Cladribine inhibits cytokine secretion by T cells independently of deoxycytidine kinase activity

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ABSTRACT

Cladribine (2-chloro-2′-deoxyadenosine) is a purine nucleoside analogue (PNA) which causes targeted and sustained reduction of peripheral lymphocyte counts. Cladribine tablets produced significant treatment benefit for patients with relapsing–remitting multiple sclerosis in the phase 3 CLARITY study. In addition to the well-characterised cell-specific phosphorylation of PNAs responsible for lymphocyte reduction, the mode of action of cladribine may encompass distinct activities contributing to its overall effects on the immune system. Here we demonstrate that clinically relevant concentrations of cladribine also inhibit cytokine secretion by human peripheral blood T cells *in vitro* through mechanisms independent of the induction of lymphocyte death.

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1. Introduction

Cladribine (2-chloro-2′-deoxyadenosine) is an adenosine deaminase (ADA)-resistant purine deoxynucleoside analogue (PNA) developed to treat certain lymphoid malignancies (Beutler, 1992). ADA-resistant deoxynucleoside analogues were developed to target lymphoid cells based on the knowledge that congenital ADA-deficiency is responsible for the toxic accumulation of triphosphorylated deoxyadenosine in lymphocytes (Buckley et al., 1997) resulting in a form of severe combined immunodeficiency (ADA-SCID) characterised by the near absence of these cells in the periphery.

Cladribine administration induces cell death in susceptible lymphoid neoplastic cells (Genini et al., 2000; Marzo et al., 2001) and also results in a targeted and sustained reduction in peripheral lymphocyte counts (Carson et al., 1983). Accumulation of the triphosphorylated cladribine nucleotide has been shown to induce the formation of DNA strand breaks (Barbieri et al., 1998; Seto et al., 1985) through the inhibition of the enzymes DNA polymerase II and ribonucleotide reductase (van den Neste et al., 2005). DNA strand breaks activate the enzyme poly-ADP ribose polymerase 1 (PARP-1), leading to the intracellular

depletion of NADH and NADPH. Depletion of these two redox agents, results in intracellular oxidative damage culminating in apoptotic cell death (Barbieri et al., 1998; Carson & Leoni, 2003). However, it should be noted that these *in vitro* experiments used concentrations of cladribine far exceeding those achieved *in vivo*, and that at clinically relevant concentrations autophagic pathways may also contribute to cell death (Hentosh & Peffley, 2010).

It has been clearly established that the induction of cell death by cladribine requires its intracellular phosphorylation (Carson et al., 1980; Seto et al., 1985). The initial phosphorylation of cladribine by the enzyme deoxycytidine kinase (DCK) is the rate-limiting step towards the formation of the active phosphorylated derivatives. Conversely, cladribine mono-phosphate is dephosphorylated by 5′-nucleotidase enzymes. The sensitivity of lymphocytes to cladribine is explained by a high ratio of DCK to 5′-nucleotidase activity, compared with other cell types, leading to the accumulation of cladribine nucleotides in these cells. Although intracellular deoxyadenosine concentrations are regulated by ADA, which deaminates deoxyadenosine to deoxyinosine, cladribine is resistant to the action of ADA (Beutler, 1992).

Gorski et al. demonstrated that cladribine inhibits T and B cell proliferation *in vitro* and antibody production both *in vitro* and *in vivo* (Gorski et al., 1993b). Their initial observations were probably an indirect result of the reduction of T and B cells associated with cladribine administration. In a subsequent report, however, these authors suggested that cladribine also has a direct inhibitory effect on T and B cell activation based on the decreased ability of these cells to express activation markers in the presence of cladribine (Gorski

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et al., 1993a). Neither study made a clear distinction between the respective contributions of apoptosis induction by cladribine and of a putative mechanism involving the inhibition of lymphocyte activation. Therefore these data did not exclude the possibility that the molecular mechanisms involved in cladribine-induced cell death were also responsible for reducing the lymphoproliferative response.

To shed some light on these issues, we investigated the effects of cladribine on T cell activation. Because the induction of apoptosis in lymphocytes by cladribine is a confounding factor in such experiments, we used a biochemical approach designed to inhibit phosphorylation of cladribine by DCK and obviate subsequent cell death induction. We show that under these conditions cladribine inhibits cytokine secretion by human T cells *in vitro* via mechanisms that are independent of the phosphorylation of cladribine and are not associated with the induction of cell death.

2. Materials and methods

2.1. Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from healthy donors by gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Human total or CD4+ memory T cells were purified by negative magnetic selection with the pan T cell isolation kit II or the human CD4+ memory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), respectively. Cells were cultured in RPMI-1640 growth medium (Gibco, Paisley, UK) supplemented with 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco).

2.2. Compounds

Cladribine and deoxycytidine were purchased as powder from Sigma-Aldrich (St Louis, MO, USA) and were dissolved in dimethylsulphoxide (DMSO) to 0.1 M. Cladribine monophosphate was purchased from Chemie Brunschwig AG (Basel, Switzerland) as an aqueous solution.

2.3. T cell stimulation and functional analysis

T cells were activated with 10 μg/ml plate-bound anti-CD3 (UCHT1) and 1 μg/ml soluble anti-CD28 (CD28.2) antibodies (BD Biosciences, San Diego, CA USA) or with 10^{-6} M phorbol myristate (PMA) and 10^{-6} M ionomycin (Sigma-Aldrich). Cladribine was added to the culture medium 15–20 min before cell stimulation. When used in combination with cladribine, deoxycytidine was diluted first in culture medium in which cells were pre-incubated for 15–20 min prior to the addition of cladribine. After the pre-incubation period, cells were transferred to flat-bottom 96-well plates pre-coated with anti-CD3 antibody in presence of soluble anti-CD28 antibody. Cytokine concentrations in supernatants were determined by flow cytometry with a FACScalibur™ (BD Biosciences) using the Human Th1/Th2 Cytometric Bead Array Kit 2 (BD Biosciences). Cellular proliferation was assessed by monitoring bromodeoxyuridine (BrdU) incorporation using the Click-iT™ EdU Flow Cytometry Assay Kit (Molecular Probes/Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions, in parallel with CD25 up-regulation and viability. Anti-CD25 and anti-CD69 antibodies were purchased from BD Biosciences. Cellular viability was assessed by staining the cells with the 7-aminoactinomycin D (7-AAD) dye and AnnexinV conjugated to allophycocyanin (BD Biosciences). In the case of intracellular cytokine staining, stimulation was carried out for 4 h in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) and of R-Phycoerythrin-conjugated anti-CD107a antibody (BD Biosciences). The LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes/Invitrogen) was

used as a marker to exclude dead cells. Antibodies to human interferon (IFN)-γ and tumour necrosis factor (TNF)-α obtained from R&D Systems (Mineapolis, USA) and eBiosciences (San-Diego, CA, USA), respectively, were used to stain for intracellular cytokines. Data acquisition was performed using a FACSAria™ flow cytometer (BD Biosciences).

2.4. Monitoring of cladribine monophosphate formation by reversed-phase high-performance liquid chromatography (HPLC)

We adapted the method described by Bierau et al., (2004) for the detection of cladribine monophosphate generated by protein homogenates made from primary T cells. Total T cells were purified from the PBMCs of healthy donors as described above, washed once in PBS and resuspended to a density of 5×10^7 cells/ml in lysis buffer (200 mM NaCl, 10 mM DTT and complete EDTA-free protease inhibitor cocktail [Roche Diagnostics Gmbh, Mannheim, Germany] in 50 mM Tris HCl pH 7.4). Following sonication, the lysate was centrifuged at $10,000 \times g$ for 15 min at 4 °C. The supernatant was collected and mixed 1:1 with reaction buffer (10 mM ATP, 10 mM MgCl$_2$, 200 mM NaCl, 20 mM NaF, 2 mM DTT in 10 mM Tris HCl pH 7.4), to which cladribine was added at a concentration of 200 μM. The reaction was carried out over 2 h at 37 °C and terminated by placing the tubes on ice and adding 1 volume of ice cold methanol. The tubes were then centrifuged at $10,000 \times g$ for 15 min at 4 °C, supernatants were collected and mixed 1:1 with 50 mM NH$_4$H$_2$PO$_4$. One hundred μl of the sample was injected on a Supelcosil LC-18-S 5 μm column (250 mm × 4 mm) connected to a HPLC system consisting of an Alliance 2695 Separation Module and a 996 Photodiode Array (Waters, Milford, MA, USA). Elution of cladribine metabolites was monitored by measuring the absorbance at 265 nm at a constant flow of 1 ml/min. Sample injection in the pre-equilibrated column was isocratically performed in a mix of 90% buffer A (50 mM NH$_4$H$_2$PO$_4$) and 10% buffer B (50% methanol in 50 mM NH$_4$H$_2$PO$_4$). These flow conditions were maintained for 10 min. A gradient was established over 10 min to a final proportion of 50% buffer A and 50% buffer B, which was then followed by another 10 min of flow in these conditions. The identity of the different elution peaks was determined based on the retention times of purified commercial compounds added to the protein homogenate prior to mixing with methanol without any incubation period.

2.5. Statistical analysis

Data were analysed using unpaired two-tailed *t*-test by Prism 4.0 (GraphPad Software, San Diego, CA, USA). All *P* values <0.05 were considered significant.

3. Results

3.1. Cladribine inhibits T cell activation following T cell receptor cross-linking

We assessed the effect of cladribine on total T cells activated with anti-CD3 and anti-CD28 antibodies. Following overnight activation, cladribine concentrations equal or superior to 0.1 μM inhibited both cytokine secretion (Fig. 1A) and CD69 up-regulation (Fig. 1B) in a dose-dependent fashion without substantially affecting cellular viability (Fig. 1C). In peripheral blood T cells from six healthy donors, 1 μM cladribine inhibited cytokine release by approximately 80% on average (Fig. 1D) for interleukin (IL)-2 and IL-10. The degree of inhibition of IFN-γ and TNF-α secretion varied more greatly among the 6 donors, with values ranging from 25% to 80%, and averaged approximately 55% for both cytokines (Fig. 1D).

In comparison with the observed reduced cytokine release in culture supernatant, incubation of T cells with cladribine decreased the proportion of T cells secreting cytokines or releasing cytotoxic mediators to a greater extent. The proportion of CD4+ IFN-γ+ and