Targeting Mitochondria with Avocatin B Induces Selective Leukemia Cell Death

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Abstract

Treatment regimens for acute myeloid leukemia (AML) continue to offer weak clinical outcomes. Through a high-throughput cell-based screen, we identified avocatin B, a lipid derived from avocado fruit, as a novel compound with cytotoxic activity in AML. Avocatin B reduced human primary AML cell viability without effect on normal peripheral blood stem cells. Functional stem cell assays demonstrated selectivity toward AML progenitor and stem cells without effects on normal hematopoietic stem cells. Mechanistic investigations indicated that cytotoxicity relied on mitochondrial localization, as cells lacking functional mitochondria or CPT1, the enzyme that facilitates mitochondria lipid transport, were insensitive to avocatin B. Furthermore, avocatin B inhibited fatty acid oxidation and decreased NADPH levels, resulting in ROS-dependent leukemia cell death characterized by the release of mitochondrial proteins, apoptosis-inducing factor, and cytochrome c. This study reveals a novel strategy for selective leukemia cell eradication based on a specific difference in mitochondrial function. Cancer Res; 75(12); 2478–88. ©2015 AACR.

Introduction

Leukemia and leukemia stem cells (LSC) possess several mitochondrial features that distinguish them from normal hematopoietic cells. Compared with normal cells, leukemia cells contain greater mitochondrial mass, have higher rates of oxidative phosphorylation (1), and depend on fatty acid oxidation for survival (2). Together, these altered mitochondrial characteristics may make drugs that target mitochondria potentially useful for the eradication of leukemia cells.

Acute myeloid leukemia (AML) is a devastating disease characterized by the accumulation of malignant myeloid precursors that fail to terminally differentiate (3). Patients diagnosed with AML are faced with poor disease prognosis. In adults (>65), 2-year survival rates are less than 10% (4). The suboptimal strategies that target both the bulk and LSC populations are needed to improve AML patient outcomes.

To identify novel AML therapeutics, we screened a natural health product library (n = 800) for compounds that induce death of TEX leukemia cells, an AML cell line with features of LSCs (1, 5, 6). From this screen, we identified avocatin B, a lipid-derived from avocados, as a novel anti-AML compound.

Materials and Methods

Cell culture

Leukemia (OCI-AML2) cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Seradigm) and antibiotics (100 U/mL of streptomycin and 100 μg/ml of penicillin; Sigma Chemical). TEX leukemia cells were cultured in 15% FBS, antibiotics, 2 mmol/L glutamine (Sigma Chemical), 20 ng/ml stem cell factor and 2 ng/ml IL3 (Peptide). Primary human samples (fresh and frozen) were obtained from the peripheral blood of AML patients who had at least 80% malignant cells among the mononuclear cells and cultured at 37°C in IMDM, 20% FBS, and antibiotics (see Supplementary Tables S2 and S3 for clinical parameters). Normal G-CSF-mobilized peripheral blood mononuclear cells were obtained from volunteers donating peripheral blood stem cells (PBSC) for allotransplant and were cultured similar to the primary AML samples. The collection and use of human tissue for this study was approved by the local ethics review board (University Health Network, Toronto, ON, Canada; University of Waterloo, Waterloo, ON, Canada).

Cell growth and viability

Cell growth and viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay.
(Promega) according to the manufacturer’s protocol and as previously described (7). Cells were seeded in 96-well plates, treated with drug for 72 hours, and optical density (OD) was measured at 490 nm. Cell viability was also assessed by the Trypan blue exclusion assay and by Annexin V and propidium iodide (PI) staining (Biovision), as previously described (7).

### Functional stem cell assays

Clonogenic growth assays with primary AML and normal hematopoietic stem cells were performed, as previously described (7). Briefly, CD34<sup>+</sup> bone marrow–derived normal stem cells (STEMCELL Technologies) or AML mononuclear cells from patients with >80% blasts in their peripheral blood (4 × 10<sup>3</sup> cells/ml) were treated with vehicle control or increasing concentrations of avocatin B and plated in duplicate by volume at 10<sup>3</sup> cells/ml per 35-mm dish (Nunclon) in MethoCult GF H4434 medium (STEMCELL Technologies) containing 1% methoculture in IMDM, 30% FBS, 1% bovine serum albumin (BSA), 3 U/mL recombinant human erythropoietin, 10<sup>−4</sup> mol/L 1,2-mercaptoethanol (2ME), 2 mmol/L glutamine, 50 ng/mL recombinant human stem cell factor, 10 ng/mL recombinant human granulocyte macrophage-colony-stimulating factor, and 10 ng/mL recombinant human IL3. After 7 to 10 days of incubation at 37°C with 5% CO<sub>2</sub> and 95% humidity, the number of colonies were counted on an inverted microscope with a cluster of 10 or more cells counted as one leukemic colony and 50 or more cells counted as a normal colony similar to previously described methods (1, 8).

Mouse xenotransplant assays were performed as previously described (1, 9). Briefly, AML patient cells were treated with 3 μmol/L avocatin B or dimethyl sulfoxide (DMSO; as a control) for 48 hours in vitro. Next, these cells were transplanted into femurs of sublethally irradiated, CD122-treated NOD/SCID mice and following a 6-week engraftment period, mice were killed and femurs excised, bone marrow flushed, and the presence of human myeloid cells (CD45<sup>+</sup>/CD33<sup>+</sup>/CD14<sup>+</sup>) was detected by flow cytometry. All animal studies were carried out according to the regulations of the Canadian Council on Animal Care and with the approval of the University Health Network, Animal Care Committee.

### High-throughput screen

A high-throughput screen of a natural product library (n = 800; Microsource Discovery Systems Inc.) was performed as previously described (1, 7). Briefly, TEX leukemia cells (1.5 × 10<sup>6</sup>/well) were seeded in 96-well polystyrene tissue culture plates. After seeding, cells were treated with aliquots (10 μmol/L final concentration) of the chemical library with a final DMSO concentration no greater than 0.05%. After 72 hours, cell proliferation and viability were measured by the MTS assay.

### Protein and mRNA detection

Western blotting was performed as previously described (10). Briefly, whole-cell lysates were prepared from treated cells, heated for 5 minutes at 95°C, and subjected to gel electrophoresis on 7.5% to 15% SDS-PAGE at 150 V for 85 minutes. The samples were then transferred at 25 V for 45 minutes to a polyvinylidene difluoride membrane and blocked with 5% BSA in Tris-buffered saline-tween (TBS-T) for 1 hour. The membrane was incubated overnight at 4°C with the primary antibody, poly(ADP) ribose polymerase (PARP) α (1:1,500; Cell Signaling Technology), ANT (1:1,000; Santa Cruz Biotechnology), ND1 (1:10,000; Santa Cruz Biotechnology), or α-tubulin (loading control; 1:5,000; Santa Cruz Biotechnology). Membranes were then washed and incubated with the appropriate secondary antibody (1:10,000) for 1 hour at room temperature. Enhanced chemiluminescence (ECL) was used to detect proteins according to the manufacturer’s instructions (GE Healthcare) and luminescence was captured using the Kodak Image Station 4000MM Pro and analyzed with a Kodak Molecular Imaging Software Version 5.0.1.27.

Quantitative PCR was performed as previously described (6) in triplicate using an ABI 7900 Sequence Detection System (Applied Biosystems) with 5 ng of RNA equivalent cDNA, SYBR Green PCR Master Mix (Applied Biosystems), and 400 nmol/L of CPT1-specific primers (forward: 5′-TCGTCACCCTTCTGCCCCTT ‑3′, reverse: 5′-ACACACCATAGCGTATCA TC ‑3′). Relative mRNA expression was determined using the ΔΔC<sub>T</sub> method as previously described (6).

### Assessment of fatty acid oxidation and mitochondrial respiration

Measurement of oxygen consumption rates (OCR) was performed using a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience). TEX cells were cultured in a 3% minimum essential medium (Life Technologies) containing 1% FBS and plated at 1 × 10<sup>5</sup> cells per well in polycl-lysine (Sigma Chemical)–coated XF24 plates. Cells were incubated with etomoxir (100 μmol/L, Sigma Chemical) or vehicle control for 30 minutes at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Next, palmitate (175 μmol/L, Seahorse Bioscience) or avocatin B (10 μmol/L) was added and immediately transferred to the XF24 analyzer. Oxidation of exogenous fatty acids was determined by measuring mitochondrial respiration through sequential injection of 5 μmol/L (final concentration) oligomycin, an ATP synthase inhibitor (Millipore), 5 μmol/L CCCP, a hydrogen ion ionophore (Sigma Chemical), and 5 μmol/L rotenone (Millipore)/5 μmol/L antimycin A, which inhibit complex III activity (Sigma Chemical). Fatty acid oxidation was determined by the change in oxygen consumption following oligomycin and CCCP treatment and prior to antimycin and rotenone treatment, according to the manufacturer’s protocol and as described by Abe and colleagues (11). Data were analyzed with XF software (Seahorse Bioscience).

ROS, NADH, NADPH, and GSH detection

Reactive oxygen species (ROS) were detected using 2,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA; Sigma Chemical) and dihydroethidium (DHE; Sigma Chemical). DCFH-DA is hydrolyzed by intracellular esterase to produce a nonfluorescent DCFH product. It can then be oxidized by ROS and other oxidizing species to produce a highly fluorescent DCF product (12). DHE is a superoxide indicator that, upon contact with superoxide anions, produces the fluorescent product 2-hydroxyethidium (13). Following drug treatment, TEX cells (5 × 10<sup>5</sup>) were collected and washed in PBS (Sigma-Aldrich). Cells were stained with 5 μmol/L (final concentration) DCFH-DA or 10 μmol/L DHE and allowed to incubate for 30 minutes in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Samples were then washed in PBS and ROS were measured by flow cytometry using the Guava EasyCyte 8HT (Millipore). Data were analyzed with GuavaSoft 2.5 software (Millipore).

Nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NAD), and glutathione (GSH) were measured by commercially available fluorimetric kits (AAT Biologicals).
Bioquest), according to the manufacturers’ protocol, following incubation of increasing duration with avocatin B (10 μmol/L). For NADPH studies, cells were also incubated with palmitate (175 μmol/L) in the presence or absence of etomoxir (100 μmol/L). For NADH and GSH studies, cells were incubated in the presence of palmitate and N-acetylcytsteine (NAC; 1 mmol/L), respectively. Data are presented as a percent NAD, NADPH, or GSH compared with control-treated cells ± SD.

**Liquid chromatography/mass spectroscopy**

Avocatin B’s presence in mitochondria and cytosolic fractions was detected using thin film solid-phase microextraction (TF-SPME; Professional Analytical System Technology) followed by liquid chromatography–high resolution mass spectrometry analysis (LC/MS; Thermo Exactive Orbitrap mass spectrometer; Thermo Scientific; refs. 14, 15). TEX cells were treated with avocatin B or a vehicle control for 1 hour, as performed for the Seahorse Bioanalyzer experiments (i.e., assessment of fatty acid oxidation), and cytosolic and mitochondrial fractions were then isolated, as previously described (16). Fraction purity was determined by Western blot analysis for the mitochondrial-specific protein ND1 (i.e., complex 1). Next, samples were prepared by TF-SPME and then subjected to LC/MS analysis. For detailed methods on sample preparation, standardization, and calibration, please refer to the Supplementary Methods.

**Apoptosis determination**

Caspase activation, PARP cleavage, Annexin V/PI, and DNA fragmentation assays were performed, as previously described (10). Release of proapoptotic mitochondrial proteins cytochrome c and apoptosis-inducing factor (AIF) were assessed using a flow cytometry–based assay, as previously described (17, 18) and these assays are further detailed in the Supplementary Methods.

**Statistical analysis**

Unless otherwise stated, the results are presented as mean ± SD. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software). P ≤ 0.05 was accepted as being statistically significant.

**Results**

A high-throughput screen for novel anti-AML compounds identifies avocatin B

To identify novel compounds with anti-AML activity, we screened a commercially available natural health products–specific library against TEX leukemia cells. These cells possess several LSC properties, such as marrow repopulation and self-renewal (1, 5, 6, 19). The compound that imparted the greatest reduction in viability was avocatin B (Fig. 1A; arrow indicates avocatin B). Avocatin B is a 1:1 mixture of two 17-carbon lipids derived from avocados and belongs to a family of structurally related lipids (Fig. 1A insert: avocatin B’s structure; refs. 20, 21). We tested four avocatin lipid analogues and determined that avocatin B was the most cytotoxic (EC_{50}: 1.5 ± 0.75 μmol/L; Supplementary Fig. S1 and Table S1).

Avocatin B’s selectivity toward leukemia cells was validated in primary AML samples and in PBSCs isolated from G-CSF–stimulated healthy donors. Avocatin B, at concentrations as high as 20 μmol/L, had no effect on the viability of normal PBSCs (n = 4). In contrast, avocatin B reduced the viability of primary AML patient cells (n = 6) with an EC_{50} of 3.9 ± 2.5 μmol/L, which is similar to other recently identified compounds with anti-AML activity (Fig. 1B; see Supplementary Table S2 for patient sample characteristics; refs. 1, 8, 9, 22).

**Avocatin B is selectively toxic toward leukemia progenitor and stem cells**

Given the selectivity toward AML patient samples over normal hematopoietic cells, we next assessed avocatin B’s effects on functionally defined subsets of primitive human AML and normal cell populations. Adding avocatin B (3 μmol/L) into the culture medium reduced clonogenic growth of AML patient cells (n = 3; Supplementary Table S3 for patient characteristics). In contrast, there was no effect on normal cells (n = 3; Fig. 1C, left). In addition, treatment of primary AML cells with avocatin B (3 μmol/L) reduced their ability to engraft in the marrow of immune-deficient mice (Fig. 1C, right). Taken together, avocatin B selectively targets primitive leukemia cells.

**Avocatin B induces mitochondria-mediated apoptosis**

We next assessed the mode of avocatin B–induced leukemia cell death. Externalization of phosphatidylinerine, an early marker of apoptosis detected by Annexin V, was observed by flow cytometry in live cells (i.e., Annexin V+/PI−) treated with avocatin B (Fig. 2A; F3,7 = 19.09; P < 0.05; see Supplementary Fig. S2 for raw data). This coincided with the occurrence of DNA fragmentation (Fig. 2B; F4,14 = 171.4; P < 0.001), caspase activation (Fig. 2C; F3,16 = 69.56; P < 0.001), and PARP cleavage (Fig. 2D), as measured by cell-cycle analysis (see Supplementary Fig. S3 for raw data), a caspase activation assay, and Western blotting, respectively.

To test whether death was dependent on caspase enzymes, we coincubated avocatin B with the pan-caspase inhibitor Z-VAD-FMK or the caspase-3–specific inhibitor QVD-OPh for 72 hours. Both inhibitors only slightly protected cells from avocatin B–induced death (F4,8 = 2.714; P < 0.01; Fig. 2E). Because cell death can occur independent of caspase enzymes through the release of mitochondria-localized proteins, such as AIF, we tested for the presence of AIF in cytosolic fractions of avocatin B–treated TEX cells. However, given that AIF release involves mitochondrial outer membrane permeability and that we detected caspase activation, we also simultaneously tested for the presence of cytochrome c, which activates caspase enzymes following its release from the mitochondrial intermembrane space. Cells treated with avocatin B showed an increase in cytoplasmic concentrations of AIF (Fig. 2F; F4,20 = 8.211; P < 0.001) and cytochrome c (Fig. 2F; F4,20 = 13.57; P < 0.001). Therefore, avocatin B–induced apoptotic death is characterized by the release of the mitochondrial proteins AIF and cytochrome c; however, AIF is likely the key mediator, as death occurred in the presence of caspase inhibitors. Future studies would be needed to confirm the functional importance of AIF in avocatin B–induced death.

**Avocatin B inhibits fatty acid oxidation**

Apoptosis was characterized by the release of mitochondrial proteins following avocatin B treatment. Because avocatin B contains 17-carbon lipids and lipids of that size can enter the mitochondria and undergo fatty acid oxidation after they have been processed by carnitine palmitoyltransferase I (CPT1), we evaluated the impact of avocatin B on fatty acid oxidation. Fatty acid oxidation produces acetyl-CoA, which enters the TCA cycle to produce...
Inhibition of fatty acid oxidation results in reduced NAD, NADPH, and GSH levels in leukemia cells. Avocatin B (10 μmol/L), similar to etomoxir (100 μmol/L), decreased NADPH, an effect that occurred even in the presence of palmitate (175 μmol/L; Fig. 4A; \( F_{3,19} = 5.129; P < 0.05 \)). Similarly, avocatin B decreased NADPH and GSH (Fig. 3D: NAD: \( F_{3,11} = 5.145; P < 0.05 \); Fig. 4B: GSH: \( F_{3,11} = 188.9; P < 0.001 \)). Inhibition of fatty acid oxidation can reduce NADPH and GSH, leading to reduced antioxidant capacity, elevated ROS, and cell death (26). ROS levels were tested in avocatin B–treated cells using DCFH-DA and DHE, which measure general oxidizing species such as ROS and superoxide, respectively. TEX or primary AML cells treated with avocatin B had a time-dependent increase in ROS levels as measured by DCFH-DA (Fig. 4C; left; \( F_{3,11} = 176.7; P < 0.01 \); see Supplementary Fig. S4 for histogram data) and DHE (\( F_{3,11} = 36.75; P < 0.01 \); Fig. 4C; see Supplementary Fig. S4 for histogram data). To test the importance of ROS in avocatin B–induced death, we coincubated cells with NAC and \( \alpha \)-tocopherol (\( \alpha \)-Toc). NAC can neutralize a number of oxidizing species, including ROS directly or indirectly through antioxidant regeneration [i.e., convert oxidized GSH (i.e., GSSG) to reduced GSH; GSH is decreased following NADPH depletion; ref. 26] and \( \alpha \)-Toc is a lipid-based antioxidant that accumulates in organelle membranes, particularly mitochondria, to prevent lipid peroxyl radicals formed by ROS-induced membrane damage (27). Coincubation with NAC (Fig. 4D: \( F_{3,7} = 70.55; P < 0.05 \); Fig. 4E: \( F_{3,10} = 70.55; P < 0.05 \)) or \( \alpha \)-Toc (Fig. 4D: \( F_{3,7} = 10.23; P < 0.05 \)
abolished avocatin B–induced death. Daunorubicin was used as a negative control, as antioxidants do not protect against its cytotoxicity (28, 29). Finally, we coincubated cells with polyethylene glycol-superoxide dismutase (PEG-SOD), an antioxidant that reduces cellular concentrations of the superoxide anion. Coincubation with PEG-SOD similarly reduced ROS and blocked avocatin B's activity (Supplementary Fig. S5). Together, these results demonstrate that avocatin B decreased levels of NAD, NADPH, and GSH and that ROS is functionally important for avocatin B's activity.

Mitochondria and CPT1 are functionally important for avocatin B–induced death

We demonstrated that avocatin B inhibits fatty acid oxidation and induces apoptosis characterized by the release of mitochondrial proteins cytochrome c and AIF. Because avocatin B is a lipid and leukemia cells possess mitochondrial and metabolic alterations that result in their dependence on fatty acid substrates for survival (2), we hypothesized that avocatin B's toxicity was related to its localization in mitochondria. To directly examine whether avocatin B accumulated into mitochondria, LC/MS was performed on mitochondria and cytosolic fractions of avocatin B–treated TEX cells. Fraction purity was confirmed by Western blot analysis of the mitochondria–specific protein ND1 (Fig. 5C; Supplementary Fig. S8). Avocatin B was detected in mitochondrial and cytosolic fractions of avocatin B or vehicle control–treated TEX cells. Two peaks [with a mass/charge (m/z) ratio of 285.2424 and 287.2580] were detected, which reflect the nature of avocatin B's two-lipid composition. Importantly, retention times (min) following 10-nonyl acridine orange (NAO) staining and by Western blotting for mitochondrial specific proteins ND1 and adenine nucleotide translocator (ANT). The significant reduction of mitochondria was confirmed, as cells cocultured in ethidium bromide containing media demonstrated a drastic reduction in NAO staining (Supplementary Fig. S6), absence of mitochondrial respiration (Supplementary Fig. S6), and a near absence of ND1 and ANT (Fig. 5A). Avocatin B's toxicity was abolished in cells lacking functional mitochondria (i.e., JURK-Rho(0) cells), as measured by the Annexin V/PI assay (Fig. 5B; F2,12 = 6.509; P < 0.001). Highlighting the utility of these cells in assessing mitochondrial participation in drug activity, we have previously shown that cells lacking mitochondria were equally sensitive to their mitochondria containing controls when subjected to a compound that activates mitochondria-independent, calpain-mediated apoptosis (10).

To further explore avocatin B's participation in the mitochondria, we examined its effects on mitochondrial protein dynamics. In mitochondria, NADPH and NADH pool was measured following 10-nonyl acridine orange (NAO) staining and by Western blotting for mitochondrial specific proteins ND1 and adenine nucleotide translocator (ANT). The significant reduction of mitochondria was confirmed, as cells cocultured in ethidium bromide containing media demonstrated a drastic reduction in NAO staining (Supplementary Fig. S6), absence of mitochondrial respiration (Supplementary Fig. S6), and a near absence of ND1 and ANT (Fig. 5A). Avocatin B's toxicity was abolished in cells lacking functional mitochondria (i.e., JURK-Rho(0) cells), as measured by the Annexin V/PI assay (Fig. 5B; F2,12 = 6.509; P < 0.001). Highlighting the utility of these cells in assessing mitochondrial participation in drug activity, we have previously shown that cells lacking mitochondria were equally sensitive to their mitochondria containing controls when subjected to a compound that activates mitochondria-independent, calpain-mediated apoptosis (10).

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for m/z 285 and 287 were nearly identical between pure compound and the cellular fractions (pure avocatin B: 4.46 and 4.76; mitochondrial fraction: 4.46 and 4.78; cytosolic fraction: 4.46 and 4.78). As expected, avocatin B was not found in vehicle control–treated cells (Supplementary Fig. S7).

Lipids of 16 to 20 carbon length enter mitochondria by the activity of CPT1 (32). To determine the role of CPT1 in avocatin B–induced death, we blocked CPT1 chemically with etomoxir and genetically using RNA interference. Etomoxir concentrations that did not reduce viability (100 μmol/L; Fig. 6A), abrogated avocatin B–induced cell death (Fig. 6B; F5,17 = 94.45; P < 0.001) and reductions in clonogenic growth (Fig. 6C; F5,17 = 94.45; P < 0.001). As a genetic approach, we generated cells with reduced CPT1 gene expression (mRNA: Fig. 6D, left; for protein see ref. 33). CPT1 knockdown cells were significantly less sensitive to avocatin B (Fig. 6D, middle; F9,32 = 23.73; P < 0.001) and were insensitive to avocatin B–induced reduction of NADPH (Fig. 6D, right; F3,16 = 65.04; P < 0.001). Together, these results show that avocatin B is a lipid that localizes to the mitochondria and impairs fatty acid oxidation.

**Discussion**

A screen of a natural health product library identified avocatin B as a novel anti-AML agent. *In vitro* and preclinical functional studies demonstrated that it induced selective toxicity toward leukemia and LSCs with no toxicity toward normal cells. Mechanistically, we highlight a novel strategy to induce selective leukemia cell death, where mitochondrial localization of avocatin B inhibits fatty acid oxidation and decreases levels of NADPH, resulting in elevated ROS leading to apoptotic cell death.

Avocatin B targets leukemia over normal cells. We propose this specificity is related to the leukemia cells’ altered mitochondrial characteristics, as a number of observations suggest avocatin B targets mitochondria. For example, (i) we directly show avocatin B
accumulates in leukemia cell mitochondria using LC/MS; (ii) cells with significantly reduced mitochondria or (iii) lacking the enzyme that facilitates mitochondrial lipid transport, CPT1, are insensitive to avocatin B; (iv) chemical treatment with etomoxir, a CPT1 inhibitor, blocked avocatin B’s activity; and (v) CPT1 only facilitates entry of lipids of avocatin B’s size into mitochondria [e.g., 16–20 carbons (32); avocatin B:17 carbons (21)]. Compared with normal hematopoietic cells, leukemia cells contain higher mitochondrial mass (1) and depend on fatty acid substrates for survival (2). Thus, given this mitochondrial phenotype, we propose that avocatin B accumulates with greater concentration in leukemia over normal cells, thus conferring its increased toxicity toward leukemia cells.

Inhibition of fatty acid oxidation by avocatin B resulted in ROS-induced apoptosis. Apoptosis was characterized by the mitochondrial proteins cytochrome c and AIF, which are commonly released following ROS-induced increases in mitochondrial outer membrane permeability (34, 35). Inhibiting fatty acid oxidation by blocking CPT1 with etomoxir resulted in ROS-dependent death of glioma cells caused by reduced concentrations of intracellular antioxidants attributed to decreased NADPH (26). Similarly, we demonstrated that avocatin B–induced inhibition of fatty acid oxidation decreased NADPH and GSH levels and that antioxidant supplementation rescued cells from death. NADPH is used for catabolic processes in proliferating cells and is able to regenerate cellular antioxidants (i.e., convert oxidized GSH,

Figure 4. Avocatin B decreased levels of NADPH and GSH and elevated ROS. A, NADPH was measured in TEX cells (left; t = 3–5 hours) or primary AML cells (n = 3; t = 24 hours, right; results for OCI-AML2 cells are shown in Supplementary Fig. S9) using the commercially available Amplite Fluorimetric Assay following treatment with avocatin B (10 μmol/L), palmitate (175 μmol/L), or etomoxir (100 μmol/L) according to the manufacturer’s protocol. Data, a percentage of NADPH compared with vehicle control ± SD. B, GSH was measured in TEX cells in the presence or absence of NAC using a commercially available fluorimetric assay following treatment with avocatin B (10 μmol/L), according to the manufacturer’s protocol. Data, percentage of GSH compared with vehicle control–treated cells ± SD. C, ROS were measured in TEX cells (left) or primary AML cells (n = 3, right; results for OCI-AML2 cells is shown in Supplementary Fig. S9) treated with 10 μmol/L avocatin B for increasing time by DHE and DCFH-DA by flow cytometry. Data, percentage of cells with increased ROS compared with vehicle control ± SD from representative experiments. D, TEX cells were treated with 10 μmol/L avocatin B in the presence or absence of NAC or α-Toc, which can neutralize ROS. Daunorubicin (DNR) was used as a negative control. Viability was measured by the Annexin V/PI assay; data, mean percentage of viable cells (i.e., Annexin V−/PI−) ± SD from representative experiments. NS, nonsignificant. E, TEX cells were treated with 10 μmol/L avocatin B in the presence or absence of NAC and colonies were counted as described in Materials and Methods. All experiments were performed three times in triplicate, and representative figures are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Avocatin B Inhibits Fatty Acid Oxidation

Figure 5.
Mitochondria are functionally important for avocatin B–induced death. A, Jurkat T cells were cultured in 50 ng/mL of ethidium bromide, 100 mg/mL sodium pyruvate, and 50 μg/mL uridine for 60 days to create Jurkat-Rho(0) cells, which lack functional mitochondrial. To confirm that Jurkat-Rho(0) cells lack mitochondria, we measured the mitochondria-specific markers ANT and complex I (ND1) by Western blotting. B, avocatin B’s activity was tested in cells with (JURK) and with reduced (Jurkat-Rho(0)) mitochondria. Viability was measured by the Annexin V/PI assay and flow cytometry; data, mean percentage of live cells (i.e., Annexin V+/PI –) ±SD from representative experiments. C, mitochondrial and cytosolic fractions were collected, as outlined in Materials and Methods, and tested for purity by staining for the mitochondria-specific protein ND1. D, LC/MS chromatographs demonstrating the presence of avocatin B in the mitochondria and cytosol fractions of avocatin B–treated cells. All experiments were performed three times in triplicate, and representative figures are shown.

***, P < 0.001.

Over, they are not efficiently or preferentially oxidized. For example, mice fed diets containing radiolabeled odd- and even-numbered fatty acids only accumulate odd-numbered fatty acids in adipose tissue (i.e., C15 and 17; ref. 39); odd-numbered fatty acids show consistent adipose accumulation (37, 40, 41). In humans, lipids of 13, 15, and 17 carbon lengths are used as serum and adipose tissue biomarkers of dietary fat intake, as these fatty acids are more slowly catabolized compared with even-numbered fatty acids (38, 42). Although they undergo the same pathway of oxidation, the terminal step of odd-numbered fatty acid oxidation produces 1 acetyl-CoA and 1 propionyl-CoA molecule, whereas even-numbered fatty acids produce 2 acetyl-CoA molecules (43). Propionyl-CoA can then be converted to methylmalonyl-CoA by propionyl-CoA carboxylase and vitamin B12, at the expense of 1 ATP, which, in turn, is converted to succinyl-CoA that can enter the TCA cycle (41). Because this alternate pathway requires energy and delays overall ATP production, the decreased metabolic activity (i.e., reduced acetyl-CoA production and/or decreased entry of fatty acid byproducts into
experiments. All experiments were performed three times in triplicate, and representative products requires ROS (45), therefore; avocatin B accumulation into the mitochondrial matrix (44). However, the generation of lipotoxic damage to mitochondrial DNA, lipids, and proteins within the are converted by ROS into lipid peroxides that, in turn, cause accumulation may contribute to death through lipotoxicity but only after suf- cient ROS production (i.e., following avocatin B accumulation).

Figure 6.
CPT1 is functionally important for avocatin B–induced death. A, TEX cells were incubated with increasing concentrations of the CPT inhibitor etomoxir for 72 hours. B and C, avocatin B’s (10 μmol/L) activity was tested using Annexin V/PI (B) or colony assays (C) in the presence of etomoxir (100 μmol/L; which does not impart toxicity). D, left, mRNA expression demonstrating knockdown of CPT1 in OCI-AML2 cells. Middle, avocatin B’s activity was tested in CPT1 knockout cells. Right, NADPH was tested in CPT1 knockout cells following avocatin B (10 μmol/L) treatment. Data, percentage of NADPH relative to control. Unless otherwise noted, viability was measured by the Annexin V/PI assay and flow cytometry; data, mean percentage of live cells (i.e., Annexin V−/PI−) ± SD from representative experiments. All experiments were performed three times in triplicate, and representative figures are shown. *** P < 0.001.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.A. Lee, L. Angka, S.-G. Rota, T. Hanlon, A. Mitchell, X.M. Wang, M. Minden, A. Datti, J.L. Wrana, J.W. Joseph, J. Quadrilatero, P.A. Spagnuolo

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References

13. Avocatin B Inhibits Fatty Acid Oxidation

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