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A Selective Novel Peroxisome Proliferator–Activated Receptor (PPAR)-α Antagonist Induces Apoptosis and Inhibits Proliferation of CLL Cells *In Vitro* and *In Vivo*

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Tumor-specific metabolic changes can reveal new therapeutic targets. Our findings implicate a supporting role for fatty acid metabolism in chronic lymphocytic leukemia (CLL) cell survival. Peroxisome proliferator–activated receptor (PPAR)-α, a major transcriptional regulator of fatty acid oxidation, was recently shown to be upregulated in CLL. To evaluate PPARα as a potential therapeutic target, we developed a highly selective, potent small molecule antagonist of PPARα, NXT629. NXT629 inhibited agonist-induced transcription of PPARα-regulated genes, demonstrating target engagement in CLL cells. Furthermore, NXT629 induced apoptosis of CLL cells even in the presence of a protective microenvironment. To mimic the proliferative lymphoid compartment of CLL, we examined the activity of NXT629 on CLL cells that were stimulated to proliferate *in vitro*. NXT629 reduced the number of leukemia cells undergoing cell division. In addition, in two xenograft mouse models of CLL (one a model for nondividing and one for dividing CLL), NXT629 reduced the number of viable CLL cells *in vivo*. Overall, these results suggest that fatty acid metabolism promotes survival and proliferation of primary CLL cells and that inhibiting PPARα gene regulation could be a new therapeutic approach to treating CLL.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world, leading to ~5,000 deaths annually (1). CLL is characterized by an accumulation of monoclonal mature B cells in blood, secondary lymphoid tissues and bone marrow. Despite major advances in the field, there is no curative therapy for CLL to date (2). Current treatment approaches aim at achieving minimal residual disease, which is associated with superior long-term outcome (3). Treatment avenues, such as those targeting pathways downstream of the B cell receptor, such as Syk, Btk and PI3Kδ (4–9), that are currently being evaluated in clinical trials or have recently been approved by the U.S. Food and Drug Administration (such as ibrutinib) are focused on kinases. Surprisingly, little attention has been given to targeting metabolic enzymes in CLL. While the traditional view has been that cancer cells are fueled by glucose, named the "Warburg effect" after Otto Warburg (10,11), recent studies demonstrate the involvement of fatty acid oxidation (FAO) in cancer cell viability. Some solid tumors including prostate, ovarian and renal cell carcinoma rely on fatty acids to satisfy their metabolic needs (12–16). Solid tumors that are initially dependent on glucose can undergo a metabolic switch upon detachment from the extracellular matrix and start depending on FAO for survival (12). In addition, hypoxia and oncogenic RAS increase fatty acid uptake by tumor cells (17). Inhibition of FAO with Etomoxir, an irreversible small molecule inhibitor of CPT1A, the rate-limiting enzyme for fatty acid import into mitochondria (18), sensitized AML cells to apoptosis induction by ABT-737, an inhibitor of Bcl-2 and Bcl-xL (19). Fur-

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thermore, some evidence points toward a critical role for FAO in the viability of leukemia-initiating cells. For instance, the CPT1A inhibitor Etomoxir decreases the number of leukemia-initiating cells in primary human AML samples (19), and FAO signaling downstream of PML was found to be critical for maintenance and function of hematopoietic stem cells and possibly leukemia-initiating cells (20).

Peroxisome proliferator–activated receptors (PPARs) are a family of ligand activated nuclear hormone receptors comprised of three isoforms: PPARα, PPARδ and PPARγ. PPARα, also known as NR1C1 (nuclear receptor subfamily1, group C, member 1) is a major transcriptional regulator of lipid metabolism. Endogenous PPARα ligands include free fatty acids and eicosanoids as well as oleoylethanolamide (OEA), a naturally occurring lipid (21). Upon ligand binding, PPARα induces transcription of a number of genes, resulting in a shift toward β-oxidation (for example, CPT1A) and away from glucose oxidation (for example, PDK4) (22).

It was recently shown that compared with normal B cells, CLL cells overexpress PPARα, rendering them dependent on β-oxidation for energy (23). This metabolic strategy helps account for some of the pathogenic characteristics of CLL, including immunosuppression and drug resistance. These reports establish PPARα as a promising molecular target for the treatment of this cancer. However, to date, no clinically relevant, selective PPARα antagonists have been available to address this idea in human trials. We have recently described the discovery and synthesis of a family of novel, selective and bioavailable PPARα antagonists that can bind reversibly to the ligand binding site and effectively compete with both synthetic and endogenous PPARα agonists (24). Herein, we evaluated the activity of the PPARα antagonist NXT629 on CLL viability and proliferation *in vitro* as well as tumor burden *in vivo*.

MATERIALS AND METHODS

Patient Samples, Isolation of CLL B cells, Cell Culture and Reagents

Blood samples were collected from patients at The Feinstein Institute for Medical Research, North Shore–LIJ Health System (Manhasset, NY, USA), and the Sunnybrook Odette Cancer Center (Toronto, Canada) who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL after providing written informed consent in compliance with the Declaration of Helsinki (25) and the Institutional Review Board of the North Shore–LIJ Health System and the Sunnybrook Health Sciences Center. Blood was collected from patients for whom clinical information and laboratory data were available. For a set of patients (from N Chiorazzi), immunoglobulin heavy-chain variable region gene (IGHV) and immunoglobulin light-chain variable region gene (IGLV) DNA sequences were also available. For some experiments, the CLL cells were purified from frozen peripheral blood mononuclear cells (PBMCs) via negative selection by using anti-CD2 and anti-CD14 magnetic beads (Miltenyi Biotech).

Isolation of B Cells from Healthy Volunteers

Peripheral blood mononuclear cells were isolated from the blood of normal volunteers over a Ficoll-Hypaque density gradient. Anonymous blood samples were purchased from the San Diego Blood Bank; therefore, no Institutional Review Board approvals were necessary. B cells were isolated by positive selection using $CD19^+$ beads (Milenyi Biotech) per the manufacturer's instructions. Cells were cultured and viability was monitored as described below.

Measurement of Cell Viability

CLL cells were cultured at 2×10^5 cells/mL in 100 μL media in 96-well plates (Costar, Corning Inc.) in RPMI 1640/10% fetal calf serum (FCS). All inhibitors were prepared in dimethyl sulfoxide (DMSO) (0.1% final concentration), which was used as vehicle control in all experiments. Determination of CLL cell viability was based on the analysis of mitochondrial transmembrane potential ($\Delta\psi_m$) using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Invitrogen), and cell membrane permeability to propidium iodide (PI) (Sigma-Aldrich). For viability assays, 100 μL of the cell culture was collected at the indicated days and mixed with media containing 100 μL of 40 μmol/L $DiOC_6$ and 10 μg/mL PI. The cells were then incubated at 37°C for 15 min and analyzed within 30 min by flow cytometry by using the flow cytometer Accuri C6 (Accuri). Data were analyzed by using the CFlowPlus software (Accuri). The percentage of viable cells was determined by gating on PI negative and $DiOC_6$ bright cells.

Cocultures

Macrophages (J774A.1) were obtained from ATCC (ATCC® TIB67™ and CRL-11882™) and plated in 96-well plates at a density of 50,000 cells per well. OP9 cells were also obtained from ATCC (ATCC® CRL-2749™) and plated at 10,000 cells/well. Macrophages and OP9 cells (26) were exposed to 10 μg mitomycin C (Sigma-Aldrich M4287) for 3 h to prevent proliferation. The cells were then washed three times with media to remove the mitomycin C. Cells were plated in RPMI/10% FCS, and CLL cells were subsequently plated over the layer of mitomycin C–treated macrophages or OP9 cells. NXT629 or vehicle control was added to the cells at the beginning of the coculture and incubated in a 37°C/5% CO_2 incubator for 7 d. After 7 d, the CLL cells were collected and stained with $DiOC_6$/PI and analyzed for viability as described above.

CLL Proliferation Assay

PBMCs were isolated from the blood of normal volunteers (purchased anonymously from the San Diego Blood Bank) over a Ficoll-Paque PLUS density gradient (GE Healthcare). $CD2^+$ T cells were isolated from PBMCs by positive selec-

tion using anti-CD2 beads (Miltenyi Biotech) following the manufacturer's instructions. To activate T cells, they were cultured on a 24-well plate (Costar/Corning Inc.) at 1×10^6 cells/mL in 1 mL of culture media (RPMI 1640 supplemented with 10 mmol/L HEPES [GIBCO-BRL], penicillin [100 U/mL]–streptomycin [100 μg/mL] (Gibco/Invitrogen) and 10% FCS [ATCC]) in the presence of 20 μL CD3/CD38 Dynabeads (Invitrogen) per 10^6 T cells for 3 d. T cells were collected, resuspended at 2×10^7 cells/mL in fresh medium containing 12 μg/mL mitomycin C and incubated for 3 h at 37°C. At this point, the T cells were washed four times with fresh media and frozen by using Recovery Cell Culture freezing media (Gibco/Life Technologies) in liquid N_2 until use. CLL proliferation induced by frozen versus fresh T cells was comparable (data not shown). Thus, frozen T cells were used for all experiments.

To set up the CLL proliferation assay, CLL PBMCs were thawed from frozen vials and cultured at 1.4×10^5 CLL cells/well in 100 μL of the above-listed culture media for 2 h in the presence of PPARα antagonist or vehicle control. Mitomycin C–treated, activated T cells were added at 6×10^4 T cells/well in 100 μL of the above-listed culture media supplemented with hrIL-4 (5 ng/mL, R&D Systems) and hrIL-10 (15 ng/mL, R&D Systems). Cells were cultured for 5–8 d. In some experiments, CLL cells were prelabeled with carboxyfluoresceinsuccinimidyl ester (CFSE) (Invitrogen) after the manufacturer's instructions before coculture. Five days after coculture, CLL cells were stained with CD19-APC (Becton Dickinson), and proliferation was assessed by flow cytometry (Accuri cytometer) gating on $CD19^+$ $CFSE^+$ cells. In the majority of the experiments, if not otherwise indicated, the number of viable CLL cells was analyzed by staining with $DiOC_6$/PI as described above and collecting each sample for 30 s via flow cytometry. The number of viable cells was determined by gating on $DiOC_6$ bright and PI negative cells.

Cell Cycle Analysis

CLL cells were cultures as above to trigger proliferation. At d 8, cells were collected and analyzed for cell cycle progression by using the FxCycle PI/RNase staining solution (Molecular Probes, Life Technologies) according to the manufacturer's instructions.

Isolation of RNA and cDNA Synthesis

Purified CLL cells were plated at 6.8×10^6 cells/mL in 1 mL media in a 12-well plate. Cells were exposed to antagonist NXT629 for 2 h, followed by an agonist for 48 h, as described in the respective figure legends. RNA was isolated from CLL cells by using the RNeasy kit (Qiagen). A total of 100 ng RNA was used in each cDNA reaction by using the IScript reaction mix (Bio-Rad) following the manufacturer's instructions.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Reverse transcription reactions were performed by using the iTaqUniversal SYBR® Green supermix (Bio-Rad) following the manufacturer's instructions. Each reaction contained 2.5 ng reverse-transcribed RNA (based on the initial RNA concentration) in 20 μL final reaction volume. The reaction conditions were as follows: 95°C for 30 s, 45 cycles of 95°C for 4 min followed by 59°C for 5 min, 65°C for 5 min and finally 95°C for 5 min. Primer sequences used were as follows: *β-actin*-F: GCT GTG CTA CGT CGC CCT G, *β-actin*-R: GGA GGA GCT GGA AGC AGC C, *PDK4*-F: GGAGC ATTTCTCGCGCTACA, and *PDK4*-R: ACAGGCAATTCTTGTCGCAAA. Primers were synthesized by Integrated DNA Technologies. The polymerase chain reaction (PCR) was carried out by using the C1000Touch Thermal Cycler (Bio-Rad).

CLL Mouse Model

Two different CLL mouse models were evaluated. NOD/Shi-scid,γc^{null} (NSG) mice, a NOD/SCID-derived strain, that lacks the IL-2 receptor family common cytokine receptor γ chain gene (γc), rendering animals completely deficient in lymphocytes, including natural killer cells, were used for this study. Female NSG mice (12–14 wks at study initiation) were purchased from The Jackson Laboratory. Animals were given food and water *ad libitum* and allowed to acclimate for at least 1 wk before initiation of experiments. All protocols were approved by the Inception Sciences Institutional Animal Care and Use Committee.

Model for resting CLL. CLL PBMCs from two patients were pooled, and CFSE was labeled and randomized among the groups. The 10^8 CFSE-labeled cells were delivered by an intravenous bolus injection (50 μL) into the tail vein of NSG mice. Immediately after injection of CLL cells, groups of five mice received daily dosing of vehicle control (saline, 10 mL/kg, intraperitoneal), NXT629 at 30 mg/kg or fludarabine at 50 mg/kg. Mice were sacrificed 4 wks after engraftment, and the splenocytes were stained with hCD19 and hCD5 and analyzed by flow cytometry.

Model for proliferative CLL. The Institutional Review Board and the Institutional Animal Care and Utilization Committee of the North Shore–LIJ Health System sanctioned these studies. T cells were purified from CLL PBMCs using Milteny anti-CD3 beads, resuspended in 1×10^6 cells/mL and stimulated with anti-CD3/CD28 Dynabeads (30 μL/mL) in the presence of IL-2 (36 U/mL) in RPMI 1640/10% FCS for 3 d. Next, beads were removed from the cultures, and the cells were cultured in media supplemented with IL-2 for an additional 4 d. Preactivated human T cells (5×10^5) were administered in 4- to 8-wk-old NSG mice (The Jackson Laboratory) by injection into the retro-orbital plexus (50 μL). After confirming the presence of human T cells in the blood of recipient mice (10 d after injection), CLL PBMCs from the same patient (2×10^7) were delivered by an intravenous (50 μL) injection into the retro-orbital plexus. At the time of CLL cell injection, mice received vehicle control or NXT629, 30 mg/kg of mouse weight, which was given by intraperitoneal injec-

Table 1. IC_{50} values (inhibition in antagonist mode) for human PPARα, PPARδ and PPARγ.

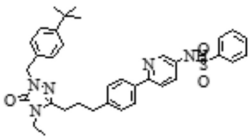
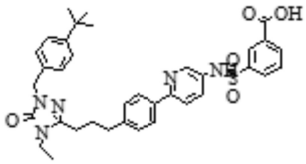
Human PPAR	NXT629	NXT962
		
PPARα	78 nmol/L	>15,000 nmol/L
PPARδ	>30,000 nmol/L	>30,000 nmol/L
PPARγ	>30,000 nmol/L	>30,000 nmol/L

Table 2. IC_{50} values μmol/L using NXT629 (inhibition in antagonist mode) for human nuclear hormone receptors?

PPARα	PPARδ	PPARγ	ERβ	GR	TRβ
0.077 ± 35	6.0 ± 3.2	15 ± 19	12 ± 8	26 ± 21	57 ± 27

tions daily for 2 wks. All mice were killed at the end of experiment, and the spleen and bone marrow (BM) were collected for flow cytometric analyses. Spleen and BM cells were stained by using anti-mCD45, anti-hCD45, anti-hCD5, anti-hCD19, anti-hCD4 and anti-hCD8 antibodies.

Statistical Analysis

Statistical significance was determined by using the Student *t* test. The *p* values <0.05 were considered significant. Median inhibitory concentration (IC_{50}) values were determined using nonlinear regression (curve fit) analysis with Prism software (GraphPad Software).

All supplementary materials are available online at www.molmed.org.

RESULTS

NXT629 Inhibits Transcription of PPARα Target Genes

We recently designed several novel small-molecule PPARα selective antagonists. One such molecule, NXT629, was used in the current set of experiments to determine the role of this nuclear hormone receptor in CLL B-cell function. NXT629 has an IC_{50} value of 78 nmol/L against PPARα in a luciferase reporter assay (Invitrogen) (Table 1) and is selective against other nuclear hormone receptors (Table 2) (24). In addition, the negative control compound NXT962 was synthesized. NXT962 has a similar chemical structure, but does not significantly inhibit PPARα in the luciferase reporter assay (IC_{50} = 15 μmol/L). A recent study demonstrated that CLL cells show increased expression levels of PPARα relative to B cells from healthy donors (23). As a transcriptional regulator, PPARα controls the expression of a number of genes, including those involved in β-oxidation (27,28). Target engagement of NXT629 in CLL cells was determined by measuring inhibition of PPARα agonist–induced expression of the PPARα target gene pyruvate dehydrogenase kinase isoform 4 (*PDK4*) by real-time quantitative PCR (QPCR). The synthetic agonist GW590763 (29) caused a three-fold increase in *PDK4* mRNA expression, which was dose-dependently inhibited by NXT629 (Figure 1A), but not by the negative control compound NXT962 (Figure 1B). The natural agonist OEA caused a six-fold upregulation of PDK4 in CLL cells, which was almost completely inhibited by 3 μmol/L NXT629 (Figure 1C). NXT629 also inhibited mRNA upregulation of the target gene carnitine/acylcarnitine translocase (CACT/SLC25A20), a rate-limiting protein for β-oxidation (Figure 1D).

PPARα Antagonist Is Cytotoxic to CLL Cells Even in the Presence of a Protective Microenvironment

PPARα was previously shown to mediate protection of CLL cells from harsh microenvironmental conditions, including the presence of cytotoxic agents (23,30). This result suggests that PPARα plays a role in CLL cell viability. To test this, we examined to what extent PPARα inhibition, by using a selective small molecule antagonist, is cytotoxic to CLL cells *in vitro*. NXT629 dose-dependently induced apoptosis of resting CLL cells (Figure 2A). The concentration at which 50% of the cells underwent apoptosis (IC_{50}) is 6.4 μmol/L (n = 5 patient samples).

CLL cells in lymphoid organs are in contact with their microenvironment, and cells of the microenvironment can protect CLL cells from spontaneous and drug-induced apoptosis (31). It is therefore critical to evaluate new compounds in the context of the cellular microenvironment. Thus, we examined whether NXT629 could kill CLL cells when cocultured with macrophages, which can protect CLL cells from spontaneous apoptosis *in vitro* (32). As expected, macrophages protected CLL cells from spontaneous apoptosis *in vitro* (Figure 2B), and addition of NXT629 to these cocultures caused a significant reduction in CLL cell viability. After 6 d, the majority of CLL cells underwent apoptosis in the presence of 10 μmol/L NXT629 (Figure 2B). Because a frontline therapy for CLL is fludarabine (2), we compared NXT629 and fludarabine for their potential to induce apoptosis of CLL cells in the presence of accessory cells. Under the same coculture conditions, CLL cells were completely protected from fludarabine-induced apoptosis (Figure 2C), at doses that were cytotoxic to CLL cells cultured in the absence of macrophages (Figure 2D), whereas NXT629 induced apoptosis of CLL cells under both conditions (Figures 2A, B).

Preadipocytes (OP9 cells [26]) were evaluated for their ability to protect CLL cells from spontaneous apoptosis, since they play a supportive role in other cancers and could be a potential source of lipids *in vivo* (33,34). Preadipocytes (OP9 cells) also increased viability of CLL cells, and this effect was completely blocked by using 10 μmol/L NXT629 (Figure 2E).

PPARα Antagonist Inhibits CLL Proliferation

While the majority of CLL cells present in blood are in a resting/nondividing state, up to 1% of the leukemia cells are "born" each day (35) in lymphoid organs (36). Although killing the nondividing cells, which represent the bulk of the tumor is important, it is beneficial to have a therapy that will kill the dividing cells or at least stop proliferation in areas such as the lymph node and spleen. To mimic the proliferative compartment of CLL, we examined the activity of PPARα inhibition on CLL cells that were induced to proliferate *in vitro*. To induce CLL cell proliferation, the CLL cells were cocultured with allogeneic T cells that were previously activated with CD3/CD28 microbeads and subsequently treated with mitomycin C to stop their division (see Materials and Methods). To demonstrate CLL B-cell division, CLL cells were labeled with CFSE before coculture with T cells, and CLL proliferation was analyzed by flow cytometry. Dilution of the CFSE stain indicates cell division. Two different T:CLL ratios were evaluated. A ratio of 1:2 caused more proliferation and hence was used in all subsequent experiments. At d 5–7, robust CLL proliferation was observed with approximately three-fold expansion (Figure 3A). To allow for comparison between CLL samples, it is preferable to use T cells from the same donor. Thus, T cells were frozen after the mitomycin C treatment and compared with fresh T cells for their ability to induce CLL cell proliferation. Because frozen and fresh T cells induced comparable levels of CLL proliferation (data not shown), frozen T cells were used in this study. This assay was developed for a 96-well plate using frozen T cells and is therefore amenable to high-throughput screening of compounds. It also allows for a better comparison between compounds and between assay runs, since the same batch of frozen CLL and T cells are used. Furthermore, this allows for a comparison of different CLL samples using the same compounds and using the same T cells. Figure 3B depicts the absolute number of CLL cells demonstrating an approximately five-fold expansion of CLL cells in this culture system and a dose-dependent inhibition of CLL proliferation with NXT629 (Figure 3A). CLL blast formation was not affected by NXT629 (Supplementary Figure 2). The control compound NXT962 did not inhibit CLL proliferation (Figure 3B). When testing the effect of PPARα inhibition on dividing CLL cells, NXT629 reduced the number of leukemia cells undergoing cell division with an IC_{50} of 9.6 μmol/L (n = 10 different CLL samples) (Figure 3C). Cell cycle analysis revealed that NXT629 significantly decreases the fraction of cells in the G2/M phase, but does not alter the fraction of cells in the G0/1 phase (Figure 3D). This result suggests that cells as they are trying to enter division are not undergoing apoptosis, but instead

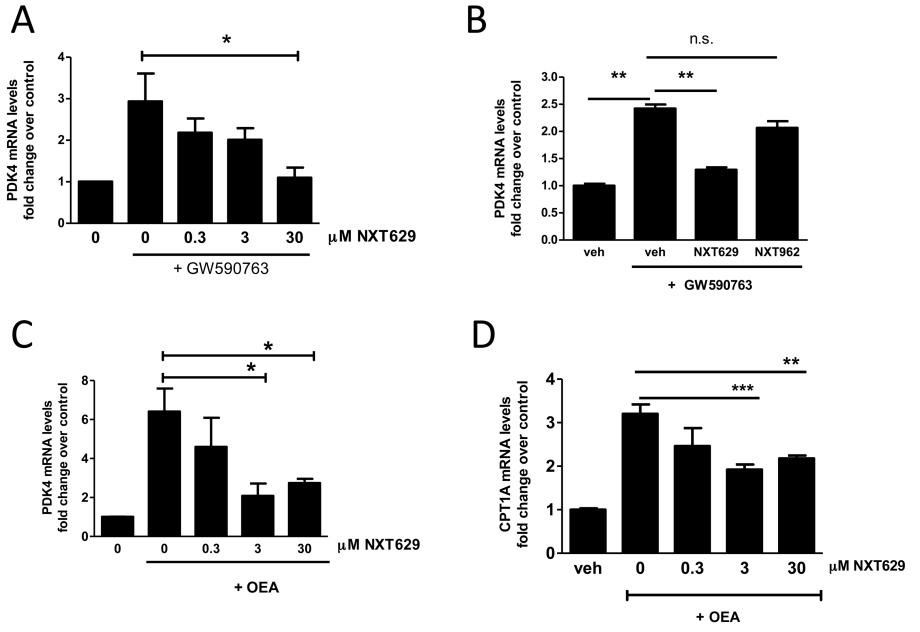


Figure 1. Target engagement in CLL cells. (A) Purified CLL cells were incubated with increasing doses of antagonist or vehicle control for 2 h. Subsequently, the synthetic PPARα agonist GW590735 (purchased from GlaxoSmithKline) was added at 1 μmol/L for 48 h. Cells were harvested for RNA isolation. PDK4 (a PPARα target gene) expression was measured by real-time PCR. Data are mean ± standard error of the mean (SEM) from six independent experiments using six different CLL donors. Significant difference: $*p < 0.05$, unpaired Student *t* test. (B) Purified CLL cells were preincubated with vehicle control (veh), NXT629 or the control compound NXT962, both at 30 μmol/L for 2 h. Subsequently, GW590735 was added at 1 μmol/L for 48 h. Cells were analyzed as above. One representative result of two independent experiments using two different CLL donors is shown. Significant difference: $*p < 0.05$, $**p < 0.005$, unpaired Student *t* test. n.s., Nonsignificant. (C, D) Purified CLL cells were incubated in serum- and glucose-free RPMI with NXT629 for 2 h. Subsequently, the natural agonist OEA was added at 10 μmol/L for an additional 4 h. Cells were analyzed for PDK4 (C) or CPT1A (D) as above. Data are the mean ± standard deviation (SD) from independent experiments using four different CLL donors. Significant difference: $*p < 0.05$, unpaired Student *t* test.

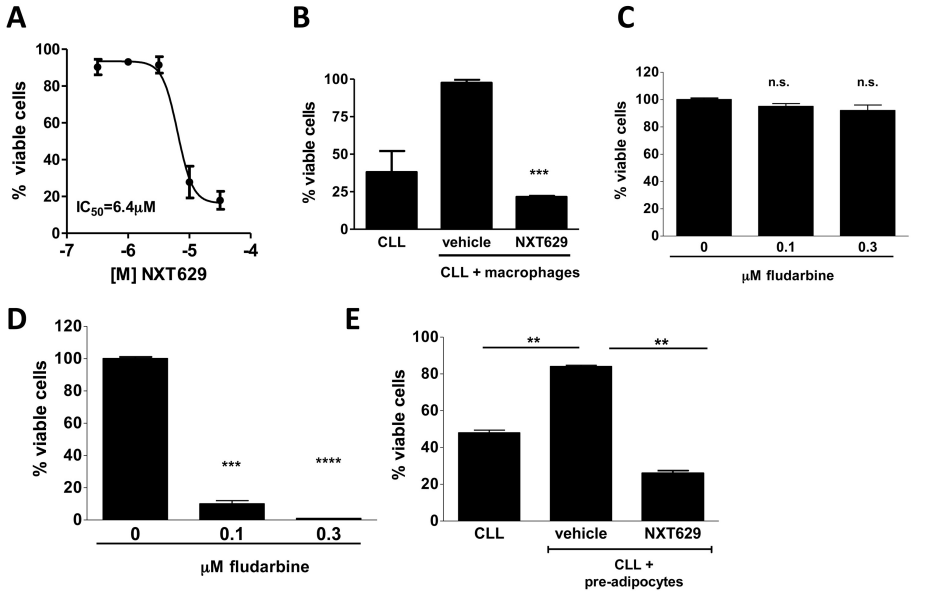


Figure 2. PPARα antagonist is cytotoxic to CLL cells, even in the presence of the microenvironment. (A) CLL cells were cultured in the presence of the PPARα antagonist NXT629 or DMSO control, added once at the beginning of the culture. CLL cells were harvested after 4 d and stained with $DiOC_6$/PI and analyzed by flow cytometry. The percentage of viable cells as determined by gating on $DiOC_6$ bright and PI-negative cells is shown. Data are mean ± SD for five different experiments with five different CLL donors. The IC_{50} was calculated using GraphPad Prism software. (B) CLL cells were cultured alone or in the presence of mitomycin C–treated J774 macrophages. NXT629 (10 μmol/L) or DMSO was added once at the beginning of the coculture. Cell viability was measured at d 6 as above. Data are mean ± SD from two independent experiments using two different CLL donors. Significant difference: ***$p < 0.0005$, unpaired Student *t* test. CLL cells were cultured in the presence (C) or absence (D) of mitomycin C–treated J774 macrophages. DMSO or fludarabine was added once at the beginning of the coculture. Cell viability was measured at d 6 as above. Data are mean ± SEM from two independent experiments using two different CLL donors. Significant difference: ***$p < 0.0005$, unpaired Student *t* test. n.s., Nonsignificant. (E) CLL cells were culture alone or in the presence of preadipocytes (OP9) cells. NXT629 (10 μmol/L) or DMSO was added once at the beginning of the coculture. Cell viability was assessed at d 6 as above. Data are mean ± SEM from two independent experiments using two different CLL donors. Significant difference: **$p < 0.005$, unpaired Student *t* test.

are stalled in G0/1 and prevented from undergoing cell division.

NXT629 Reduces CLL Tumor Burden in Two Adoptive Transfer Mouse Models

To model the CLL microenvironment *in vivo*, the effect of NXT629 on CLL cells in lymphoid tissue was evaluated using a xenograft model described by Herman *et al.* (37). CFSE-labeled CLL PBMCs were injected intravenously and busulfan was given intraperitoneally (IP) on d 1. Groups of mice received daily dosing of saline, NXT629 at 30 mg/kg IP, or fludarabine at 50 mg/kg IP. Studies in rat showed that the oral bioavailability of NXT629 is only 10.7%; therefore, intraperitoneal dosing was performed in our mouse studies. Prior experiments showed that administration of 10 mg/kg (IP) NXT629 (called compound 33 by Bravo *et al.* [24]) as a saline solution in mouse yielded good drug coverage across 24 h (that is, C_{trough} = 0.05 μmol/L) with a calculated plasma area under the curve of 2.4 μmol/L * h. This amount should be adequate for target engagement, since the IC_{50} value in the luciferase assay is 78 nmol/L. Furthermore, the maximum plasma concentration was 0.38 μmol/L at 1 h postdose (24). Mice were killed 4 wks after engraftment, and the splenocytes were stained with hCD19 and analyzed by flow cytometry. The engrafted population represented 3% of total splenocytes. The NXT629 treated mice showed a significant reduction in disease burden (Figure 4A). Mice were dosed for 1 month daily, during which time the mice were observed and body weights were recorded. The mice treated with NXT629 showed normal behavior and insignificant reduction in body weight (Figure 4A), indicating that there was no systemic toxicity. On the other hand, fludarabine-treated mice showed a dramatic reduction in body weight. In this mouse model, the CLL cells homed to the spleen, but did not divide, since we did not observe CFSE dilution. Thus, this model provided an in *in vivo* microenvironment for resting CLL cells.

Because a fraction of CLL cells proliferate *in vivo* (35), it is critical to evaluate new treatment modalities on the proliferative compartment. It has been shown that autologous T cells promote CLL cell survival and proliferation in NSG mice (38). To model the proliferative CLL compartment *in vivo*, a modified protocol of the xenograft model described by Bagnara *et al.* (38) was applied. In brief, first, *in vitro* activated CLL T cells were adoptively transferred into NSG mice. Subsequently, autologous CLL B cells were given to those mice in which T-cell engraftment was documented by the presence of $CD3^+$ cells in the blood. This approach is a model of CLL B-cell growth *in vivo*, since the transferred leukemic cells proliferate extensively, often exceeding six to seven divisions on the basis of CFSE dilution analyses during the course of the study. However, the approach is not a model of CLL disease, since the transferred clone survives for 4–12 wks; this time interval varies on the basis of the CLL sample analyzed. Nevertheless, during this window, the effects of various

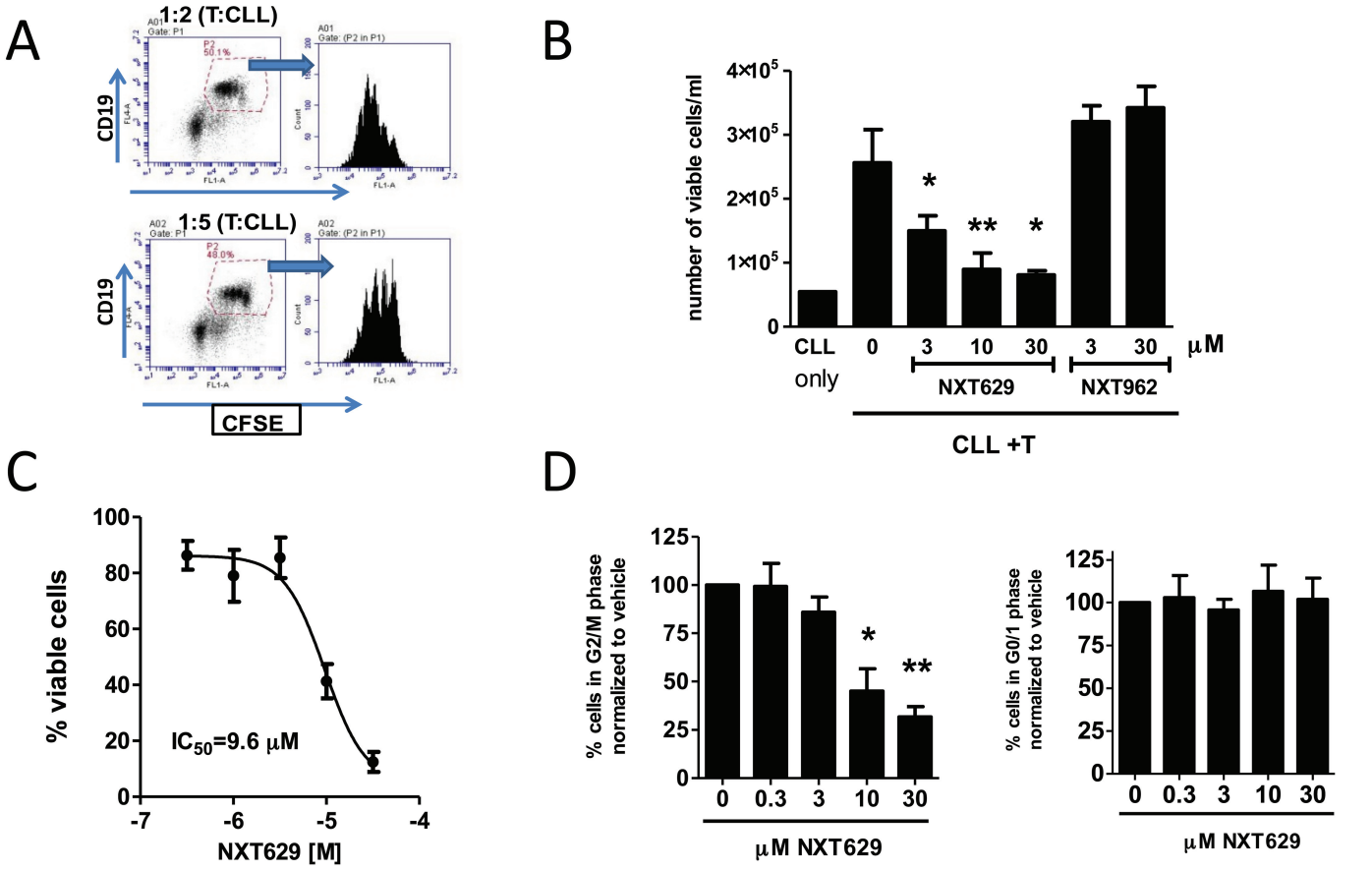


Figure 3. PPARα antagonist inhibits CLL proliferation. (A) To demonstrate CLL proliferation under the described culture conditions, CLL cells were CFSE-labeled and cocultured with activated allogeneic T cells as described in Materials and Methods. Five days after coculture, CLL cells were stained with CD19-APC and proliferation was assessed by flow cytometry. The histograms to the right show CFSE profile gated on $CD19^+$ cells. (B) CLL cells were incubated with either PPARα antagonist NXT629 or the negative control compound NXT962 for 2 h. Subsequently, T cells were added and CLL proliferation was assessed after 5 d. The number of viable cells determined by gating on $DiOC_6$ bright and PI negative cells with the use of Accuri software is depicted. Data are mean ± SD from three independent experiments using CLL cells from the same donor. Significant difference: $*p < 0.05$, $**p < 0.005$, unpaired Student *t* test. (C) CLL cells were incubated with either PPARα antagonist NXT629 or vehicle control for 2 h. Subsequently, T cells were added, and CLL proliferation was assessed after 8 d. The number of viable cells ($DiOC_6$ bright and PI negative) was quantified by flow cytometry and normalized to the vehicle control, set as 100%. Data are mean ± SEM from 10 different CLL patient samples. (D) CLL cells were incubated as in (C) and, after 8 d, cell cycle analysis was performed using PI staining as described in Materials and Methods. Percentage of cells in G2/M and G0/1 phase was normalized to vehicle control. Data are mean ± SEM from four different CLL donors. Significant difference: $*p < 0.05$, $**p < 0.005$, unpaired Student *t* test.

therapies on CLL growth can be effectively studied. Proliferating CLL B cells are found most often in the spleen and to a lesser extent in the bone marrow. Lymph node infiltration is rarely seen during the time frame of these studies.

Mice treated in this manner were dosed daily with 30 mg/kg NXT629 IP, and 2 wks after B-cell administration, recipients were killed and splenocytes were stained with mCD45/hCD45/hCD19/hCD5. As seen in the other CLL model, a marked reduction in both the percentage and the absolute number of $hCD19^+/hCD5^+$ CLL cells was observed in NXT629-treated animals (Figure 4B). These results suggest that NXT629 delays disease progression of CLL *in vivo*.

DISCUSSION

Despite major advances in the field, there is no curative therapy for CLL to date (2). All patients inevitably relapse and retreatment is often limited by resistance to chemotherapy. Thus, new therapies are needed. Current treatment approaches aim at achieving minimal residual disease, which is associated with superior long-term outcome (3). A major focus in the field has been on inhibition of kinases, and little attention has been given to metabolic pathways. Recent reports have highlighted the role of PPARα and FAO in cancer (14,19,39–41). PPARα KO mice completely suppress metastasis and growth

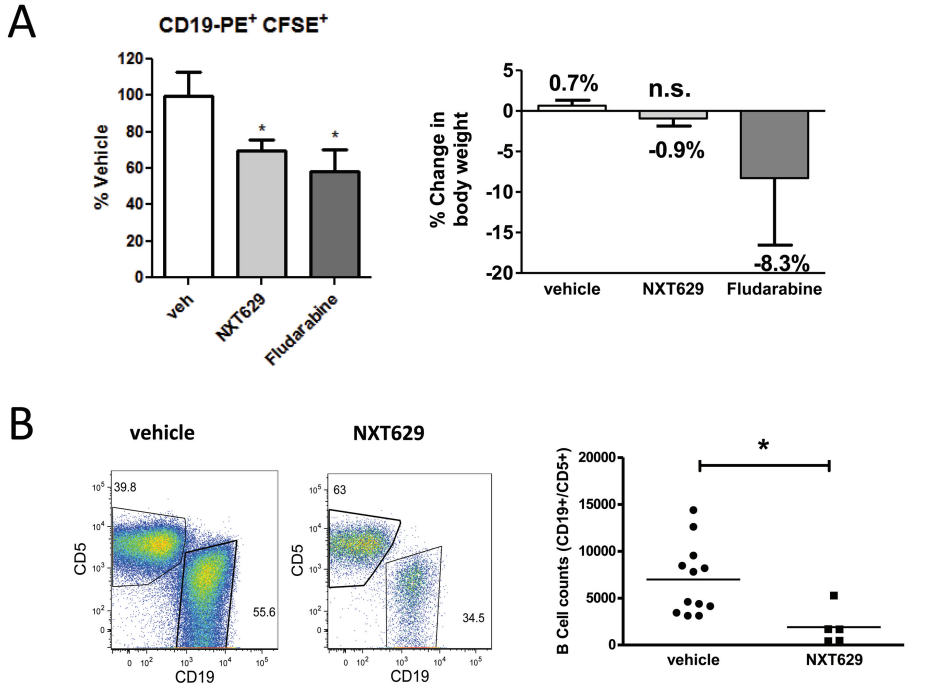


Figure 4. CLL mouse model. (A) CLL PBMCs from two patients were labeled with CFSE and randomized among the groups. The 10^8 CFSE-labeled cells were injected intravenously into NSG mice (lacking T, B and NK cells). Groups of five mice received daily dosing of saline: NXT629 at 30 mg/kg IP or fludarabine at 50 mg/kg IP. Mice were killed 4 wks after engraftments, and the splenocytes were stained with hCD19 and analyzed by flow cytometry. Data are $hCD19^+/CFSE^+$ cells normalized to vehicle control. (B) Proliferative model. Groups of mice received daily dosing of saline, NXT629 at 30 mg/kg IP. Mice were killed 2 wks after engraftments of CLL cells. CLL cells in spleen of NSG mice were quantified by gating on human $CD45^+$ cells, and a second gate was applied to determine $CD19^+/CD5^+$ CLL cells. Representative flow cytometry plots are depicted to the left. The absolute number of CLL cells is significantly decreased in NXT629-treated animals. Data are mean ± SEM. Significant difference: $^*p < 0.05$, unpaired Student *t* test. n.s., Nonsignificant.

of primary tumor in Lewis lung carcinoma and melanoma models >100 d after tumor implantation (42). PPARα is overexpressed in CLL cells and helps to protect them from harsh microenvironmental conditions. MK886, a small molecule that is reported to have PPARα antagonist properties, exhibits anti-CLL activity *in vitro* and *in vivo* (23,30). Taken together, these observations made us consider that PPARα may be an important therapeutic target for CLL and other cancers that use FAO as a metabolic strategy. We have also performed in-house experiments showing that, unlike NXT629, MK886 is not selective, since it inhibits the following nuclear hormone receptors: ERβ (7.5 μmol/L), TR (11 μmol/L) and GR (10 μmol/L) at IC_{50} levels that are lower than or similar to PPARδ (17 μmol/L), PPARγ (14 μmol/L) and PPARα (18 μmol/L). We evaluated MK886 in the luciferase reporter assay and found that its IC_{50} for inhibition of PPARα-driven luciferase expression overlapped with the IC_{50} for cellular cytotoxicity in the CHO cells. The strong overlap in toxicity prevents a clear interpretation regarding whether this molecule does indeed inhibit PPARα, leading to a decrease in luciferase signal or that the cytotoxic effect leads to a decrease in luciferase signal. NXT629 does not suffer from the same problem, since it is not cytotoxic in CHO cells and is highly selective for PPARα (100-fold or more selective) (Table 1). To date, there are no highly selective PPARα antagonists that are available for clinical studies. Taken together, these results strongly suggest that MK886 is not a good molecule to study PPARα inhibition, and therefore our study is the first one to show the effect of PPARα inhibition on CLL cells.

The small molecule PPARα antagonist NXT629 induced apoptosis in resting CLL cells and inhibited proliferation of CLL cells *in vitro* (Figures 2, 3), but was less cytotoxic to B cells isolated from healthy volunteers (Supplementary Figure S1). Because accessory cells can rescue CLL cells from spontaneous and drug-induced apoptosis (43–45), as well as protect CLL cells from fludarabine-induced apoptosis *in vitro* (31), it is essential to evaluate potential therapeutics in CLL accessory cell cocultures. Importantly, NXT629 induced apoptosis of CLL cells in the presence of the microenvironment, whereas fludarabine was completely inactive (Figure 2C).

The doses needed to see an effect in CLL experiments are significantly higher than the IC_{50} value in the luciferase reporter assay. We can only speculate as to why this is the case. The luciferase reporter assay also requires less agonist to induce gene expression and generally appears more sensitive to both PPARα induction and inhibition. This method is an artificial system with CHO cells overexpressing a reporter construct. It is not known which and how many molecules of corepressor and coactivator are present in CHO cells in comparison to CLL cells. All of this could play are role. However, the dose that is needed to engage the target in CLL cells *in vitro* is also the dose that leads to cell death, indicating that the observed effect on CLL cells in on target.

Furthermore, NXT629 was also found to lower CLL tumor burden in two different CLL mouse models *in vivo* (Figure 4). One CLL mouse model was adopted

from Herman *et al.* (37). In contrasts to Herman *et al.*, who showed proliferation of CLL cells 3–4 wks postxenograft in the spleen, we did not observe proliferation of CLL cells in the spleen. These differences could relate to technical differences or differences in the patient samples used in our studies. Therefore, our *in vivo* experiment tested the effect of PPARα inhibition on resting CLL cells, and the decrease in tumor burden is most likely due to cytotoxic effects on the tumor, which is in concordance with the *in vitro* results. The second tumor model (38), also an adoptive transfer model of CLL, was initially described by Bagnara *et al.* In this model, CLL cells proliferate because of the presence of activated autologous T cells. In concordance with the *in vitro* proliferating CLL cultures, NXT629 lowered tumor burden of proliferating CLL cells *in vivo* (Figure 4B). In this mouse model, the majority of CLL cells reside in the spleen; therefore, the splenocytes were analyzed, and both the percentage as well as the absolute number of CLL B cells were reduced in NXT629-treated animals. In addition to the spleen, bone marrow was also analyzed. Although significant engraftment of CLL cells in bone marrow only occurred in two to three mice per group, the same trend was observed as in the spleen. NXT629-treated mice contained a lower number of $CD19^+/CD5^+$ human B cells in the bone marrow (data not shown). Interestingly, trough levels as low as 50 nmol/L NXT629 measured in plasma were sufficient to cause a significant reduction in tumor burden, whereas *in vitro,* micromolar levels are needed. One explanation for the discrepancy between the active dose *in vitro* and *in vivo* could be that CLL cells in their *in vivo* microenvironment are more dependent on β-oxidation compared with *in vitro* culture conditions and thus are more sensitive to its inhibition. On the other hand, cell culture media contain high levels of glucose, which could provide one possible alternative energy source to fatty acids *in vitro* and therefore lower the dependency on FAO and reduce the sensitivity to PPARα inhibition. However, removal of glucose *in vitro* did not increase sensitivity of CLL cells to PPARα inhibition (data not shown), ruling out the possibility of a metabolic switch to glucose *in vitro*. Another alternative energy source could be glutamine, whose role remains to be investigated. A further consideration was that CLL cells might encounter hypoxic conditions *in vivo* and thus be more sensitive to inhibition of PPARα. However, culture of CLL cells *in vitro* at low oxygen failed to increase sensitivity to PPARα inhibition (data not shown).

Alternatively, NXT629 could affect certain cells of the microenvironment *in vivo*, which after exposure to NXT629 could withdraw support factors. As a consequence of this, CLL cells could undergo apoptosis. Indeed, it is likely a combination of factors where *in vivo* the antagonist acts on both the CLL cells and the microenvironment. All these questions remain to be investigated.

CONCLUSION

Overall, our preclinical data demonstrate sensitivity of CLL cells to PPARα inhibition and show PPARα antagonist–mediated cytotoxicity *in vitro* and *in vivo*, suggesting that antagonism of PPARα might be a potent and safe new therapeutic target for CLL. Spaner *et al.* (23) showed that PPARα expression was highly associated with advanced-stage disease. Thus, PPAR expression could be used to select the patient population that would benefit from treatment. Blocking a fundamental fuel source might not allow for escape mutants as, for example, inhibition of kinases does. However, combination therapies will most likely be more efficacious.

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DISCLOSURE

All authors who are Inception employees have a share in the company.

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