bjh short report

The anti-histaminic cyproheptadine synergizes the antineoplastic activity of bortezomib in mantle cell lymphoma through its effects as a histone deacetylase inhibitor

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Summary

Cyproheptadine, an inhibitor of the H1 histamine receptors, has recently shown activity in models of leukaemia and myeloma, presumably through inhibition of cyclin-D expression. Mantle cell lymphoma (MCL) is an aggressive subtype of non-Hodgkin lymphoma characterized by overexpression of cyclin-D1. We investigated the effect of cyproheptadine alone and in combination with the proteasome inhibitor bortezomib in models of MCL. The combination of these drugs was mathematically synergistic, producing significant reductions in the mitochondrial membrane potential leading to apoptosis. In a severe combined immunodeficient beige mouse model, cyproheptadine plus bortezomib demonstrated a statistically significant advantage compared to either agent alone.

Keywords: cyproheptadine, mantle cell lymphoma, bortezomib, HDAC inhibitor, cyclin D1.

Cyproheptadine is a known inhibitor of H1 histamine and 5-HT serotonin receptors, leading to its use for the treatment of migraines, anorexia and atopic dermatitis (Rao *et al*, 2000; Kardinal *et al*, 1990; Klein & Galant, 1980). Recently, using a chemical biology approach, Mao *et al* (2007, 2008) identified cyproheptadine as an inhibitor of D-cyclin expression. They demonstrated that cyproheptadine decreased D-cyclin expression, and induced apoptosis in models of myeloma and leukaemia *in vitro* and *in vivo* (Mao *et al*, 2007, 2008). Mantle cell lymphoma (MCL) represents a distinct subtype of aggressive lymphoma, characterized by dysregulated cyclin D1 gene (*CCND1*) expression secondary to the t(11;14) translocation. This reciprocal translocation places *CCND1* under the control of the *IGH* promoter, leading to constitutive expression (Williams & Swerdlow, 1994). The proteasome inhibitor bortezomib was approved for the treatment of relapsed or refractory MCL based on five phase 2 studies, and has been shown to complement the activity of a host of other drugs, including BH3-only mimetics (O'Connor *et al*, 2005, 2006; Paoluzzi & O'Connor, 2006; Fisher *et al*, 2006; Goy *et al*, 2008). Based on the cyclin-D1 rationale noted above, we investigated the cytotoxicity of cyproheptadine alone and in combination with the proteasome inhibitor bortezomib in models of MCL.

Materials and methods

The mantle cell lymphoma cell lines HBL-2, Granta-519 and Jeko-1 were obtained and maintained as described previously (Paoluzzi *et al*, 2008a,b). Cyproheptadine was obtained from Tocris Bioscience (Ellsville, MO, USA) and diluted in Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) which was maintained at a final concentration of <0·5%. All reagents were obtained as previously described (Paoluzzi *et al*, 2008a,b). For all *in vitro* assays (cytotoxicity, flow cytometry and immunoblotting), cells were counted, incubated and processed as previously described (Paoluzzi

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et al, 2008a,b). Acetyl-histone H3 and cyclin D1 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

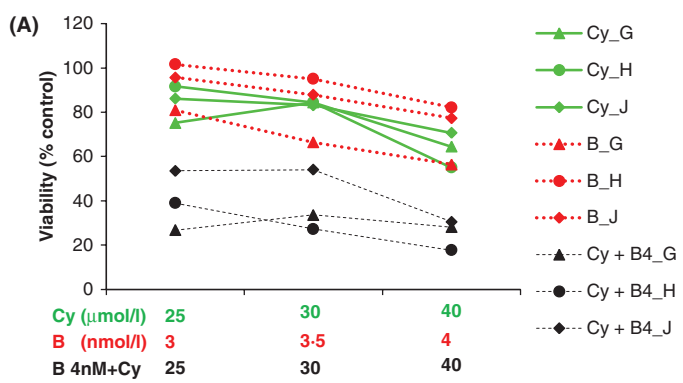
In vivo experiments were performed as described previously (Paoluzzi *et al*, 2008a,b). After dilution in sterile water, cyproheptadine was given by intraperitoneal (IP) injection at a dose of 36 mg/kg per d days 1–4 and at 30 mg/kg days 11–14. Bortezomib was given by IP injection at 0·5 mg/kg on days 1, 4, 11 and 14.

The $IC50_s$ (the concentrations inhibiting 50% of cells) were calculated by fitting dose response curves. Drug–drug interactions were computed using the relative risk ratio analysis (RRR, GraphPad, http://www.graphpad.com) with RRR < 1 defining synergism, RRR = 1 additivity, RRR > 1 antagonism. Data from the *in vitro* assays were analysed using a *t*-test with a robust variance estimate. For the mouse experiments, the tumour volumes and area under the curves (AUCs) were log transformed and evaluated using analysis of variance for four-way comparison and Wilcoxon test for pairwise comparisons (Paoluzzi *et al*, 2008a,b). *P* values <0·05 were considered significant.

Results and discussion

The IC_{50} values for cyproheptadine alone at 24 h were in the high micromolar range, at 42, 52, 55 μmol l^{-1} for HBL-2, Jeko-1 and Granta-519, respectively. The duration of exposure to cyproheptadine did not appear to be a major determinant of activity. Formal synergy analyses were performed on the three MCL cell lines treated with cyproheptadine and bortezomib. A synergistic cytotoxic effect was observed after 24 h at varying concentrations of the two drugs (cyproheptadine from 25 to 40 μmol l^{-1}, bortezomib from 2·5 to 4 nmol l^{-1}, Fig 1A and B) as follows: RRR = 0·43–0·93 for HBL-2, 0·62–0·85 for Granta-519, 0·4–0·96 for Jeko-1.

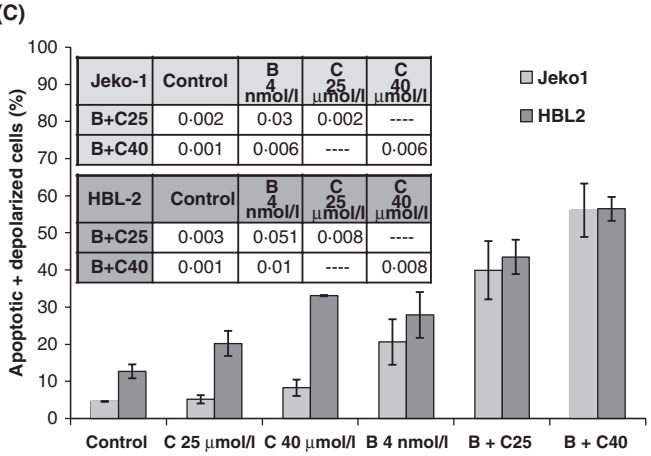
Treatment of Granta-519 and HBL-2 cells with cyproheptadine alone for up to 72 h decreased the normalized mitochondrial membrane potential (Δψm) at relatively high concentrations (more than 25 μmol l^{-1}). A simultaneous staining of cells with JC-1 (for quantification of Δψm) and annexin V-allophycocyanin (for apoptosis) established that the mitochondrial depolarization was associated with induction of apoptosis, validating a role for the mitochondrial pathway of



(B)

Cyproheptadine		BORTEZOMIB			
		2·5 nmol/l	3 nmol/l	3·5 nmol/l	4 nmol/l
Granta-519	25 μmol/l	0·62	0·71	0·77	0·63
	30 μmol/l	0·79	0·78	0·76	0·68
	40 μmol/l	0·79	0·85	0·74	0·74
Jeko-1	25 μmol/l	0·91	0·83	0·48	0·4
	30 μmol/l	0·89	0·77	0·9	0·84
	40 μmol/l	0·93	0·57	0·96	0·56
HBL-2	25 μmol/l	0·84	0·86	0·74	0·61
	30 μmol/l	0·85	0·74	0·62	0·43
	40 μmol/l	0·93	0·55	0·44	0·46

(C)



Jeko-1	Control	B 4 nmol/l	C 25 μmol/l	C 40 μmol/l
B+C25	0·002	0·03	0·002	----
B+C40	0·001	0·006	----	0·006

HBL-2	Control	B 4 nmol/l	C 25 μmol/l	C 40 μmol/l
B+C25	0·003	0·051	0·008	----
B+C40	0·001	0·01	----	0·008

Fig 1. Synergism of cyproheptadine combined to bortezomibin MCL. (A) Cyproheptadine (Cy, green) at 25, 30 or 40 μmol l^{-1}, Bortezomib (B, red) at 3, 3·5 or 4 nmol l^{-1}, Cy + B at 4 nmol l^{-1} (black) in three cell lines of MCL (G, Granta-519; J, Jeko-1; H, HBL-2). Viability was assessed by luminometricdetection at 24 h. (B) Relative risk ratio analysis at 24 h for cyproheptadine (25–40 μmol l^{-1}) plus bortezomib (2·5–4 nmol l^{-1}). All ratios were <1 (synergy). (C) Assessment of apoptosis and mitochondrial membrane potential (Δψm) by JC-1 and annexinV-APC. Treatment of Jeko-1 and HBL-2 cells with cyproheptadine (C) at 25 or 40 μmol l^{-1} and bortezomib (B) at 4 nmol l^{-1} induced significant apoptosis and decrease in Δψm compared to any group alone ($P < 0·05$). All comparisons are shown in the upper left part.

apoptosis in these MCL cell lines, and consistent with previous observation in leukaemia and myeloma cell lines (Mao *et al*, 2008). The combination of cyproheptadine at 25 or 40 μmol l^{-1} plus bortezomib at 4 nmol l^{-1} showed statistically significant decreases in the mitochondrial membrane potential ($\Delta\psi m$) and apoptosis compared to the two drugs given alone in Jeko1 ($P \leq 0{\cdot}03$) and HBL2 ($P \leq 0{\cdot}05$, Fig 1C).

The immunoblotting analysis for cyclin-D1 expression after exposure to cyproheptadine at 20 or 30 μmol l^{-1} for 6, 12 or 24 h showed a time- and concentration-dependent decrease of cyclin-D1 in HBL-2 and a concentration- but not time-dependent decrease in Granta-519 (Fig 2A). Given that these observations were reminiscent with those previously reported by Sakajiri *et al* (2005), we evaluated the cells for the accumulation of acetylated histone-H3, demonstrating an increase of this acetylated H3 after treatment with cyproheptadine alone at 20 and 30 μmol l^{-1} for 12 and 24 h in HBL-2 and Granta-519 (Fig 2B).

The ability to repress cyclin D1 and to increase the acetylation of histone proteins is indicative of histone deacetylase inhibitor (HDACI) activity (Sakajiri *et al*, 2005). HDACIs, such as vorinostat and valproic acid have been shown to repress cyclin D1, possibly through the phosphoinositide 3-kinase – mammalian target of rapamycin (mTOR) pathway. Sakajiri *et al* (2005) demonstrated that vorinostat repressed cyclin D1 and D2 expression in MCL, but not in myeloid leukaemia cell lines. Recently, Heider *et al* (2008) demonstrated that vorinostat was synergistic with the proteasome inhibitor bortezomib in *in vitro* models of MCL.

The *in vivo* efficacy of cyproheptadine was investigated in combination with bortezomib in a xenograft model of MCL (HBL-2). In the study by Mao *et al* (2008), cyrpoheptadine was administered daily at 36 mg/kg per d for 7 weeks in a multiple myeloma xenograft non-obese diabetic severe immunocombined deficiency (NOD/SCID) mouse model. At 42 d post-treatment, the group of mice treated with cyproheptadine alone showed statistically significant reduction in tumour volume compared to the control. In the *in vivo* MCL model, SCID beige mice were treated with both drugs alone, and in combination. The cohort receiving the combination of cyproheptadine and bortezomib was statistically superior to bortezomib alone ($P = 0{\cdot}01$), cyproheptadine alone ($P = 0{\cdot}0002$) and the control ($P = 0{\cdot}002$) on day 4 (Fig 2C). While this advantage was not sustained beyond the first week of treatment, it was the only cohort where a complete remission was seen. A partial explanation may be related to

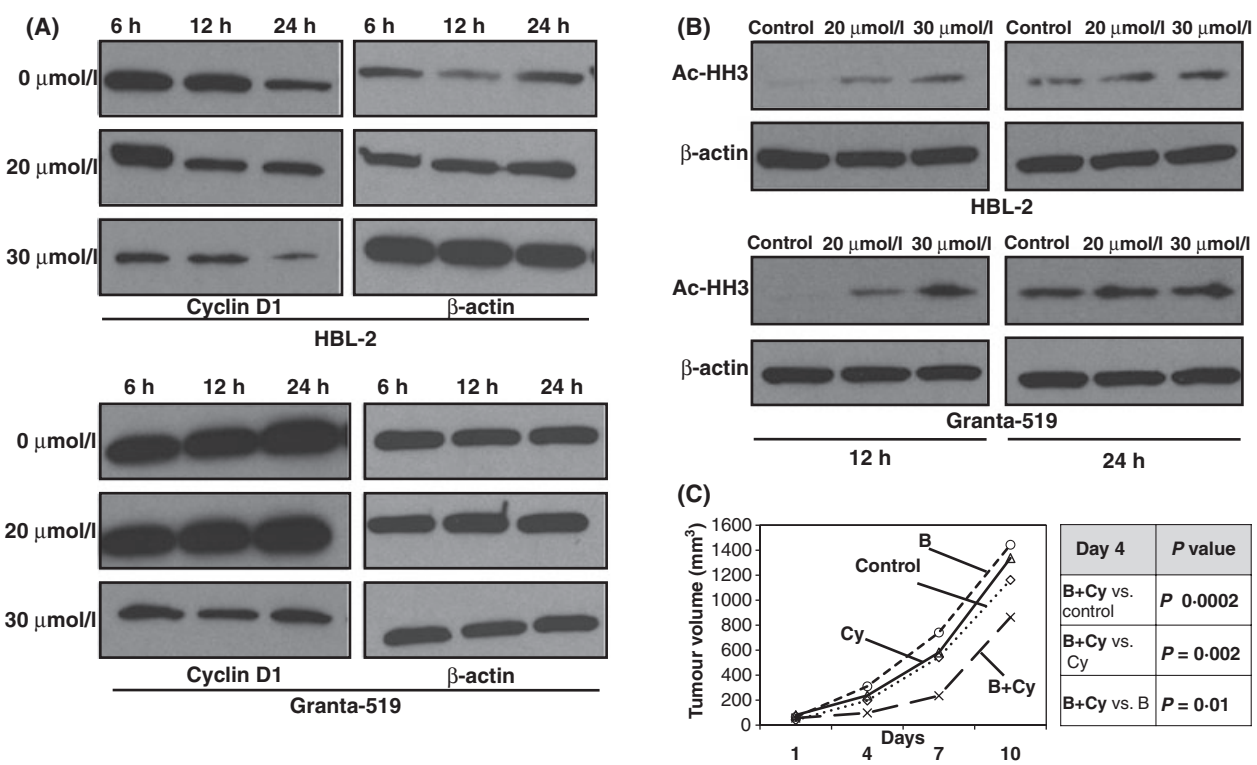


Fig 2. Expression of Cyclin D1 and acetylated histone H3 before and after treatment with cyproheptadineat 20 and 30 μmol l^{-1} and *in vivo* SCID beige xenograftmodel for MCL (HBL-2). (A) Cyclin D1 expression was decreased in Granta-519 after treatment with 30 *versus* 20 μmol l^{-1} with no time dependency, In HBL-2 the decrease in cyclin D1 appears time and concentration dependent. (B) Increased acetylationof histoneH3 (Ac-HH3) after treatment with cyproheptadine was more significant at 12 h, when concentration dependency was also observed. (C) Cyproheptadine (Cy) + bortezomib (B) showed significant tumour volume control compared to any other treatment group. The multiple comparison analysis (right panel) shows the superiority of the combination treatment to each other group ($P \leq 0{\cdot}01$). B + Cy was the only cohort where a complete remission was observed.

the interruption of treatment for 1 week after day 4 because of excessive toxicity (8/9 mice in the combination group, 4/10 mice in the cyproheptadine alone group experienced early weight loss of more than 10% of initial weight). Treatment was resumed on day 11 (cyproheptadine was reduced from 36 to 30 mg/kg per d) with four additional days of cyproheptadine and two additional days of treatment with bortezomib (on days 11 and 14). Interestingly, the cyproheptadine group demonstrated a statistically significant advantage over the control ($P \leq 0{\cdot}01$).

Very few clinical studies have been reported regarding the *in vivo* pharmacokinetic profile of cyproheptadine in humans. The peak plasma concentration after a single oral dose of 5–8 mg is between 0·14 and 0·32 μmol l^{-1}, although intravenous doses up to 48 mg have been used. Commonly reported side effects are mild and reversible and attributed to the antihistaminic effects.

In conclusion, cyproheptadine demonstrated activity in *in vitro* and *in vivo* models of MCL with a mechanism of action resembling a classic HDACI. In combination with bortezomib, cyproheptadine displayed a synergistic interaction, inducing significant apoptosis and promising *in vivo* activity. The combination of cyproheptadine with bortezomib further substantiates the merits of combining these two mechanisms (histone deacetylase and proteasome inhibition) for the treatment of haematological malignancies, with a particularly strong rationale in MCL. While more potent HDACI are now available, the potential use of a safe and readily accessible agent like cyproheptadine may be associated with some clinical advantage, though clearly a better understanding of the pharmacokinetic behaviour is warranted.

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