Supplementary Materials

Supplementary Methods

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Supplementary Methods

Study Evaluations

FISH for the common CLL abnormalities, namely deletions 13q, 11q or 17p, trisomy 12, or rearrangements of chromosome 14, was performed according to standard techniques by the Brigham and Women's Hospital Clinical Cytogenetics Laboratory (1). *IGHV* mutational status and ZAP-70 were performed by the CLL Research Consortium Tissue Core according to previously described methods (2). Unmutated *IGHV* was considered to be greater than or equal to 98% homologous to the closest germline match, and positivity for ZAP-70 was considered to be >20% (2). Bone marrow minimal residual disease was assessed by four color flow cytometry at Genzyme Genetics, Westborough, MA.

Peripheral blood B, T and NK cell subsets were followed serially on therapy and for up to one year following, using multicolor flow cytometry. Serum samples for determining trough concentrations of rituximab and alemtuzumab were collected before initiating treatment and every 2 weeks during therapy immediately before dosing. Blood was allowed to clot for at least 60 minutes and then centrifuged at 1800RPM, 10min, 20ºC to collect serum, which was stored at -80°C until assayed.

Pharmacokinetic Assays for Alemtuzumab and Rituximab

A standard curve was made using the pretreatment serum obtained from each patient and adding alemtuzumab at concentrations of 0.25 to 16 µg/mL in two-fold increments. Biotinylated alemtuzumab binding peptide Cp-1 (3), sequence ACGSLSPSSCGGL-Biotin, in phosphate buffered saline (50 µg/mL,100 µl) was added to neutravidin coated ELISA strip wells and incubated with shaking for one hour at room temperature. The wells were washed with Tris-buffered saline

with Tween 20, blocked with 5% bovine serum albumin, and washed. Study samples diluted 1:500 in TBS containing 2.5% BSA (100 µl) and calibration standards were added to triplicate wells and the plate was incubated for 1 hr. The wells were washed, goat anti-human IgG peroxidase was added, washed, and 3,3′,5,5′-Tetramethylbenzidine substrate was added. The reaction was stopped after 15 min and UV absorbance at 450 nM was measured. The mean absorbance of the triplicate determinations for each sample was calculated. The best-fit equation determined by linear regression of the mean absorbance and known concentration of the calibration standards was used to calculate the drug concentration in study samples. The lower limit for the blank was 0.26 µg/mL, the limit of detection 1 µg/mL and the limit of quantitation (defined as a coefficient of variation <20%) 2 µg/mL.

A similar assay was used to measure rituximab levels (3). The rituximab binding peptide Rtx-10 was used (ACPYSNPSLCGGL-Biotin) to coat the plates and the samples were diluted 1:50,000 due to the higher levels of rituximab that were assayed.

Statistical Methods

All eligible enrolled patients who received a single dose of therapy were evaluable for toxicity. Those patients who completed at least two weeks of study therapy were evaluable for efficacy. Overall survival (OS) was defined as the time from initiation of study therapy to death from any cause, or censoring on the date last known alive. Progression free survival (PFS) was defined as the time from initiation of study therapy to disease relapse, progression or death in remission, whichever occurs first, or censoring on the date last known alive without progression. Time to treatment failure (TTF) was defined as the time from initiation of study therapy to time of disease progression, to initiation of new therapy in the absence of disease progression, or to death

without progression, or to censoring on the date last known alive without one of the above events. TTF, PFS and OS curves were obtained using the Kaplan-Meier method, with 95% confidence intervals calculated using Greenwood's formula (4, 5). Exact 90% confidence intervals were calculated for response rates.

The maximum trough concentrations of alemtuzumab and rituximab in serum were determined using data from all samples obtained from each patient one week after the prior dose was given. The two-tailed t-test was used to compare trough concentrations of the two drugs between patients grouped according to response after logarithmic transformation of the pharmacokinetic data. A p-value <0.05 was considered significant. In addition, Wilcoxon's rank sum test was used to assess the relationship between maximum trough serum concentrations of the two drugs and bone marrow clearance. A logistic regression model was also constructed to control for dosing cohort and similar results were obtained.

References

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Supplementary Table 1: Dosing Cohorts and Results

Dose Level	Rituximab Weekly	Alemtuzumab Wks 1-2	Alemtuzumab Wks 3-4	Alemtuzumab Wks 5-8/16	N	DLTs?
1	375 mg/m^2	30 mg d1,3,5	45 mg d1	45 mg d1	3	
2	375 mg/m^2	30 mg d1,3,5	45 mg d1,4	45 mg d1	8	Gr 3 Rituximab Rxn
3	375 mg/m^2	30 mg d1,3,5	45 mg d1,4	60 mg d1	3	
4	375 mg/m^2	30 mg d1,3,5	45 mg d1,4	90 mg d1	14	

Supplementary Table 2

Median Cell Counts During and After Therapy with Alemtuzumab and Rituximab

Weeks on Study	N	CD5+ CD19+	CD20+	CD3+	CD4+	CD4+ CD45RO+	CD4+ CD45RO-	CD8+	CD8+ CD45RO+	CD8+ CD45RO-	CD56+ CD3-
0	17	9481	7699	394	238	228	39	202	95	86	161
3	23	9	0	4	3	2	0	2	2	1	10
5	23	2	0	5	3	3	0	1	0	0	9
7	20	0	0	15	14	13	0	1	0	0	5
8	23	1	0	13	14	10	0	1	1	0	4
14	17	1	1	26	23	21	0	3	2	0	3
28	14	2	1	48	39	40	0	7	4	4	25
40	8	76	5	175	149	125	33	24	15	12	93
52	4	2630	500	2005	785	638	147	767	254	446	189

Supplementary Table 3

Drug Levels Decreased in Setting of Packed Bone Marrow

	N	Median Highest Trough R Level	N	Median Highest Trough A Level
CLL <80% IT space	17	282 (115, 434)	18	4.5 (1.1, 14.1)
CLL >80% IT space	6	134 (2.5, 417)	6	1.8 (0.1, 6.2)
P Values		0.06		0.05

N, number of patients; R, rituximab; A, alemtuzumab.