the fate of glucose carbons after 4 hours of treatment (Fig. 3c). Uprosertib treatment
regardless of lactic acid did not change the $^{13}$C labelling pattern of pyruvate or
lactate in both HCT116 (Fig. 3d) and LS174T (Fig. 3e) cells. However, labelling into
citrate M+2 was reduced significantly by uprosertib (50\%) in the absence of lactic
acid in both lines, consistent with reduced contribution of glucose carbon into the
TCA cycle via pyruvate dehydrogenase (PDH) flux. In untreated HCT116 and
LS174T cells, lactic acid supplementation decreased the relative abundance of M+3
isotopologues of pyruvate and lactate and reduced the proportion of citrate M+2,
consistent with unlabelled lactate diluting out label contributions from $^{13}$C glucose to
pyruvate. Under these conditions no subsequent effect of uprosertib on citrate M+2
enrichment was observed in HCT116 cells and only a minor reduction in the LS174T
cell line.

These data indicate that while uprosertib can inhibit glycolysis irrespective of lactic
acid supplementation, in the absence of lactic acid uprosertib also limits glucose-
derived pyruvate entry into the TCA cycle via PDH. By contrast, during lactic
acidosis, the contribution of glucose to citrate production is lower and is not
significantly reduced further by uprosertib compared to vehicle.
The metabolic fate of exogenous lactic acid is unaffected by uprosertib treatment.

As lactic acid supplementation appeared to change glucose metabolism and the effect of uprosertib treatment, we tested whether exogenous lactate uptake and metabolism could be detected in these conditions. To investigate this, we used $^{13}$C$_3$-lactic acid to trace the fate of lactate carbons in cells treated with uprosertib for 4 hours before intracellular metabolites were extracted and analysed using GC-MS (Fig 4a). Mass isotopologue distributions (MIDs) show that despite net production of lactate, $^{13}$C from exogenous lactic acid contributed significantly to the generation of pyruvate M+3 and citrate M+2 in both lines (Fig. 4b and Fig. 4c). Significantly higher enrichment of $^{13}$C$_3$-lactic acid into pyruvate M+3 and citrate M+2 compared to $^{13}$C$_3$-sodium lactate occurred in LS174T cells (Fig. S7) consistent with prior reports that lowering pH enhances $^{13}$C-lactate uptake and TCA cycle entry$^{31}$. This observation may also explain why sodium lactate in the absence of acidosis was not protective against uprosertib. Upon uprosertib treatment no significant alteration to the incorporation of $^{13}$C$_3$-lactic acid carbon into pyruvate or citrate was observed. These data, together with our $^{13}$C glucose labelling experiments, suggest that lactic acid supplementation decouples the TCA cycle from glycolysis in both cell lines tested and that the metabolism of exogenous lactate is unaffected by treatment with uprosertib.

Since the co-factor NAD$^+$ is necessary for lactate dehydrogenase to catalyse the conversion of lactate to pyruvate, the NAD$^+$/NADH ratio was determined. No significant changes in the NAD$^+$/NADH ratio in the presence of lactic acid or
uprosertib treatment were observed in HCT116 (Fig. S8a) or LS174T cells (Fig S8b), although a moderate reduction in HCT116 cells occurred.

**Inhibiting MCTs or oxidation is sufficient to rescue uprosertib-induced apoptosis in cancer cells exposed to lactic acidosis.**

Since exogenous lactate contribution to the TCA cycle was maintained in cells treated with uprosertib, we hypothesised that inhibiting lactate uptake via MCTs or oxidation could re-sensitise cells to uprosertib in the presence of lactic acid. Thus cells were treated with uprosertib (10 µM) in combination with either the MCT1 inhibitor, AZD3965 (1 µM), or the mitochondrial complex I inhibitor metformin\(^{32,33}\) (0.5 mM to 10 mM) for 24 hours, before assessing sensitivity using a Caspase-Glo 3/7 assay (Fig. 5). MCT1 and MCT4 are functionally redundant\(^{34}\), therefore LS174T cells with MCT4\(^{-/-}\) status were used for experiments with AZD3965 treatment. MCT4 knockout was confirmed using western blotting (Fig. S9a).

Inhibition of lactate uptake using AZD3965 in LS174T-MCT4\(^{-/-}\) cells significantly rescued sensitivity to uprosertib in the presence of lactic acid (Fig. 5a). \(^{13}\)C\(_3\)-lactic acid labelling confirmed AZD3965 treatment blocked lactate carbon entry into cells (Fig. S9b). In contrast, in LS174T-wild type cells, AZD3965 treatment did not block \(^{13}\)C\(_3\)-lactic acid entry or rescue sensitivity to uprosertib in the presence of lactic acid (Fig. S9c and S9d).

Metformin as a monotherapy up to 10 mM did not induce apoptosis in either cell line (Fig. 5b and Fig. 5c). However, metformin treatment at the same doses in combination with uprosertib potentiated apoptosis in both lines irrespective of lactic
acid supplementation. Notably in both lines, metformin treatment under lactic acidosis was sufficient to restore caspase-3/7 activity induced by uprosertib to levels observed in cells treated with uprosertib alone in the absence of lactic acid. The same rescue in sensitivity was observed using the Complex I and III inhibitors rotenone and antimycin A in both lines (Fig. S10), supporting the importance of OXPHOS in the protective effect of lactic acid.

To confirm that metformin was inhibiting respiration, the OCR was measured using the MitoXpress Xtra oxygen probe in LS174T cells (Fig. 5d). FCCP was used to measure the maximum oxygen consumption capacity. Results showed that metformin treatment reduced the OCR in all conditions tested, thus confirming that inhibiting oxidation is associated with enhanced sensitivity to uprosertib treatment. Furthermore, uprosertib treatment did not significantly alter the OCR, whereas lactic acid supplementation enhanced the basal OCR and significantly increased the maximum respiratory capacity of cells, as indicated in FCCP treated conditions. Overall, these data confirm that targeting mitochondrial oxidation using metformin, OXPHOS inhibitors or blocking lactate uptake can potentiate apoptosis and restore sensitivity to uprosertib treatment under lactic acidosis.

**DISCUSSION**

In the present study, we demonstrated that lactic acid supplementation is sufficient to induce resistance to the Akt inhibitor uprosertib in LS174T and HCT116 colon cancer cell lines and present evidence that metabolic factors may play a role (Fig. 6). Clinical reports indicate that uprosertib treatment as a monotherapy is primarily associated with tumour growth inhibition and stable disease at the recommended
phase II dose (75 mg) in patients with solid tumours\textsuperscript{22}. Since lactate concentrations
and acidity are often elevated in the tumour microenvironment, the cytostatic
response to uprosertib in the presence of lactic acid in our experimental system
potentially mimics the stable disease response that has been observed in patients.

Glycolysis inhibition occurred upon treatment with uprosertib in the presence and
absence of lactic acid supplementation. Similarly, uprosertib treatment has been
shown to cause dose-dependent hyperglycaemia and reduced glucose uptake in
patients with gynaecological malignancies\textsuperscript{22,23}. Glucose carbon entry into the TCA
cycle via PDH was also inhibited by uprosertib, consistent with previous work that
suggests a direct interaction between PDH subunit B and AKT1 and that silencing of
AKT1 could reduce PDH flux from \textsuperscript{13}C labelled glucose\textsuperscript{35}. By contrast exogenous
lactate incorporation into citrate was maintained in cells treated with uprosertib.
Furthermore, lactic acidosis stimulated an increase in respiration and respiratory
capacity in our cell models. Thus, a decoupling of glycolysis from the TCA cycle and
enhanced oxidative metabolism induced by lactic acid might be a mechanism of
resistance to uprosertib.

The proportion of citrate M+2 derived from \textsuperscript{13}C\textsubscript{3}-lactate was greater than that derived
from \textsuperscript{13}C\textsubscript{6}-glucose, consistent with previous observations that isotope labelling from
circulating lactate to the TCA cycle exceeds that from glucose in most tissues \textit{in vivo}\textsuperscript{15,16}. Despite this, isotope exchange fluxes can lead to overestimations of the net
contribution of lactate into the TCA cycle\textsuperscript{31}. Several models describing intracellular
compartimentalisation of lactate/ pyruvate metabolism have been proposed, including
the presence of intracellular mitochondrial shuttles for lactate oxidation\textsuperscript{36–38}; this may also explain our observations.

Several studies have previously described associations between altered tumour metabolism and treatment efficacy. Notably, estrogen-related receptor alpha dependent lactate oxidation renders breast cancer cells resistant to phosphatidylinositol-3-kinase (PI3K) and mammalian target of rapamycin (mTOR) inhibitors cultured in the absence of glucose\textsuperscript{39}. Additionally, enhanced mitochondrial OXPHOS coupled with suppressed glycolysis is associated with therapeutic resistance to several other targeted therapies in cancer cells\textsuperscript{40–42}. Overall, these data combined with findings from the present report highlight the importance of metabolic flexibility in cancer cells in response to targeted therapies.

Clinical investigations combining uprosertib with the MEK inhibitor trametinib in several cancers types have been underway, however, similar studies in multiple myeloma, melanoma and cervical cancer have revealed no clinical benefits of this combination\textsuperscript{43–46}. Although uprosertib treatment as a monotherapy has been well tolerated\textsuperscript{22,23}, the adverse toxicity profile of phase II combination studies has been deemed unacceptable, thus, uprosertib is not currently under further development\textsuperscript{46}. Since we have demonstrated that enhanced OXPHOS in the presence of lactic acid is related to uprosertib resistance, and previous reports highlight that resistance to MEK combination therapies also corresponds with enhanced OXPHOS\textsuperscript{41}, this metabolic phenotype should be considered as a possible contributor to the lack of clinical efficacy observed during phase II trials with uprosertib. Thus, combining uprosertib with inhibitors of mitochondrial OXPHOS might present a valid therapeutic
strategy to improve responses at tolerated doses. In support of this, metformin has been demonstrated to cause metabolic adaptations in human leukemic cells that increases sensitivity to Akt inhibition\(^{47}\).

We observed that the higher the lactic acid concentration in media, the more resistant LS174T cells were to uprosertib and also that lactate addition or media acidification alone were insufficient for protection. MCTs are proton-dependent transporters\(^{48,49}\), as a result, the direction of MCT function is dependent on the pH gradient across the membrane. Therefore, the requirement for exogenous lactic acid supplementation is consistent with enhanced lactate uptake being a necessary condition for uprosertib resistance. Such conditions have also been shown to promote reverse lactate dehydrogenase flux\(^{50}\). Although we cannot rule out completely that intracellular acidosis contributes to the protective effect, combining uprosertib with the phase I clinical candidate MCT1 inhibitor AZD3965\(^{51-53}\) in MCT4 knockout cells, rescued sensitivity to uprosertib under lactic acidosis. This suggests that interference with lactate transport could also enhance tumour response to uprosertib in the clinic.

Uprosertib treatment induced apoptosis, which was significantly reduced in the presence of lactic acid. However, other PI3K/ Akt/ mTOR inhibitors tested did not stimulate apoptosis and no lactic acid induced resistance was observed (Fig. S11 and Fig. S12). Therefore, apoptosis and cytotoxicity appear to be necessary for lactic acid induced resistance to occur. Alternative, high affinity targets of uprosertib\(^{20,21}\), other than Akt, have been proposed, and it is possible that these
interactions may drive the cytotoxic effect observed. However, a full investigation of off-target effects was beyond the scope of the current investigation.

Finally, although the present study has primarily considered lactate utilisation as a mechanism of resistance to uprosertib, lactic acidosis may also induce resistance through other mechanisms. For instance, lactate has been shown to act as an agonist for the G-protein coupled receptor 81, which upregulates expression of genes associated with lactate metabolism and survival. Lactate has also been demonstrated to increase expression of the anti-apoptotic protein Bcl-2 via the PI3K/Akt/mTOR signalling pathway, promoting survival under glucose deprivation.

Additionally, the conversion of lactate to pyruvate by lactate dehydrogenase B has been shown to promote lysosomal acidification and autophagy, which is sufficient for survival under conditions of nutrient stress, such as glucose deprivation.

In conclusion, we have demonstrated that lactic acidosis protects colon cancer cells from the cytotoxic effects of uprosertib treatment. Lactate uptake and enhanced oxidation in the presence of uprosertib was associated with resistance and inhibiting either re-sensitised cells to treatment. The results presented here highlight the potential influence of metabolic plasticity to mediate response to targeted therapy against oncogenic pathways that also regulate glucose metabolism. Further, our findings provide new insight into a potential mechanism of resistance to uprosertib, which may in part explain the limited efficacy that has been observed in clinical trials.
Supplementary information is available at the British Journal of Cancer’s website.

**Ethical approval and consent to participate**

No human or animal ethics approval was required for this study.

**Consent for publication**

No consent for publication was required for this study.

**Data availability**

Data generated for the current study are available from the corresponding author on reasonable request.

**Conflicts of interest**

The authors declare no competing financial interest.

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**Authors’ contributions**
H.C.K conceived and designed the study and managed the project. E.M.E.B designed and performed experiments, conducted analysis and drafted the manuscript. Y.X supported data collection and data analysis. A.N contributed to analysis of microscopy and respiration measurements. L.H supported metabolomics experimentation. A.P.S conducted drug uptake experiments. A.B, A.N & E.O.A contributed to interpretation of study data and experimental design. All authors discussed results and interpretation, contributed to reviewing the manuscript and approved the final submitted version.

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REFERENCES


Lactic acid induces resistance to the pan-Akt inhibitor uprosertib in colon cancer cells. **a, b** Effects of uprosertib on survival in the presence or absence lactic acid. HCT116 and LS174T cell lines were treated for 72 h with uprosertib (1 µM to 15 µM) in the presence or absence of lactic acid (0 to 20 mM) and cytotoxicity was determined using SRB assays (**a**). LS174T cells were treated with uprosertib (10 µM) for 72 h before cells were counted (**b**). DMSO (0.1%) was used as a vehicle control. The results shown are normalised to the relative 0 h controls. **c** The effect of uprosertib on apoptosis in the presence or absence of lactic acid. Cells were treated for 24 h with uprosertib (5 µM or 10 µM) in the presence or absence of lactic acid (10 or 20 mM) and apoptosis was measured using a Caspase-Glo 3/7 assay (**c**). Results are shown as caspase 3/7 induction relative to cell biomass measured using SRB and the relevant vehicle controls. **d** The effect of uprosertib treatment (5 µM, 10 µM and 15 µM) on ATP levels in the presence or absence of lactic acid in LS174T cells (**d**). Results are shown as ATP levels normalised to cell biomass measured using SRB and to the relevant vehicle controls. **e** Effect of uprosertib treatment and lactic acid on 3-D spheroids. HCT116 spheroids were dosed with uprosertib (1 µM to 15 µM) in the presence or absence of lactic acid (10 mM) for 72 h. Spheroid viability was quantified using a CellTitre-Glo 3D assay and representative images of spheroids from one independent experiment are shown (**e**). The scale bar represents 100 µm. Quantified results are shown as fold change in ATP normalised to the relevant vehicle control. The dotted line at y=1 in **b** indicates the initial 0 h cell number. The results shown are the mean ± SEM from three independent experiments (n=3). *p<0.05, **p<0.01 and ***p<0.001.
Fig. 2

Uprosertib inhibits Akt signalling in the presence and absence of lactic acid. a-c Effect of uprosertib treatment in the presence or absence of lactic acid on Akt signalling. The proteome profiler human phosphokinase array kit was used to investigate the phosphorylation of downstream Akt signalling proteins in LS174T cells dosed with uprosertib (10 µM) for 1 h in the presence or absence of lactic acid (10 mM) (a). DMSO (0.1%) was used as a vehicle control. Densitometry using Image-J was done to quantify the expression of relevant phosphoproteins and results are shown as the Log2 of the fold change in phosphorylation compared to the 0 mM lactic acid vehicle controls (mean of technical duplicates) (b). Numbers on the array correspond to the numbered protein in the graph. Phosphorylation and total Akt and PRAS40 expression were examined using western blotting in LS174T cells after 1 h of uprosertib treatment (10 µM) in the presence or absence of lactic acid (10 mM) (c). β-actin was used as a loading control. Blots shown are from one representative experiment from a minimum of three independent replicates. Densitometry was performed to quantify expression of phospho-Akt and phospho-PRAS40. Data were normalised to the relevant total protein and graphs were plotted as fold change relative to the 0 mM lactic acid vehicle control. Data were presented as mean ± SEM from three independent replicates (n=3). *p<0.05.
Uprosertib inhibits glucose utilisation in the presence and absence of lactic acid. a, b
Effect of uprosertib and lactic acid on glucose uptake and lactate release rates.

LS174T and HCT116 cells were treated with uprosertib (10 µM) in the presence or
absence of lactic acid (10 mM) for 24 hours before media were collected and cells
counted. Extracellular glucose and lactate concentrations were determined using
NMR spectroscopy and the rate of glucose uptake (a) or lactate release (b) in fmol/
cell/ hour was calculated. c Schematic representation of enrichment of $^{13}$C derived
from $^{13}$C$_6$-glucose into glycolytic and TCA cycle intermediates (c). d, e Effect of
uprosertib treatment in the presence or absence of lactic acid on glucose utilisation
in HCT116 (d) and LS174T (e) cells. Fraction labelled from $^{13}$C$_6$-glucose into
pyruvate (M+3), lactate (M+3) and citrate (M+2) in the presence or absence of lactic
acid (10 mM) after 4 hours of treatment with uprosertib (10 µM). The results shown
are the mean ± SEM from three independent experiments ($n=3$). *p<0.05, **p<0.01
and ***p<0.001.
Enrichment of $^{13}$C$_3$-lactic acid into cells is maintained in the presence of uprosertib. a Schematic representation of $^{13}$C$_3$-lactic acid enrichment into pyruvate and TCA cycle intermediates. b, c Mass isotopologue distributions of $^{13}$C$_3$-lactic acid enrichment into pyruvate (b) and citrate (c) after 4 hours of incubation in the presence of uprosertib (10 µM) in LS174T and HCT116 cell lines. Cells were incubated in 10 mM of $^{13}$C$_3$-lactic acid. DMSO (0.1%) was used for vehicle controls. The results shown are the mean ± SEM from three independent experiments ($n=3$). *$p<0.05$, **$p<0.01$ and ***$p<0.001$. 

Fig. 4
Targeting lactate transport and oxidative metabolism re-sensitises cells to uprosertib in the presence of lactic acid. **a** MCT1 inhibition using AZD3965 in combination with uprosertib in LS174T-MCT4 \(^+\) cells. LS174T-MCT4 \(^-\) cells were treated with uprosertib (10 µM) in combination with AZD3965 (1 µM) for 24 hours (**a**). Apoptosis was measured using the Caspase-Glo 3/7 assay. Results were normalised to cell density measured using SRB assays and also to the vehicle controls. The results shown are the mean ± SEM from five independent experiments (n=5). **b, c** The effect of combining metformin with uprosertib on apoptosis. HCT116 (**b**) and LS174T (**c**) cell lines were dosed with metformin (0.5 mM to 10 mM) alone or in combination with uprosertib (10 µM) for 24 hours before apoptosis was measured using a Caspase-Glo 3/7 assay. Results are shown as caspase 3/7 induction relative to cell biomass measured using SRB and also to the relevant vehicle controls. **d** The effect of metformin and uprosertib treatment on the OCR in the presence and absence of lactic acid. LS174T cells were treated with metformin and uprosertib in the presence and absence of lactic acid for 2 hours (**d**). FCCP was used as a positive control to measure the maximum respiratory capacity. The MitoXpress Xtra reagent was added to wells before fluorescence was measured over 2 hours. The OCR was calculated from the slope of the fluorescent lifetime calculated using MARS analysis software. Graphs were plotted as OCR (lifetime, µs/hr). The results shown are the mean ± SEM from four independent experiments (n=4). In **a** and **d** statistical significance is indicated by a line with \(*p<0.05\), \(**p<0.01\) or \(***p<0.001\). In **b** and **c** statistical significance compared to the 0 mM lactic acid uprosertib only condition is denoted as \(#p<0.05\), \(##p<0.01\) or \(###p<0.001\) and compared to the 10 mM lactic acid uprosertib only condition is denoted as \(*p<0.05\) or \(***p<0.001\).
Fig. 6

Schematic representation of the impact of uprosertib treatment combined with OXPHOS or MCT inhibition in cancer cells exposed to lactic acidosis. **a** Alteration of cellular fate in the presence or absence of lactic acid. **b** Schematic of the possible metabolic effects associated with altered response to treatment. Exogenous lactic acid decouples glycolysis from OXPHOS as lactate (black arrows) is preferentially utilised via the TCA cycle over glucose (green arrows). ETC, electron transport chain. This figure was produced and adapted using Servier Medical Art licensed under the Creative Commons Attribution 3.0 Unported License (https://www.servier.com).
Fig. 2

a

0 mM lactic acid

10 mM lactic acid

Vehicle

Uprosentib

b

10. AK1/2/3 (T090)
9. AK1/2/3 (S473)
8. TOR (S2448)
7. p70 s6 kinase (T389)
6. p70 s6 kinase (T421/S424)
5. GSK-3β (S21/93)
4. eNOS (S1177)
3. PRAS40 (T246)
2. CREB (S133)
1. WNK1 (T86)

Log2(Fold change relative to the 0 mM lactic acid vehicle)

0 0.5 1.0

-3 -2 -1.5 -1 -0.5 0 0.5 1.0

c

LS174T

Uprosentib

Lactic acid

pAkt S473

Pan Akt

pPRAS40 T246

Total PRAS40

β-actin

Vehicle

Uprosentib

Vehicle

Uprosentib

Vehicle

Uprosentib

0 mM lactic acid

10 mM lactic acid

Vehicle

Uprosentib

Vehicle

Uprosentib

0 mM lactic acid

10 mM lactic acid
Fig. 3

a

HCT116

Glucose uptake (final cell/hour)

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

LS174T

Glucose uptake (final cell/hour)

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

b

HCT116

Lactate release (final cell/hour)

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

LS174T

Lactate release (final cell/hour)

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

c

13C-glucose

Pyruvate

(M+3)

Lactate

(M+3)

CO2

Acetyl-CoA

Oxaloacetate

Citrate

(M+2)

13C-glucose derived pyruvate (M+3)

Fraction labelled M+3

HCT116

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

13C-glucose derived lactate (M+3)

Fraction labelled M+3

HCT116

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

13C-glucose derived citrate (M+2)

Fraction labelled M-2

HCT116

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

d

LS174T

13C-glucose derived pyruvate (M+3)

Fraction labelled M+3

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

13C-glucose derived lactate (M+3)

Fraction labelled M+3

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid

13C-glucose derived citrate (M+2)

Fraction labelled M-2

LS174T

Vehicle
Upsonutin

0 mM lactic acid
10 mM lactic acid
Fig. 4

(a) 

(b) HCT116
\[ ^{13}\text{C}_2\text{lactic acid derived pyruvate} \]

(c) HCT116
\[ ^{13}\text{C}_2\text{lactic acid derived citrate} \]

LS174T
\[ ^{13}\text{C}_2\text{lactic acid derived pyruvate} \]

\[ ^{13}\text{C}_2\text{lactic acid derived citrate} \]

Fraction labelled

M+0 M+1 M+2 M+3 M+4 M+5 M+6

M+0 M+1 M+2 M+3 M+4 M+5 M+6

Vehicle Uprosertib

Vehicle Uprosertib
Fig. 5

(a) LS174T-MCT4

(b) HCT116

(c) LS174T

(d) LS174T
Fig. 6

(a) Uprosertib (GSK2141795) and lactic acid inhibition of OXPHOS or MCT inhibition.

(b) Lactate, glucose, and pyruvate metabolism with Metformin and Antimycin A.
NAD/NADH-Glo assay
To measure the levels of NAD and NADH in cells LS174T and HCT116 cell lines, the NAD/NADH-Glo™ (Promega, Southampton, UK) bioluminescent assay was used according to the manufacturer’s protocol. Cells were plated at a density of 4 x 10⁴ and incubated for 24 hours before media were changed to test media containing glucose (5.6 mM), glutamine (2 mM), 10% FBS and lactic acid (0 mM or 10 mM). Cells were also dosed with uprosertib (10 µM) or DMSO (0.1%) before plates were analysed after 24 hours. The NAD⁺ signal was normalised to the NADH signal so graphs were plotted as the ratio of NAD⁺/ NADH.

pH measurements
The pH of DMEM was measured immediately after glucose (5.6 mM), glutamine (2 mM), 10% FBS or lactic acid (0, 10 or 20 mM) was added to media. To adjust media to pH 6.5, hydrochloric acid was added dropwise to DMEM containing glucose (5.6 mM), glutamine (2 mM) and 10% FBS until the pH measured 6.5. To measure pH, a Mettler Toledo InLab Routine Pro electrode was used.

Intracellular uprosertib measurements using liquid-chromatography mass-spectrometry (LC-MS).
A density of 8 x10⁵ cells per well were plated into 6-well plates and incubated for 24 hours before media were changed to DMEM (0 hours) supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS and lactic acid (0 or 10 mM) with or without uprosertib (0 or 10 µM). Untreated and vehicle (0.1% DMSO) controls were also
used. After 24 hours of treatment, media were aspirated, wells were washed twice with Ringer's buffer and cells were quenched using ice cold methanol. Samples were subsequently dried under nitrogen gas, before the samples were deproteinised using 800 µL MeOH/H₂O 3/1 v/v (LC-MS grade), incubated in -20 °C for 30 min and then centrifuged (8500 rcf for 10 min, 8°C). A volume of 700 µL of the supernatant was transferred into high recovery LC-MS vials and evaporated under nitrogen flow, before being reconstituted in 100 µL of AcCN/H₂O 1/9 v/v (LC-MS grade).

LC-MS analysis was performed by an ultra-performance liquid chromatography system (1290 Agilent) hyphenated to a 4000 QTrap mass analyser (AB Sciex) equipped with a Turbo V electrospray source in the positive ionisation mode. Direct infusion of a 1 µg/mL uposertib solution in AcCN/H₂O 1/1 v/v with 0.1 % formic acid was performed to define the MRM transitions specific for uposertib analysis and also to define the optimum DP and CE parameters for each MRM transition.

Reversed phase (RP) chromatographic separation was performed for the quantification of uposertib. An Acquity HSS T3 C18 column (2.1 mm x 100 mm, 1.8 µm) at a flow rate of 0.6 mL/ min maintaining the temperature at 40°C and using 5 µL of injection volume. The mobile phase consisted of A (LC-MS grade H₂O with 0.2% formic acid) and B (LC-MS grade ACN with 0.2% formic acid). The elution gradient profile was as follows (minute / % of B): 0/0.5, 2/0.5, 5/15, 10/99.5, 13/99.5, 13.1/0.5, 15/0.5). The following five MRM transition were recorded: (Q1/Q3 Masses m/z): 429.40/412.40 Da, 429.40/243.00 Da, 429.40/153.20 Da, 429.40/133.30 Da and 429.40/127.30 Da. The m/z 429.40/412.40 Da was used for quantification and the other four for confirmation. Each cell sample was run in duplicate and standards of
uprosertib were run for quantification, identification and confirmation of retention time.

Analysis of MCT4 protein expression using western blotting

Protein extraction was performed as described in the main article. Cell lysates containing 40 µg of protein were loaded onto 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, California, US) before being separated by gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated in anti-MCT4 ((H-90) #50329) or α-tubulin ((H-235) #9104) purchased from Santa Cruz Biotechnology (Dallas, Texas, US). Chemiluminescent signals were enhanced using West Pico plus chemiluminescent substrates (Thermo Scientific, Rockford, US) and detected using the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences). Blots were analysed using Image Studio Lite (LI-COR software).
Fig. S1 The effect of lactic acid on growth of HCT116 cells

HCT116 cells were cultured for three days in DMEM supplemented with 0, 10 or 20 mM lactic acid for 72 hours. Cell density was determined using SRB assays and graphs were plotted as the OD at 72 hours normalised to the OD at 0 hours. The results shown are the mean of three technical replicates from one independent experiment (n=1).
Fig. S2 Uprosertib treatment in the presence of lactic acid or pyruvic acid

a The effect of lactic acid or pyruvic acid on cytotoxicity measured using SRB assays after 72 hours of uprosertib (10 µM) treatment in LS174T cells (a). b, c The effect of lactic acid or pyruvic acid on caspase 3/7 activity after 24 hours of uprosertib (10 µM) treatment in LS174T (b) and HCT116 (c) cells. d, e The effect of lactic acid or pyruvic acid on ATP levels in LS174T (d) and HCT116 (e) cells after 24 hours of uprosertib treatment (5 µM, 10 µM or 15 µM). Controls without lactic acid or pyruvic acid supplementation and vehicle controls containing DMSO (0.1%) were used for all experiments. Data were presented as mean ± SEM from three independent experiments in a, c, d and e (n=3), and from four independent experiments in b (n=4). *p<0.05, **p<0.01 and ***p<0.001. In b, statistical significance compared to the control in the same treatment condition is shown as ###p<0.001 above bars.
Uprosertib treatment in the presence and absence of sodium lactate

Fig. S3 Uprosertib treatment in the presence and absence of sodium lactate

a, b The effect of uprosertib (0.1 μM to 15 μM) treatment on HCT116 (a) and LS174T (b) cells in the presence or absence of sodium lactate (10 mM) after 72 hours was measured using SRB assays. Media were replenished every 24 hours to control for changes in the extracellular metabolite concentrations. DMSO (0.1%) was used for vehicle controls. The results shown are the mean ± SEM from three independent experiments (n=3).
Fig. S4 The effect of low pH on sensitivity to uprosertib.

a The pH of DMEM supplemented with glucose (5.6 mM), glutamine (2 mM), 10% FBS and lactic acid (0, 10 or 20 mM) was measured using a Mettler Toledo InLab Routine Pro electrode. b The effect of low pH induced by adding hydrochloric acid to DMEM supplemented with glucose (5.6 mM), glutamine (2 mM) and 10% FBS in LS174T cells after 72 hours of treatment with uprosertib (10 µM). Media were changed every 24 hours to control for changes in extracellular metabolite concentrations. SRB cytotoxicity assays were performed at 72 hours and the optical density was normalised to the optical density at 0 hours. DMSO (0.1%) was used for vehicle controls. The results shown are the mean ± SEM from three independent experiments (n=3). *p<0.05 or ***p<0.001.
Fig. S5 Intracellular uprosertib concentration in the presence and absence of lactic acid in HCT116 cells.

HCT116 cells were treated with uprosertib (10 μM) in the presence or absence of lactic acid (10 mM). Untreated and vehicle (0.1% DMSO) controls were also used. Intracellular material was extracted after 24 hours of treatment using methanol quenching and intracellular uprosertib levels were measured using LC-MS. The concentration of uprosertib was subsequently calculated and normalised to the cell number, so graphs were plotted as the intracellular uprosertib concentration per cell (μg/ cell). Data were presented as mean ± SEM from two biological replicates (n=2) from one independent experiment, except the vehicle controls which were presented as the mean from one biological replicate (n=1).
Fig. S6 The rate of glutamine uptake in the cells treated with uprosertib in the presence and absence of lactic acid.

HCT116 (a) and LS174T (b) cells were dosed with uprosertib (10 µM) for 24 hours in the presence or absence of lactic acid (10 mM). Media were collected and analysed using NMR spectroscopy. Cell counts at 0 hours and 24 hours were also obtained. The changes in the glutamine concentration were calculated and normalised to the cell number so graphs were plotted as the rate of glutamine uptake in fmol/cell/hour. The results shown are the mean ± SEM from three independent experiments (n=3).
Fig. S7 MIDs of pyruvate and citrate derived from $^{13}$C$_3$-lactic acid and $^{13}$C$_3$-sodium lactate.

**a, b** MIDs of pyruvate and citrate derived from $^{13}$C$_3$-lactic acid and $^{13}$C$_3$-sodium lactate in LS174T cells. Cells were incubated in DMEM supplemented with glucose (5.6 mM) and glutamine (2 mM) with either $^{13}$C$_3$-lactic acid (10 mM) or $^{13}$C$_3$-sodium lactate (10 mM) for 1 hour. Intracellular metabolites were subsequently extracted and analysed using GC-MS. MIDs of pyruvate (**a**) and citrate (**b**) were plotted. Data were presented as mean ± SEM from two independent experiments ($n=2$). *p<0.05 or ***p<0.001.
Fig. S8 Effect of uprosertib on NAD\(^{+}\)/NADH in the presence and absence of lactic acid.

\textbf{a, b} The effect of uprosertib (10 µM) in the presence or absence of lactic acid (10 mM) after 24 hours of treatment on NAD\(^{+}\)/NADH. Levels of NAD\(^{+}\) and NADH were measured using the NAD/NADH-Glo\(^{TM}\) assay. The NAD\(^{+}\) signal was normalised to the corresponding NADH signal, so graphs were plotted as the ratio of NAD\(^{+}\)/NADH. The results shown are the mean ± SEM from three independent experiments (\(n=3\)).
Fig. S9 Effect of AZD3965 treatment in LS174T-MCT4<sup>-/-</sup> and wild-type LS174T cells.

a MCT4 protein expression in wild-type (-WT) and LS174T-MCT4<sup>-/-</sup> cells. Alpha-tubulin was used for loading controls. Blots shown are from one experiment representative of three independent replicates.

b, c Effect of MCT1 inhibition using AZD3965 (1 µM) on <sup>13</sup>C<sub>3</sub>-lactic acid incorporation into LS174T-MCT4<sup>-/-</sup> (b) and the LS174T-WT cells (c) after 2 hrs of incubation. Pyruvate M+3 and citrate M+2 were plotted. DMSO (0.1%) was used for vehicle controls. Data were presented as mean of two technical repeats from one independent experiment (n=1).

d Effect of uprosertib (10 µM) treatment on caspase 3/7 activity in combination with AZD3965 (1 µM) in the wild-type parental LS174T cell line after 24 hours of treatment. Data were presented as mean ± SEM from three independent experiments (n=3). All conditions with uprosertib were significantly increased (p<0.001) compared to vehicle or AZD3965 only treated cells (not indicated on graph). All other significant differences were indicated by a line, **p<0.01 or ***p<0.001.
Fig. S10 The OXPHOS inhibitors rotenone and antimycin A rescue sensitivity to uprosertib treated cells exposed to lactic acidosis.

**a-d** Rotenone and antimycin A treatment re-sensitise LS174T and HCT116 cells to uprosertib under lactic acidosis. LS174T (**a, b**) and HCT116 (**c, d**) cells were treated with rotenone (1 and 5 µM) or antimycin A (1 and 5 µM) alone or in combination with uprosertib (10 µM) in the presence or absence of lactic acid (10 mM). Caspase-Glo 3/7 assays were performed after 24 hours, before results were normalised to cell density measured using SRB assays and subsequently to the vehicle controls. Data were presented as mean ± SEM from three independent experiments (n=3). *p<0.05 or **p<0.01.
Fig. S11 Effect of lactic acid on response to PI3K/ Akt/ mTOR pathway inhibitors.

a-c LS174T and HCT116 cells were treated with AZD5363 (0.1 µM to 50 µM) (a), pictilisib (0.001 µM to 10 µM) (b) or INK128 (0.001 µM to 10 µM) (c) for 72 hours in the presence or absence of lactic acid (10 mM). SRB cytotoxicity assays were performed and results were normalised to the optical density at 0 hours. DMSO (0.1%) was used for vehicle controls. The results shown are the mean ± SEM from three independent experiments (n=3).
The influence of PI3K/Akt/mTOR pathway inhibitors on apoptosis in the presence and absence of lactic acid.

**Fig. S12** The influence of PI3K/ Akt/ mTOR pathway inhibitors on apoptosis in the presence and absence of lactic acid.

**a**, **b** HCT116 (**a**) and LS174T (**b**) cells were treated with AZD3563 (1 μM and 5 μM), pictilisib (1 μM and 10 μM) or INK128 (1 μM and 10 μM) for 24 and 48 hours in the presence or absence of lactic acid (10 mM). Media were replenished every 24 hours to control for changes in the extracellular metabolite concentrations. Uprosertib (10 μM) was used as a positive control. DMSO (0.1%) was used for vehicle controls. The results shown are the mean ± SEM from three independent experiments (n=3).

**p<0.01** or **p<0.001**.