Methods

Cells. Primary CLL cells and peripheral blood mononuclear cells (PBMC) from healthy donors were obtained from the Pasquarello Tissue Bank at the Dana-Farber Cancer Institute (DFCI) under an Institutional Review Board–approved protocol.

Gene expression analysis. Total RNA was harvested using the RNeasy Plus Mini Kit (Qiagen), reverse transcribed using random hexamers, and assayed by quantitative reverse transcription polymerase chain reaction using the indicated primers, as previously described (19). Gene expression was analyzed in triplicate and normalized by 18S ribosomal RNA. The STAT3 gene expression signature was derived from an arithmetic mean of the five genes.

Viable cell number and apoptosis quantitation. Viable cell number was measured by ATP-dependent bioluminescence using the Cell Titer Glo assay (Promega). Apoptosis was measured using flow cytometric staining for annexin V and propidium iodide (PI) using Apoptosis Detection Kit I (BD Biosciences).

Clinical trial design. In order to assess the effects of pyrimethamine in this novel patient population, we designed a typical 3+3 dose escalation study, beginning at 12.5 mg daily and escalating to 50 mg daily. Eligibility criteria included relapsed CLL with measurable disease after at least one prior regimen and currently meeting IWCLL criteria for therapy; no threshold values of absolute neutrophil count (ANC), hemoglobin or platelets were required. This study was completed prior to the widespread availability of targeted kinase inhibitors for CLL, with enrollment between May 2010 and April 2012.

The study was approved by the Dana-Farber Harvard Cancer Center Institutional Review Board, and all patients signed informed consent prior to initiation of therapy. This study is registered at ClinicalTrials.gov as NCT01066663.

Clinical Trial Treatment Plan. Patients received continuous oral daily dosing, and samples were drawn for pharmacokinetic and pharmacodynamic analyses weekly during cycle 1 and every other week in cycle 2. Patients were prescreened for folate deficiency and repleted if necessary prior to initiating drug. Growth factor support was permitted as was infectious prophylaxis, although trimethoprim-sulfamethoxazole was discouraged due to possible synergistic toxicity. Dose limiting toxicity (DLT) was evaluated in the first cycle of treatment only, and was defined as any non-hematologic toxicity of grade 3 or greater severity (excluding asymptomatic grade 3 laboratory abnormalities that are not life-threatening and respond to treatment; grade 3 fatigue; grade 3 nausea, vomiting or diarrhea occurring without optimal prophylaxis). Infectious toxicities were only considered dose-limiting if grade 4 or greater. Any grade 4 non-hematological toxicity, as well as any irreversible grade 2 cardiac, renal or neurologic toxicities, was considered dose-limiting. Grading of non-hematologic toxicities was according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4. Hematologic toxicity was graded according to the 2008 IWCLL criteria. Patients who were removed from study for reasons other than DLT prior to completion of one month of dosing were replaced.

Study Evaluations. CT scans for disease evaluation were performed at baseline and at 1, 3, and 6 months on therapy. A bone marrow evaluation was performed at baseline and thereafter only to confirm complete response (CR).

Statistical Considerations. The primary endpoint of the clinical trial was to determine the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D) of pyrimethamine in relapsed CLL/SLL. Once the recommended phase 2 dose was established, the original plan was to move to phase 2 with a primary endpoint of ORR in a two stage design, requiring 2 responses

in the first 10 patients, and considering the drug promising if at least 5 responses were seen in 26 patients, for a target response rate of 35%. This plan was modified as accrual dropped significantly with the availability of the targeted signaling inhibitors in CLL. Secondary objectives included to describe the toxicity in CLL/SLL patients, to determine the overall response rate (ORR) and progression-free survival (PFS) and whether any disease prognostic factors predicted them, to determine pyrimethamine levels *in vivo* in the patients, and to determine whether pyrimethamine inhibits STAT3 *in vivo* by assessing downregulation of STAT3-dependent gene expression in CLL cells and/or peripheral blood mononuclear cells.

Drug Level Monitoring. Blood samples were collected in 8 mL BD Vacutainer CPT cell preparation tubes with sodium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ) during the screening visit and shortly before the first dose of pyrimethamine was taken, before dosing on any one day from days 2 through 5 and days 8, 15, and 22 of cycle 1, days 1 and 15 of cycle 2, and on any single day of each subsequent cycle of therapy. Patients were instructed not to take the daily dose of the drug before they arrived at the outpatient clinic on the scheduled sample collection days. They were also asked to maintain a diary to record the date and time that each daily dose was taken. The CPT tubes were processed according to the manufacturer's recommended instructions. Briefly, the plasma was removed from the tube after the initial centrifugation (1,700 g, 25°C, 20 min) and transferred into a cryovial for storage at -80°C. The buffy coat residing above the gel barrier of the CPT tube was removed and washed by gently mixing with 10 mL of cold phosphate buffered saline. The cell pellet afforded by centrifuging (300 g, 4°C, 15 min) and aspirating the supernatant was resuspended in 1.0 mL of cold phosphate buffered saline, from which a 10 µL aliquot was removed to determine the total cell

count. After centrifuging the cell suspension once again, as much of the supernatant as possible was removed without disturbing the cell pellet, which was stored at -80°C.

The concentration of pyrimethamine in plasma and PBMC lysates was measured by high performance liquid chromatography with mass spectrometric detection. The sample preparation procedure and chromatographic conditions were adapted from previously reported bioanalytical methods for pyrimethamine (20, 21). PBMC lysates were prepared by resuspending the thawed cell pellet in 500 µL of water and homogenizing the mixture for 4-min using an Ultra-Turrax T8 disperser with an S8N-5G dispersing element (IKA Works, Inc., Wilmington, NC). Subsequently, the homogenate was sonicated for 5-min and subjected to three freeze-thaw cycles to insure complete lysis of the cells. The resulting PBMC lysate was assayed in the same manner as plasma samples. The analytical method was validated and applied to the analysis of study samples in accordance with recommendations that were applicable at the time (22). Pyrimethamine was determined at concentrations ranging from 1.0 to 100.0 ng/mL with an inter-day accuracy of -0.6 to 9.3% and precision ranging from 2.2 to 10.1%. Inter-day accuracy and precision at the 0.50 ng/mL lower limit of quantitation were -15.2% and 11.2%, respectively.

Previous clinical investigations with pyrimethamine have demonstrated that the onset of steady state pharmacokinetics is achieved within 2-3 weeks for a repetitive dosing regimen because the apparent biological half-life of the drug is approximately 100 h (17). In addition, steady state plasma levels of pyrimethamine at any time between successive doses are essentially constant when given once every day as peak concentrations are only 15% greater than trough concentrations on average (17), which is typically not considered to be a clinically significant difference. Consequently, the steady state drug concentration in each patient was calculated as the geometric mean of all samples obtained on and after day 15 of cycle 1 that met the following

criteria: (1) the date and time for both drawing the blood sample and taking the preceding dose were documented; (2) the sample was collected within 32 h of the prior dose; (3) there were no missed doses or interruptions in dosing during the prior two weeks. Pyrimethamine steady state concentrations in plasma and PBMCs are reported as the geometric mean (geometric %CV) of the values for individual patients at each dose level (23). GraphPad Prism for Windows, version 8.3.0 (GraphPad Software, La Jolla, CA) was used for the statistical analysis and graphical presentation of the pharmacokinetic data.

Pharmacodynamic analyses. Blood was collected in heparin-coated tubes for pharmacodynamics analyses, and mononuclear cells (principally CLL cells) were isolated using Ficoll separation. Cells were viably frozen until each patient was removed from study. The cells were then thawed and counted, and mRNA was harvested and cDNA was generated. STAT3 target gene expression was measured by RT-PCR, as described above. Pre-treatment cells were also used for *ex vivo* analyses of gene expression and viability in response to pyrimethamine, using the above-described techniques.