**Figure 3.**

Avocatin B inhibits fatty acid oxidation, resulting in decreased levels of NADH. A, illustration of fatty acid oxidation in mitochondria. Long-chain fatty acids (LCFA) enter the mitochondria via CPT1 for fatty acid oxidation to yield NADH and acetyl-CoA. Acetyl-CoA enters the TCA cycle to generate NADPH. ME, malic enzyme; IDH, isocitrate dehydrogenase; α -KG, α -ketoglutarate. B, oxidation of exogenous fatty acids was assessed by measuring the OCR in TEX cells treated with palmitate (175 μ mol/L), avocatin B (10 μ mol/L), avocatin B, and palmitate or palmitate and etomoxir (100 μ mol/L). Arrows, the time when oligomycin, CCCP, and antimycin/rotenone were added to the cells. Effects on fatty acid oxidation were measured with the Seahorse Bioanalyzer and quantified (C) by peak area after oligomycin and CCCP treatment, as described in the manufacturer's protocol and detailed in Materials and Methods. Data, percentage of OCR compared with palmitate-treated cells \pm SD. BSA was also used as a control. D, NADH was measured in TEX cells (left; $t = 3$ -5 hours) or primary AML cells (right; $n = 3$; $t = 24$ hours; results for OCI-AML2 cells are shown in Supplementary Fig. S9) using the commercially available Amplitude Fluorimetric Assay following treatment with avocatin B (10 μ mol/L), palmitate (175 μ mol/L), or avocatin B and palmitate according to the manufacturer's protocol. Data, percentage of NADH compared with vehicle control-treated cells \pm SD.

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 μ mol/L; Fig. 6A), abrogated avocatin B-induced cell death (Fig. 6B; $F_{5,17} = 94.45$; $P < 0.001$) and reductions in clonogenic growth (Fig. 6C; $F_{5,17} = 94.45$; $P < 0.001$). 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; $F_{9,32} = 23.73$; $P < 0.001$) and were insensitive to avocatin B-induced reduction of NADPH (Fig. 6D, right; $F_{3,16} = 65.04$; $P < 0.001$). 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

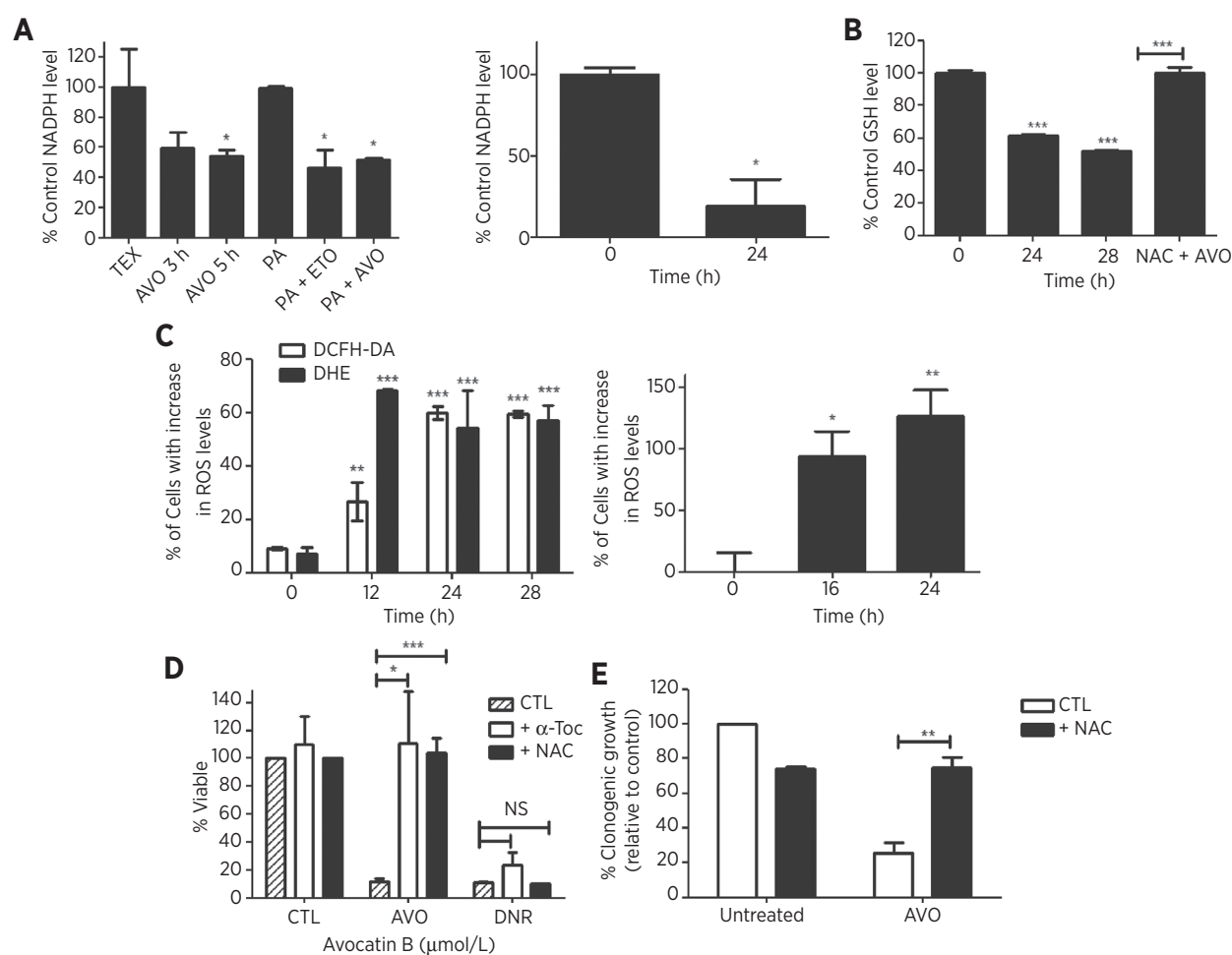


Figure 4.

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 Amplitude Fluorimetric Assay following treatment with avocatin B (10 $\mu\text{mol/L}$), palmitate (175 $\mu\text{mol/L}$), or etomoxir (100 $\mu\text{mol/L}$) according to the manufacturer's protocol. Data, a percentage of NADPH compared with vehicle control-treated cells \pm 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 $\mu\text{mol/L}$), according to the manufacturer's protocol. Data, percentage of GSH compared with vehicle control-treated cells \pm 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 $\mu\text{mol/L}$ avocatin B for increasing time by DHE and DCFH-DA by flow cytometry. Data, percentage of cells with increased ROS compared with vehicle control \pm SD from representative experiments. D, TEX cells were treated with 10 $\mu\text{mol/L}$ 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⁻) \pm SD from representative experiments. NS, nonsignificant. E, TEX cells were treated with 10 $\mu\text{mol/L}$ 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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,

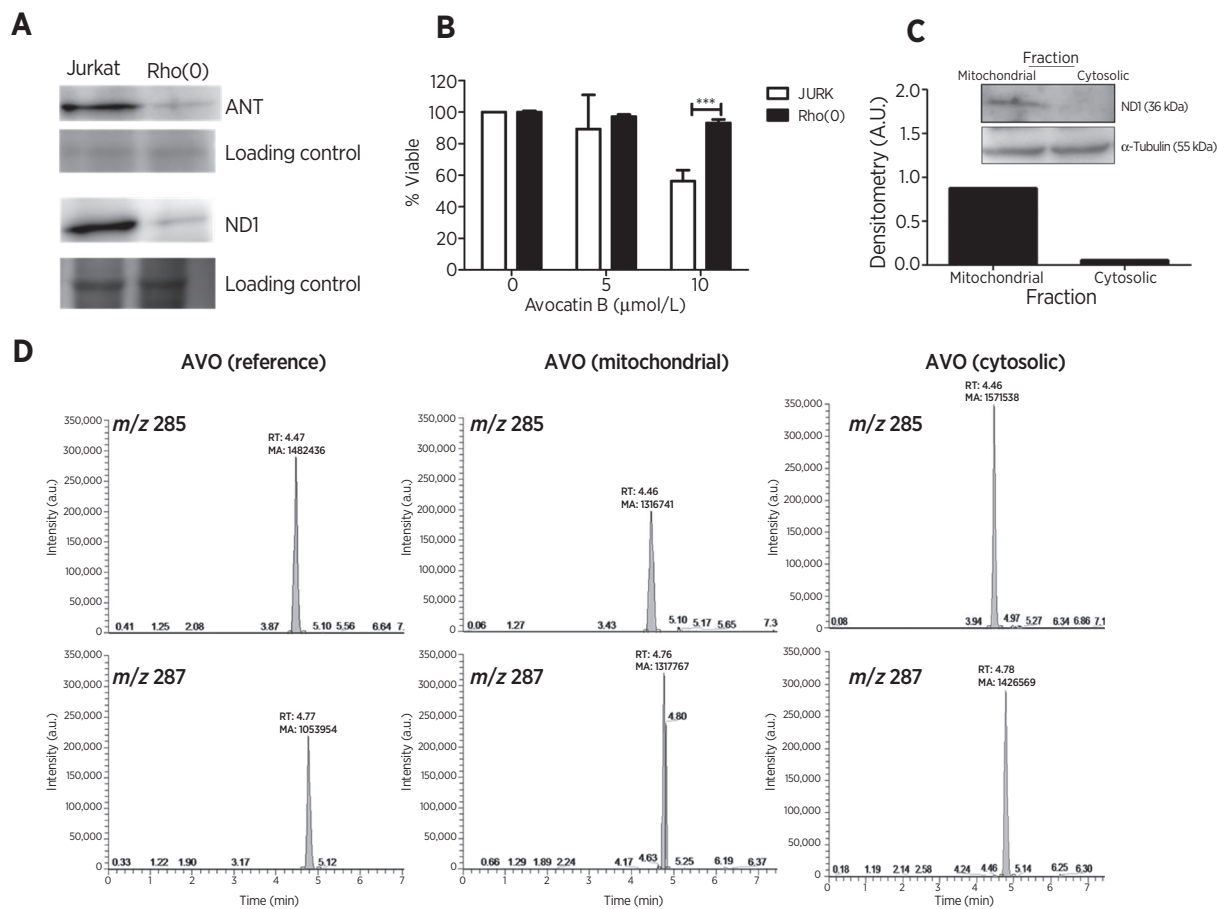


Figure 5. Mitochondria are functionally important for avocatin B-induced death. A, Jurkat T cells were cultured in 50 ng/mL of ethidium bromide, 100 mg/mL sodium pyruvate, and 50 $\mu\text{g/mL}$ uridine for 60 days to create Jurkat-Rho(O) cells, which lack functional mitochondria. To confirm that Jurkat-Rho(O) cells lack mitochondria, we measured the mitochondria-specific markers ANT and complex I (NDI) by Western blotting. B, avocatin B's activity was tested in cells with (JURK) and with reduced [Jurkat-Rho(O)] mitochondria. Viability was measured by the Annexin V/PI assay and flow cytometry; data, mean percentage of live cells (i.e., Annexin V⁻/PI⁻) \pm 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 NDI. 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. ***, $P < 0.001$.

thioredoxins, and peroxiredoxins to their reduced equivalents), which counteract the detrimental effects of free radicals, including ROS; GSH specifically converts hydrogen peroxide to water (23, 36). Our observed NADPH decrease ($t = 5$ hours; Fig. 4A) preceded ROS elevation ($t = 12$ hours; Fig. 4C), further confirming the relationship between inhibition of fatty acid oxidation, NADPH, and ROS-dependent leukemia cell death. Of note, in our experiments, avocatin B accumulated in mitochondria to inhibit fatty acid oxidation and reduced NADPH at 10 $\mu\text{mol/L}$, whereas other studies used etomoxir, which blocks fatty acid entry into mitochondria and reduces NADPH at 100 $\mu\text{mol/L}$ (2) or 1,000 $\mu\text{mol/L}$ (26). Together, these results point to a mechanism in which avocatin B enters the mitochondria and potentially inhibits fatty acid oxidation, resulting in decreased NADPH and GSH leading to elevated ROS and apoptotic cell death.

Avocatin B is a 1:1 ratio of two 17-carbon lipids derived from methanol extracted from avocado pear seeds (*Persea gratissima*; ref. 20). Odd-numbered carbons are rare, not produced endogenously and obtained only from dietary sources (37, 38). More-

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

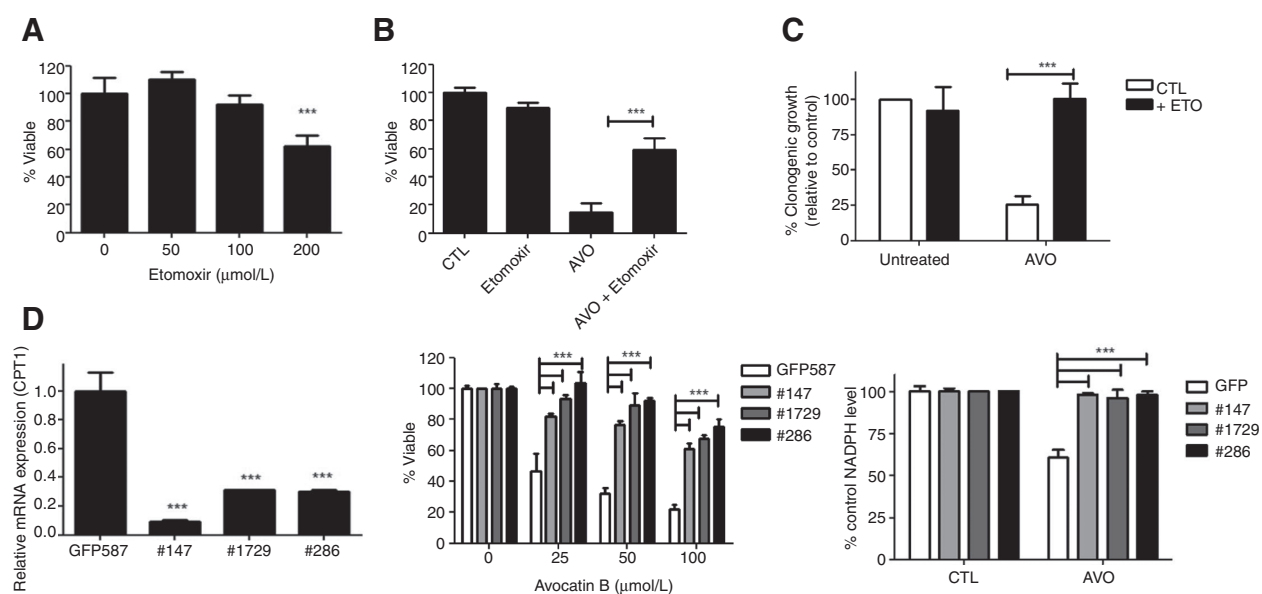


Figure 6. CPT1 is functionally important for avocatin B–induced death. A, TEX cells were incubated with increasing concentrations of the CPT1 inhibitor etomoxir for 72 hours. B and C, avocatin B's (10 $\mu\text{mol/L}$) activity was tested using Annexin V/PI (B) or colony assays (C) in the presence of etomoxir (100 $\mu\text{mol/L}$; 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 $\mu\text{mol/L}$) 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⁻) \pm SD from representative experiments. All experiments were performed three times in triplicate, and representative figures are shown. ***, $P < 0.001$.

the TCA cycle) likely explains our observed decrease in NAD and NADPH. As such, decreased levels of NADPH may not only result in elevated ROS, but also indicate a decrease in overall metabolic activity. Thus, a novel pathway by which fatty acid oxidation can be inhibited in leukemia cells is by the odd-numbered carbon lipid, avocatin B stalling or rendering less efficient the fatty acid oxidation pathway. This highlights a novel strategy to induce selective leukemia cell death by which preferential mitochondrial localization of avocatin B reduces leukemia cell metabolism and decreases NADPH, leading to ROS-mediated cell death.

Alternatively, mitochondrial accumulation of fatty acids could have lipotoxic effects. When in excess, fatty acids can accumulate inside the mitochondrial matrix where they are deprotonated, because of the proton gradient, creating fatty acid anions. These are converted by ROS into lipid peroxides that, in turn, cause damage to mitochondrial DNA, lipids, and proteins within the mitochondrial matrix (44). However, the generation of lipotoxic products requires ROS (45), therefore; avocatin B accumulation by itself would be insufficient to impart lipotoxicity. Thus, once inside the mitochondria, avocatin B or avocatin B derivatives may be converted to lipotoxic byproducts and contribute to death but only after sufficient ROS production (i.e., following avocatin B–induced inhibition of fatty acid oxidation). Thus, mitochondrial accumulation may contribute to death through lipotoxicity but this is not the underlying mechanism of avocatin B's activity.

Few compounds that inhibit fatty acid oxidation are currently approved for clinical use (23). CPT1 inhibitors, such as etomoxir and perhexiline, are associated with hepatotoxicity (46) and neurotoxicity (47), respectively, and are not approved for clinical use in North America. Other inhibitors, such as trimetazidine, which inhibits 3-ketoacyl-CoA thiolase, an enzyme involved in fatty acid

catabolism and ranolazine, which blocks late sodium currents, have had clinical success for the treatment of angina (48, 49). None of these compounds are approved for use in AML or other hematologic malignancies. Future studies are needed to assess avocatin B's pharmacology and pharmacokinetics; however, initial assessment of avocatin B's physicochemical properties suggests favorable tissue distribution. In particular, it possesses a high estimated partition coefficient ($\text{LogP} = 8.9$; ref. 21), indicating that it will accumulate in lipid-rich tissues such as adipose tissue and bone marrow. Given that LSCs reside in bone marrow, this could significantly enhance avocatin B's therapeutic efficacy. Nonetheless, future studies are needed to test the pharmacokinetics and safety of avocatin B in human trials.

In conclusion, avocatin B accumulated in mitochondria to inhibit fatty acid oxidation and decrease NADPH, resulting in ROS-mediated cell death characterized by the mitochondrial release of cytochrome *c* and AIF. Given the observed leukemia cell specificity, inhibiting fatty acid oxidation following avocatin B accumulation represents a novel therapeutic strategy that targets an important cellular pathway involved in leukemia cell activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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