Original Article

MOLECULAR METABOLISM

Antimetabolic cooperativity with the clinically approved L-asparaginase and tyrosine kinase inhibitors to eradicate CML stem cells

Anne Trinh [1,9], Raeeka Khamari [1,9], Quentin Fovez [1,9], François-Xavier Mahon [2], Béatrice Turcq [2], Didier Bouscary [3,5], Patrice Maboudou [4], Marie Joncquel [4], Valérie Coiteux [1], Nicolas Germain [1], William Laine [1], Salim Dekiouk [1], Sandrine Jean-Pierre [7], Veronique Maguer-Satta [8], Bart Ghesquiere [6], Thierry Idziorek [1], Bruno Quesnel [1], Jerome Kluza [1,,10], Philippe Marchetti [1,4,*,10]**

ABSTRACT

Objective: Long-term treatment with tyrosine kinase inhibitors (TKI) represents an effective cure for chronic myeloid leukemia (CML) patients and discontinuation of TKI therapy is now proposed to patient with deep molecular responses. However, evidence demonstrating that TKI are unable to fully eradicate dormant leukemic stem cells (LSC) indicate that new therapeutic strategies are needed to control LSC and to prevent relapse. In this study we investigated the metabolic pathways responsible for CML surviving to imatinib exposure and its potential therapeutic utility to improve the efficacy of TKI against stem-like CML cells.

Methods: Using complementary cell-based techniques, metabolism was characterized in a large panel of BCR-ABL+ cell lines as well as primary CD34+ stem-like cells from CML patients exposed to TKI and L-Asparaginases. Colony forming cell (CFC) assay and flow cytometry were used to identify CML progenitor and stem like-cells. Preclinical models of leukemia dormancy were used to test the effect of treatments.

Results: Although TKI suppressed glycolysis, compensatory glutamine-dependent mitochondrial oxidation supported ATP synthesis and CML cell survival. Glutamine metabolism was inhibited by L-asparaginases such as Kidrolase or Erwinase without inducing predominant CML cell death. However, clinically relevant concentrations of TKI render CML cells susceptible to Kidrolase. The combination of TKI with Lasparaginase reactivates the intinsic apoptotic pathway leading to efficient CML cell death.

Conclusion: Targeting glutamine metabolism with the FDA-approved drug, Kidrolase in combination with TKI that suppress glycolysis represents an effective and widely applicable therapeutic strategy for eradicating stem-like CML cells.

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Keywords Synthetic lethality; Metabolic addiction; LSC; Metabolic stress; Stem-like cells

1. INTRODUCTION

Because of the development of tyrosine kinase inhibitor (TKI) therapy, the outcome of chronic myeloid leukemia (CML) patients has drastically improved. Unfortunately, TKI, such as Imatinib mesylate and other second or third generation BCR-ABL1 inhibitors, preferentially target differentiated cells and leave some of the CML stem cells alive [1–4]. A fraction of LSC can survive independently from BCR-ABL1 signaling and as such are totally insensitive to Imatinib [2,5]. TKIs are effective in inducing a long-term response and treatment discontinuation is proposed to patients with persistent deep molecular response [1,3]. The STIM study (Multicenter, nonrandomized Stop Imatinib Trial) underlined that 40% of patients successfully reached treatment-free remission with no disease recurrence. However, resuming treatment is necessary for patients

[1]Univ. Lille, CNRS, Inserm, CHU Lille, Institut de Recherche contre le Cancer de Lille, UMR9020 — UMR-S 1277 - Canther — Cancer Heterogeneity, Plasticity and Resistance to Therapies, F-59000, Lille, France [2]Institut Bergonié, Université de Bordeaux, CNRS SNC5010, Inserm, U1218 ACTION, F — 33076, Bordeaux, France [3]Université de Paris, Institut Cochin, CNRS UMR8104, INSERM U1016, Paris, France [4]Centre de Bio-Pathologie, Banque de Tissus, CHU Lille, France [5]Assistance Publique-Hôpitaux de Paris. Centre-Université de Paris, Service d'Hématologie clinique, Hôpital Cochin, Paris, France [6]Department of Oncology and VIB, KU Leuven, Leuven, Belgium [7]Centre de Recherche en Cancérologie de Lyon, Inserm U1052, CNRS UMR5286, Centre Léon Bérard, 69008, Lyon, France [8]Centre de Recherche en Cancérologie de Lyon, Inserm U1052, CNRS UMR5286, France

[9] These authors contributed equally to this work.

[10] JK and PM shared co-ownership to this work.

*Corresponding author. Institut de Recherche contre le Cancer de Lille, UMR9020 — UMR-S 1277, F-59000, Lille, France. E-mail: philippe.marchetti@inserm.fr (P. Marchetti).

**Corresponding author. Institut de Recherche contre le Cancer de Lille, UMR9020 — UMR-S 1277, F-59000, Lille, France. E-mail: jerome.kluza@inserm.fr (J. Kluza).

Abbreviations: CML (chronic myeloid leukemia), TKI (tyrosine kinase inhibitors); BCR-ABL1 (B Cell Receptor-Abelson), LSC (leukemic stem cell)

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exhibiting BCR-ABL1 transcript increase following Imatinib discontinuation. The inability of TKI to kill LSCs and/or progenitor cells is at the origin of those relapses [5]. There is a strong need for strategies targeted at improving TKI efficacy against LSC in CML in order to definitely eradicate the disease and enable long-term definite TKI discontinuation in most patients.

Cancer cell metabolism has opened up a new avenue in cancer treatment because it has become possible to target specific metabolic features of cancer cells, thus providing a potential therapeutic window [6]. Many oncogenes and oncogenic pathways driving cancer development also drive metabolism. Classically, it is assumed that cancer metabolism depends on glycolysis, which is predominant even in normoxic conditions (a phenomenon called the Warburg effect). The aberrant activation of the MAPK pathway via the BRAFV600E mutation increases glucose uptake and glycolysis enabling intense cell proliferation (for review [6–8]). Consistent activation of the PI3K/Akt/mTOR pathway by BCR-ABL1 also increases glucose metabolism in leukemic cells [9–11]. In light of these observations, it is not surprising that the exposure to oncogenic "driver" inhibitors such as BRAFV600E inhibitors [7,8] or BCR-ABL1 inhibitors [11,12] dramatically reduces glucose uptake and glycolysis to promote cell cycle arrest.

It has become evident that cancer metabolism cannot be reduced solely to the Warburg effect and it consists of a more complex network linking glucose metabolism and others nutrients like glutamine. A large body of evidence indicates that mitochondrial oxidative metabolic pathways play a crucial role in cancer development, particularly to immediately compensate for glucose deprivation. This dependence on mitochondrial oxidative metabolism allows cells to avoid cell death induced by MAPK inhibitors [7,13] or TKI [6,12]. Moreover, CML stem cells are particularly sensitive to mitochondrial oxidative metabolism inhibitors [13,14]. One interesting therapeutic strategy in cancer could be the combination of molecular-targeted drugs with antiglycolytic activities and inhibitors of the compensatory mitochondrial oxidative pathways thereby creating an "antimetabolic cooperativity" [6,15]. Herein, our objective was to develop a preclinical proof of concept for antimetabolic cooperativity against CML stem cells. We demonstrated that the combination of Kidrolase, a L-asparaginase used as treatment of acute lymphoblastic leukemia, with Imatinib possesses antimetabolic cooperativity and acts synergistically to eradicate CML stem cells *in vitro* and *ex vivo*.

2. MATERIALS AND METHODS

2.1. Chemicals

Imatinib mesylate was purchased from Sigma–Aldrich, Nilotinib and Dasatinib were purchased from Selleckchem. Kidrolase and Erwinase were provided by CHU Lille, [$^{13}C_6$] Glucose and [$^{13}C_5$] Glutamine were obtained from Professor Bart Ghesquière.

2.2. Patient samples

CML samples consisted of blood or bone marrow samples obtained from individuals in chronic or acute CML stages recruited from the Hematology Department (Lille CHU, France), after having obtained a signed informed consent in accordance with the Declaration of Helsinki and approval of the institutional ethics committee (CPP Lille). CD34+ cells were isolated from umbilical cord blood using the EasySep™ Human CD34 Positive Selection Kit II (Stemcell Technologies). Cytapheresis of CML patients were kindly provided by Pr. François-Xavier Mahon (CHU Bordeaux, France). CD34+ cells were cultivated at 37 °C and 5% CO_2 in RPMI medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin, and a growth factor cocktail containing 10 ng/ml of interleukins (IL)-3, (IL)-6, (IL)-7 and granulocyte colony-stimulating factor (G-CSF), 5 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF), and 25 ng/ml of stem cell factor (SCF) (Peprotech).

2.3. Cell lines

The leukemic DA1-3b cell line was generated by stable transfection of BCR-ABL1 [14–16]. Isolation of tumor cells d60 and d365 were previously described [14–17]. K562, KCL-22 and KU812 cell lines were grown in the same conditions. MS-5 mesenchymal cells were grown in α-MEM medium w/o nucleosides (Gibco) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 2 mM pyruvate. The identity of HBL, LND, and Mel-4M was also confirmed by karyotyping and array comparative genomic hybridization testing.

2.4. OCR and ECAR measurement, determination of cellular ATP

Extracellular acidification rate measurements (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse XFe24 analyzer (Seahorse Bioscience, Billerica, MA, USA). Detailed methods are provided in Supplementary Information files.

2.5. Colony forming cell (CFC) assay

Clonogenic assay was realized with cells seeded into 35 mm petri dish in semi-solid methylcellulose medium (Methocult™ M3231 for murin cells or Methocult™ H4230 for human cells, Stemcell Technologies). Cells were treated with the drugs for 72 h and then centrifugated. The pellet was resuspended in Iscove's modified Dulbecco medium (Lonza) supplemented with 2% fetal calf serum and 50 U/ml penicillin, 50 mg/ml streptomycin at 10,000 cells/ml, and cell suspension was added to methylcellulose medium (1000 cells/1ml/dish) and left at 37 °C and 5% CO_2. Colony forming efficiency was determined after 7 days using Leica DMI8 inverted microscope (Leica Microsystems) and quantified using Image J software.

2.6. Metabolite flux

Two hundred thousand cells were supplemented with media containing uniformly labelled U-$13C_6$ glucose (25 mM) or 13C-glutamine (2 mM) for 24 h. Detailed methods are provided in the Supplementary Information files.

2.7. Immunoblot analysis and real-time quantitative reverse transcription

For immunoblot, cell lysates were prepared as described previously [14,17]. mRNA quantititaive detection was performed with real-time PCR using the Lightcycler 480 detector (Roche Applied Science, Manheim Germany) as previously published [15,17].

2.8. Amino acid measurements

Amino acids concentration assay (μmol/l) was performed via high-performance liquid chromatography (Shimadzu C18 column, Kyoto, Japan) associated with tandem mass spectrometry (Sciex 3200 Qtrap, Framingham, MA) using the aTRAQ kit for amino acid analysis of physio-logical fluids (Sciex). Acquisition in the mass spectrometer was achieved by multiple reaction monitoring. Data recording and analysis were performed with Analyst software, v.1.6 (Sciex). Internal controls were systematically analyzed for each sample series.

2.9. In vivo studies

The DA1-3b/C3H tumor dormancy mouse model was previously described [14–17]. Seven-to eight-week-old C3H/HeOuJ female mice (Charles River Laboratories, Lyon, France) were intraperitoneally injected with 1×10^6 DA1-3b, DA1-3b d60 or DA1-3b/d365. All animal experiments were approved by the Animal Care Ethics Committee CEEA.NPDC (agreement no. 2017022716306305).

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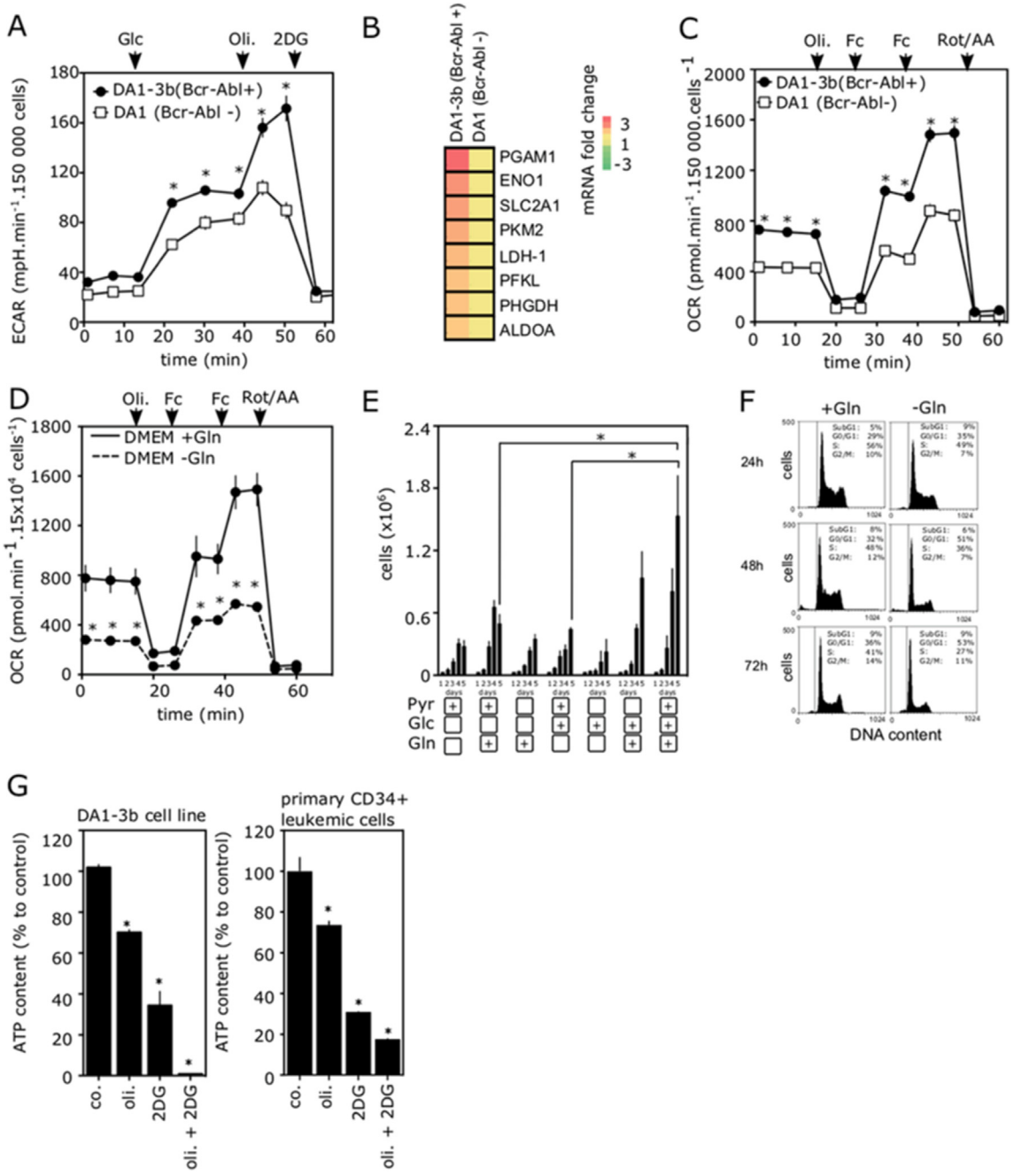


Figure 1: Glucose metabolism and mitochondrial respiration through glutamine oxidation are necessary for optimal metabolism and cell proliferation in CML cells. (A) Glycolytic activity and (C) oxygen consumption of BCR-ABL1+ (DA1-3b) and BCR-ABL1- (DA1) measured with Seahorse XFe24 extracellular flux analyzer after the injection of indicated drugs (Glc for glucose, Fc for FCCP, Oli for oligomycin A, 2DG for 2-deoxy-glucose, Rot/AA for rotenone and antimycin A) ($n = 3$, $^*p < 0.05$). (B) Glycolysis enzymes mRNA expression were quantified by RT-qPCR. 18S mRNA was used as housekeeping gene and data were expressed as mean of fold change ($n = 3$, $^*p < 0.05$). (D) DA1-3b leukemic cells were incubated in DMEM medium with glucose supplemented or not with glutamine for 24 h and oxygen consumption rate (OCR) was then measured. (E) DA1-3b cells were cultured in medium with or without glutamine, glucose or pyruvate as indicated. Proliferation was assessed by cell count from 1 to 5 days (mean +/-SD, $n = 3$). (F) DA1-3b cells were cultured in medium with or without glutamine. After 24, 48 and 72 h, cell death was determined by measuring sub-G1 population using propidium iodide staining. Pictures are representative of three independent experiments. Percentages of cells belonging to each phase of the cell cycle (G1, S and G2/M) were calculated using FlowJo software. (G) DA1-3b cells (left panel) and primary $CD34^+$ leukemic cells isolated from CML patient blood (right panel) were untreated (co.) or treated with oligomycin A (1 μM), 2-DG (10 mM) and a combination of both molecules for 4 h. ATP content was measured by luminescence (mean +/-SD, $n = 3$. $^*p < 0.05$).

2.10. Statistical analysis

All data points are represented as means ± SD. A two-tailed Student's t test was used to compare mean values between two groups. One-way or two-way analyses of variance (ANOVA) followed by Dunnett's or Sidak post hoc testing were used to compare mean values between multiple groups. The statistical analysis was performed using Prism version 6.0f (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered statistically significant.

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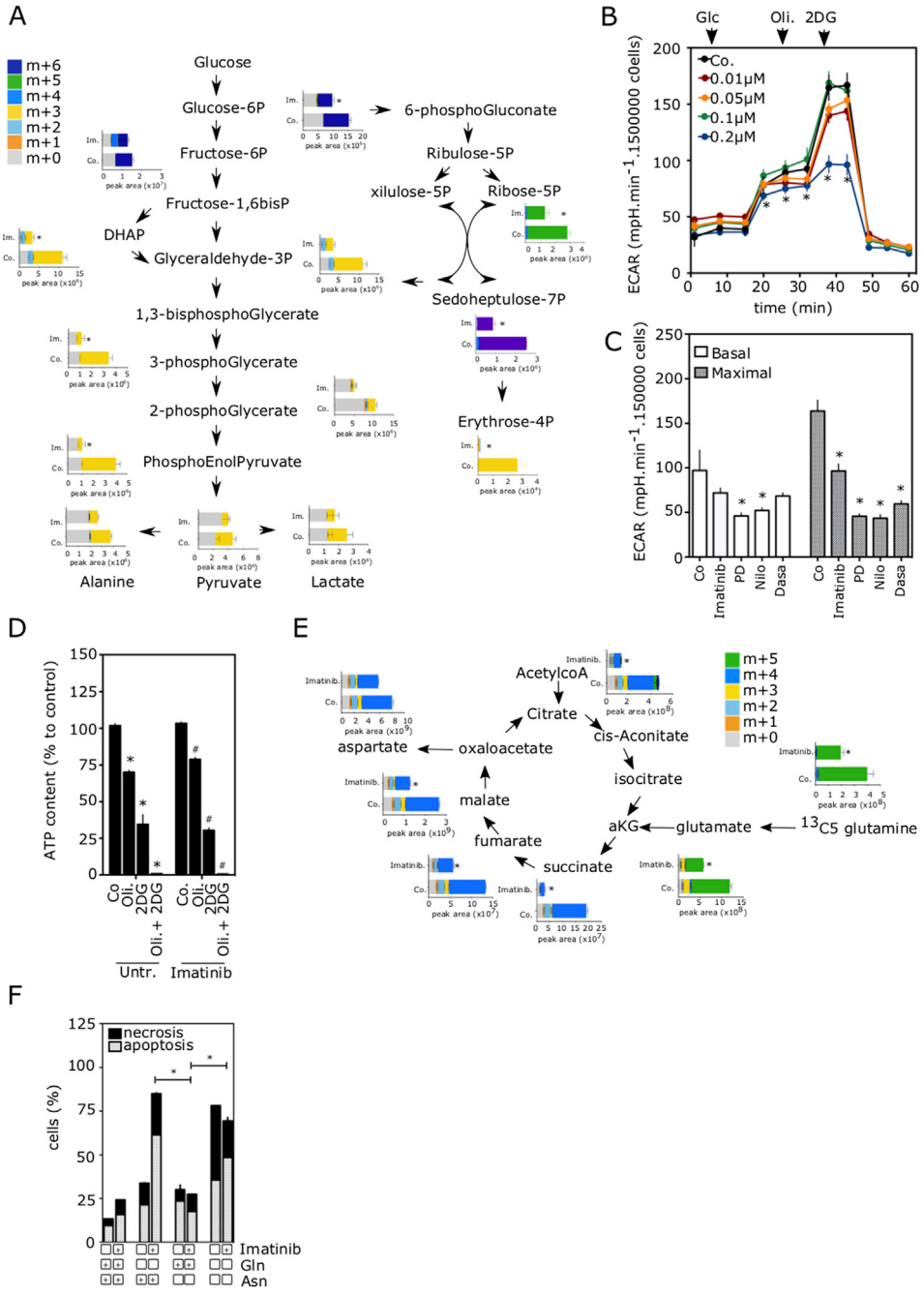


Figure 2: BCR-ABL1+ cells exhibit mitochondrial addiction and glutamine dependency under TKI exposure. (A) Isotopolog quantification of glycolysis intermediates were measured by liquid chromatography-mass spectrometry analysis in DA1-3b cells treated with Imatinib 0.2 µM (Im.) for 24 h or not treated (Co.) and grown in media containing U-$13C_6$ glucose (mean ± SD, n = 3, *p = 0.05). The symbol ''m+'' indicates the number of carbon atoms of each metabolite labeled with 13C. (B) Glycolytic activity (ECAR) of DA1-3b cells treated with Imatinib (0.01−1 µM) for 24 h measured with Seahorse XFe24 extracellular flux analyzer after the injection of indicated drugs (Glc for glucose, Oli for oligomycin A, 2DG for 2-deoxy-glucose) (C) Variations of glycolytic activity (ECAR) was determined after addition of inhibitors (Glc for glucose, Oli for oligomycin A, 2DG for 2-deoxy-glucose) using Seahorse XFe24 extracellular flux analyzer (left panel). Leukemic cells were treated by sub-lethal concentration of TKI of BCR-ABL1 (Imatinib 0.2 µM, PD180970 0.01 µM, Nilotinib 5 nM, Dasatinib 2 nM) for 24 h, and basal and maximal ECAR were assessed. Basal glycolysis ans maximal glycolysis are measured following glucose or oligomycin injection respectively (mean +/-SD, n = 3. *p < 0.05). (D) DA1-3b leukemic cells were treated with Imatinib (0.2 µM) for 72 h or with oligomycin A (1 µM) or/and 2-DG (10 mM) for 4 h. ATP levels were then measured by luminescence (mean +/-SD, n = 3. * or #P < 0.05 respectively compared to control). (E) Isotopolog quantification of TCA cycle intermediates levels by liquid chromatography-mass spectrometry analysis in DA1-3b cells grown in media containing U-$13C_5$ glutamine and treated or not with Imatinib (0.2 µM for 24 h) (mean ± SD, n = 3, *p < 0.05). (F) DA1-3b cells were cultured in medium containing a combination as indicated of Imatinib (0.5 µM), glutamine (2 mM) and asparagine (4 mM) for 48 h. Necrosis and apoptosis were determined by flow cytometry analysis of Annexin V and Sytox blue staining (mean ± SD, n = 3, *p < 0.05).

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3. RESULTS

3.1. Metabolic organization of CML involves both glycolysis and glutamine dependent mitochondrial OXPHOS

We first compared the metabolism of DA1-3b leukemic cells expressing BCR-ABL1 to the isogenic cell line DA1 not expressing BCR-ABL1 (Figure 1A—C). There was a significant increase in glycolysis (as validated by ECAR and gene expression) (Figure 1A,B) as well as mitochondrial respiration (Figure 1C) in cells transfected with p210 BCR-ABL1 compared to control cells. In BCR-ABL1-expressing DA1-3b cells, the mitochondrial respiration was largely sustained by glutamine (Figure 1D), indicating that CML cells consume both glucose and glutamine. In the presence of glutamine, glucose, and pyruvate, DA1-3b exhibited the highest proliferative rates (Figure 1E). Under glucose or glutamine deprivation conditions, DA1-3b cells were still able to proliferate, albeit at a lower rate than in conditions with both nutrients (Figure. 1E). Glucose or glutamine starvation did not trigger an obvious increase in cell death but promoted G0/G1 cell cycle arrest (Figure. 1F). This result is consistent with the observation that inhibition of either glycolysis with 2DG or mitochondrial respiration with oligomycin A was insufficient to totally deplete DA1-3b (Figure 1G, left panel) or primary CD34+ leukemic (Figure 1G, right panel) cells in ATP. This is probably due to the development of compensatory mechanisms to prevent energy collapse. These results suggest that CML cells have a metabolic flexibility, enabling them to survive during carbon source deprivation periods. Oonly the inhibition of the two metabolic pathways was able to deplete cells in ATP and kill BCR-ABL1 expressing cells. Overall, these results strongly suggest that the depletion of carbon sources, glucose and glutamine, is required for energy crisis and subsequent CML killing.

3.2. Upon Imatinib exposure BCR-ABL1+ cells are dependent on mitochondrial metabolism for survival

Previous studies showed that Imatinib displays a sustained inhibitory effect on glucose uptake and glycolysis via a reduction in the expression of key proteins such as GLUT-1 or PKM2 [10,18—20]. In this study, we specifically examined the metabolism of leukemic cells that survive Imatinib exposure at sub-lethal (Supplementary Figs. S1A—D), and clinically relevant anti-proliferative concentrations [11,21]. At concentrations below 0.2 μM, Imatinib induced a strong antiproliferative effect in DA1-3b cells (Supplementary Fig. S1A) but did not induce mitochondrial apoptosis as evidenced by the absence of translocation of phosphatidyl-serines, the retained high mitochondrial membrane potential ($\Delta\psi m$) and low level of mitochondrial ROS (Supplementary Fig. S1 B-E).

We performed metabolic flux analysis using 13C-labeled glucose in CML cells treated with vehicle or Imatinib for 24 h before the incubation with labeled glucose. Cells treated with Imatinib exhibited a major impairment of glucose uptake and glycolysis as judged by the decrease in labeled glycolytic intermediates and labeled lactate (Figure 2A). Moreover, it was associated with a reduction in the glucose flux via the nonoxidative pentose phosphate pathway (Figure 2A). In agreement with the inhibition of glucose uptake and lactate production (Supplementary Figs. S2A and S2B), we also observed a decrease in glycolysis-associated protein expression (Supplementary Fig. S2C), as well as a decrease in ECAR in BCR-ABL1+ leukemic cells exposed to Imatinib (Fig. 2B) or to other clinical BCR-ABL1 inhibitors (Figure 2C). Despite the glycolysis decrease, Imatinib exposure did not deplete in the high-energy molecule, ATP indicating that cells that survive to Imatinib can maintain energy state in the absence of efficient glycolysis (Figure 2D). This situation is compatible with mitochondrial metabolism maintenance despite Imatinib exposure, and a slight decrease in basal OCR under TKI treatment (Supplementary Fig. 2E). To explore whether glutamine was used to fuel mitochondrial activity in such conditions, we cultured cells in [13C5] L-glutamine in the presence or absence of Imatinib and analyzed intracellular metabolites via mass spectrometry. As shown in Figure 2E, cells that survived Imatinib continued to use glutamine to produce the TCA intermediates alphaKetoGlutarate in the absence of efficient glycolysis (Figure 2B). We observed that leukemic cells exposed to Imatinib used glutamine-dependent mitochondrial activity to survive. These cells became highly sensitive to the withdrawal of glutamine but not asparagine (Figure 2F). To survive Imatinib, BCR-ABL1+ cells require glutamine-derived carbon maintaining the TCA cycle in the absence of glycolysis.

3.3. L-asparaginases inhibit glutamine metabolism and reduce leukemic cell growth but are insufficient to eradicate BCR-ABL1+ cells

We previously demonstrated that targeting glutamine metabolism bears potential anti leukemic effects on myeloid leukemia cells [19,20,22]. We sought to target glutamine addiction in Imatinib-surviving BCR-ABL1+ cells. L-asparaginases (*e.g.* the *E.coli-asparaginase*, Kidrolase) are used to treat pediatric and adult forms of acute lymphoblastic leukemia and are also used in pediatric AML. These therapeutically relevant components are able to deaminate L-asparagine into aspartate. These enzymes also deplete glutamine and the antileukemic activity correlates with their ability to deplete extra cellular asparagine better than glutamine at lower doses, and to deplete both amino acids at higher doses [21,23]. We studied the metabolic effects of the FDA-approved Kidrolase in BCR-ABL1 + leukemic cells (Figure 3). In conditions depleting extracellular glutamine and asparagine (Figure 3A—B and Supplementary Fig. S3A), the flux experiment indicates that L-asparaginase depleted drastically the anaplerotic flux of glutamine into the TCA in BCR-ABL1+ cells (Figure 3C). This was accompanied by a significant reduction in OCR (Figure 3D—E), confirming that glutamine is a major energy source to fuel mitochondrial respiration in BCR-ABL1+ cell lines, but also in CD34+ CD38- leukemic cells from CML patients. As a result of mitochondrial inhibition, Kidrolase displayed strong anti-proliferative effects without predominant cytotoxic activity in BCR-ABL1+ cells (Supplementary Fig. S3B and Fig. S3G). This absence of important cell death in BCR-ABL1+ cells was compatible with the maintenance of high ATP level in Kidrolase-treated cells (Figure 3F). L-asparaginase increased intracellular glycolytic intermediates in BCR-ABL1+ cells (Figure 3G). We tested whether the glycolysis increase with Kidrolase treatment allowed leukemic cells to cope with metabolic stress. We found that the compensatory increase in glycolysis supported the survival of cells exposed to Kidrolase since glycolysis inhibition with 2DG did synergize with Kidrolase to induce BCR-ABL1+ cell death (Figure 3H). To determine whether Kidrolase sensitivity is a general feature of leukemic cells, we tested a large panel of BCR-ABL1 + or - leukemic myeloid cell lines. The antileukemic effects of Kidrolase were dose dependent and occurred in all tested cell lines. However, the antileukemic responses were highly heterogeneous in term of cell death observed up to 72 h (Supplementary Fig. S3C). We discovered a reverse correlation between glycolysis and Kidrolase-induced leukemic cell death (Figure 3I). The FDA-approved glutamine inhibitor Kidrolase hinders glutamine-dependent mitochondrial metabolism but is insufficient to eradicate BCR-ABL1+ cells due to glycolytic compensation.

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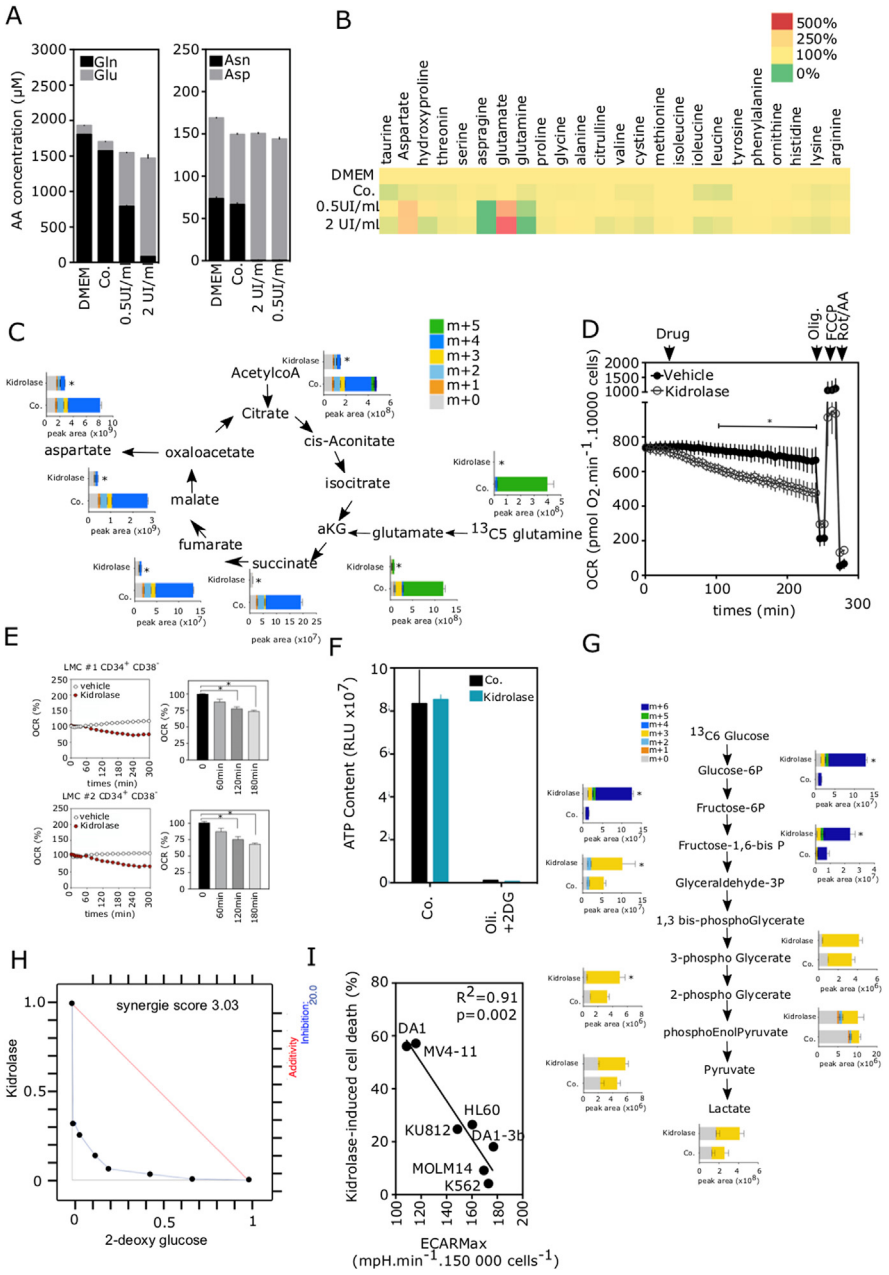


Figure 3: Myeloid leukemic cells are able to survive to Kidrolase-induced glutamine depletion through glycolysis. (A) and (B) DA1-3b cells were cultured in medium containing Kidrolase 0.5 or 2 UI/mL (Kid.) for 24 h. Cells were centrifugated, supernatent was removed and amino acids concentration as indicated was measured by HPLC/MS. (C) Isotopolog quantification of TCA cycle intermediates levels were measured by LC-MS analysis in DA1-3b cells grown in media containing U-13C_5 glutamine and treated or not with Kidrolase (0.5 UI/mL) for 24 h (mean ± SD, n = 3, *p = 0.05). The symbol "m+" indicates the number of carbon atoms of each metabolite labeled with 13C. (D) Assessment of mitochondrial respiration in DA1-3b cells by measuring OCR with XF24e Seahorse. The following molecules were injected subsequently: drug (Kidrolase or vehicle control), oligomycin (Olig.), FCCP and rotenone/antimycine A (Rot/AA). (E) Assessment of mitochondrial respiration in CD34+ CD38+/− patient primary leukemic cell sub-population from 2 CML patients by measuring OCR with XFe24 Seahorse. Molecules were injected as indicated: vehicle or Kidrolase. Percentages OXPHOS inhibition were calculated after 60, 120 and 180 min of exposure to kidrolase (mean ± SD, n = 3, *p = 0.05). F) Intracellular ATP level of DA1-3b cells exposed to oligomycin (oli.), 2-deoxy-glucose (2DG) or a combination of the two inhibitors for 4 h, with or without 72 h pre-incubation with Kidrolase 1 UI/mL (mean ± SD, n = 3, *p = 0.05). (G) Isotopolog quantification of glycolysis intermediates measured by LC-MS analysis in DA1-3b cells treated with Kidrolase 0.5 UI/mL for 24 h and grown in media containing U-13C_6 glucose (mean ± SD, n = 3, *p = 0.05). (H) DA1-3b cells were cultured with combination of 2-DG (0.033−100 mM) and Kidrolase (0.00033−10 UI/mL) for 72 h and cell proliferation inhibition was quantified by fluorescence using CyQUANT Cell Proliferation Assay. The response of the combination was compared with its single agents against the widely used Loewe model for drug-with-itself dose additivity using Chalice software and presented as an isobologram. (mean ± SD, n = 3, *p = 0.05). (I) Correlation between maximal glycolytic activity deterrmined by XFe24 Seahorse after glucose/oligomycin injection (ECAR Max) and cell death (determined by PI staining) induced by 48 h Kidrolase treatment (0.5 UI/mL) in 8 myeloid leukemic cell lines (p = 0.002, R^2 = 0.91).

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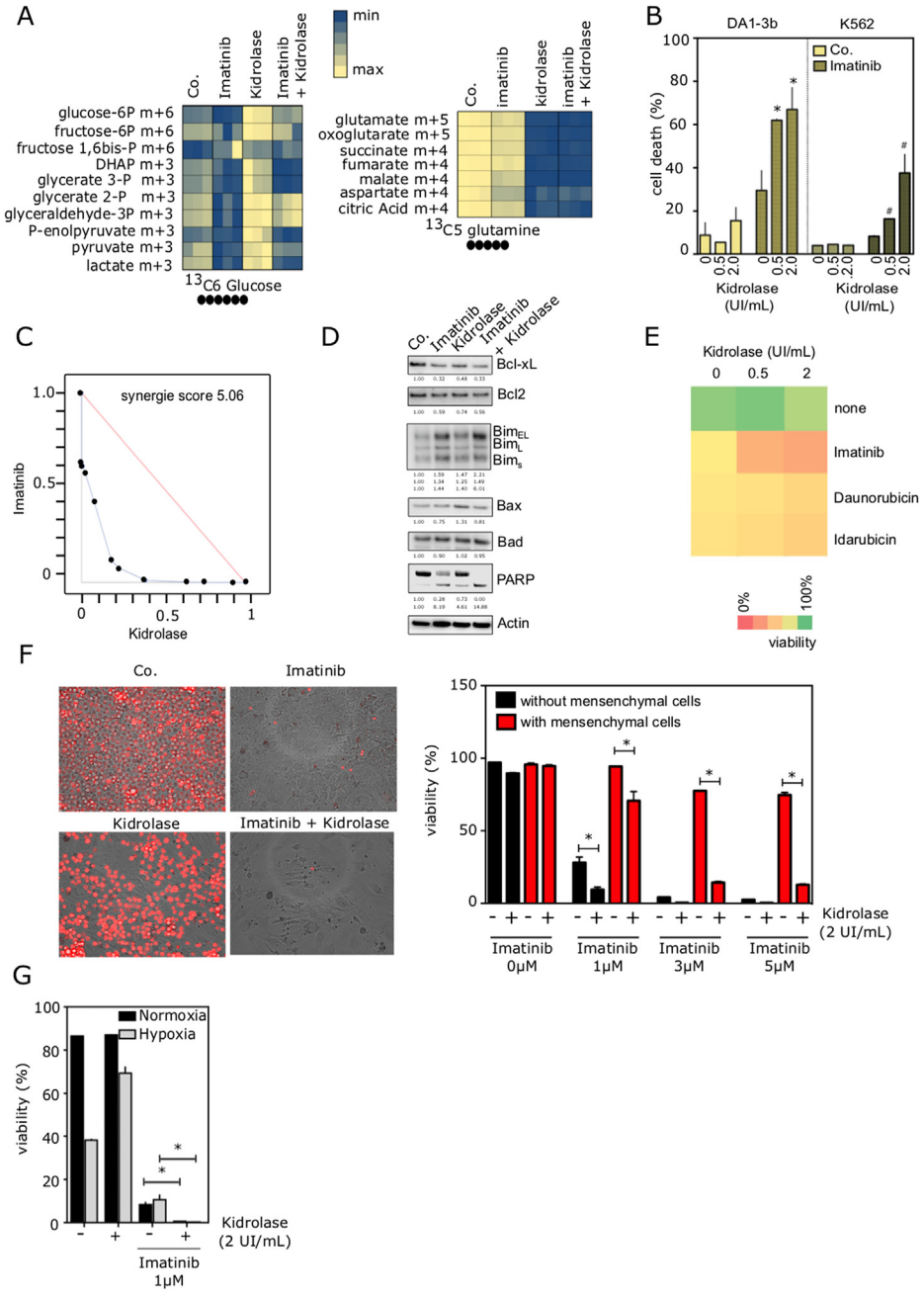


Figure 4: Imatinib and Kidrolase drug combination is effective to target glycolysis and mitochondrial metabolism and to induce cytotoxic effects. (A) Isotopolog quantification of glycolysis (left panel) and TCA cycle (right panel) intermediates calculated as percentage of the total metabolite pool following liquid chromatography-mass spectrometry in DA1-3b cells treated with Imatinib (0.2 µM) and Kidrolase (0.5 UI/mL) for 24 h and grown in media containing U-13C_6 glucose (left panel) or U-13C_5 glutamine (right panel) (n = 3). The symbol ''m+'' indicates the number of carbon atoms of each metabolite labeled with 13C. (B) Determination of DA1-3b and K562 cell death after 48hrs exposure to Imatinib (0.5 µM) and Kidrolase treatments as indicated (mean ± SD, n = 3, * or #p < 0.05 respectively compared to control). (C) DA1-3b cells were cultured with combination of Imatinib (0.0001−100 µM) and Kidrolase (0.00033−10 UI/mL) for 72 h and cell proliferation inhibition was quantified by fluorescence using CyQUANT Cell Proliferation Assay). Isobologram was determined as seen in Figure 3(D) Immunoblotting of pro- and anti-apoptotic proteins (as indicated) in DA1-3b cells treated with Imatinib (0.5 µM), Kidrolase (2 UI/mL) or a combination of both drugs for 24 h. Pictures are representative of three independent experiments. Actin was used as loading control. (E) DA1-3b cells were treated with a combination of Kidrolase and anticancer drugs (Imatinib 0.5 µM, Daunorubicin 0.01 µM, Idarubicin 0.001 µM) for 48 h and viability was assessed by cytometric analysis of annexin V and Sytox stainings. (F) Phase-contrast analysis of DA1-3b cells transfected with DsRed co-cultured with MS-5 mesenchymal cells and treated by Imatinib (3 µM) and Kidrolase (2 UI/mL) for 7 days (left panel). Viablity of DA1-3b in mono-culture or in co-culture with mesenchymal cells after 48hrs treatments with Imatinib (1−5 µM) and Kidrolase (2 UI/mL) after Sytox blue stainings (right panel). Qauntification was expressed as mean ± SD (n = 3, *p = 0.05). (G) DA1-3b cell lines were exposed to Imatinib (1 µM) and Kidrolase (2 UI/ml) treatments in normoxia (20% O_2) or hypoxia (1% O_2) for 72 h. Viability was assessed by flow cytometry after PI stainings (mean ± SD, n = 3, *p < 0.05).

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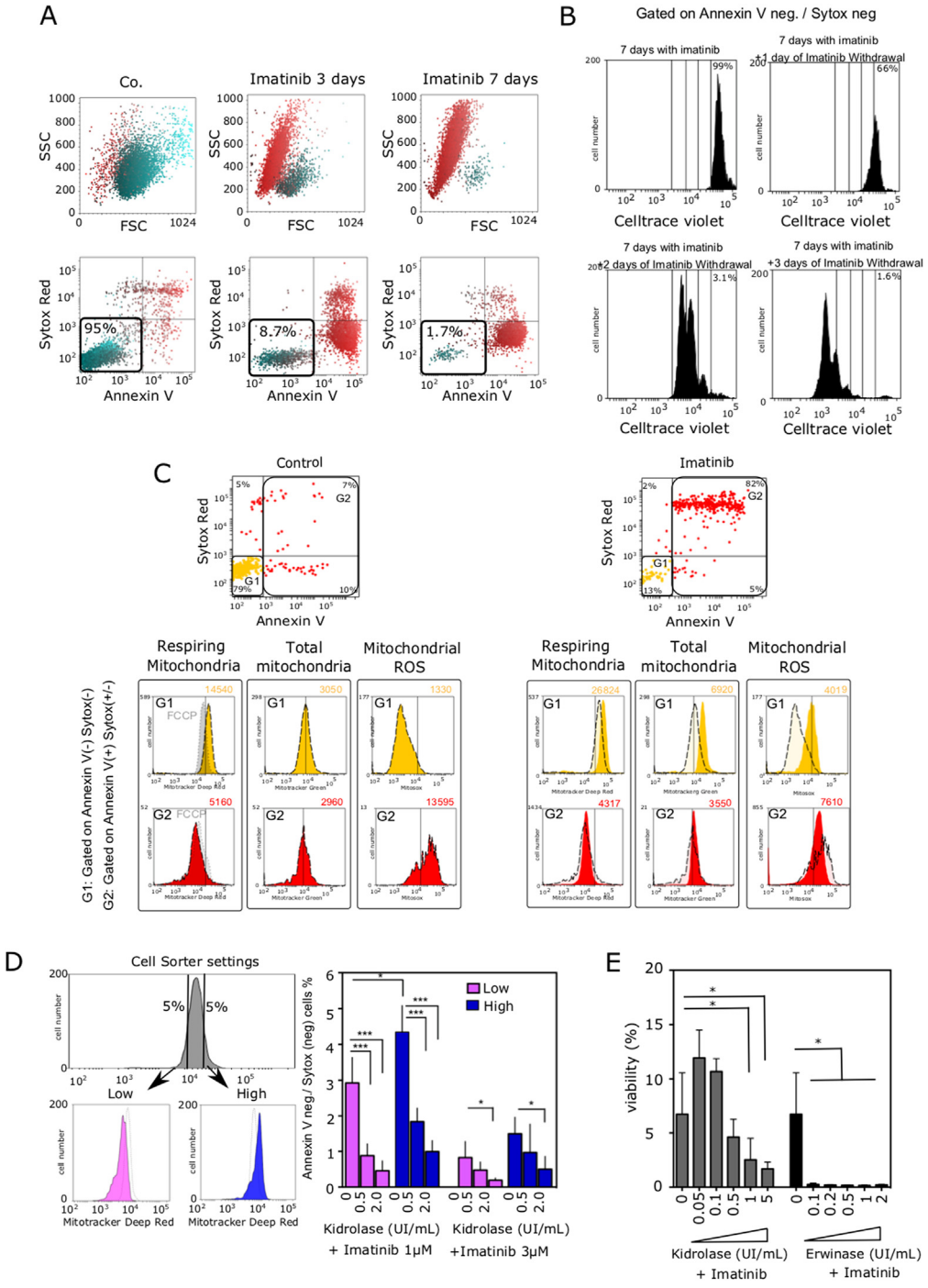


Figure 5: TKI and Kidrolase combination reduces Bcr Abl persistant cells following TKI treatment. (A) DA1-3b cells were treated by Imatinib and viability was assessed by cytometric analysis using Annexin V and sytox blue stainings. (B) DA1-3b cells were treated by Imatinib for 7 days and medium was changed for Imatinib-free medium (Imatinib withdrawal condition) containing CellTrace Violet probe. CellTrace Violet fluorescences were measured by flow cytometry following 1, 2 and 3 days after Imatinib withdrawal. (C) DA1-3b cells were untreated or treated by Imatinib (0.5 µM) for 7 days. Mitochondrial membrane potential, mitochondrial mass and mitochondrial ROS production were measured in living (yellow histograms) and dead (red histograms) cells using flow cytometry analysis of mitotracker deep red, mitotracker green and mitosox red stainings respectively, and annexin V and sytox stainings. For membrane potential analysis, FCCP was used as positive control for depolarization of mitochondrial membrane (grey histogram). The mean of fluorescence is indicated at the top right corner of each histogram. Cytofluorimetric profiles are representative of three independent experiments. (D) Using cell sorting by flow cytometry, DA1-3b cells were separated in two subpopulations characterized by low or high mitochondrial membrane potential ($\Delta\psi$m) in comparison with unsorted population. Both populations were treated with Imatinib (at the indicated concentrations) for 7 days and the percentage of persistant cells (annexin V neg./sytox neg) was determined by flow cytometry. (mean $\pm$ SD, n = 3, *p $<$ 0.05; ***p $<$ 0.005). (E) Cell death (by PI staining) in DA1-3b cells treated by Kidrolase or Erwinase (from 0 to 2 UI/mL) in combination with Imatinib (0.5uM) was determined after 7 days (mean $\pm$ SD, n = 3, *p $<$ 0.05).

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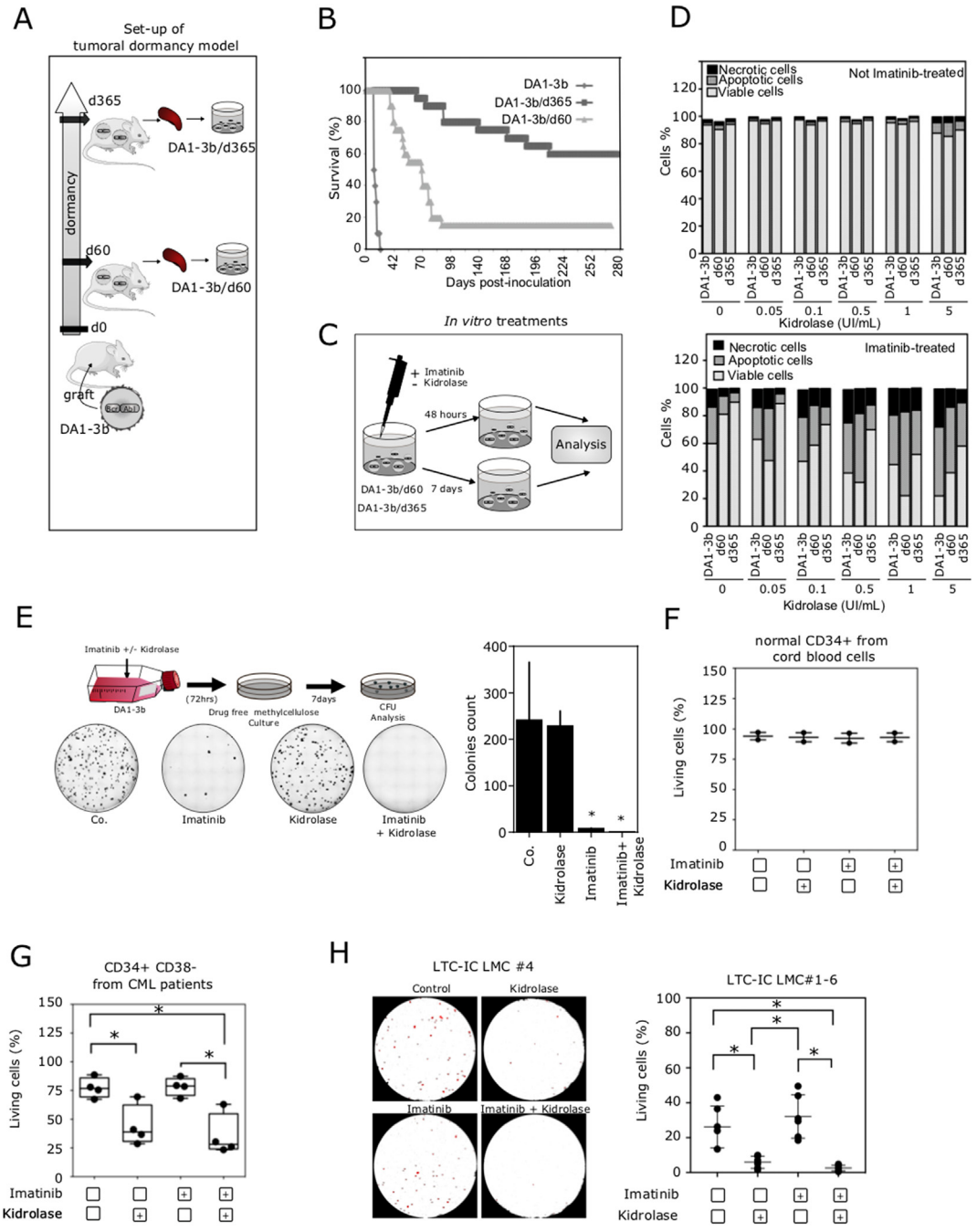


Figure 6: TKI and Kidrolase combination increases chemosensitivity in different models of TKI resistance. (A) Schematic representation of tumor dormancy model set up and treatments on leukemic dormant cells d60 and d365. Previously, mice were immunized with irradiated IL12—transduced DA1-3b cells, challenged with wild-type DA1-3b cells and randomly killed after 60 days or 365 days follow-up [2,24]. Leukemic residual cells were collected from bone marrow of sacrificed mice and used to generate stable cell lines (DA1-3b/d60 or DA1-3b/d365). (B) Lethal leukemia developped in mice injected intraperitoneally with DA1-3b wt, DA1-3b/d60 or DA1-3b/d365 cells. (C—D) DA1-3b, DA1-3b/d60 and DA1-3b/d365 cells were treated by Imatinib and Kidrolase for 48 h and cell death was assessed using flow cytometry analysis of annexin V and PI stainings. (n = 3). (E) Schematic representation of colony forming cell (CFC) assay set up (see materials and methods for more details) (upper panel). DA1-3b cells were treated *in vitro* with Imatinib 1 μM (Ima.) and/or Kidrolase 2 UI/mL (Kidro) for 72 h. Cells were then seeded in semi-solid medium and left to incubate for 7 days. Images representative of 3 experiments are shown (lower panel). The clonogenic potential of leukemic cells after undergoing treatments are measured by colonies count (right panel) (mean ± SD, n = 3, *p < 0.05). (F) Assessment of $CD34^+$ progenitors from cord blood cells of 2 healthy donors (CBCs) viability (by PI assay) after 48 h exposure to Imatinib (3 μM) and Kidrolase (2 UI/mL). (G) Assessment of the viability of CD34+/CD38-primary CML cells (freshly collected from 4 newly diagnosed CML patients) after 48 h exposure *ex vivo* to Imatinib (3 μM) and Kidrolase (2 UI/mL). (H) Long-Term Culture Initiating Cell assay of postculture CML progenitors were performed. CD34+/CD38-primary leukemic cells (cryopreserved samples from 6 CML patients) were incubated in myelocult medium containing vehicle, imatinib (2 μM), kidrolase (2 UI/ml) or imatinib and kidrolase. Then, CML cells were then plated in contact with MS5 stromal feeders for 5 weeks. The number of Imatini-resistant LTC-IC was determined by replating in methylcellulose (mean ± SD, n = 3, *p < 0.05).

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3.4. Dual treatment with imatinib and kidrolase significantly induces death of BCR-ABL1+ leukemic cells

Given the metabolic flexibility of BCR-ABL1+ cells observed above, we hypothesized that the combination of Imatinib and L-asparaginases such as Kidrolase could be therapeutically relevant by blocking both glycolysis and mitochondrial metabolism. Metabolic flux analyses indicate that cells treated with the Imatinib and Kidrolase combination caused much more impairment of the carbon flux into the TCA cycle, than either of these drugs alone (Figure 4A right panel). This decreased TCA activity was not linked to changes in the respiratory chain protein expression as evidenced by immunoblot of several proteins of each complex (Supplementary Fig. 3E). This drug combination also effectively impeded the glycolytic flux (Figure 4A left panel). In BCR-ABL1+ cell lines, Kidrolase synergized with Imatinib to induce cell death (Figure 4B-C and supplementary 3F). The combination of Imatinib with Kidrolase enhanced cell death by potentiating the intrinsic pathway of apoptosis through the downregulation of Bcl-2 and Bcl-XL protein levels and Bim upregulation (Figure 4D). The combination of Kidrolase and Imatinib showed the highest killing effect compared to the association of Kidrolase and other anti-leukemic drugs (daunorubicin or idarubicin) (Figure 4E). Kidrolase acted in synergy with Imatinib in BCR-ABL1 + leukemia cells even when cells were cultured on the MS5 stroma cell line (Figure 4F) or in hypoxic conditions (Figure 4G), situations known to protect leukemia cells from L-asparaginase [22] or Imatinib effects [23,24]. These results suggest that Imatinib plus Kidrolase elicit death synergistically in BCR-ABL1 + leukemic cells.

3.5. Imatinib synergized with kidrolase to eradicate BCR-ABL1+ persistent stem cells and leukemia-initiating cells in CML patients

We explored the potential of the synergistic combination of Imatinib plus Kidrolase against subpopulations of short-term relapse-inducing CML cells. First, the subpopulation of cells that survived in the presence of Imatinib for 7 days was enriched in persistent CML (Figure 5A) and Imatinib inhibition led to the proliferation of blast cell clones (Figure 5B). This persistent population, which survived during TKI exposure, showed a high level of mitochondrial metabolism as determined by their mitochondrial potential, mitochondrial mass and the overproduction of mitochondrial ROS (Figure 5C). To confirm the predominant role of mitochondrial metabolism, we isolated two populations characterized by low ($\Delta\psi_m$ low) or high mitochondrial ($\Delta\psi_m$ high) membrane potential using cell sorting. As expected, the percentage of persistent leukemia cells was higher in $\Delta\psi_m$ high DA1-3b cells in comparison with $\Delta\psi_m$low cells, whereas the combination of Imatinib and Kidrolase efficiently eradicated both subpopulations. Finally, we showed that Erwinase, another L-asparaginase, also synergized with Imatinib to kill persistent BCR-ABL1+ cells (Figure 5E).

We studied the effect of this drug combination on long-term persistent BCR-ABL1+ cells originated from a mouse model of *in vivo* leukemia dormancy [15]. In our model, the DA1-3b/d60 and DA1-3b/d365 cells were derived from the BCR-ABL1 + DA1-3b cells injected in mice and isolated after 2 months or 1 year of tumor dormancy, respectively [16,24] (Figure 6A). DA1-3b/d60 and DA1-3b/d365 were injected intraperitoneally in mice to develop a lethal leukemia (Figure 6B). As expected, death of DA1-3b/60 and DA1-3b/d365-bearing mice was delayed in comparison with DA1-3b WT mice, confirming the maintenance *in vivo* of the dormant phenotype (Figure 6B). Persistent-leukemic DA1-3b/d60 and DA1-3b/d365 cells were completely refractory to Kidrolase monotherapy and also partially resistant to treatment with Imatinib (Figure 6C,D). However, the combination of both drugs eradicated long-term persistent cells as evidenced by the induction of apoptosis (annexin V staining) (Figure 6D) and loss of colony-forming potential (Figure 6E). Kidrolase in combination with Imatinib reduced colony formation of DA1-3b cells by more than 90% compared to Imatinib alone (Figure 5E). We assessed the *ex vivo* effects of Kidrolase plus Imatinib in different CD34+ and CD38-subpopulations of primary CML CD34+ progenitor cells (freshly collected from newly diagnosed patients) (n = 4; Figure 6G and Supplementary Fig. S4) and from 6 cryopreserved primary CML cells from patients at diagnosis (Supplementary Fig. S5). We underlined that the killing effect was more pronounced on the stem cell-enriched CD34+ CD38-subpopulation as compared to more differentiated CML cells (Figure 6G and Supplementary Fig. S4). As expected, the combination had no effect on CD34+ progenitor cells from healthy individuals (n = 2; Figure 6F). We used LTC-IC assays to confirm that cells surviving BCR-ABL inhibition retained the *in vitro* functional capacity of stem cells. As seen in Figure 6H and Supplementary Fig. S6, we confirmed the efficacy of Kidrolase in combination with Imatinib in reducing the stem cell potential of primary CML cells.

The combined use of Imatinib and Kidrolase synergistically increase selective cytotoxicity in CML stem cells.

4. DISCUSSION

We characterized the metabolic effects of the FDA-approved drug Kidrolase in association with TKI in myeloid leukemia and demonstrated that they displayed synergistic antimetabolic effects eradicating CML stem cells. It is well established that TKI such as Imatinib possess a potent anti-Warburg effect. Imatinib efficiently hampers glucose metabolism through the reduction of GLUT-1 surface localization [10], inhibition of BCR-ABL1-mediated PKM2 phosphorylation or the modulation of PKM isoforms [18]. Glycolysis inhibition alone is often insufficient to eradicate cells due to compensatory activating metabolic pathways [6,13]. Upon Imatinib exposure, mitochondrial oxidative metabolism is maintained at high levels due to fatty acid [18] and/or glutamine oxidation (Figure 2). This suggests that remaining TKI-tolerant cells become addicted to mitochondrial activity for survival [13,25]. In agreement with our results, mitochondrial metabolism is spared by $FLT3^{ITD}$ TK inhibitors in myeloid cells [25,26]. The mechanisms that result from glycolytic metabolism shift toward oxidative metabolism remain largely unknown. However, we and others [25,27] observed a significant increase in mitochondrial mass upon TKI exposure suggesting important changes in mitochondrial biogenesis. As a consequence of mitochondrial addiction, TKI-tolerant cells were highly sensitive to the antileukemia effects of mitochondrial-targeting drugs. Myeloid leukemia presents numerous mitochondrial-druggable targets [25,26,28–30]. CPT1 (carnitine O-palmitoyltransferase 1) inhibitors, which reduce fatty acid oxidation and mitochondrial OXPHOS, significantly decrease the number of quiescent leukemia progenitor cells [27,31]. The combination of mitochondrial drugs and TKI consists in a rational approach where the therapeutic objective is to consider complementary mechanisms of action. TKI and mitochondrial inhibitors address the two compensatory aspects of the metabolism, glycolysis and mitochondrial oxidation, which neither of the monotherapy is capable of achieving in order to kill leukemia cells. Several preclinical studies evidenced that TKI, such as $FLT3^{ITD}$ inhibitors, can synergize with mitochondrial inhibitors for potent antileukemia effects [13,25,28–30]. Inhibition of the mitochondrial "booster" STAT3 [25,31], or the mitochondrial translation [13,29] can synergize with BCR-ABL1 inhibitors in CML. We completed the frame including the

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inhibition of glutamine-dependent mitochondrial metabolism. Genetic and pharmacological inhibition of the first enzyme in glutamine metabolism is synthetically lethal with FLT3-ITD-TKI in myeloid leukemia cells [25,32]. This effect seems related to glutathione depletion and enhancement of mitochondrial oxidative stress resulting in leukemia cell death [29,30]. We used L-asparaginase to deplete extracellular glutamine and therefore mitochondrial metabolism. To best translate *in vitro* results into clinical application, we selected the existing FDA-approved drug Kidrolase. However, L-asparaginase clinical use has been associated with a number of dose-dependent toxicities [32,33]. When combined with TKI, L-asparaginase should be less toxic since exposure to BCR-ABL1 inhibitors specifically increases the sensitivity of BCR-ABL1+ cells to mitochondrial targeting drugs (results and [30,34]). Imatinib has already been added to intensive combination chemotherapy, which included asparaginase, to treat newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia [35]. Toxicities of the combined treatment were manageable and included grade 4 myelosuppression (with severe neutropenia) and reversible grade 3 hyperbilirubinemia, which was more likely due to treatment with L-asparaginase alone. The toxicities of the combination could probably be largely attributed to the L-asparaginase effects.

As demonstrated (Figure 3A), L-asparaginase depleted the extracellular AA, glutamine and asparagine. Several studies uncovered a crucial role of asparagine in cancer cell growth and proliferation [33,36] as well as cell survival to glutamine deprivation [34,37]. Thus, asparagine is required for anabolism and cell proliferation in the absence of glutamine [13,36,38,39]. For these reasons, the synergistic effect of L-Asparaginase observed here is probably based on the depletion of both extracellular asparagine and glutamine. It is noteworthy that LSCs relies on AA including glutamine and glutamate to maintain OXPHOS for survival [37].

Targeting mitochondrial metabolism was suggested as a therapeutic approach against CML stem cells [13,38,39]. Recently a therapeutic drug combination targeting mitochondrial metabolism demonstrated its efficacy against LSCs in patients with AML [2,40]. We evidenced that both glycolysis and glutamine-dependent mitochondrial metabolism had to be impaired to eradicate LSCs. Targeting compensatory pathways of glutamine metabolism in CML stem cells can improve the efficacy of cancer treatments that impair glucose utilization. We provided preclinical evidence that the antimetabolic cooperativity by the combination of oncogene tyrosine kinase inhibitors and mitochondrial inhibitors constitutes a novel interesting therapeutic approach to eradicate LSC. This antimetabolic strategy can target CML stem cells and could therefore limit therapeutic failure in patients following the discontinuation of TKI therapy.

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AUTHOR CONTRIBUTIONS

AT, RK, QF, PM MJ, NG, WL, SD, BG and TI performed experiments and interpreted data. BT, DB, TI, BQ, JK and PMA designed experiments, interpreted data and wrote the manuscript. FX-M, BT and VC provided the patients samples. All authors approved the paper.

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CONFLICT OF INTEREST

All other authors declare no competing financial interests

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101410.

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