INHIBITION OF ALANINE AMINOTRANSFERASE (ALAT) IN SILICO AND IN VIVO PROMOTES MITOCHONDRIAL METABOLISM TO IMPAIR MALIGNANT GROWTH*
Gregor Beuster*1, Kim Zarse*1, Christoph Kaleta†, René Thierbach‡, Michael Kiehntopf§, Pablo Steinberg§, Stefan Schuster†, Michael Ristow*2
* Dept. of Human Nutrition, Inst. of Nutrition, University of Jena, Jena, D-07743, Germany, † Dept. of Bioinformatics, School of Biology and Pharmaceutics, University of Jena, Jena, D-07743, Germany, ‡ Dept. of Food Toxicology and Complementary Methods to Animal Testing, University of Veterinary Medicine Hannover, D-30173, Germany, § Inst. of Clinical Chemistry and Laboratory Medicine, University of Jena, Jena D-07743, Germany, ª Dept. of Clinical Nutrition, German Inst. of Human Nutrition Potsdam-Rehbrücke, Nuthetal, D-14558, Germany
Running head: ALAT and cancer growth
1 These authors contributed equally to this work.
2 To whom correspondence should be addressed: Dept. of Human Nutrition, Inst. of Nutrition, University of Jena, Jena, D-07743, Germany; Email: mristow@mristow.org

Cancer cells commonly exhibit increased non-oxidative D-glucose metabolism whereas induction of mitochondrial metabolism may impair malignant growth. We have first used an in silico method named elementary mode analysis to identify inhibition of ALAT (L-alanine aminotransferase) as a putative target to promote mitochondrial metabolism. We then experimentally show that two competitive inhibitors of ALAT, L-cycloserine and β-chloro-L-alanine, inhibit L-alanine production and impair D-glucose uptake of LLC1 Lewis lung carcinoma cells. The latter inhibition is linked to an initial energy deficit, as quantified by decreased ATP content, which is then followed by an activation of AMPK (AMP-activated protein kinase) and subsequently increased respiration rates and mitochondrial production of reactive oxygen species (ROS), culminating in ATP replenishment in ALAT-inhibited LLC1 cells. Moreover, we observe altered phosphorylation of p38 MAPK (mitogen-activated protein kinase 14), ERK (mitogen-activated protein kinase 1/2) and Rb1 (retinoblastoma 1) proteins, as well as decreased expression of Cdc25a (cell decision cycle 25 homolog A) and Cdk4 (cyclin-dependent kinase 4). Importantly, these sequelae of ALAT inhibition culminate in similarly reduced anchorage-dependent and anchorage-independent growth rates of LLC1 cells, altogether suggesting that inhibition of ALAT efficiently impairs cancer growth by counteracting the Warburg effect due to compensatory activation of mitochondrial metabolism.

Compared to non-malignant entities, cancer cells commonly exhibit increased non-oxidative D-glucose metabolism (glycolysis) (1,2) while mitochondrial activity and in particular respiration rates are severely impaired in malignant cells (3-9). Based on these facts, Otto Warburg proposed that an inappropriate increase in glycolysis due to impaired respiratory capacity may be the cause of malignant growth (1,2), a concept that was named Warburg’s hypothesis in subsequent decades. This effect has been supported on theoretical grounds by the comparably inefficient utilisation of available nutrients in cancer cells (10).

While it is questionable whether the Warburg effect actually causes cancer, impairing D-glucose uptake or D-glucose metabolism in cancer cells unequivocally induces oxidative metabolism, and has been shown to effectively impair malignant cell growth in vitro and in vivo (11,12). In this regard, a typical inhibitor of proximal glycolysis, 2-deoxy-D-glucose (2-DOG), has been shown to be particularly effective in impairing cancer growth (13). This effect was in later years additionally used to increase the efficacy of chemotherapeutic agents (14).

Consistent with these promising effects of glycolytic inhibitors like 2-DOG, forcing cancer cells into increased mitochondrial metabolism independently of D-glucose metabolism, e. g. by over-expressing rate-limiting mitochondrial proteins, efficiently reduces both anchorage-
dependent and – independent growth, as well as tumor growth in nude mice (15). However and unlike in cultured cells, selective activation of mitochondrial metabolism in vivo is difficult to achieve.

Recently established in silico methods, and in particular the so-called elementary mode analysis (EMA) of metabolic networks can be used to identify novel pathways and alternate biochemical routes, including those that may selectively promote mitochondrial metabolism. EMA is capable of predicting so-called elementary modes (EMs), which are the smallest possible subsets of biochemical reactions connecting to points of a steady-state metabolic network (16-18).

In the present study we have used this method to identify biochemical pathways that may increase oxidative metabolism of cancer cells when inhibited by appropriate compounds. Specifically and by applying EMA, we have identified the conversion of L-pyruvate into L-alanine by the enzyme ALAT (L-alanine aminotransferase) as a putatively crucial step, and provide experimental support for this hypothesis primarily generated in silico. By applying ALAT inhibitors to malignant cells we observe fundamental metabolic changes that culminate in increased oxidative metabolism and cell cycle arrest, leading to impaired anchorage-dependent and – independent cancer cell growth.

**EXPERIMENTAL PROCEDURES**

**Chemicals**- All chemicals used were obtained from Sigma-Aldrich (Taufkirchen, Germany). L-Cycloserine (Cyclo) and β-chloro-L-alanine (Cl-Ala) were dissolved in DMSO to 10, 50 and 250 mM stock solutions that were then aliquoted and stored at −80°C prior to use. For cell treatment, stock solutions were diluted 1:1000 in medium to a final concentration of DMSO of 0.1%. If not stated otherwise, Cyclo and Cl-Ala were used at a final concentration of 250 µM.

**Elementary mode analysis (EMA)**- Determination of energy-producing EMs was conducted as previously described (16-18) using the non-commercial software YANAsquare 0.98 (downloaded at http://yana.bioapps.biozentrum.uni-wuerzburg.de/).
Scintillation was measured according to the protocol of the manufacturer (Beckton and Dickinson, Oxford, UK). Aliquots of NaOH lysates were used to determine cellular protein content per well for normalization.

**D-Glucose / L-lactate-ratio** - D-glucose uptake from supernatant media was determined as previously described (20). L-lactate was measured according to the method of Sweetmann et al. (21) modified as described below. After deproteinization of samples by addition of perchloric acid (1:1), 500 µl of H2O, 50 µl of internal standard (4 mmol/l 2-hydroxybutyric acid) and 500 µl of saturated NH4Cl-solution were added to 100 µl of the deproteinized supernatant. Subsequently, sample extraction was performed by addition of 2 x 5 ml ethyl acetate. The organic phase was collected, dried over anhydrous Na2SO4, evaporated to dryness under a gentle stream of nitrogen in a new tube and derivatized (100 µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 30 min, 60°C). One microlitre of the derivatized solution was subjected to analysis by gas chromatography-mass spectrometry using a ZB-5 column (Phenomenex, Aschaffenburg, Germany) and a Shimadzu QP2010 GC-MS (Shimadzu, Duisburg, Germany).

**Cellular ATP content** - Cellular ATP content was measured by using a luciferin/luciferase-based bioluminescence assay (CellTiter-Glo, Promega, Madison, USA) as described before (20). Briefly, cells were seeded in a 96-well plate (2 x 10^4 per well), washed with PBS following treatment, lysed (CellTiter-Glo buffer), and aliquots were taken for protein determination prior to the addition of bioluminescent substrate / enzyme-solution (CellTiter-Glo substrate). Luminescence was measured using a 96-well plate luminometer (Fluostar, BMG, Offenburg, Germany). Cellular ATP content was calculated by an ATP standard curve and normalized to cellular protein content per well.

**Cellular respiration** - Briefly, 2 x 10^3 cells per well were seeded in a 96-well OxoPlate (PreSens, Regensburg, Germany), sealed using adhesive sealing foil (Thermo Fischer Scientific, Rockford, USA), kept at 37°C, and fluorescence / phosphorescence was measured every 60 min up to 48 h according to the protocol of the manufacturer (PreSens, Regensburg, Germany). In parallel equally treated plates for different time points were used to determine integrated cellular protein for normalization.

**L-Glutamine utilization** - Briefly, 1 x 10^4 cells per well were seeded in a 24-well plate and supernatant medium was collected after 48 h treatment. L-glutamine and L-glutamate concentrations were determined in the supernatant by using an enzyme-based kit according to the protocol provided by the manufacturer (Glutamine and Glutamate Determination Kit GLN-1, Sigma-Aldrich, Taufkirchen, Germany). L-glutamine utilization was calculated as L-glutamine uptake minus L-glutamate production both normalized to integrated cellular protein content per well.

**Mitochondrial reactive oxygen species (mtROS) production** - The measurement was performed according to the protocol provided by the manufacturer (CMX-Ros, Invitrogen, Carlsbad, California). Briefly, 1 x 10^6 cells per well were seeded in a 24-well plate. After treatment cells were incubated with fresh medium containing 1 µM Mitotracker-Red (CMX-ROS) for 30 min, washed twice with PBS, supplied with fresh medium, and fluorescence was measured after 10 min incubation at 37°C at 578 nm excitation and 599 nm emission wavelengths. CMX-Ros fluorescence was normalized to cellular protein content per well.

**Protein determination** - Following lysis of cells with 1 M NaOH protein contents were determined by using a bicinchoninic acid assay kit according to the protocol provided by the manufacturer (BCA Protein Assay, Thermo Fischer Scientific, Rockford, USA).

**Immunoblotting and Western blot analysis** - Methods for immunoblotting were performed as previously described (15) using the following primary antibodies: anti-phospho-AMPK (Thr172), anti-AMPK, anti-tubulin, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-Erk1/2, anti-Cdk4 and anti-phospho-Rb (Ser780) supplied by Cell Signaling (Boston, USA), anti-Rb and anti-ALAT (anti-GPT) obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Western blots were tested for equal protein loading by both ponceau red staining of the...
membrane, and anti-tubulin blotting (data not shown). Densitometric analysis of western blots was conducted using ImageJ software (National Institute of Health, Bethesda, USA) according to the program’s manual.

Soft agar assay- Soft agar assays were performed in a semi-automated manner in 96-well plates using an epMotion 5075 Liquid Handling Workstation (Eppendorf AG, Hamburg, Germany) as described previously (22). Briefly, in each well 100 μl top agar containing 1 x 10^3 LLC1 cells and test compounds or solvent were placed on top of 100 μl previously solidified base layer. After 6 days of incubation, Alamar Blue (resazurin) was added and colony growth was quantified fluorometrically.

Animal experiments- Nude mice (Crl:CD1-Foxn1nu, 5 weeks of age) were obtained from Charles River Laboratories (Sulzfeld, Germany). One million LLC1 cells (viability >90 %) were re-suspended in 1 ml DMEM medium (without antibiotics and fetal bovine serum) and injected subcutaneously in the left hind area using an insulin syringe (Becton-Dickinson, Heidelberg, Germany). Starting on the next day, 10 μl of 0.9 % sodium chloride solution per gram body weight (BW) with or without test compounds (Cyclo: 100 mg/kg BW; Cl-Ala: 20 mg/kg BW) were injected subcutaneously in the right hind area once per day at the same time. On day 13 mice were sacrificed, tumors were excised and the tumor mass was determined. Mice were kept in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, and all experiments were approved by the corresponding institutional review boards.

Statistical analyses- All calculations were performed with SPSS, version 13. Kolmogorov-Smirnov-Test was used to test for normal distribution, which was confirmed in all experiments. The unpaired Student’s t-test was used to determine the statistical significance of the inhibitor effects. A p-value below 0.05 was considered statistically significant. If not indicated otherwise significance of differences in treated groups compared to control groups are shown by asterisks located above the respective treatment group in the corresponding figures.

RESULTS

Identifying ALAT as a putative target to force cancer cells into mitochondrial metabolism. To identify a previously unidentified target for the metabolic inhibition of cancer cell growth we used software-based elementary mode analysis (EMA). We constructed a stoichiometric metabolic model containing enzymes and metabolites likely to be related to the energy production in cancer cells (Fig. 1). We then used this metabolic model as input for an EMA software package named YANAsquare which proposed six major energy-producing elementary modes (EMs) (Fig. 1, see also Suppl. Fig. 1A & B for more details).

Only two of these six pathways do not employ mitochondrial enzymes: anaerobic ATP generation from D-glucose producing L-lactate (Fig. 1, EM I.) or ATP generation from D-glucose producing L-alanine (Fig. 1, EM II.). Regarding anaerobic D-glucose metabolism ample evidence exists that inhibiting this pathway both blocks cancer cell growth and induces mitochondrial energy conversion (see Discussion). The pathways utilizing mitochondrial enzymes correspond to the aerobic generation of ATP by converting L-glutamine into L-lactate (Fig. 1, EM III) or L-alanine (Fig. 1, EM IV) and the aerobic generation of ATP by completely oxidizing L-glutamine (Fig. 1, EM V) or D-glucose (Fig. 1, EM VI) along the tricarboxylic acid cycle. Conversely and as identified here by EMA, inhibiting the conversion of L-lactate into L-alanine (EM II and IV) may shift ATP production towards mitochondrial pathways (Fig. 1, EM V and VI) and may thereby inhibit cancer growth.

Inhibition of ALAT impairs L-alanine production of cancer cells. The cytosolic enzyme ALAT catalyses the terminal step in L-alanine production from D-glucose, or other L-pyruvate-contributing carbon sources. By using two different and previously established inhibitors of ALAT (23,24), namely Cyclo and Cl-Ala, we were able to abolish the production of L-alanine in the highly transformed lung cancer cell line LLC1 (Fig. 2A). However, the
expression levels of ALAT in LLC1, revealed by immunoblotting, were found to be unaltered by inhibitor treatment (Fig. 2B). It should be noted that untreated cells produce rather large amounts of L-alanine (Fig. 2C), as previously described for cultured cancer cells as well as human cancers in situ (25-27), Moreover and in contrast to untreated cells, inhibitor-treated LLC1 cells showed only minor consumption of the L-alanine present in the cell culture medium at the beginning of the experiment (Fig. 2C).

Cyclo and Cl-Ala have previously been reported to potentially exhibit inhibitory effects on other enzymes than ALAT, especially aspartate-aminotransferase (ASAT), in particular at significantly higher concentrations compared to those used in this study. However, in clear contrast to the reduction of L-alanine production after treatment (Fig. 2A), Cyclo and Cl-Ala did not inhibit L-aspartate turnover in LLC1 cells when used at a concentration of 250 µM, indicating the lack of any inhibitory effect on ASAT activity (Suppl. Fig. 2).

Inhibition of ALAT alters D-glucose metabolism of cancer cells. Excretion of L-alanine from cancer cells causes a net loss of energy for the individual cell. Hence, blocking L-alanine synthesis from L-lactate may reduce the need for exogenous energy equivalents, particularly D-glucose. To investigate whether the inhibition of L-alanine production from L-lactate and hence D-glucose may indirectly inhibit D-glucose import into the cell, we determined uptake of 14C-labeled 2-DOG. Both ALAT inhibitors entailed a significant decrease in D-glucose uptake (Fig. 2D), thus indicating that a decrease in overall D-glucose metabolism does in fact occur.

While control cells showed a nearly stoichiometric conversion of D-glucose into L-lactate (i.e. one mol of D-glucose being converted into two moles of L-lactate, leading to a D-glucose/L-lactate ratio of approx. 0.5), we unexpectedly found that inhibitor-treated cells exhibit D-glucose/L-lactate ratios significantly below 0.5 (Fig. 2E). This strongly suggests that ALAT inhibition causes a relative increase in L-lactate production from carbon sources other than D-glucose.

ALAT inhibitors cause an initial energy deficit in cancer cells. Glycolysis in cancer cells provides adequate supply of ATP even in states of severely reduced respiration. Conversely, we questioned whether impairing both the disposal of L-lactate into L-alanine (Fig. 2A and Suppl. Fig. 3) as well as D-glucose uptake (Fig. 2D) would subsequently cause a cellular energy deficit. Not surprisingly, after a 24 h treatment with either ALAT inhibitor we observed cellular ATP levels to be decreased (Fig. 3A).

Inhibitor-initiated energy deficit activates AMPK and replenishes ATP. Decreased ATP availability is known to culminate in activation of a key energy sensor of the cell, AMP-activated kinase (AMPK) (28), which subsequently induces mitochondrial metabolism. Since decreased availability of ATP following inhibitor treatment causes an energy deficit (Fig. 3A), we quantified phosphorylation of the alpha-subunit of AMP-activated kinase (AMPKα) at Thr172, which is known to be indicative of AMPK activity. Immunoblotting of both the basal as well as the phosphorylated forms of AMPKα indicated that this energy sensor is activated after a 24 h treatment with Cyclo or Cl-Ala (Fig. 3B). In accordance with these findings, ATP content in inhibitor-treated cells was identical to control cells 48 h after addition of the inhibitor (Fig. 3C), thus indicating that the initial ATP deficit (Fig. 3A) is transient and efficiently compensated by the subsequent activation of AMPK. Concurrent with the observed ATP restoration (Fig. 3C) we also found the initial AMPK activation to be abolished after 48 h (Fig. 3D).

Inhibitor-initiated activation of mitochondrial L-glutamine metabolism. The findings so far indicate that inhibitor-treated LLC1 cells generate amounts of ATP similar to control cells (Fig. 3C). This occurs despite the fact that D-glucose uptake is reduced (Fig. 2D) and L-alanine turnover is reduced (Fig. 2C). Also given the previously established role for AMPK in activating mitochondrial metabolism, we quantified cellular oxygen uptake, which was found to be dramatically increased in inhibitor-treated LLC1 cells (Fig. 3E).

However, the relative increase in L-lactate accumulation suggests that D-glucose-derived L-pyruvate may not be the predominant mitochondrial substrate compensating for D-glucose deficiency. To find out whether
increased respiration is possibly due to mitochondrial oxidation of carbon sources other than L-pyruvate, L-lactate and D-glucose, we quantified the turnover of L-glutamine. L-glutamine is known to be an important fuel for fast growing cells in vitro and is therefore the second most abundant nutrient in cell culture media (Suppl. Fig. 3). Utilization of this amino acid was found to be strongly increased following inhibitor treatment (Fig. 3F). Since ALAT inhibitors appear to impair D-glucose uptake and to promote L-glutamine turnover in parallel, we conclude that L-glutamine is the main substrate of increased mitochondrial metabolism in states of ALAT inhibition.

**Inhibitor-initiated activation of respiration promotes mitochondrial formation of ROS.** Activation of mitochondrial oxygen metabolism, particularly in cancer cells, has been frequently connected to increased production of mitochondrial ROS (mtROS). We therefore used a rhodamine-based, redox-sensitive, fluorescent and cell-permeable dye to investigate mtROS levels in inhibitor-treated cells, and observed increased mtROS-related fluorescence after a 24 h treatment with ALAT inhibitors (Fig. 3G).

Taken together, the findings so far indicate that inhibition of ALAT leads to increased mitochondrial activity in an AMPKα-dependent manner to replenish ATP levels while also increasing mtROS formation.

**ALAT inhibition promotes several growth-inhibiting signaling pathways.** Activation of AMPKα, increased mitochondrial activity and increased mtROS levels have independently been shown to impair cancer cell growth. These three metabolic states have also been shown to activate p38 MAP kinase signaling (15,29,30). Accordingly, we here observed increased phosphorylation and hence activation of p38 following treatment with ALAT inhibitors for 24 h (Fig. 4A). In line with this observation, expression of the phosphatase Cdc25a, known to be regulated by p38, was found to be decreased (Fig. 4B). Cdc25a-dependent dephosphorylation of mitogenic stress kinase ERK was consistently found to be decreased (Fig. 4C), suggesting increased degradation of the protein kinase Cdk4. Correspondingly, expression of protein kinase Cdk4, which in addition has been previously established to be regulated by Cdc25a, was also decreased after treatment with ALAT inhibitors (Fig. 4D). Consistent with the above-mentioned alterations a decreased phosphorylation of retinoblastoma protein (Rb1) at the Cdk4-specific phosphorylation site Tyr780 was lastly observed (Fig. 4E).

Taken together, these findings suggest activation of a signaling cascade that might cause growth inhibition of LLC1 cells treated with either Cyclo or Cl-Ala.

**ALAT inhibitors impair growth of cancer cells in vitro and in vivo.** The aforementioned alterations of growth signaling may cause reduced growth rates following treatment with ALAT inhibitors. In a first step we investigated the effects of Cyclo and Cl-Ala on anchorage-dependent growth after a 0, 24 and 48 h treatment. Cyclo showed stronger inhibitory effects than Cl-Ala, while both substances significantly reduced protein content after 48 h in a concentration-dependent manner in the range from 10 to 250 µM (Fig. 4F and 4G).

We subsequently questioned whether the inhibitors would similarly affect anchorage-independent growth. Using a recently developed, semi-automated soft-agar assay (22) we quantified colony formation by LLC-1 cells in the absence and presence of ALAT inhibitors at different concentrations. Both inhibitors were capable of reducing colony formation in a concentration-dependent manner in the range from 10 to 250 µM (Fig. 4H and 4J).

To test whether the observed growth inhibitory effects of Cyclo and Cl-Ala are specific for malignant cells, and not simply due to an unspecific toxicity, we simultaneously treated non-malignant BJ fibroblasts (BJ1) and highly malignant BJ fibroblasts (BJ4) with the respective inhibitors. After 48 h treatment, both inhibitors exerted significantly stronger reduction of cellular protein content of malignant BJ4 cells compared to a relatively mild effect on BJ1 cells, indicating a malignancy-dependent growth inhibitory effect of Cyclo and Cl-Ala (Fig. 4K).

Lastly, we injected LLC-1 cells into immune-compromised nude mice and quantified absolute tumor masses after two weeks of exposure to ALAT inhibitors. In the groups treated with inhibitors we found tumor masses to be reduced by approx. 40% when compared to
tumor masses in saline-injected control mice. The statistical analysis revealed a trend towards a significantly reduced LLC1-derived cancer growth in nude mice (Cyclo: \( p=0.059 \); Cl-Ala: \( p=0.085 \)) (Fig. 4L).

Taken together, these findings indicate that inhibition of ALAT impairs malignant growth by inducing mitochondrial metabolism.

**DISCUSSION**

In the current study we have used *in silico* EMA to identify a previously unknown biochemical approach to re-instate mitochondrial metabolism in a highly malignant cancer cell line, culminating in the inhibition of both anchorage-dependent and -independent growth and thereby reducing the malignancy of such cells.

Cancer cells are known to exhibit extremely high rates of glycolysis and concurrently reduced mitochondrial activity, as shown repeatedly in the past. These observations are supported by our findings in untreated LLC1 cells (Fig. 2E), which indicate that incorporated D-glucose is mainly converted into L-lactate. Based on EMA predictions we have tested the possibility to impair glycolysis indirectly by preventing transamination of highly acidic L-lactate into less acidic L-alanine (Fig. 1), which - by applying the corresponding inhibitors - indeed causes reduced L-alanine production and turnover (Fig. 2A and 2C). Notably and as predicted, this inability to convert L-lactate into L-alanine would cause excessive and therefore detrimental acidification of the cellular environment, would subsequently reduce D-glucose uptake (Fig. 2D), presumably to prevent excess L-lactate production, and would lead to corresponding decreases in cellular pH. Consistently, both L-lactate production as well as L-alanine content of tumor tissues have previously been shown to positively correlate with tumor malignancy (27,31). Moreover, high amounts of nutritive D-glucose may cause lactic acidosis in humans suffering from cancers (32), and, most interestingly, alanine is the only amino acid produced by human colon carcinomas *in situ* (25).

Furthermore, the non-oxidative conversion of D-glucose and the oxidation of L-glutamine both contribute to the massive production of L-lactate and L-alanine in cancer cells (9,33-35).

In cells that are highly dependent on glycolysis, decreased D-glucose uptake should initially cause an energy deficit, as reflected by the decreased intracellular ATP content observed 24 h after addition of the inhibitors (Fig. 3A). However, 48 h after addition of these inhibitors no energy deficit was detected anymore (Fig. 3C), thus suggesting that the cell compensates for the initial deficit by activation of AMPK, as experimentally shown in Fig. 3B. Notably, this kinase has repeatedly been reported to be involved in the control of cancer cell growth (36,37). Given the initially shown reduction of glycolytic energy conversion, it appears that the cell initiates a compensatory switch to mitochondrial energy conversion, as reflected by increased oxygen consumption rates (Fig. 3E), increased L-glutamine consumption (Fig. 3F) and lastly increased production of mtROS (Fig. 3G). This activation of mitochondria is paralleled by the activation of previously established pathways (Fig. 4A-E) and inhibits cancer cell growth in an anchorage-dependent (Fig. 4F and 4G) and anchorage-independent (Fig. 4H and 4J) manner. As previously shown, other anti-cancer agents, including dichloroacetate (12,38) and 2-DOG (39,40), appear to promote mitochondrial metabolism in a similar way. Moreover, genetic approaches to stimulate mitochondrial activity similarly impair cancer growth (15). Finally, there is limited evidence that ALAT inhibitors may affect prokaryotic (41) or malignant cell growth (42-45). These latter effects, however, have been linked to impaired ceramide synthesis, which may occur independently from our current findings. Taken together, we here provide evidence that *in silico* predictions of inhibitor-based alterations of nutrient metabolism are capable of anticipating their effects on cancer cell metabolism and growth, namely ALAT inhibition to cause an induction of mitochondrial metabolism and subsequently reduced malignancy.
REFERENCES


**FOOTNOTES**

* The authors especially thank William C. Hahn at the Dana-Farber Cancer Institute Boston, MA (USA) for providing the BJ-hTERT and BJ-hTERT-st-LT-Hras cell lines. The authors thank Beate Laube for excellent technical assistance, and Daniel Scharlau and Michael Glei for helpful advice. This work is part of the research program of the Jena Centre for Systems Biology of Ageing — JenAge funded by the German Ministry for Education and Research (Bundesministerium für Bildung und Forschung – BMBF; support code: 0315581).

The abbreviations used are: 2-DOG, 2-deoxy-D-glucose; ALAT, L-alanine aminotransferase; BSTFA,N,O-bis(trimethylsilyl)trifluoroacetamide; Cdc25a, cell division cycle 25 homolog A; Cdk4, cyclin-dependent kinase 4; Cl-Ala, β-chloro-L-alanine; Cyclo, L-Cycloserine; DMEM, Dulbecco’s modified Eagle medium; EMA, elementary mode analysis; EMs, elementary modes; ERK, mitogen-activated protein kinase 1; FBS, fetal bovine serum; LLC1 Lewis lung carcinoma cells; mtROS, mitochondrial reactive oxygen species; p38 MAPK, mitogen-activated protein kinase 14; PBS, phosphate.
buffered salt solution; Rb1, retinoblastoma 1; RHB, Krebs-Ringer-Hepes-Buffer; ROS, reactive oxygen species.

**FIGURE LEGENDS**

**Fig. 1.** Identification of energy-producing pathways in cancer cells using YANAsquare-based elementary mode analysis. I.-VI. Elementary modes (EM) representing pathways with the least amount of enzymes involved to produce ATP from D-Glucose or L-Glutamine. All framed metabolites are intended to underlie a net production or consumption. Further involved metabolites and co-factors are considered to be compensated by anaplerotic and cataplerotic reactions to form a steady state metabolite flux and therefore remain hidden, namely: Acetyl-CoA, Citrate, Coenzyme Q, FAD, FADH$_2$, Fumarate, GDP, L-Glutamate, GTP, D-Isocitrate, L-Malate, NAD$^+$, NADH/H$, Oxaloacetate, Pyruvate, Succinate, SuccinylCoA, α-Ketoglutarate. Following enzymes and simplified enzyme systems were used for EM determination: (1) ATP-GTP-Mutase, (2) Alanine aminotransferase (ALAT) (dashed arrows), (3) Citrate synthase, (4) Aconitase, (5) Isocitrate dehydrogenase, (6) α-Ketoglutarate dehydrogenase, (7) SuccinylCoA synthase, (8) Succinate dehydrogenase, (9) Fumarase, (10) Malate dehydrogenase, (11) Glutamate dehydrogenase, (12) Glutaminase, (13) Glycolysis (simplified to an one-step reaction from D-glucose to pyruvate), (14) Lactate dehydrogenase, (15) Malic enzyme, (16) Pyruvate dehydrogenase, (17) “NADH/H$^+$ = 2.5 ATP”, (18) “FADH$_2$ = 1.5 ATP”. All enzymes are intended to be unregulated and only dependent on energy producing substrates.

**Fig. 2.** Inhibitors of ALAT prevent L-alanine production and reduce D-glucose metabolism in cancer cells. Panel A depicts a western blot of ALAT after a 24 h treatment, B depicts L-alanine concentration in supernatant medium after a 48 h treatment with inhibitors, C shows L-alanine turnover defined as changes in medium L-alanine referred to integral of cellular protein over a 48 h treatment (n = 4), D indicates $^{15}$C-2-deoxy-D-glucose uptake per µg protein after a 48 h treatment with inhibitors (n = 4), E depicts ratio of D-glucose uptake and L-lactate production (n = 4). Inhibitors were used at a final concentration of 250 µM; error bars represent SD; *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 3.** Inhibitors of ALAT modulate energy metabolism and promote respiration and L-glutamine utilisation. Panel A shows cellular ATP concentration after a 24 h treatment with inhibitors, B indicates densitometric analysis of western blots of phospho-AMPK$\alpha$ protein (pAMPK$\alpha$) and basal AMPK$\alpha$ protein (n=5) (A and B after a 24 h treatment with inhibitors), C shows cellular ATP concentration per µg protein (n = 8), D indicates densitometric analysis of western blots of phospho-AMPK$\alpha$ protein (pAMPK$\alpha$) and basal AMPK$\alpha$ protein (n = 3) (C and D after a 48 h treatment), E shows cumulative oxygen uptake during a 36 h treatment with inhibitors referred to integral of cellular protein (n = 8), F summarizes L-glutamine utilisation, defined as L-glutamine uptake minus L-glutamate excretion during a 48 h treatment referred to integral of cellular protein (n = 4), and G depicts mitochondrial ROS-related fluorescence after a 24 h treatment normalised for cellular protein content (n = 8). For all panels, inhibitors were used at a final concentration of 250 µM; error bars represent SD; *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 4.** Inhibitors of ALAT activate stress kinase-dependent pathways and inhibit growth of highly malignant cancer cells. Panel A depicts western blots of basal and phosphorylated p38 protein after a 24 h treatment with inhibitors, B shows a representative western blot of Cdc25a protein after a 24 h treatment with inhibitors (n = 3), Ca depicts a representative western blots of basal and phosphorylated ERK protein, Cβ shows the densitometric analysis of basal and phosphorylated ERK (n=4) (both after a 24 h treatment with inhibitors), D depicts a representative western blot of Cdk4 protein after a 24 h treatment with inhibitors (n = 3), E indicates representative western blots of basal and phosphorylated Rb protein
after a 24 h treatment with inhibitors (n = 3), F & G shows protein content per well of anchorage-dependent growing cells after a 0, 24 and 48 h treatment with L-cycloserine (Panel F) and β-chloro-L-alanine (Panel G). H & J indicate relative fluorescence units measured per soft agar well of anchorage-independent growing cells treated for 6 days with L-cycloserine (Panel H) and β-chloro-L-alanine (Panel J). K shows protein content per well of anchorage-dependent growing BJ1 versus BJ4 cells relative to respective control after a 48 h inhibitor treatment and L summarizes tumor masses in nude mice after two weeks of treatment with a daily dose of Cyclo 100 mg/kg BW or Cl-Ala 20 mg/kg BW (Panels A – E and K): Inhibitors were used at a final concentration of 250 µM; error bars represent SD; *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 2

A) Alanine Conc. [µM] 
B) ALAT
C) Alanine Turnover [nmol/µg]
D) 14C-DOG Uptake [pmol/min/µg]
E) Glucose/Lactate-Ratio [mM/mM]

Control Cyclo Cl-Ala

Medium Control Cyclo Cl-Ala

47kDa

Control Cyclo Cl-Ala

Control Cyclo Cl-Ala
Figure 3
Figure 4

A

B

C α

D

E

F

G

H

J

L
Inhibition of alanine aminotransferase (ALAT) in silico and in vivo promotes mitochondrial metabolism to impair malignant growth
Gregor Beuster, Kim Zarse, Christoph Kaleta, Rene Thierbach, Michael Kiehntopf, Pablo Steinberg, Stefan Schuster and Michael Ristow

*J. Biol. Chem. published online May 3, 2011*

Access the most updated version of this article at doi: 10.1074/jbc.M110.205229

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
[http://www.jbc.org/content/suppl/2011/05/03/M110.205229.DC1](http://www.jbc.org/content/suppl/2011/05/03/M110.205229.DC1)

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/early/2011/05/03/jbc.M110.205229.full.html#ref-list-1](http://www.jbc.org/content/early/2011/05/03/jbc.M110.205229.full.html#ref-list-1)