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Elimination of Chronic Lymphocytic Leukemia Cells in Stromal Microenvironment by Targeting CPT with an Anti-Angina Drug Perhexiline

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Abstract

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western countries and is currently incurable due in part to difficulty in eliminating the leukemia cells protected by stromal microenvironment. Based on previous observations that CLL cells exhibit mitochondrial dysfunction and altered lipid metabolism and that carnitine palmitoyltransferases (CPT) play a major role in transporting fatty acid into mitochondria to support cancer cell metabolism, we tested several clinically relevant inhibitors of lipid metabolism for their ability to eliminate primary CLL cells. We discovered that Perhexiline, an anti-angina agent that inhibits CPT, was highly effective in killing CLL cells in stromal microenvironment at clinically achievable concentrations. These effective concentrations caused low toxicity to normal lymphocytes and normal stromal cells. Mechanistic study revealed that CLL cells expressed high levels of CPT1 and CPT2. Suppression of fatty acid transport into mitochondria by inhibiting CPT using Perhexiline resulted in a depletion of cardiolipin, a key component of mitochondrial membranes, and compromised mitochondrial integrity leading to rapid depolarization and massive CLL cell death. The therapeutic activity of Perhexiline was further demonstrated *in vivo* using a CLL

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Conflict of interest

The authors declare no conflict of interest

transgenic mouse model. Perhexiline significantly prolonged the overall animal survival by only 4 drug injections. Our study suggests that targeting CPT using an anti-angina drug is able to effectively eliminate leukemia cells in vivo, and is a novel therapeutic strategy for potential clinical treatment of CLL.

Keywords

Chronic lymphocytic leukemia; Lipid metabolism; CPT; perhexiline; stromal microenvironment

Introduction

Chronic lymphocytic leukemia is a lymphoproliferative disorder with variable clinical courses.[1] Despite significant progress in the recent years, CLL still remains an incurable disease due in part to the persistence of residual leukemia cells after therapy.[2–5] The stromal microenvironment or tissue niches that promote leukemia cell viability and drug resistance is a major factor contributing to drug resistance *in vivo*. Although most drugs currently used in clinical treatment of CLL are effective in inducing leukemia cell death *in vitro* when CLL cells are cultured alone, their therapeutic activity decreases substantially *in vivo* when stromal cells are present[6]. The tissue microenvironment protects leukemia cells by multiple mechanisms, including signaling through direct cell-cell contact, secretion of stromal factors, and metabolic interactions. [6–10] Thus, development of new therapeutic strategies to effectively eliminate CLL cells in tissue microenvironment is extremely important in overcoming *in vivo* drug resistance and improving therapeutic outcomes.

Our recent studies showed that an important mechanism by which bone marrow stromal cells protect CLL cells is by promoting glutathione (GSH) synthesis in CLL cells, and that disabling this protective mechanism by inhibition of the cystine transporter (Xc-) in stromal cells or by direct depletion of GSH in CLL cells are effective in killing CLL cells in the presence of stromal cells.[6,11,12] Another strategy to overcome stromal mediated drug resistance is to disrupt stromal-leukemia cell interaction and promote the release of CLL from their tissue microenvironment into the blood circulation, where leukemia cells might be more vulnerable to chemotherapeutic agents. The CXCR4 inhibitor AMD3100 seems able to function as a chemo-sensitizing agent through such mechanism.[13] However, due to the multiple mechanisms involved in the stromal protection of leukemia cells, inhibition of one protective mechanism might not be sufficient to effectively overcome drug resistance *in vivo*. We speculated that an effective strategy to overcome this problem would be to induce CLL cells to undergo an intrinsic cell death that cannot be protected by stromal cells. The main goal of the current study was to test this possibility based on the unique metabolic properties of CLL cells.

Metabolic abnormalities are a hallmark of transformed cells that may provide growth advantages.[14–17] Beyond the well-known Warburg effect,[18] alterations in fatty acids (FAs) metabolism have also been noted in cancer. In particular, deregulation of lipid metabolism seems involved in CLL pathogenesis, as suggested by a frequent up-regulation of lipoprotein lipase (LPL) in CLL cells and its association with poor prognosis.[19] FAs are essential

building blocks of biomembranes, and function as important nutrients for energy metabolism and as the precursors of certain signaling molecules. As such, pharmacological inhibition of fatty acid synthase (FASN) to suppress the *de novo* synthesis of FAs and inhibition of cellular uptake of exogenous FAs are considered as potential therapeutic strategies.[20–23] In this study, we used both *in vitro* and *in vivo* experimental systems to test several drugs that inhibit different steps of FA metabolism for their impact on CLL viability in stromal microenvironment. Our study identified Perhexiline, a carnitine palmitoyltransferase inhibitor that suppresses the transport of FA into mitochondria, as a highly effective compound capable of selective killing CLL cells in the presence of bone marrow stromal cells and in vivo.

Results

Perhexiline effectively killed CLL cells in the presence of bone marrow stromal cells

Previous studies showed that CLL cells have multiple metabolic alterations, including mitochondrial dysfunction with high ROS generation[6,12,24–26] and elevated expression of lipoprotein lipase (LPL), which hydrolyzes triglycerides in lipoproteins to release free fatty acids.[27,28] The deregulation of LPL expression and altered lipid metabolism seems to play a significant role in CLL pathogenesis.[24,29–37] Consistent with these observations, our study using transmission electron microscopy (TEM) revealed that CLL cells contained significantly more mitochondria with an accumulation of lipid droplets compared to normal B lymphocytes ($P<0.01$, Supplementary Figure 1). To evaluate the role of lipid metabolism in CLL cell viability, we tested several clinically relevant lipid metabolic inhibitors for their ability to kill primary CLL cells. Figure 1a shows several key steps of lipid metabolism and the respective inhibitors used in this study. Cerulenin, an antifungal antibiotic that inhibits FASN,[38] was used to suppress *de novo* FA synthesis. Perhexiline, an anti-angina drug that inhibits carnitine palmitoyltransferases 1 and 2 (CPT-1 & CPT-2), was employed to block fatty acids (LCFAs) transport into mitochondria. Ranolazine, another anti-angina drug, was used to suppress mitochondrial fatty acid β-oxidation. As shown in Figure 1b, inhibition of *de novo* FA synthesis by Cerulenin exhibited cytotoxic effect in CLL cells cultured alone. However, in the presence of bone marrow stromal cells, Cerulenin lost its cytotoxic effect against CLL cells (Figure 1b, lower panels), suggesting that this drug would not be effective *in vivo* as an anti-CLL agent. Another anti-agina drug ranolazine only exhibited limited cytotoxic effect in CLL cells even at the drug concentrations up to 500–1000 μM (Figure 1c), suggesting that inhibition of mitochondrial FAs β-oxidation was not a major cytotoxic event in primary CLL cells.

In contrast, blocking FA transport into mitochondria by Perhexiline showed potent cytotoxic effect against CLL cells when the leukemia cells were cultured alone or co-cultured with bone marrow stromal cells (Figure 1d). This compound is highly effective in eliminating CLL cells at the drug concentrations of 5–10 μM, which could be achieved clinically in angina patients.[39] The potent anti-CLL activity of perhexiline was consistently observed in multiple CLL patient samples in the presence of StromaNktert cells (Figure 1d, right panel). We also tested the ability of perhexiline to kill CLL cells co-cultured with two other stromal cell lines (HS-5, KUSA-H1) and obtained similar results (data not shown). In a control

experiment, the drug solvent dimethyl sulfoxide (DMSO, final concentration 0.1%) did not cause any significant cytotoxicity (Supplementary Figure 2). These results together showed that perhexiline was effective in killing CLL cells in the presence of bone marrow stromal cells, which usually confer resistance to many anticancer agents.

Rapid depletion of mitochondrial cardiolipin in CLL cells by perhexiline as a key mechanism of cytotoxic action

Because blocking the transport of FAs into mitochondria by Perhexiline was highly effective against CLL cells whereas inhibition of FA β-oxidation by Ranolazine had only modest cytotoxicity, we reasoned that generation of ATP via β-oxidation was unlikely the critical mechanism by which mitochondrial FAs support CLL cell survival. Indeed, measurement of cellular oxygen consumption showed that Perhexiline did not cause any significant decrease in oxygen consumption (Figure 2a). These data suggest that mitochondrial FAs in CLL cells might not be used as a major fuel for β-oxidation (which consumes oxygen). This prompted us to further explore the other possible mechanisms underlying the cytotoxic effect of Perhexiline.

Considering that cardiolipin is a special lipid component of the mitochondrial membranes and is its synthesis in the mitochondria requires FAs as precursors, we reasoned that inhibition of FA transport into mitochondria by Perhexiline might impact the synthesis of cardiolipin. To test this possibility, we used 10-N-nonyl acridine orange (NAO), a fluorescent dye for staining of cardiolipin[11,12,40] and flow cytometry analysis or quantitation. As shown in Figure 2b, the majority of primary CLL cells rapidly lost their cardiolipin after incubation with 10 μM Perhexiline. Approximately 60% and 80% of the drug-treated CLL cells lost their normal cardiolipin contents at 4 h and 6 h, respectively, as indicated by a substantial left-shift of the NAO fluorescent peak. These data suggest that inhibition of FA transport into mitochondrial by Perhexiline caused a rapid depletion of cardiolipin due to a lack of metabolic precursors.

We then tested if incubation of CLL cells with Perhexiline would lead to damage to mitochondrial membrane integrity. We used rhodamine-123 to measure transmembrane potential as an indicator of mitochondrial membrane integrity. As shown in Figure 2c, more than 40% of CLL cells lost their mitochondrial integrity 4 h after Perhexiline incubation. This was associated with in a release of cytochrome *c* from the mitochondria, as indicated by a left-shift of the mitochondrial cytochrome *c* signal in flow cytometry analysis (Figure 2d, log scale) and a substantial increase in cytosolic cytochrome *c* at 4 h and beyond (Figure 2e). Consequently, the release of cytochrome *c* led massive apoptosis (Figure 2f).

Analysis of cellular ATP showed that there was a time-dependent decrease (Figure 2g). The loss of ATP appeared concurrent with the time-course of cell death. This loss of ATP was likely due to its leakage from the damaged cells and not due to a decrease in β-oxidation, since there was no significant decrease in oxygen consumption when the cells were treated with Perhexiline (Figure 2a). Interestingly, CLL cells exhibited similar sensitivity to Perhexiline regardless of the present/absent of glucose (Supplementary Figure 3 a and b). These results also support the conclusion that Perhexiline-induced cell death mainly by depletion of mitochondrial cardiolipin, and not by inhibition of ATP generation through β-

oxidation of FAs. Consistently, there was no compensatory increase in glucose uptake when CLL cells were treated with Perhexiline (Supplementary Figure 3c).

Selective cytotoxicity of Perhexiline against CLL cells with high expression of CPT

The potent cytotoxic effect of Perhexiline against primary CLL cells in the presence of stromal cells suggests that this compound could be effective in killing CLL *in vivo*. We then tested if this compound might also be toxic to normal lymphocytes. As shown in Figure 3a, Perhexiline at a relatively low concentration (5 μM) exhibited potent cytotoxic effect against CLL cells with only 14% viable cells at 48 h, but the same drug concentration caused only modest cytotoxicity in normal lymphocytes (68% viable cells). In the presence of bone marrow stromal cells, 5 μM Perhexiline was still able to induce massive apoptosis (64%) in CLL cells, but only cause minimum cell death in normal lymphocytes (9%, Figure 3b). Consistently, analysis of mitochondrial cardiolipin (Figure 3c) and transmembrane potential (Figure 3d) showed that Perhexiline induced only minimum changes in normal lymphocytes, even at a higher drug concentration (10 μM). This was in a sharp contrast with the observations in CLL cells, which showed a rapid depletion of cardiolipin (Figure 2b) and a substantial loss of transmembrane potential (Figure 2c). We also compared the effect of Perhexiline (5 μM) in CLL and normal lymphocytes at multiple time points (12, 24 and 48 h), and consistently observed preferential toxicity against CLL cells (Supplementary Figure 4a–4b).

To confirm the therapeutic selectivity of Perhexiline, we tested multiple CLL samples (n =11) and normal lymphocytes isolated from healthy individuals (n=5) with various concentrations of Perhexiline in the presence and absence of bone marrow stromal cells. The results again demonstrated that this compound preferentially impacted CLL cells when stromal cells were either absent (Figure 3e) or present (Figure 3f). We noted that the therapeutic selectivity was optimal when perhexiline concentrations were between 5–7.5 μM. At a higher concentration, Perhexiline exhibited some toxicity in normal lymphocytes.

We further examined the effect of Perhexiline on bone marrow stromal cells (StromaNKtert), and showed that this compound did not cause any significant loss of stromal cell viability at concentrations up to 10 μM (Figure 4a), whereas this drug concentration caused a loss of over 90% of CLL cell viability (Figure 4b).

In an attempt to understand why primary CLL cells exhibited much higher sensitivity to Perhexiline than to normal cells, we compared the expression of several enzymes involved in fatty acid metabolism in the leukemia cells and normal cells. As shown in Supplementary Figure 5 c–f, CLL cells showed higher expression (3–4 folds increase) of most carnitine palmitoyltransferase isoforms including CPT-1A, CPT-1B, CPT-2, with an exception of CPT1C (Figure 4c–4f). The expression of fatty acid synthase (FASN) and lipoprotein lipase (which involves in uptake of exogenous FAs) was also higher in CLL (Figure 4g–4h). These data together suggest that CLL cells were highly active in *de novo* FA synthesis as well as in uptake of exogenous FAs, likely due to a high requirement of FAs for cardiolipin synthesis.

Comparison of lipoprotein lipase expression in CLL cells and normal lymphocytes revealed that CLL cells expressed significantly higher levels of LPL (Figure 5a), which has

previously been implicated as a potential prognostic factor for CLL patients whose high expression is correlated with poor clinical outcome.[19,29,30,32,34,37–39,41] We also noted that among the 12 CLL patient samples tested, the LPL expression levels exhibited substantial heterogeneity among individual patients. Interestingly, *in vitro* treatment of CLL cells with Perhexiline consistently induced a further increase in LPL expression, especially in those CLL samples with relatively lower basal LPL expression (Figure 5b), suggesting a possible compensatory response in CLL cells after drug treatment. Similarly, the expression of the A2 phospholipase isoform iPLA2α, an enzyme that catalyzes the release of FAs from phospholipids, was also increased in CLL cells compared with normal lymphocytes (Figure 5c). The expression of iPLA2α could also be further up-regulated by Perhexiline treatment (Figure 5d). The expression of FASN in CLL cells and normal lymphocytes appeared similar (Figure 5e), and Perhexiline incubation also led to an increase in FASN expression in all 3 CLL samples tested (Figure 5f). These data together suggest that CLL cells were highly active in utilizing exogenous FAs through uptake via LPL and PLA2α for cardiolipin synthesis, and that inhibition of FA transport by Perhexiline triggered a compensatory up-regulation of LPL, PLA2α, and FASN.

In vivo therapeutic activity of Perhexiline in transgenic CLL mice

Since Perhexiline was able to effectively kill CLL cells in the presence of bone marrow stromal cells, we postulated that this compound would be effective against CLL cells *in vivo*. To test this possibility, we generated CLL transgenic mice with Tcl-1^{Tg}:$p53^{-/-}$ genotype [42] to evaluate the *in vivo* therapeutic activity of Perhexiline. Mice that had developed CLL were first measured for basal leukemia cell burden in their peritoneal cavities as described previously [6]. After a week of recovery period, the mice were treated with perhexiline (8 mg/kg, i.p.) every other day for a total of four injections. Peritoneal cells were collected one week and two weeks after the last drug treatment to determine total cell counts and CLL cell surface markers. As shown in Figure 6a–6b, there was a significant decrease in leukemia cell counts in all mice after drug treatment ($P<0.01$). Importantly, flow cytometry analysis of the cell for expression of CD5 and IgM revealed that 59% of the cells were $CD5^+/IgM^+$ leukemia cells in the pre-treatment samples, whereas only 0.9% cells were $CD5^+/IgM^+$ after drug treatment (Figure 6c), indicating that the leukemia cell population ($CD5^+/IgM^+$ population) were selectively eliminated *in vivo* by Perhexiline, with most (99%) of the remaining cells were CD5/IgM negative cells.

To evaluate the impact of Perhexiline treatment on the overall animal survival, Tcl-1Tg:p53−/− mice that started to develop CLL at the age of approximately two months were treated with Perhexiline (8 mg/kg, i.p.) every other day for a total of four injections. The mice were then observed for survival time without further drug treatment. As shown in Figure 6d, there was a significant increase in median survival time of the drug-treated mice (5.0 months) compared to the untreated mice (3.8 months, $P=0.0046$). Considering that the mice were treated with only 4 injections of Perhexiline as a single agent, these in vivo data suggest that this drug might be potentially effective for clinical treatment of CLL.

Discussion

Among the many challenges in cancer treatment, development of drug resistance and low therapeutic selectivity of cytotoxic anticancer agents are two major problems. The protective effect of stromal microenvironment that promotes cancer cell survival and drug resistance further contributes to the difficulty in elimination of cancer cells *in vivo*. Thus, identification of new therapeutic strategies and novel compounds capable of effectively and selectively kill cancer cells in stromal microenvironment would have significant clinical implications and potentially improve the treatment outcome of cancer patients. One logical way to improve therapeutic selectivity is to target the key biological differences between cancer cells and normal cells. Metabolic alterations in cancer cells have emerge as a promising area that has gained significant new insights into the biological differences between malignant and normal cells, and provided new basis for selective targeting cancer cells.

In addition to increase in aerobic glycolysis known as Warburg effect,[18] other metabolic alterations including high dependency on glutamine and abnormal lipid metabolic pathways are also observed in cancer cells. Inhibition of *de novo* FA synthesis by targeting FASN has been considered as an attractive anticancer strategy.[43,44] Deregulation of FA metabolism seems to play an important role in CLL pathogenesis.[29,31,33,41,43,45] In particular, increased expression of LPL and PLA2 are often observed in CLL, and the high expression of LPL has been considered as a poor prognosis factor.[33] Since LPL and PLA2 facilitate the release of FAs from lipoproteins and phospholipids for cellular utilization, it seems that CLL cells are highly active in using exogenous lipids to meet their metabolic needs, although the exact underlying reasons remain unclear.

In this study, we showed that inhibition of FAs transport into mitochondria by perhexiline was able to effectively kill CLL cells, even when the leukemia cells were under the protection by bone marrow stromal cells. This suggests that CLL cells are highly dependent on the transport of FAs into mitochondria to maintain viability. Our study suggests that a key role of mitochondrial FAs is to support the synthesis of cardiolipin as a critical component of mitochondria membranes to maintain their integrity, and inhibition of FAs transport into the mitochondria would lead to a severe depletion of cardiolipin and loss of mitochondrial integrity, leading to a release of apoptotic factors and cell death. Importantly, such intrinsic cell death process triggered within the mitochondria could not be rescued by stromal cells.

Although it is possible that the long-chain fatty acids transported into mitochondria could be used as an energy source to generate ATP via β-oxidation to support CLL viability, it is unlikely that this could be the key mechanism to maintain CLL cell survival, since the toxic concentrations of perhexilin did not inhibit oxygen consumption, indicating that the FAs transported into mitochondria were not used as a main fuel source. This conclusion is further supported by the observation that inhibition of β-oxidation by high concentrations of ranolazine only exhibited modest cytotoxic effect in CLL cells, suggesting that β-oxidation of FAs might not be a critical energy source in primary CLL cells.

Cardiolipin is a structurally unique dimeric phospholipid in the inner mitochondrial membranes, where it is required for maintaining the integrity of mitochondria. A loss of

cardiolipin may lead to mitochondrial dysfunction. Cardiolipin is also necessary for cytochrome *c* anchorage to the mitochondrial membranes for proper function. Loss of cardiolipin could arise from impaired cardiolipin synthesis due to a decrease in precursors such FAs for its synthesis.[46] Perhexiline, by its known ability to inhibit carnitine palmitoyltransferases, suppresses the transport of FAs into mitochondria and thus depletes the precursors for cardiolipin biosynthesis.

One important question is why inhibition of FA transport into mitochondria is selectively toxic to CLL with low cytotoxicity to normal lymphocytes and stromal cells. A possible explanation is that the turnover rate of cardiolipin in CLL cells may be higher than normal cells, due to intrinsic oxidative stress in CLL cells and high expression of cardiolipin degradation enzymes such as phospholipase iPLA2.[47,48] The high intrinsic mitochondrial ROS generation in CLL cells [6,12,24–26] might accelerate the degradation of cardiolipin, which is highly sensitive to ROS-mediated damage and degradation.[11,12] Thus, the high turnover of cardiolipin in CLL cells would render them highly dependent on the transport of FAs into mitochondria for cardiolipin synthesis. As such, CLL cells are more vulnerable to inhibition of FA transport into mitochondria by perhexiline. This may also explain why CLL cells often show elevated expression of LPL, which facilitates the uptake of exogenous FAs for use in active synthesis of cardiolipin.

Our *in vivo* study showed that Perhexiline was able to effectively and selectively eliminate CD5+/IgM+ leukemia cells in the CLL mouse model (Figure 6c). This is consistent with its ability to selectively kill CLL cells in stromal tissue microenvironment. It is also important to note that the CLL mice used in this study harbored Tcl-^{Tg}:p53$^{-/-}$ genotype. Since a subset of CLL patients with chromosome 17p-deletion (loss of p53 gene in 17p) are difficult to treat due to drug resistance and aggressive disease progression. The ability of Perhexiline to eliminate leukemia cells in the Tcl-^{Tg}:p53$^{-/-}$ mice and to significantly prolong the mouse survival by only 4 injections of the drug seems very promising. It should be noted that Perhexiline is an anti-angina drug used since the 1970s and its clinical pharmacokinetic parameters have been well characterized. This makes it feasible to reposition this drug for new applications in CLL treatment. This possibility warrants further evaluation.

Methods

Reagents

Perhexiline maleate salt, Ranolazine dihydrochloride, Cerulenin, fludarabine, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonyl acridine orange (NAO) and Rhodamine-123 were obtained from Invitrogen Molecular Probes (Carlsbad, CA, USA). Annexin-V-FITC was supplied by BD Biosciences (San Jose, CA, USA). Protein assay kit was from Pierce Biotechnology (Rockford, IL, USA). Mouse anti-β-actin was purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-cytochrome C and goat anti-HSP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and primary CLL cells

Bone marrow stromal cell lines (HS5, StromaNKtert, and KUSA-H1) were cultured as described previously[6], and were tested to be free of mycoplasma contamination. Blood samples were obtained from CLL patients with confirmed diagnosis of CLL using the National Cancer Institute Criteria.[49] Proper informed consents under a research protocol approved by the Institutional Review Board (IRB) of MD Anderson Cancer Center were obtained from all patients before collection of blood samples. CLL cells were isolated from blood samples as described previously.[50] All viable samples were used in this study without exclusion. The clinicobiologic characterisitics of the patients are summarized in Table S1.

Transmission electron microscopy (TEM)

TEM imaging was carried out as previously described.[51] Cell viability was determined by double staining with annexin-V-FITC/PI followed by flow cytometry analysis as described previously.[6]

Glucose uptake and oxygen consumption assays

CLL cells were first treated with 5 μM Perhexiline in triplicates for 3 h in glucose-free medium. [^{3}H]2-deoxyglucose (0.4 μCi/ml) was then added and incubated for 60 min. The cells were collected, washed twice with PBS, and suspended in 200 μl of water, then mixed with 1 ml scintillation fluid. Radioactivity was detected by scintillation countering. Cellular oxygen consumption was measured by an Oxytherm system (Hansatech Instrument, Norfolk, UK).

Quantitation of cardiolipin and mitochondrial membrane integrity

The cellular cardiolipin contents were measured by flow cytometry analysis, using NAO as a specific fluorescence dye as described previously.[40] Changes in mitochondrial membrane potential were monitored by incubating cells with 1 μM Rho-123 for 1 h, followed by flow cytometry.

Animal study

The Tcl-1^{Tg}:p53$^{-/-}$ mouse model of CLL we recently established [42] was used to evaluate the *in vivo* therapeutic effect of Perhexiline. Before drug treatment, leukemia cells were obtained from the peritoneal cavity of Tcl-1^{Tg}:p53$^{-/-}$ mice that had developed CLL disease by peritoneal washing as described[6], and the basal leukemia burden was measured. After a week recovery period, the mice were treated with Perhexiline (8 mg/kg, i.p.) every other day for 1 week (total 4 injections). Peritoneal cells were again collected one and two weeks after the last drug treatment, and total cell counts as well as expression of surface IgM and CD5 were analyzed by flow cytometry as described.[42] To evaluate animal survival, the mice (age 2 months) were treated with Perhexiline (8 mg/kg, i.p.) every other day for a total of 4 injections. The mice were then observed for survival without further drug treatment. The mice with confirmed genotype (Tcl-1^{Tg}:p53$^{-/-}$) at age of 2 months were enrolled in this study without exclusion, and randomly assigned to the control and treated groups with clear label (no blinding). The animal study was performed in compliance with federal and

institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC).

Statistical analysis

Each experiment was repeated at least three times with CLL samples from different patients. The in vitro data were expressed as mean±SD (standard deviation). Statistical analysis was performed by Student's t-test to compare the difference between groups, using the Graphpad Prism software (GraphPad, San Diego, CA, USA). Animal survival curves between the control and drug-treated groups were compared using Kaplan-Meier plots generated by Graphpad Prism software (GraphPad, San Diego, CA), and the statistical significance was analyzed by the log-rank (Mantel-Cox) test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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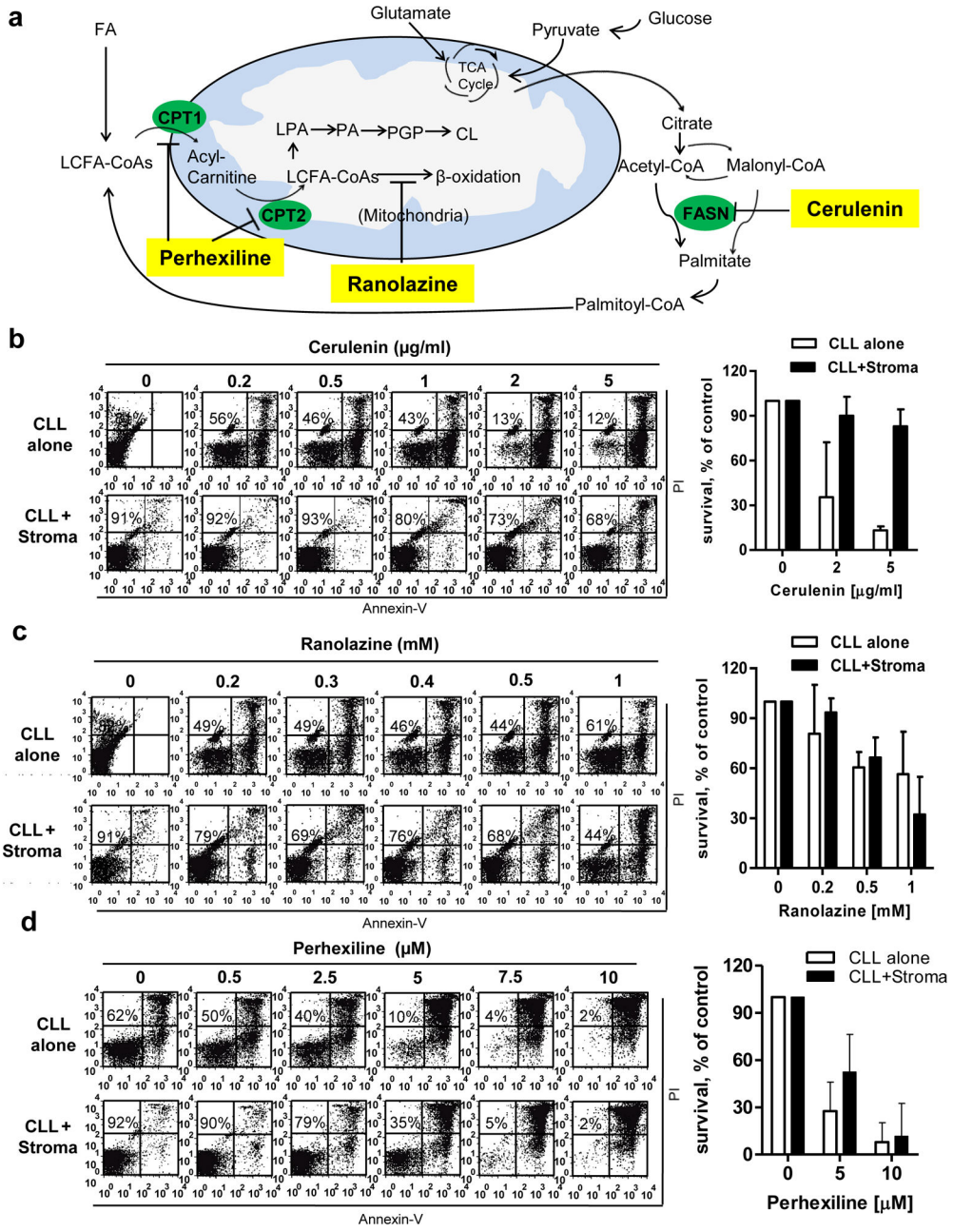

Figure 1.

Identification of perhexiline as a potent drug that effectively killed CLL cells in the presence of bone marrow stromal cells. (a) Schematic illustration of major lipid metabolic pathways and the target enzymes (green) of the three drugs (yellow) examined in this study. TG, triacylglycerol; LPL, lipoprotein lipase; FA, fatty acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; PGP, phosphatidylglycerophosphate; CL, cardiolipin; LCFA-CoAs, long-chain fatty acyl Coenzyme A; FASN, fatty acid synthase; CPT, carnitine palmitoyl transferase; TCA cycle, tricarboxylic-acid cycle. (b) Primary CLL cells were incubated with various concentrations of cerulenin (FASN inhibitor) for 48 h in the presence and absence of StromaNKtert cells as indicated, and cell viability was measured by annexin-V/PI staining

and flow cytometry analysis. The results from a representative CLL sample are shown on the left panels. The number (%) within each flow cytometry panel indicates % of viable cells (annexin-V/PI double-negative). Quantitative results of 3 CLL patient samples are shown on the right panel. Each bar shows mean ± SD of three separate experiments with 3 patient samples. (c) Primary CLL cells were incubated with various concentrations of ranolazine (β-oxidation inhibitor) for 48 h in the presence and absence of StromaNKtert cells as indicated, and cell viability was measured as described in b, n=3 patient samples. (d) Primary CLL cells were incubated with various concentrations of pehexiline for 48 h in the presence and absence of StromaNKtert cells as indicated, and cell viability was measured as in described in b. n=19 patient samples. In the co-culture experiments, the ratio of stromal cells:CLL cells was 1:25. In the control experiments in *b*, *c*, and *d*, cells were incubated with solvent (0.1% DMSO) for 48 h, which was not toxic to CLL cells as shown in Supplementary Figure S2.

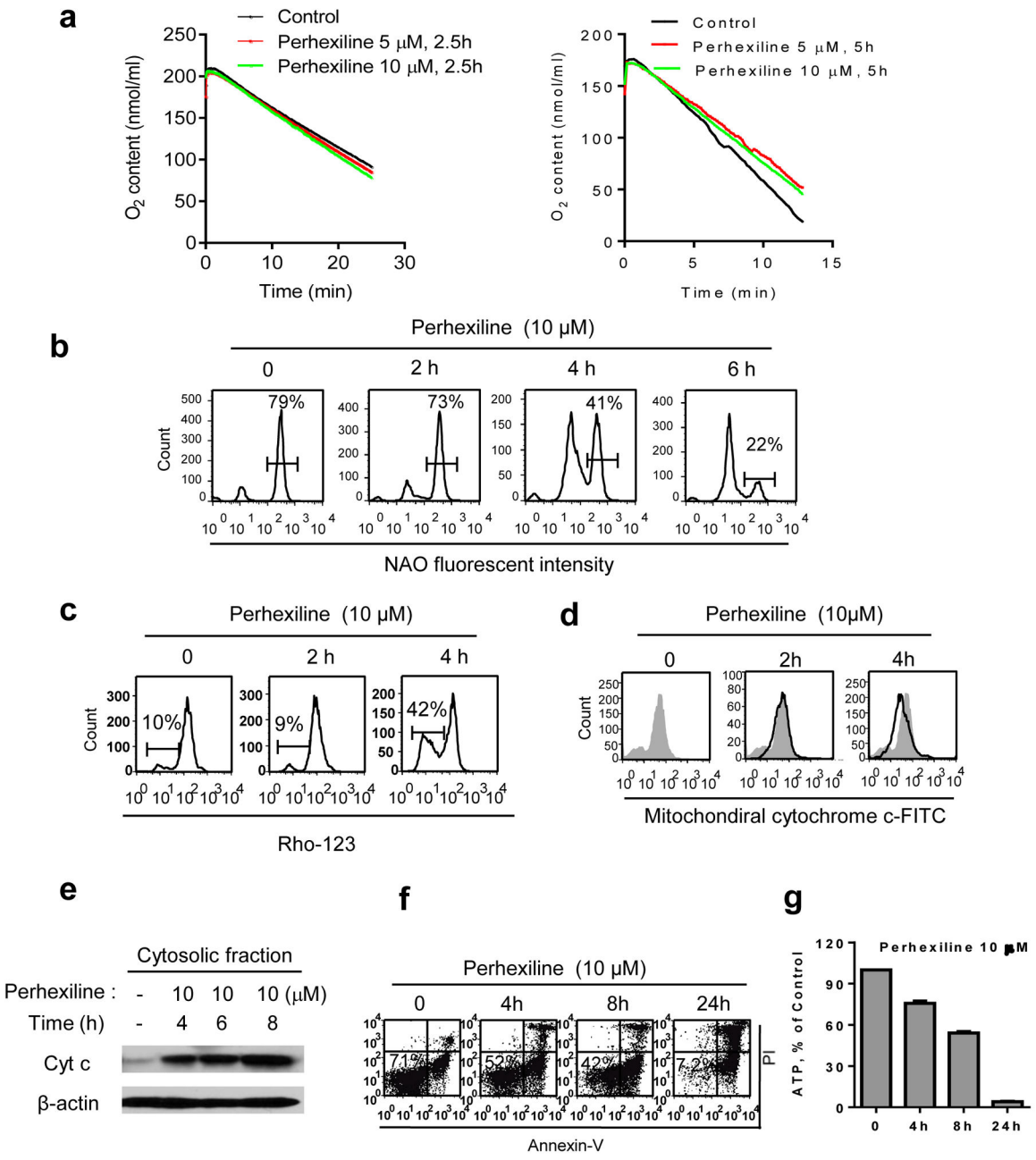

Figure 2.

Effect of perhexiline on cardiolipin and mitochondrial membrane integrity in CLL cells. (a) Primary CLL cells were treated with or without 5–10 μM perhexiline as indicated. CLL cells were suspended in fresh medium (10^8 cells/ml) and oxygen consumption was measured using an Oxythem system as described under Method. Data were representative of experiments using two separate patient samples. (b) Rapid depletion of cardiolipin induced by perhexiline. CLL cells were incubated with 10 μM perhexiline as indicated, and cardiolipin contents were measured using NAO staining and flow cytometry analysis. The number (%) in each panel indicates the % of cells with normal cardiolipin content. Data

were representative of experiments using 3 separate patient samples. (c) Induction of mitochondrial transmembrane potential loss by 10 μM perhexiline in primary CLL cells, detected by flow cytometry analysis using Rho-123 staining. The % cells that lost transmembrane potential are indicated by the number (%) within each panel. Data were representative of experiments using 3 separate patient samples. (d) Loss of mitochondrial cytochrome *c* induced by perhexiline (10 μM) in CLL cells. Mitochondrial cytochrome *c* was measured by flow cytometry analysis as described under Method. The control sample is shown in gray (shaded) and the perhexiline-treated samples are shown in black curves. Data were representative of experiments using 3 patient samples. (e) Western blot analysis of cytosolic cytochrome *c* released from mitochondria before and after perhexiline treatment. CLL cells were incubated with 10 μM perhexiline for the indicated times, and cytosolic proteins were isolated for western blot analysis of cytochrome *c* release from mitochondria. (f) Time-dependent cell death induced by 10 μM perhexiline. Cell viability was analyzed by flow cytometry after cells were double stained with annexin-V/PI. G, primary CLL cells were incubated with 10 μM perhexiline for 4–24 h as indicated; $5x10^5$ cells from each sample were collected for ATP analysis as described under Method, n=3 patient samples.

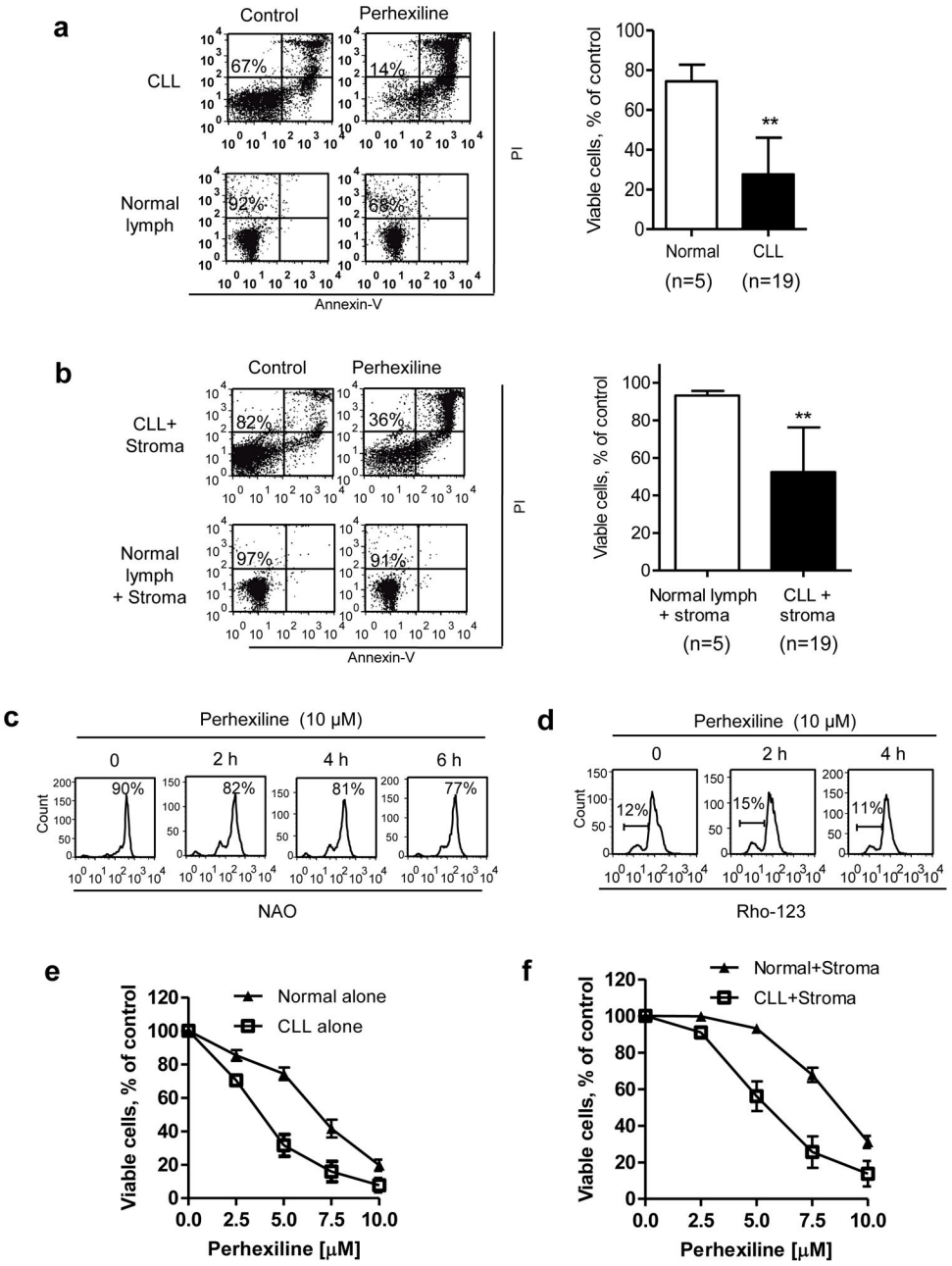

Figure 3.

Selective killing of CLL cells by perhexiline in comparison with normal lymphocytes. (a) Primary CLL cells or normal lymphocytes were treated with or without 5 μM perhexiline for 48 h, and cell viability was analyzed by annexin-V/PI staining. The number (%) within each panel indicates % of viable cells (Annexin-V/PI double-negative). The quantitative results of 19 CLL patient samples and normal lymphocytes from 5 healthy donors are shown on the right panel. Each bar shows mean ± SD; **, $P<0.01$. (b) CLL cells or normal lymphocytes were treated with or without 5 μM perhexiline in the presence of bone marrow stromal cells (StromaNKtert) for 48 h. Cell viability was analyzed annexin-V/PI staining. The quantitative results of 19 CLL patient samples and 5 normal lymphocyte samples are shown on the right

panel. Each bar shows mean ± SD; **, $P<0.01$. (c) Normal lymphocytes from healthy donors ($n=3$) were incubated with 10 μM perhexiline for 2–6 h as indicated. Cardiolipin was measured by flow cytometry using NAO staining. The number within each panel indicates % of cells with normal NAO fluorescent signal. (d) Normal lymphocytes ($n=3$) were incubated with 10 μM perhexiline for 2–4 h as indicated; mitochondrial transmembrane potential was then measured by flow cytometry after cells were stained with Rho-123. The number within each panel indicates % of cells that lost transmembrane potential. (e) Comparison of sensitivity of CLL cells and normal lymphocytes to perhexiline. CLL cells isolated from 11 CLL patients or normal lymphocytes from 5 healthy donors were incubated with the indicated perhexiline for 48 h. Cell viability was analyzed by flow cytometry. (f) Effect of perhexiline on CLL cells and normal lymphocytes in the presence of stromal cell co-culture. CLL cells isolated from 11 patient samples or normal lymphocytes from 5 healthy donors were incubated with the indicated concentrations of perhexiline in the presence of StromaNKtert cells for 48 h. Cell viability was analyzed by flow cytometry after the cells were double-stained with annexin-V/PI.

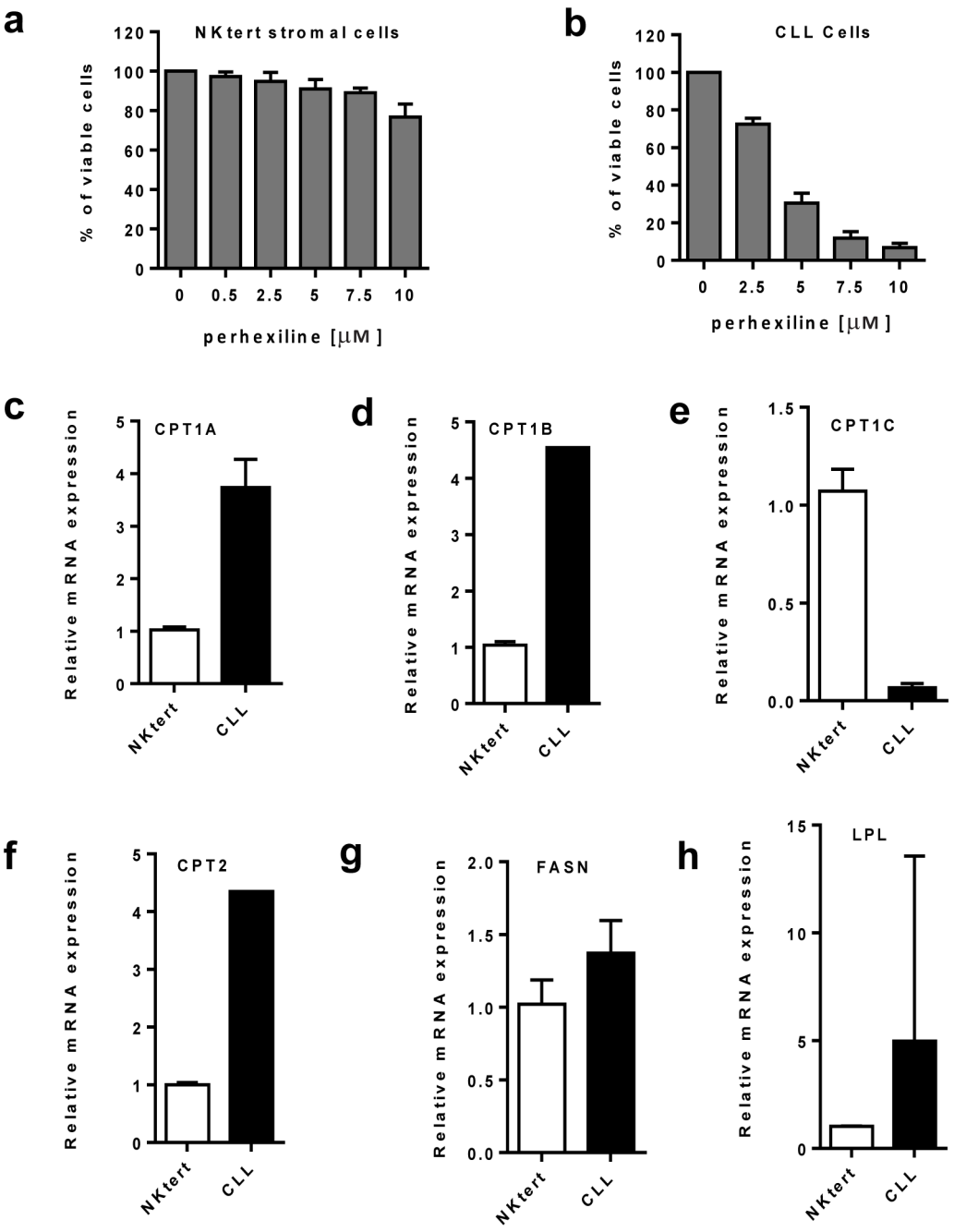

Figure 4.

Comparison of stromal cells and CLL cells for their sensitivity to perhexiline and expression of enzymes involved in lipid metabolism. (a–b) Nktert stromal cells or CLL cells were incubated with the indicated concentrations of perhexiline for 48 hours. Cell viability was analyzed by annexin-V/PI staining and flow cytometry analysis. The bars represent mean ± SD of 3–6 separate measurements using different patient samples. (c–h) Comparison of basal mRNA expression of genes involved in fatty acid metabolism between CLL cells and StromaNKtert cells. mRNA expression was quantified by qRT-PCR. The bars represent

mean ± SD of at least 3 separate measurements. CPT1A, CPT1B, CPT1C and CPT2 are carnitine palmitoyltransferas isozymes; FASN, fatty acid synthase; LPL, lipoprotein lipase.

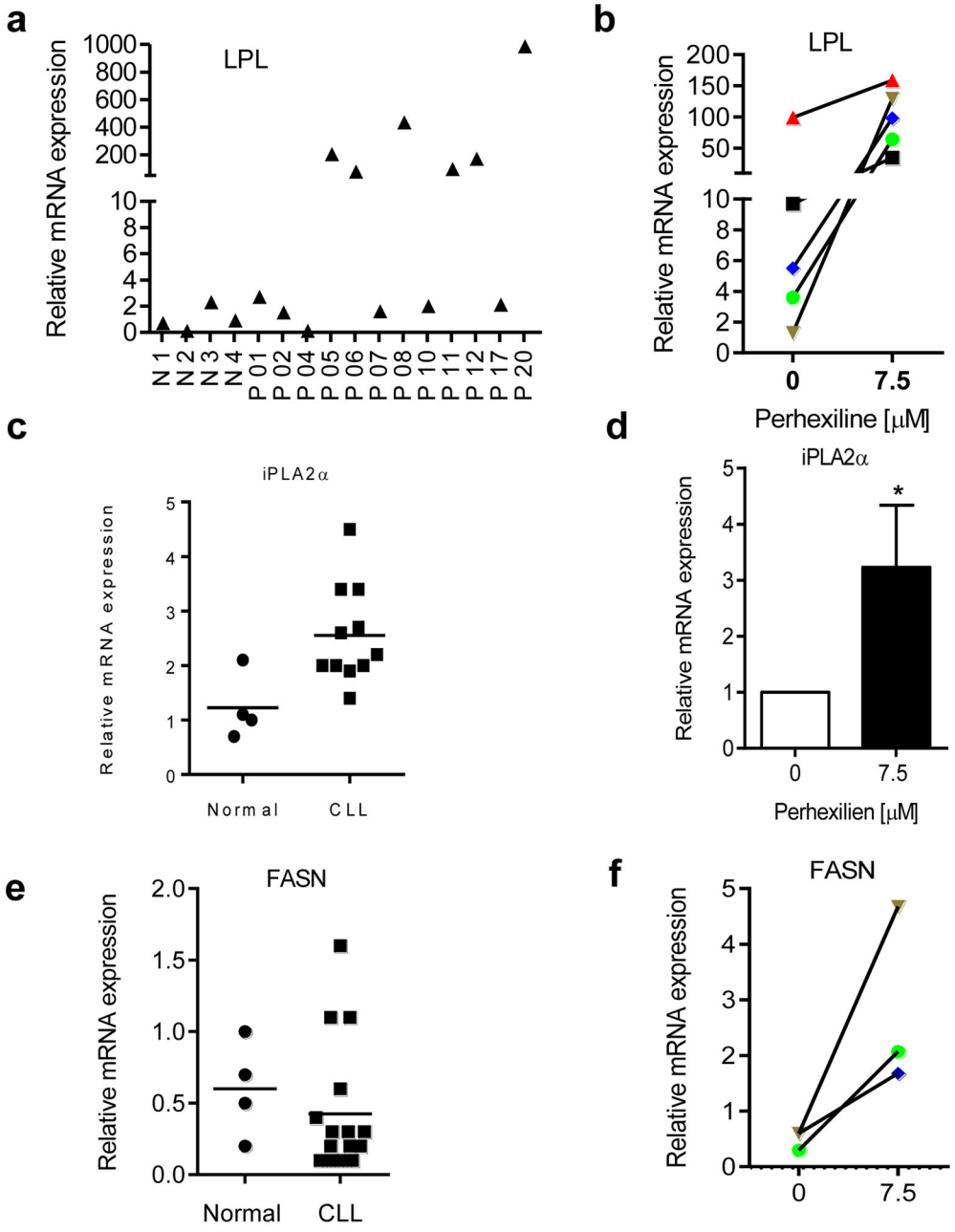

Figure 5.

Quantitative RT-PCR analysis of expression of LPL, iPLA2, and FASN in CLL cells and purified normal B lymphocytes. (a) Comparison of basal expression of LPL mRNA in purified normal B lymphocytes (n=4 donors) and CLL cells (n=12 patients) using real-time PCR analysis, N, purified normal B lymphocytes from healthy donors; P, CLL patient samples (the patient numbers corresponding to the patients listed on Table S1). (b) qRT-PCR analysis of LPL expression in CLL cells (n=5) treated with or without perhexiline (7.5 μM, 24 h). Each data point represents the mean of triplicate separate measurements. (c) Comparison of basal expression of iPLA2α mRNA in purified normal B lymphocytes (n=4) and CLL cells (n=11), P<0.05. (d) Effect of perhexiline (7.5 μM, 24 h) on iPLA2α gene

expression in CLL cells (n=3); bars represent mean ±SD; *, P<0.05. (e) Comparison of the basal expression of fatty acid synthase (FASN) in purified normal B lymphocytes (n=4) and CLL cells (n=12). (f) Quantitative RT-PCR for FASN expression in CLL cells before and after perhexiline treatment (7.5 μM, 24 h); n=3 patient samples; each data point represents the mean of triplicate measurements. The primers used in this study were listed in Table S2.

a

	Leukemia cell burden		
	Before treatment	After treatment (week 1)	After treatment (week 2)
Mouse #1	12.44 × 10^6	9.07 × 10^6	9.17 × 10^6
Mouse #2	35.26 × 10^6	12.47 × 10^6	15.03 × 10^6
Mouse #3	47.20 × 10^6	13.44 × 10^6	13.40 × 10^6
Mouse #4	16.85 × 10^6	3.16 × 10^6	2.01 × 10^6
Mouse #5	15.52 × 10^6	1.95 × 10^6	3.29 × 10^6

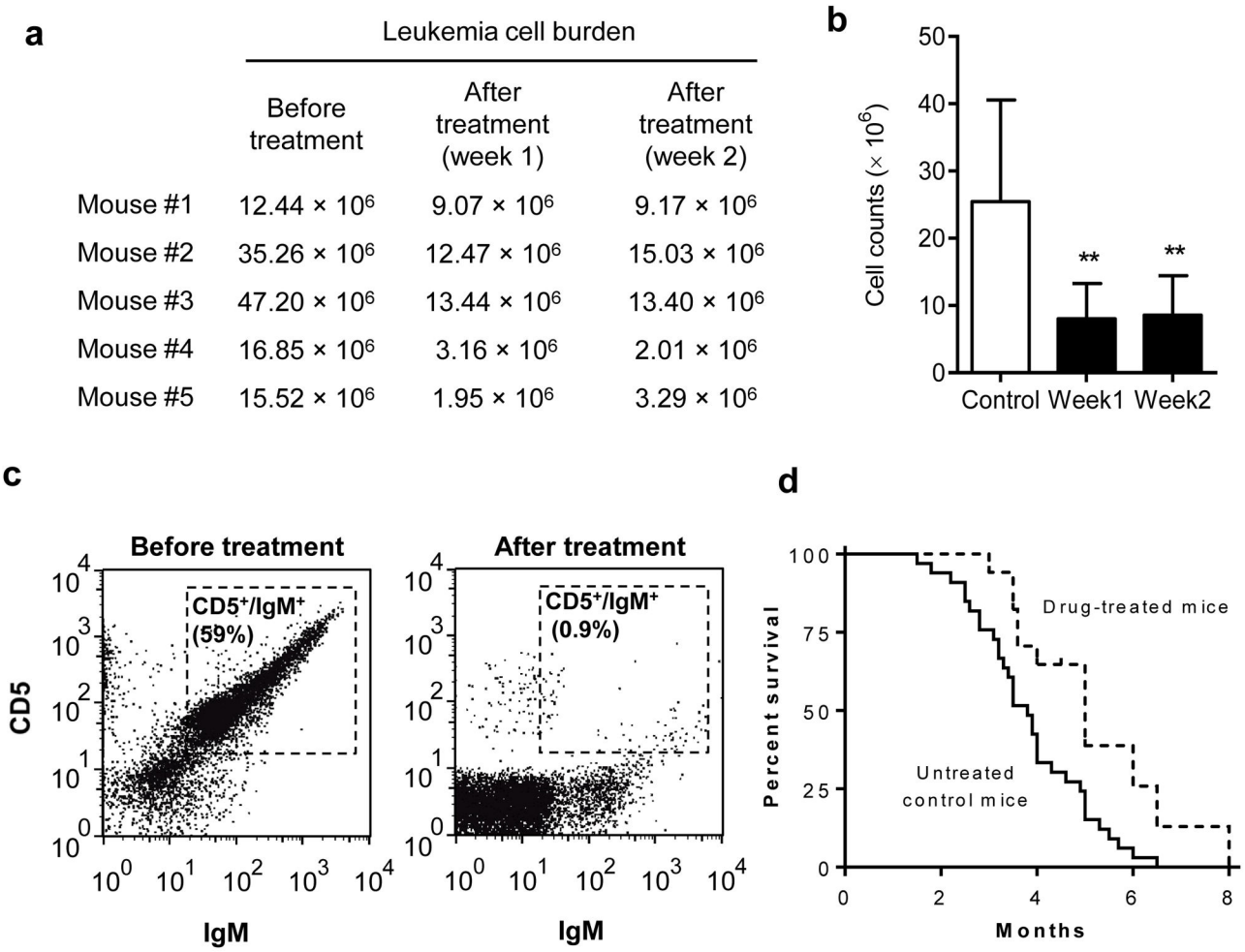

Figure 6.

In vivo therapeutic activity of perhexiline in CLL mice with Tcl-1Tg:p53−/− genotype. (a) Five mice that had developed CLL disease were first measured for the basal leukemia cell burden in their peritoneal cavities as described under Method. After a week of recovery period, the mice were then treated with Perhexiline (8 mg/kg, i.p., every other day for 4 injections). Peritoneal cells were again collected one week and two weeks after the last drug treatment, and total cell counts were determined. (b) Comparison of peritoneal cell counts in all 5 mice before and after treatment with perhexiline as described in a, ** P<0.01. (c) Perhexiline selectively eliminated $CD5^+/IgM^+$ CLL cells in Tcl-1-Tg:p53−/− transgenic mice (n=5). Cell surface CD5 and IgM expression was measured by flow cytometry analysis. (d) Four injections of perhexiline significantly prolonged the survival time of CLL mice (n=11, P < 0.01).