Abstract no. 47

Cladribine Decreases CD95 Expressing CD4$^+$ and CD8$^+$ Cells in Lymphoid Organs in Naïve Marmosets *(Callithrix jacchus)*

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INTRODUCTION

- Cladribine reduces circulating T- and B-lymphocytes in the treatment of multiple sclerosis (MS) due to the selective activation in cells with a preferential ratio of deoxycytidine kinase (DCK) and 5-nucleotidase (5 NT).[1]
- Previously, cladribine has been analysed in the blood of MS patients only, but not of healthy volunteers. Thus, the level of immune cell subtype reduction in blood is unknown, as is the effect of cladribine on lymphoid organs.
- Marmosets have been used for pharmacodynamic studies in naïve or experimental autoimmune encephalomyelitis (EAE) models to test peripheral lymphocyte-targeting MS drugs.[2]
- The impaired apoptotic signalling pathways in active lymphocytes of MS patients are regulated by CD95/FAS (first apoptosis signal receptor) cell surface receptors.[3] Cladribine phosphates can induce apoptosis *in vitro* via the FAS pathway.[4]

OBJECTIVE

- This pharmacodynamic analysis aims to assess lymphocyte subtype reduction in blood and lymphoid organs of naïve marmosets after cladribine treatment.

METHODS

- Four sets of marmoset twins (n = 8) were randomised to four groups comprising different cladribine concentrations (n = 2 each).
- Three doses of subcutaneous cladribine (0.2, 0.4 and 0.8 mg/kg/day for 5 consecutive days in 2 weekly periods) were assessed.
  - The control group received 0.9% NaCl only.
- Blood samples were taken weekly starting 2 weeks before, and up to 12 weeks after the first dose of cladribine for analysis of serum chemistry and immune monitoring (**Table 1**).

Table 1. Treatment Schedule

	Week														
	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12
Administration of cladribine (s.c)			5x *				5x *								
Venous blood (100 µl)			3x †				3x †								
Venous blood (0.5 ml)	X ‡	X ‡		X	X	X		X	X	X	X	X	X	X	X
Weighing	Twice a week														
Necropsy															X

*Five consecutive days. †15 min, 1h and 4h after s.c. dosing, the build-up of cladribine in blood was assessed only on day 1 of each week and on day 5 to determine dose accumulation. ‡Two baseline time points, T-cells, B-cells and subtypes will be averaged and used as baseline.
s.c., subcutaneous.

- For analysis, the maximum amount of blood was collected, followed by euthanasia and necropsy at week 12.
- Blood and lymphoid organs were sampled and immunophenotyped using flow cytometry and immunohistochemistry (**Table 2**).
- Statistical analysis was performed using an unpaired parametric t-test when all treated animals were pooled ($P < 0.05$).

Table 2. Organ Collection at Necropsy

	Organ				
Technique	Blood	Spleen and Lymph nodes	Thymus	Bone marrow	Heart, lung, gut, intestines, kidney, liver
Flow cytometry with MNC	X	X	X	X	
Freeze cell suspension†	X	X	X	X	
Formalin		X			X

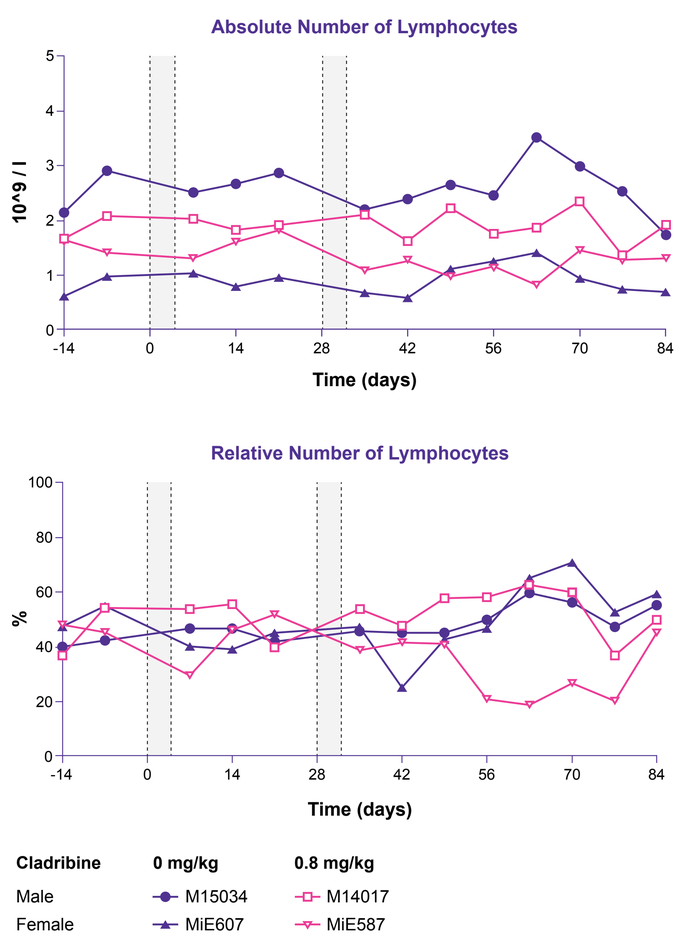
*Axillary, inguinal and mesenteric lymph nodes were harvested.
†Remaining cells that are not needed for the assays will be frozen.

RESULTS

Haematology and Serum Chemistry

- There was no change in blood lymphocyte count (**Figure 1**).
  - The number of cells remained stable over time.
  - At necropsy, no differences in serum chemistry were observed between animals at different cladribine doses (data not shown).

Figure 1. Absolute and Relative Number of Lymphocytes



Day zero = first injection of cladribine. Vertical grey areas indicate treatment periods. Twins are indicated by symbol (filled and hollow), cladribine treatment by colour.

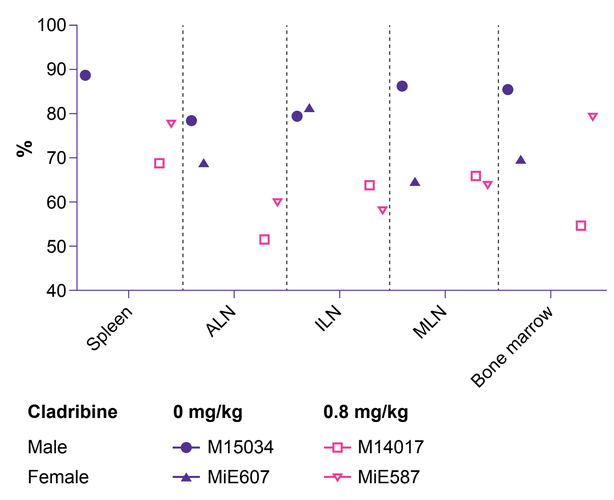
Supplementary Appendix 1: Absolute and Relative Number of Lymphocytes – All Doses

B- and T-Cell Subsets

Supplemental Appendix 2: B- and T-cell Subsets

- Blood samples showed no profound reduction of absolute B- or T-lymphocytes or their subsets over time following cladribine treatment.
  - There were fluctuations in B-cell subsets over time, but for most time points the cladribine treated animals were comparable to the control animals.
  - Cladribine induced a reduction of CD8$^+$ T-cells in blood analysed at necropsy, 52 days after the last injection of cladribine.
- In lymphoid organs the percentage of CD95-expressing CD4$^+$ (**Figure 2A**) or CD8$^+$ T-cells (**Figure 2B**) in the CD3$^+$ population were lower in cladribine-treated animals than in controls.
  - These findings are in line with the observations that in naïve lymphoid organs, the CD95/CD95L pathway is expressed only in scattered lymphocytes and that cladribine induced apoptosis may be triggered via the FAS/FASL pathway *in vitro*.[3,4]
  - This difference was statistically significant when cladribine-treated animals were pooled (**Figure 3**).

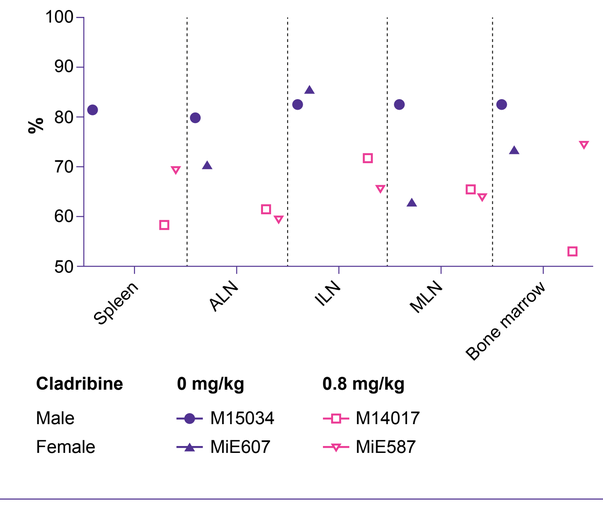
Figure 2A. Percentage of CD4$^+$ T-cells in Lymphoid Organs



Twins are indicated by symbol (filled and hollow), cladribine treatment by colour. **ALN**, axillary lymph nodes; **ILN**, inguinal; **MLN**, mesenteric; **PBMC**, peripheral blood mononuclear cells.

Supplementary Appendix 3: Percentage of CD4$^+$ T-cells in Lymphoid Organs – All Doses

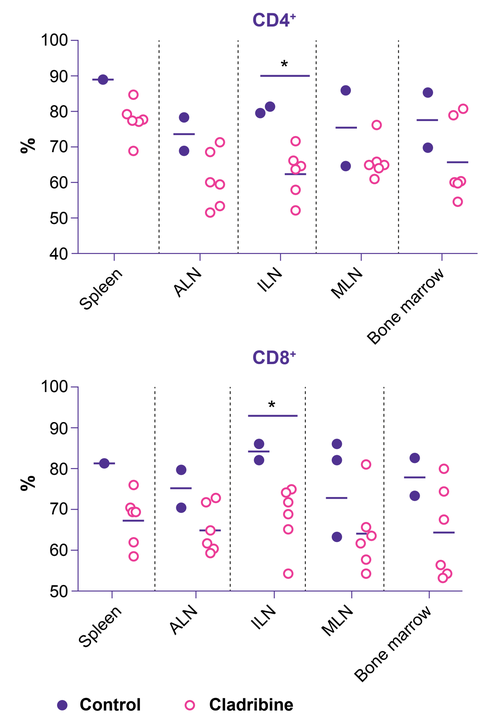
Figure 2B. Percentage of CD8$^+$ T-cells in Lymphoid Organs



Twins are indicated by symbol (filled and hollow), cladribine treatment by colour. **ALN**, axillary lymph nodes; **ILN**, inguinal; **MLN**, mesenteric; **PBMC**, peripheral blood mononuclear cells.

Supplementary Appendix 4: Percentage of CD8$^+$ T-cells in Lymphoid Organs – All Doses

Figure 3. Pooled Cladribine Samples Versus Control T-cell Samples in Lymphoid Organs



*$P < 0.05$. **ALN**, axillary lymph nodes; **ILN**, inguinal; **MLN**, mesenteric; **PBMC**, peripheral blood mononuclear cells.

CONCLUSIONS

- **No profound reduction of total lymphocytes, T- or B-cells was observed following exposure to cladribine in a setting where the immune system is not activated.**
- **At necropsy, CD95-expressing CD4$^+$ or CD8$^+$ cells were reduced in lymphoid organs in animals receiving cladribine, potentially identifying a novel *in vivo* mechanism of action of cladribine involving the CD95/CD95L pathway.**
- **The limited effect on other immune cells is in line with observations in normal lymphoid organs that the CD95/CD95L pathway is only expressed in scattered lymphocytes.[2,3]**
- **This pathway is dysregulated in MS patients and future studies will investigate cladribine's effect in an EAE and MS disease setting.**

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DISCLOSURES

YK does not declare any conflicts of interest. **UB** is an employee of Merck Serono SA, Eysins, Switzerland, a business of Merck KGaA, Darmstadt, Germany. **BtH** does not declare any conflicts of interest.

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