Tyrosine Kinase Inhibition in Leukemia Induces an Altered Metabolic State Sensitive to Mitochondrial Perturbations

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Abstract

Purpose: Although tyrosine kinase inhibitors (TKI) can be effective therapies for leukemia, they fail to fully eliminate leukemic cells and achieve durable remissions for many patients with advanced BCR-ABL+ leukemias or acute myelogenous leukemia (AML). Through a large-scale synthetic lethal RNAi screen, we identified pyruvate dehydrogenase, the limiting enzyme for pyruvate entry into the mitochondrial tricarboxylic acid cycle, as critical for the survival of chronic myelogenous leukemia (CML) cells upon BCR-ABL inhibition. Here, we examined the role of mitochondrial metabolism in the survival of Ph+ leukemia and AML upon TK inhibition.

Experimental Design: Ph+ cancer cell lines, AML cell lines, leukemia xenografts, cord blood, and patient samples were examined.

Results: We showed that the mitochondrial ATP-synthase inhibitor oligomycin-A greatly sensitized leukemia cells to TKI in vitro. Surprisingly, oligomycin-A sensitized leukemia cells to BCR-ABL inhibition at concentrations of 100- to 1,000-fold below those required for inhibition of respiration. Oligomycin-A treatment rapidly led to mitochondrial membrane depolarization and reduced ATP levels, and promoted superoxide production and leukemia cell apoptosis when combined with TKI. Importantly, oligomycin-A enhanced elimination of BCR-ABL+ leukemia cells by TKI in a mouse model and in primary blast crisis CML samples. Moreover, oligomycin-A also greatly potentiated the elimination of FLT3-dependent AML cells when combined with an FLT3 TKI, both in vitro and in vivo.


Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by the t(9;22)(q34;q11) translocation producing the Philadelphia chromosome (Ph; ref. 1). The resultant fusion protein, BCR-ABL, is causative for the disease and is also present in 20% to 30% of acute lymphoblastic leukemia (ALL; ref. 2). Although the treatment of CML with BCR-ABL tyrosine kinase inhibitors (TKI), like imatinib mesylate and dasatinib, has revolutionized therapy for these leukemias, BCR-ABL TKIs typically fail to fully eliminate the leukemia, and thus most patients require lifetime therapy. Moreover, adult Ph− ALL and patients with advanced stage CML exhibit only transient responses to BCR-ABL TKI (3).

Acute myelogenous leukemia (AML) is a heterogeneous group of leukemias. AML is the most common adult leukemia and the second most common childhood leukemia (4). About a third of AMLs express mutations in the FMS-like tyrosine kinase 3 receptor (FLT3). The presence of activating FLT3 internal tandem duplications (FLT3ITD) is associated with reduced overall survival (5, 6). Like BCR-ABL, FLT3 signaling provides prosurvival and antiproliferative signals to AML cells. Although FLT3 TKIs as monotherapy have shown promising initial responses in AML, clinical trials with FLT3 inhibitors have so far failed to show durable responses in AML (7–9).
processes that can be exploited therapeutically. Resistant CML cells de novo restricting fl transporters) away from the surface of CML cells, increasing the member 1 (SLC2A1) transporters (alternatively named GLUT-1) function of solute carrier family 2 (facilitated glucose transporter), protein kinase B (AKT) downstream signaling (12, 13).

The role of the TCA cycle and oxidative phosphorylation in the Warburg effect. Bypassing the TCA cycle provides a constant end product of metabolism is lactate fermentation, also known as the glycolytic phenotype, in which glycolysis is the main source of energy in the cell and the end product of metabolism is lactate fermentation, also known as the Warburg effect. Bypassing the TCA cycle provides a constant supply of metabolic intermediates for macromolecule biosynthesis without jeopardizing an adequate source of ATP. BCR-ABL confers this phenotype to CML cells, in part via activation of PI3K protein kinase B (AKT) downstream signaling (12, 13).

Treatment of CML cells with imatinib leads to decreased glucose uptake by suppressing glycolysis, causing the translocation of solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1) transporters (alternatively named GLUT-1) transporters away from the surface of CML cells, increasing the flux of residual glucose through the mitochondrial TCA cycle, restricting de novo nucleotide production, and inhibiting fatty acid synthesis (14–16). Furthermore, imatinib resistance can be mediated in part by hypoxia-inducible factor 1-α (HIF1α)-dependent upregulation of glycolysis (17, 18).

The role of the TCA cycle and oxidative phosphorylation in the survival of Ph+ and FLT3ITD leukemia is not fully characterized. Oxthiamine, a thiamine analogue that inhibits thiamine-dependent TCA cycle and pentose-phosphate pathway enzymes, enhances the efficacy of imatinib toward imatinib-resistant CML cells in vitro and reduces tumor burden in a mouse model of BCR-ABL+ leukemia (17). Recent studies also indicate that AML cells have altered mitochondrial dependencies, including uncoupling of oxygen consumption from ATP production and enhanced dependence on mitochondrial translation (19, 20). These studies suggest that TK-dependent leukemia cells may exhibit specific metabolic dependencies, and that the characterization of these dependencies could reveal processes that can be exploited therapeutically.

We previously performed a large-scale loss of function RNAi screen to identify genes whose inhibition synergizes with imatinib to kill Ph+ leukemia cells (21). This screen identified multiple enzymes involved in glucose metabolism as synthetic lethal. Here, we demonstrate that Ph+ and FLT3ITD leukemia cells become exquisitely sensitive to perturbations in mitochondrial function, specifically upon treatment with TKI.

Materials and Methods

Cell culture and generation of knockdown cell lines

KB M7 and KBM5 CML cells were obtained from M. Beran at MD Anderson (Houston, TX); Ba/F3 from B. Deininger at Oregon Health & Science University (Portland, OR); MV-4–11, MOLM-13, and Kasumi-1 cells from R. Arceci at Phoenix Children’s Hospital (Phoenix, AZ); NOMO-1 and OCI-AML-3, K562, and SUP-B15 cells were purchased from the DSMZ and ATCC, respectively. Cells were grown in standard culture conditions. MV-4–11 cells express the homozygous insertion D600_L601>HVDFREYED in FLT3, whereas MOLM-13 cells express the heterozygous insertion F601_K602>REYELD. Kasumi-1 cells express the ligand-independent N822K KIT activating mutation. Lentiviruses generated using pLKO.1 vectors (Sigma-Aldrich; Supplementary Table S1) were used to transduce cells as previously described (21). Cells were selected in 2.5 µg/ml puromycin. Ba/F3 murine pro-B cells expressing BCR-ABL and p210 BCR-ABL Reff ALL cells were generated as previously described (21–23). Cell lines were authenticated by short tandem repeat examination and tested negative for mycoplasma using the intiRON e-Mycro plus Mycoplasm PCR detection Kit in July 2012.

Cell viability experiments

For cell line growth curve experiments, 2 × 10^4 cells were seeded in 96-well plates and treated with the indicated drugs. After 1 to 3 days of treatment, cells were washed in PBS, and replated in fresh medium for an additional 2 to 5 days. At the indicated time, an aliquot was stained with 10 µg/mL propidium iodide and counted using a Beckman Coulter Quanta SC or Guava 8HT flow cytometer. For K562 clonogenic assays, after 3 days of treatment, cells were washed and plated in 1.2% methylcellulose (R&D Systems) and the number of colonies counted under a microscope 7 to 14 days later.

Pharmacologic agents

Imatinib mesylate and dasatinib were obtained from the University of Colorado Hospital Pharmacy, lestaurtinib was obtained from Cephalon, and oligomycin-A, cytarabine, doxorubicin, and 2-deoxy-D-glucose (2-DG) were purchased from Sigma-Aldrich. Quizartinib (AC220) was synthesized by the University of Colorado Medicinal Chemistry Core (see Supplementary Methods).

Mouse leukemia models

All mouse experiments were approved by our Institutional Animal Care and Use Committee (protocol# 41411[07]1E). The primary human AML samples were obtained under Institutional Review Board protocol # 12–0173. Female C37BL/6 mice (4–6-week-old) were obtained from the National Cancer Institute. Arf+/−/BCR-ABL/GFP+ B-ALL cells (5 × 10^5) in 100 µL of PBS were injected via tail vein. After 3 days,
mice were treated with vehicle [80 mmol/L citric acid, pH 2.1, for oral gavage (21) and PBS for intraperitoneal injections, i.p.], 10 mg/kg dasatinib (oral gavage), 100 μg/kg oligomycin-A (i.p.), or combination of dasatinib and oligomycin-A. Dasatinib was prepared as previously described (21), and the 10 mg/kg dose has been shown to replicate clinical experience in humans (23). For monitoring leukemic burden in mice, blood collected into heparin was hemolyzed and stained with B220-PE and Mac1-PE-Cy7 and monitored for GFP expression. See Supplementary Table S1 for antibody information. Samples were run using a Beckman Coulter Gallios Flow Cytometer and analyzed using FlowJo software.

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from The Jackson Laboratory and bred in house. The patient xenograft sample came from a 54-year-old female with AML expressing FLT3ITD and NPM1 mutations. The vials of primary leukemia were thawed in a water bath, washed in PBS, counted by flow cytometry, and resuspended in normal saline for injection. Four- to 6-week-old NSG mice were pretreated with 25 mg/kg of busulfan (i.p.) 24 hours before transplantation. After expansion in vivo, the secondary leukemia was harvested from the spleen and bone marrow, and subsequently transplanted into cohorts of mice for drug treatment. Cells (3 × 10^6) were injected i.v., and treatment started when peripheral blast count was at least 5% (mean, 7.85%). Leukemic burden was monitored by flow cytometry staining for human HLA-ABC and CD45.

Statistical analyses

Statistical significance was determined using one-way ANOVA followed by the Tukey post-test using GraphPad Prism. The Kaplan–Meier survival curves were analyzed using GraphPad Prism (log-rank test). Combination indices were calculated using the median-effect principle and the Combination Index-Isobologram Theorem (CompuSyn software), and summarized in Supplementary Table S2. Data shown reflect multiple independent biologic replicates, not technical replicates. See Supplementary Methods for additional details.

Results

Our large-scale RNAi screen in K562 CML blast crisis cells identified multiple metabolic enzymes (Supplementary Fig. S1A), including dihydrolipoamide S-acetyltransferase (DLAT), a component of the pyruvate dehydrogenase (PDH) complex, as synthetic lethal with imatinib (21). DLAT is located in the mitochondria and is a limiting component of PDH, which is...
essential for pyruvate entry from glycolysis into the TCA cycle.

DLAT knockdown sensitizes CML cells to BCR-ABL TKI

To validate DLAT as synthetic lethal with imatinib, K562 cells were transduced with shRNA constructs designed against DLAT or a nonsilencing shRNA control (see Supplementary Table S1 for sequences). Effective DLAT knockdown was demonstrated by Western blot (>90%; Fig. 1A and Supplementary Fig. S1B) and biochemically using acidification of the culture medium as a readout for changes in lactic acid production, a by-product of blocking pyruvate entry into the TCA cycle (Supplementary Fig. S1C). In addition, DLAT knockdown cells exhibit reduced superoxide levels reflecting decreased oxidative phosphorylation (Supplementary Fig. S1D).

Although knocking down DLAT had no detectable impact on the proliferation of vehicle-treated K562 cells (Fig. 1B, top; Supplementary Fig. S1E, top), knockdown greatly sensitized K562 cells to BCR-ABL inhibition.

Figure 2.
Oligomycin-A (OA) sensitizes BCR-ABL+ leukemia cells to BCR-ABL inhibition. A, K562 cells were treated with the indicated concentrations of oligomycin-A (0, 1, 2, 4, 6 nmol/L) in combination with vehicle (top) or 1 μmol/L imatinib (IM; bottom) for 3 days, followed by replating without drug. At the indicated time points, an aliquot was stained with propidium iodide and the number of viable cells determined by flow cytometry. The black lines indicate the duration of treatment. Statistical comparison of 0 nmol/L with 2, 4, and 6 nmol/L oligomycin-A at each imatinib concentration is shown over each bar, and combination indices are shown in Supplementary Table S2. B, a primary blast crisis CML sample was treated with oligomycin-A (2, 6, or 10 nmol/L) alone or in combination with 50 nmol/L dasatinib for 24 hours, washed, and seeded into 1.2% methylcellulose. Numbers of colonies were assessed after 14 days. C, Ba/F3 murine pro-B cells expressing vector or BCR-ABL were treated with vehicle, 0.5 or 1 μmol/L imatinib, in combination with increasing concentrations of oligomycin-A (0, 1, 2, 4, 6 nmol/L, indicated by triangle). After 3 days, the number of viable cells was determined by flow cytometry. Statistical pairwise comparisons of oligomycin-A–mediated changes are noted above each bar. D, K562 cells were treated with vehicle or 4 nmol/L oligomycin-A and increasing concentration of 2-DG (0, 2, 5 mmol/L) for 3 days and viable cells counted by flow cytometry. *, P < 0.05; **, P < 0.001.
cells to imatinib treatment (Fig. 1B, bottom; Supplementary Fig. S1E, bottom). These data suggest that upon imatinib treatment, CML cells must meet their energetic and anabolic demands via increased reliance on the TCA cycle. Indeed, treatment with methyl-pyruvate, an exogenous substrate for PDH, enhances survival of imatinib-treated CML cells (Fig. 1C), supporting the hypothesis that inhibition of glucose utilization is at least partially responsible for the antiproliferative effects of imatinib but that mitochondrial pyruvate oxidation can provide some protection from BCR-ABL inhibition. Consistent with reduced entry of pyruvate into the TCA cycle for ATP production, knocking down DLAT significantly reduced basal levels of ATP in leukemia cells (Fig. 1D). Treatment with imatinib also reduces the levels of ATP in these cells (Fig. 1D). Although treatment with imatinib at 5 μmol/L has a modest additional effect on ATP levels in combination with DLAT knockdown, synergistic effects of imatinib and DLAT knockdown on leukemia cell killing likely derive from the multiple combined catabolic and anabolic effects of inhibiting glucose metabolism.

Oligomycin-A sensitizes leukemia cells to BCR-ABL TKIs

Given that PDH is not targeted by available therapeutics, we asked how perturbing mitochondrial metabolism using oligomycin-A might sensitize leukemia cells to imatinib. Oligomycin-A binds to mitochondrial ATP-synthase C-subunits, which effectively block ATP generation (24, 25). K562 cells were treated with imatinib (0.5 or 1 μmol/L), in the range of doses required to inhibit most BCR-ABL kinase activity (21), in combination with increasing concentrations of oligomycin-A. Notably, although 0.5 to 2 μmol/L oligomycin-A synergistically eliminated K562 cells when combined with imatinib (Supplementary Fig. S2A–S2C), we found that treatment with concentrations of oligomycin-A as low as 2 to 6 nmol/L was equally effective at sensitizing CML cells to imatinib-mediated killing (Fig. 2A and Supplementary Fig. S2D). Moreover, low-dose oligomycin-A prevented the recovery of imatinib-treated K562 cells after drug removal (Fig. 2A, bottom), and combined treatment with imatinib and oligomycin-A synergistically reduced colony formation (Supplementary Fig. S2E; see Supplementary Table S2 for combination indices). Similar results on viability were observed in other Ph+ lines, including SUP-B15 B-ALL, the CML blast crisis lines KBM7 and KBM5 (Supplementary Fig. S2F–S2H), and in two primary blast crisis CML patient samples in colony forming assays (Fig. 2B and Supplementary Fig. S2I). Although oligomycin-A enhanced imatinib-mediated apoptosis (Supplementary Fig. S3A and S3B), it did not affect phosphorylation of targets downstream of BCR-ABL, including phospho-ERK1/2 and phospho-STAT5 (Supplementary Fig. S3C–S3E), indicating that the two drugs have independent mechanisms of action.

To determine whether efficacy of the combination therapy of imatinib and oligomycin-A is specific to BCR-ABL+ cells, we treated the pro-B-cell line Ba/F3, expressing either vector or p210 BCR-ABL, with increasing concentrations of imatinib and oligomycin-A. Expression of BCR-ABL in Ba/F3 cells confers IL3 independence (26). As expected, imatinib had no effect on Ba/F3-vector cells, but effectively eliminated Ba/F3-BCR-ABL cells (Fig. 2C). We also observed a modest effect (<2-fold inhibition) of oligomycin-A alone in both cell lines. However, oligomycin-A synergized with imatinib only in Ba/F3 cells expressing BCR-ABL, indicating that TK dependency is required for sensitization to the combined treatment. In addition, inhibition of glycolysis using 2-deoxyglucose, a competitive glucose analogue that cannot be metabolized through glycolysis, also sensitized CML cells to oligomycin-A treatment (Fig. 2D). These data suggest that the effectiveness of the combination therapy of oligomycin-A and imatinib is in part related to the ability of imatinib to inhibit glycolysis.

FLT3 inhibition synergizes with oligomycin-A to eliminate FLT3ITD AML cells

Given that the driving TK in a subset of AML, FLT3 expressing an internal tandem duplication (FLT3ITD), shares similar downstream effectors with BCR-ABL (including AKT; ref. 27), we asked whether the combination of the FLT3 inhibitor quizartinib and oligomycin-A could synergize to eliminate AML cells driven by aberrant FLT3ITD. We treated both FLT3WT (NOMO-1 and OCI-AML-3) and FLT3ITD (MV-4–11 and MOLM-13) cell lines with 0 to 4 nmol/L quizartinib (AC220) in combination with increasing doses of oligomycin-A (0.5–4 nmol/L).

As expected, only the two FLT3ITD cell lines, MV-4–11 and MOLM-13, were sensitive to quizartinib (Fig. 3A, left; Supplementary Fig. S4A). These cell lines also showed modest reductions in cell numbers with oligomycin-A alone. Importantly, the combination of quizartinib and oligomycin-A synergized to eliminate FLT3ITD cells. In contrast, the two FLT3WT cell lines showed no sensitivity to either quizartinib and/or oligomycin-A (Fig. 3A, right; Supplementary Fig. S4B). In addition, we observe similar combinatiorial efficacy for the primary patient FLT3ITD and FLT3ITDI sample (Fig. 3B and Supplementary Fig. S4H) when treated with quizartinib and oligomycin-A. Moreover, synergism in FLT3ITD cells can be observed with a different FLT3 inhibitor lestaurtinib (Supplementary Fig. S4C) and with treatment duration as short as 24 hours with quizartinib (Supplementary Fig. S4D and S4E; note log10 scale). Similar results were obtained by combining oligomycin-A with imatinib-mediated inhibition of KIT in Kasumi-1 AML cells, which express the ligand-independent N822K activating KIT mutation (Supplementary Fig. S4F).
To assess whether normal human hematopoietic progenitors would be affected by the combination therapy, we treated whole cord blood or CD34^+ selected cord blood cells from healthy newborns with oligomycin-A and/or quizartinib for 24 hours and plated cells in methylcellulose for clonogenic assays. Treatment with quizartinib or oligomycin-A alone showed a modest reduction in colony numbers, but the effects were not further reduced by the combination therapy (Fig. 3C and Supplementary Fig. S4G), suggesting that oligomycin-A has minimal effects on healthy hematopoietic progenitors.

To assess whether the addition of oligomycin-A to a TKI is specific to TK inhibition, we treated MOLM-13 (FLT3ITD cells) with doxorubicin and cytarabine, two standard chemotherapies in AML treatment regimens, in combination with oligomycin-A (Fig. 3D). Doxorubicin and cytarabine impaired AML cell survival as expected, but these chemotherapies failed to synergize with oligomycin-A. These data further indicate that oligomycin-A becomes effective only upon inhibition of a driving tyrosine kinase.

**Synergistic concentrations of oligomycin-A do not impair mitochondrial TCA cycle or respiration**

Oligomycin-A inhibits respiration with an IC_{50} of 500 nmol/L to 1 µmol/L (24, 28). To confirm this, we measured the oxygen consumption rate (OCR) in K562 cells treated with oligomycin-A or two additional electron transport chain (ETC) inhibitors, antimycin (complex III inhibitor) and rotenone (inhibitor of electron transfer from complex I to ubiquinone; Fig. 4A, top). All three ETC drugs inhibited the OCR at the expected concentrations. For oligomycin-A, doses below 500 nmol/L of oligomycin-A have no effect on the OCR (Fig. 4A, bottom). Thus, we observed no appreciable effect of low nmol/L oligomycin-A treatment on respiration, despite potent synergistic killing of leukemia cells when combined with TKI at these concentrations. In contrast, antimycin treatment of CML cells at concentrations that inhibit respiration showed only additive effects when combined with imatinib (Supplementary Fig. S5A), further substantiating that the effects of oligomycin-A on leukemia cell killing when combined with TKI are separable from its ability to inhibit respiration.

We also performed nuclear magnetic resonance (NMR) spectroscopy on the cellular extracts and medium of K562 cells treated with imatinib and/or oligomycin-A. As expected, imatinib decreased levels of glycolytic and TCA cycle intermediates, but oligomycin-A had no discernable additional effects (Supplementary Fig. S5B–S5E and Supplementary Table S3). Taken together, these data indicate that oligomycin-A greatly potentiates imatinib-dependent CML cell killing at concentrations that do not appear to impact either the TCA cycle or respiration.

**Oligomycin-A impairs mitochondrial function**

We next asked whether the mechanism of action of low-dose oligomycin-A is still through impairment of mitochondrial function or integrity. Cells with dysfunctional mitochondria are unable to efficiently utilize galactose as a primary carbon source and rely on glycolysis for energy production (29). In the presence of glucose, treatment with oligomycin-A alone only modestly affected the expansion of K562 cells (Fig. 4B). In contrast, in the presence of galactose, treatment with oligomycin-A led to effective killing of K562 cells. Furthermore, both imatinib and oligomycin-A treatments increase ATP levels in K562 within 30 minutes, with greater decreases observed in cells treated with both drugs (Fig. 4C). This inhibition of ATP levels is not sustained, and ATP levels are restored by 6 hours of treatment (Supplementary Fig. S6A). The decrease in ATP levels correlated with an increase in phosphorylated AMPK, indicating increases in the AMP:ATP ratio (Supplementary Fig. S6B).

Notably, treatment with concentrations of oligomycin-A as low as 1 nmol/L, with or without imatinib, rapidly decreased the mitochondrial membrane potential (ΔΨ_m) in CML cells, as early as 1 hour after treatment (Fig. 4D and Supplementary Fig. S6C). Interestingly, although low nanomolar concentrations of oligomycin-A caused depolarization of ΔΨ_m, higher concentrations (>500 nmol/L) caused hyperpolarization of ΔΨ_m (Fig. 4D). ΔΨ_m depolarization was observed with two different dyes, from 1 to 24 hours after oligomycin-A treatment (before any appreciable cell death occurs), and was not affected by cotreatment with imatinib (Supplementary Fig. S6D and S6E). Similar depolarization is observed in MOLM-13 (FLT3ITD) AML cells treated with oligomycin-A (Supplementary Fig. S6F). In all, these results indicate that although low-dose oligomycin-A does not appreciably inhibit the TCA cycle or respiration, key mitochondrial functions, including ATP production and maintenance of ΔΨ_m are significantly impaired in oligomycin-A–treated leukemia cells.

**Oligomycin-A in combination with TKI increases superoxide levels**

The process of electron transfer in the ETC, which is coupled to ATP synthase, is not completely efficient, resulting in generation of ROS that can oxidize cellular components. K562 cells were treated with imatinib and/or oligomycin-A for different periods of time, and the levels of total and mitochondrial superoxide levels were measured using flow cytometry (Fig. 5A and B). The combination of oligomycin-A with imatinib caused an immediate and sustained increase in superoxide, suggestive of a perturbation in ETC function. The immediate increases in superoxide observed in the combination therapy suggest that the superoxide levels are affected by actions of the drugs themselves and not by the subsequent apoptotic process. We observed similar effects in Ph^+ SUP-B15 ALL cells (Supplementary Fig. S6G). In addition, we observed increased levels of the oxidized derivative of deoxyguanosine, 8-oxo-dG (a major product of DNA oxidation; ref. 30), in imatinib and oligomycin-A–treated cells (Fig. 5C, and hydrogen peroxide dose–response controls in Supplementary Fig. S6H). Thus, the combination of oligomycin-A with imatinib leads to significant increases in superoxide and subsequent oxidative damage.

To further characterize the role of superoxide, we treated K562 cells with increasing concentrations of the superoxide dismutase (SOD) inhibitor 2-methoxyestradiol (31). SODs catalyze the conversion of superoxide into oxygen and hydrogen peroxide, and are the major enzymes responsible for detoxification of ROS in mammalian cells. Concentrations of 2-methoxyestradiol that increase the levels of superoxide in K562 cells (Supplementary Fig. S6I) to levels similar to those observed in the combination therapy were sufficient to elicit cell death (Supplementary Fig. S6J). Finally, treating cells with methoxy-polyethylene glycol-coupled SOD1 reduced the ability of oligomycin-A to potentiate imatinib-mediated killing (Fig. 5D). These data suggest that the mechanism of action of oligomycin-A in promoting apoptosis in TKI-treated leukemia cells is partially mediated by enhanced superoxide production.
Combined treatment with TKI and oligomycin-A effectively eliminates leukemia cells in vivo

To test whether oligomycin-A could enhance the efficacy of TKI to eliminate BCR-ABL⁺ leukemia cells in vivo, we tested the combination therapy in a mouse model of BCR-ABL⁺ B-ALL. This model has previously been shown to closely mimic human BCR-ABL⁺ B-ALL, including responsiveness to the BCR-ABL inhibitor dasatinib (22, 23). Mice were inoculated with Arf⁻/⁻ p185-BCR-ABL/GFP leukemia, and after 3 days, started on daily treatments with vehicle, dasatinib, oligomycin-A, or the combination therapy of dasatinib plus oligomycin-A. After 4 days of therapy, leukemic burden in the combination therapy group was significantly reduced compared with the dasatinib alone group (Fig. 6A and Supplementary Fig. S7A). Mice treated with either vehicle or oligomycin-A alone succumbed to leukemia on day 10 with postmortem analysis showing extensive leukemia in the blood, bone marrow, and spleen. Dasatinib-treated mice had a median survival of 32 days, but all mice succumbed to leukemia by day 80 (Fig. 6B). To determine if all leukemia cells had indeed been eliminated in the remaining 8 of 9 mice in the combination therapy, therapy was stopped on day 85. Although three of the mice eventually relapsed with leukemia, five of the remaining mice remained healthy up to day 189 when the experiment was terminated. Postmortem analysis revealed no detectable leukemia cells in peripheral blood, bone marrow, or spleens by flow cytometry and in bone marrow samples by RT-PCR analysis for BCR-ABL mRNA (Supplementary Fig. S7B), indicating that the combination therapy of dasatinib and oligomycin-A completely eliminated leukemia cells in these five mice.
We next asked whether similar efficacy could be observed for FLT3ITD AML. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were transplanted with a primary human AML sample (FLT3ITD), monitored by flow cytometry, and after one month randomized into groups with similar peripheral blood burdens (Supplementary Fig. S7C and S7D). The mice were treated with quizartinib at a clinically relevant dose (32) and/or oligomycin-A, and sacrificed when the mice lost over 15% of their body weight or showed signs of disease. As shown in Fig. 6C, we observed a significant extension of survival in the group treated with the combination of quizartinib and oligomycin-A (median survival of 54.5 vs. 88.5 days). Mice treated with quizartinib alone or in combination with oligomycin-A had a dramatic initial response, but they eventually relapse with AML (Supplementary Fig. S7E). In all, these data show that low doses of oligomycin-A can improve therapeutic outcomes for TKI for both Ph+ and FLT3ITD leukemias in vivo. Note also that mice treated with TKI (dasatinib or quizzartinib) and oligomycin-A for over two months appeared healthy.

**Oligomycin-A treatment exhibits minimal toxicity in vivo**

To determine potential toxicity of low-dose oligomycin-A, we treated mice with either vehicle or oligomycin-A for 15 days. After 15 days of therapy, the mice receiving oligomycin-A exhibited no evident changes in appearance or behavior, but did show a slight decrease in weight (<1% from initial weight) compared with the vehicle controls (Supplementary Table S4). Complete blood counts revealed small differences in the number of monocytes and red blood cells (RBCs), and hematocrit and hemoglobin levels in oligomycin-A–treated mice, but these parameters remained within the reference normal ranges (Fig. 6D, top; Supplementary Table S4). Thus, oligomycin-A does not appear to be myelosuppressive in vivo. In addition, we measured markers of hepatic and renal toxicity. Serum levels of total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and blood urea nitrogen (BUN) remained the same for the two treatment groups. Minor differences in albumin levels (Fig. 6D, bottom) were observed but values remained within the reference range (Supplementary Table S4).

Because of concerns of mitochondrial toxicity from treatment with oligomycin-A, we tested mitochondrial integrity in vivo. The mitochondrial permeability transition pore (PTP) and cyclophilin-D regulate platelet activation, and thus mitochondria play a key role in the function of platelets (33). If oligomycin-A disrupts mitochondrial Δψm in platelets, we would expect treatment with oligomycin-A to disrupt platelet activation. However, we did not observe changes in the number of circulating platelets, their activation state, or their ability to respond to platelet agonists in oligomycin-A–treated mice (Supplementary Fig. S7F and S7G and Supplementary Table S4). These data suggest that therapy with low-dose oligomycin-A has minimal impact, at least in the short term, on normal mouse physiology at a concentration that exhibits potent antileukemic effects.

**Discussion**

Our results reveal that perturbing mitochondrial function can severely limit the survival of leukemia cells upon TKI treatment (Supplementary Fig. S8). First, we identified that pyruvate entry into the TCA cycle becomes important upon BCR-ABL inhibition, providing these cells an alternative mechanism of glucose metabolism to compensate for the inhibition of glycolysis driven by BCR-ABL. PDH, the gatekeeping enzyme linking glycolysis to the
TCA cycle, converts pyruvate to acetyl-CoA, which is decarboxylated via the TCA cycle to generate the electron donors used to generate the mitochondrial membrane potential. Still, our results contrast with those observed by Bonnet and colleagues (34), in which forcing entry of pyruvate into the TCA cycle by dichloroacetate-mediated inhibition of pyruvate dehydrogenase kinase impairs the survival of cancer cells in vitro and tumor growth in mice. In fact, cancer cells generally limit pyruvate entry into the TCA, such as by negative regulation of pyruvate kinase, which generates ATP and pyruvate from phosphoenolpyruvate and ADP (11). Moreover, pyruvate kinase activators can inhibit tumor growth (35). We propose that imatinib treatment of BCR-ABL+ leukemia cells reverses the leukemic cell dependency on pyruvate entry into the TCA cycle. It is well established that imatinib treatment in leukemia cells reduces glucose uptake and flux through glycolysis, which in turn limits the amount of pyruvate available in the cell (14). Under these conditions, our data suggest that upon TKI treatment CML cells become dependent on pyruvate entry into the TCA to sustain metabolic demands for continued survival.

Importantly, our studies have identified low nmol/L concentrations of oligomycin-A as an effective adjuvant therapy to TKI in the treatment of BCR-ABL+ and FLT3ITD+ leukemias. Oligomycin-A binds the C-subunit of the ATP synthase and inhibits ATP production (24, 25, 28). Although low nmol/L oligomycin-A concentrations do not inhibit oxygen consumption, these concentrations of oligomycin-A lead to transient decreases in ATP levels and Δψm depolarization in BCR-ABL+ cells. Moreover, combining oligomycin-A with TKI increases the generation of superoxides, which have been shown to result in oxidation of critical cellular macromolecules and cell death (36, 37). We show that increases in superoxides contribute to, but do not fully account for, the ability of oligomycin-A to synergize with BCR-ABL TKIs to eliminate leukemia cells.

Recent studies have shown that ATP synthase subunits also function as the mitochondrial PTP (38, 39). Opening of the PTP causes increased ion flux, leading to Δψm depolarization across the inner mitochondrial membrane and matrix swelling (40, 41), which can promote outer mitochondrial membrane disruption during apoptosis. Indeed, given that oligomycin-A leads to Δψm depolarization at low nmol/L concentrations similar to those that synergize with BCR-ABL and FLT3 TKI to eliminate leukemia cells, ATP synthase’s function as the PTP may be the key target of oligomycin-A in leukemia cells. Δψm depolarization would also be expected to decrease ATP production, which is dependent on the electrochemical gradient across the inner mitochondrial
membrane, and increase superoxide production by uncoupling the ETC (40, 41). Thus, investigations presented here have potentially discovered an exciting means to selectively promote partial depolarization and PTP opening in leukemia cells, leading to apoptosis when combined with TKI treatment.

Based on the known inhibitory effects of TKI on glucose uptake and glycolysis, and that either 2-DG treatment or glucose substitution with galactose sensitizes leukemia cells to oligomycin-A, we propose that inhibition of glycolysis is key to sensitization of leukemia cells to oligomycin-A. In fact, leukemia cells are quite tolerant of low levels of oligomycin-A (and the consequent modest mitochondrial depolarization) in their usual highly glycolytic states. The inhibition of glycolysis will force cells to depend on mitochondrial carbon metabolism (as evidenced by the dependency on PDH engendered by TKI treatment), and this new state of mitochondrial dependency makes the leukemia cells highly sensitive to oligomycin-A treatment (Supplementary Fig. S8). Although our evidence points to mitochondrial action of oligomycin-A (including the ability of galactose to sensitize leukemia cells to oligomycin-A, as well as oligomycin-A–mediated induction of superoxides, transient reductive of ATP levels, and ΔΨm depolarization), we cannot definitively conclude that the PTP is the relevant target of oligomycin-A. That said, given that the PTP has recently been shown to be composed of ATP synthase subunits (including the oligomycin-sensitive F0 subunit; refs. 38, 39), that ATP synthase is the conserved target for oligomycin-A from yeast to humans (42), and that ΔΨm depolarization shows a concentration dependence for oligomycin-A that closely mirrors the ΔΨm depolarization observed in leukemia cells, leading to mitochondria opening in the PTP, the ATP synthase in the PTP is currently the strongest candidate as the relevant target for low concentrations of oligomycin-A.

Importantly, clonogenic assays with healthy human cord blood cells and studies of treated mice reveal minimal side effects of oligomycin-A on normal cells, which highlight its therapeutic potential for the elimination of TK-addicted leukemia. TK inhibition in leukemia cells makes the cells hypersensitive to oligomycin-A in mouse models, at concentrations that have no substantial negative impact on mouse physiology, perhaps due to reduced glycolysis and/or reduced dependency of noncancer cells for the targeted TK. Although oligomycin-A has been previously reported to induce cell death in leukemia, the effects were observed at concentrations (~12 μmol/L) at which the ATP synthase is completely inhibited (43). At this concentration, the toxicity of such therapy will outweigh any clinical benefit. In contrast, the concentrations of oligomycin-A that synergize with TKI are approximately 1,000-fold less and do not impede oxygen consumption. Interestingly, other studies have shown that inhibition of mitochondrial ATP synthase, by increasing ROS-dependent NF-kB activation, is essential for cancer cell survival (44, 45). Again, we observe the opposite effects of low concentration oligomycin-A in leukemias, but specifically in the context of TK inhibition. It is notable that low mmol/L oligomycin-A causes ΔΨm depolarization, whereas μmol/L oligomycin-A causes hyperpolarization (Fig. 4F). Other groups have also reported hyperpolarization of ΔΨm at μmol/L concentrations of oligomycin-A (46).

Thus, we do not believe that our studies are in conflict with these previous studies, but reveal how inhibition of a dominant TK oncogene in a cancer can radically change mitochondrial dependencies, with clear therapeutic implications. Indeed, a recent study showed that inhibition of mitochondrial respiration (including with oligomycin-A) blocked the emergence of a slow-cycling tumor maintaining melanoma cell subpopulation in response to various therapies (47).

Understanding how glucose utilization and energy production pathways are altered in cancers upon treatment with both conventional and targeted therapeutics will be critically important for the development of metabolically targeted drug therapies. Our studies reveal that inhibition of the dominant oncogene in Ph+ and FLT3ITD leukemias can engender mitochondrial dependencies that can be exploited therapeutically. Notably, the Warburg effect is shared by many tumor types (10, 11, 48, 49), which may suggest that perturbing mitochondrial function could be a ubiquitous target for improved elimination of cancer cells upon therapy-mediated reversal of the glycolytic phenotype.

No potential conflicts of interest were disclosed.

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