Dual inhibition of PIM and PI3-kinase by IBL-202 is highly synergistic compared to monomolecular inhibition and represents a novel treatment strategy for Chronic Lymphocytic Leukemia.

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1. To investigate the efficacy of a novel inhibitor of PIM kinase (pPIMi) against CLL cells in vitro.
2. To provide a rationale for dual targeting of PIM and PI3-kinase in CLL.
3. Investigate the efficacy of a novel dual inhibitor of PIM and PI3-kinase (IBL-202) against CLL cells cultured under conditions that mimic the tumour microenvironment.

Patient samples
All samples were collected with informed consent and with local ethical approval. Peripheral blood mononuclear cells were isolated by centrifugation through a ficoll-density gradient and cryogenically stored in LN until required.

Cytotoxicity assays
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effects of pPIMi, IBL202, SGI-1776 and CAL101 on CLL cell survival. All values are relative to vehicle-treated control cultures.

Analysis of synergy between pPIMi and CAL101
Synergy was assessed using the methodology of Chou and Talalay (1984). Briefly, pPIMi and CAL101 were combined at a ratio determined by their IC50 values as single agents, determined by MTT assay. Combination indices were calculated at a range of fractional effects, where 0.5, for example, indicates a 50% cell kill. Combination indices of <1, >1 and >1 are indicative of synergy, additivity and antagonism respectively.

CLL / CD40L-fibroblast co-culture
CLL patient cells were treated as indicated while in culture with a confluent layer of CD40L-expressing mouse L-fibroblasts. After co-culture for 48h CLL cells were harvested and incubated with the microalbumin membrane potential dye DICI1(5) and propidium iodide (PI). The integrity of the feeder layer was confirmed at the end of the culture period by microscopic inspection. CLL cell viability was determined by analysis of the percentage of DICI1(5) positive/PI negative cells remaining.

Introduction
Trials of ibrutinib and idealisib for Chronic Lymphocytic Leukemia (CLL) targeting Btk and PI3-kinase respectively, illustrate the potential of targeting components of the B-cell receptor (BCR) signalling pathway. Emerging evidence suggests that subgroups of CLL patients develop resistance to these agents. In particular late relapses on ibrutinib appear to be associated with a high frequency of acquired mutations in the PLCg2. Identification of novel combination therapies or agents that target multiple molecules in key signaling pathways represents a rational approach in the development of novel treatment strategies. Pim (provirus integration site for Moloney murine leukemia virus) family proteins are proto-oncogenic and involved in B-cell development and lymphoid malignancies. They are highly conserved serine/threonine kinases and are overexpressed in CLL. Given the clinical efficacy of idealisib and results of preclinical studies of the PIM kinase SGI-1776 [Chen et al., 2009], we sought to investigate the potential of simultaneous inhibition of PIM and PI3-kinase for CLL therapy.

Methods

Fig 1. pPIMi (Inflection Biosciences) is as effective as the PIM inhibitor SGI-1776.
The efficacy of the PIM inhibitors, pPIMi and SGI-1776, were compared by MTT assay. CLL samples (n=3), all with del 17p, and the MEC1, MEC2, RAJI, RAMOS, MOLT4 cell lines were cultured in media alone and treated with the indicated dose of each drug for 48 h. Both pPIMi and SGI-1776 induced a dose dependent decrease in the proportion of viable cells remaining at the end of the culture period.

Fig 2. pPIMi exerts strong synergy with the PI3-kinase inhibitor CAL101.
Synergy between the pan PIM inhibitor pPIMi and CAL101 was calculated by MTT assay. The strong synergy between pPIMi and CAL101 demonstrated in both primary cells (n=3) and haematological cell lines (MEC1, RAJI and RAMOS) provides the rationale for dual targeting of these molecules in CLL.

Fig 3. Dual inhibition of PIM and PI3-kinase is effective against CLL cells in co-culture with CD40L fibroblasts.
A. The efficacies of the pan PIM inhibitor pPIMi, CAL101 and the dual inhibitor of PIM and PI3-kinase IBL-202 were compared by MTT assay against primary CLL cells from 3 patients with deletion of 17p cultured in media alone. IBL-202 was significantly more effective than either pPIMi or CAL-101.
B. Co-culture of CLL cells (n=3) with a CD40L-expressing fibroblast layer conferred resistance to the PIM inhibitor pPIMi.
C. Stroma-mediated resistance to PpIMi in primary CLL cells (n=3) can be overcome by dual inhibition of PIM and PI3-kinase by IBL-202.

Summary
1. The pan-PIM kinase inhibitor pPIMi (Inflection Biosciences) proved as effective as SGI-1776 against CLL cells cultured in media alone.
2. The synergy between pPIMi and CAL-101 provides a strong rationale for dual targeting of the PIM and PI3-kinases as a treatment strategy for CLL.
3. CLL cells cultured in contact with a CD40L-expressing stromal layer were resistant to pPIMi.
4. Dual inhibition of PIM and PI3-kinase by IBL-202 (Inflection Biosciences) was effective and overcame the resistance to inhibition of PIM alone under conditions that mimic the tumour microenvironment.

Conclusions
1. Our data provide a strong rationale for dual targeting of the PIM and PI3-kinases as a therapeutic option for CLL.
2. In vitro modeling of the CLL tumour microenvironment suggests that dual targeting of PIM and PI3-kinases by IBL-202 (Inflection Biosciences) may be particularly effective at targeting the proliferative compartment of CLL disease.