Differential Regulation of T cells by PI3K delta inhibitors in a CLL Murine Model

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ABSTRACT

The purpose of the current study was to compare the effects of **umbralisib** (TGR-1202), duvelisib, and idelalisib on T cells in a CLL mouse model and analyze immune-mediated adverse events following oral administration. We hypothesized that umbralisib may preserve the number and function of the regulatory T cell (Treg) population, translating to decreased immune-mediated side effects after treatment. Leukemic euTCL1 splenocytes were adoptively transferred into wildtype mice to induce CLL disease and treated via oral administration with vehicle, umbralisib, idelalisib or duvelisib. Tcon:Treg and Teff:Treg ratios in periphery and spleen were measured by flow cytometry were found to be spared in the umbralisib-treated group and decreased on duvelisib and idealisib groups. Expression of functional Treg markers TGFB-1, CD39, CD103, PD-1 and CTLA-4 was also spared in the umbralisib group but affected in the other two. To assess immune-mediated toxicity, GI tract and liver tissues were collected and H&E stained. Pathology analysis was performed and immune-mediated toxicity was scored according to the following parameters: inflammatory cell infiltrate, kuppfer cell hyperplasia, microvesicular steatosis, cell degeneration (liver) and denuded mucosa, chronic inflammation, (GI tract). Overall toxicity grade was significantly lower in umbralisib-treated group compared to duvelisib-treated group in both liver and GI tract. Toxicity grade in these tissues negatively correlated with total Treg count in periphery (R-squared~0.5). Immunohistochemistry staining of FoxP3+ Tregs in liver and GI tract was concordant with the assessment of Treg count in spleen by flow cytometry, as the number of FoxP3+ cells was closer to normal in umbralisib-treated mice compared to duvelisib-treated mice. Next, we investigated whether co-inhibition of CK1e by umbralisib may be involved in the differential regulation of T cells. Combination of a selective CK1e inhibitor, SR-4471, with duvelisib, prevented the reduction of total Treg number and functional markers in *ex vivo* culture of murine euTCL1 T cells, mimicking the effect of umbralisib. We have found that canonical Wnt signaling is inhibited dose-dependently in euTCL1 T cells treated with umbralisib; demonstrated by lower levels of B-catenin and downstream TCF-1/7. These data determine Tregs to be a major player involved in immune-mediated toxicity characteristic of the PI3K inhibitor class of drugs. *Umbralisib may differentially regulate CLL T cells through complimentary inhibition of both PI3Kδ and CK1e, potentially preserving Treg number and function to provide protection from immune-mediated severe adverse events.*

BACKGROUND

The role of PI3K signaling is widely acknowledged as a key component of cell survival in many hematological malignancies. The PI3K molecule recruits important downstream effector signaling proteins directly following BCR ligation. For example, recruitment of BTK and AKT leads to promotion of cell survival by activating NF-kB and inhibiting apoptotic signals. The p110 delta expressing isoform of PI3K is restricted to hematopoietic cell types; therefore p110 delta represents a viable target for the treatment of B-cell malignancies with little cytotoxicity in other cell types. However, drugs targeting p110 delta may have potential off-target effects in other immune cell types. For example, off-target effects in the T-cell compartment may have important implications for immunosuppressive or immunostimulatory mechanisms which can contribute to the progression, or elimination of disease.

Idelalisib (aka "CAL-101") and Duvelisib (aka "IPI-145") are two orally available PI3K inhibitors that show selectivity for p110 delta. In the clinic, rates of objective response for these drugs are 40-60% and nodal responses exceed 70% in R/R CLL. They also show high rates of response in high-risk CLL (e.g. 17p and 11q deletions). In vitro, idelalisib inhibits p110 delta at a concentration 40 to 300-fold lower than the other class 1 PI3K isoforms and exhibits selectivity when profiled against other protein and lipid kinases.

In the phase 1 study of single-agent idelalisib in 54 R/R CLL patients who were previously heavily treated, the therapy was well-tolerated generally but 15% of participants discontinued therapy due to adverse effects. **Umbralisib** (aka **TGR-1202**) is a selective inhibitor of p110 delta with some CK1e activity. Notably, umbralisib exhibits a different structure than idelalisib and duvelisib, which are very similar compounds chemically. Thus far, umbralisib has shown promising activity in B cell lymphomas without significant severe adverse effects. It has been shown to induce cytotoxicity, and inhibit AKT phosphorylation at submicromolar concentrations in both del 17p and non del 17p primary CLL cells *in vitro*.

OBJECTIVE

In this series of studies we sought to investigate (1) how PI3K inhibitors differentially regulate CLL T cells (2) determine the effect of complimentary caesin kinase 1 epsilon (CK1e) and PI3K delta inhibition by umbralisib (TGR-1202) in normal and CLL T cells.

MATERIALS AND METHODS

CLL Murine Model

25x10^6 splenocytes from leukemic aged euTCL1 mice were injected via tail vein into C57BL/6 mice (Jackson Laboratories). After confirmation of disease induction (increased peripheral lymphocyte count) mice were gavaged once per day with TGR-1202, duvelisib, idelalisib or vehicle for a total of 21 days.

Magnetic Cell Purification

EasySep T cell isolation kits or Rosette Sep T cell isolation kits (StemCell Tech.) were utilized for the enrichment of >95% purity of cells of interest. Company supplied protocols were followed and flow cytometry was performed to elucidate purity. T cell stimulation was achieved with CD3/CD28 soluble cytokines (BD Bioscience, San Jose CA) or Immunocult (StemCell Tech.)

Inhibitors

Idelalisib and duvelisib were obtained from SellleckChem. Umbralisib (TGR-1202) was kindly supplied by TG Therapeutics. SR-4471 (CK1e-selective inhibitor) was kindly donated by the Cleveland lab at the Moffitt Cancer Center. All inhibitors were dissolved in DMSO for *in vitro* assays.

Flow Cytometry Immunophenotyping

Flow cytometric analysis was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-CD3, -CD4, -CD8, -CD25, -CD127, -CD279 (PD-1), -CTLA-4, -FOXP3, BD Bioscience, San Jose CA, eBioscience, San Diego CA, -TGFB-1, -CD103, -GITR Biolegend, San Diego CA) and the vitality dye Zombie NIR. Data was acquired on an LSRII cytometer (Beckman Coulter), and analyzed with FlowJo software (Tree Star, Ashland, OR). Absolute cell numbers calculated using AccuChek Counting beads (Invitrogen).

Phospho Flow/Intracellular Flow

Isolated normal T cells were stimulated with PMA for 15 min. ~pAKT (S473) (BD Biosciences) was used to determine AKT phosphorylation on an iQue cytometer and analyzed with accompanying software. Flow cytometric analysis was performed using ~B-catenin and ~TCF1/7 antibodies (Invitrogen) following fixation and permebilization of isolated cells indicated. Stimulation was performed for 18h prior to analysis with Wnt3A ligand (R&D Systems) at 0.5ug/mL.

We would like to acknowledge the Flow Cytometry Core & Tissue Core at the H. Lee Moffitt Cancer Center for their support.

Figure 1. *In vivo* efficacy of PI3K inhibitors in CLL murine model

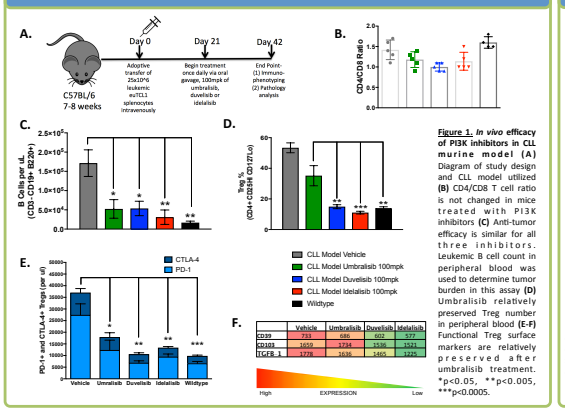

Figure 1. *In vivo* efficacy of PI3K inhibitors in CLL murine model (A) Diagram of study design and CLL model utilized **(B)** CD4/CD8 T cell ratio is not changed in mice treated with PI3K inhibitors **(C)** Anti-tumor efficacy is similar for all three inhibitors. Leukemic B cell count in peripheral blood was used to determine tumor burden in this assay **(D)** Umbralisib relatively preserved Treg number in peripheral blood **(E-F)** Functional Treg surface markers are relatively preserved after umbralisib treatment. *p<0.05, **p<0.005, ***p<0.0005.

Figure 2. Incidence of adverse events is associated with Treg count

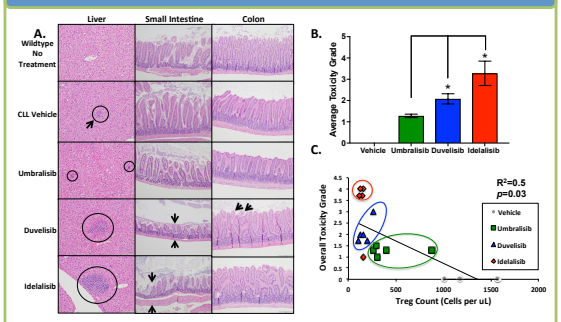

Figure 2. Incidence of drug-related immune-mediated adverse events in indicated tissues is associated with Treg count. Toxicity grade was determined after H&E stain by blinded histological analysis of liver and GI tract tissues using known indicators of immune-mediated adverse events. **(A)** Representative histologic findings. Left Column: Liver tissue showing inflammation. Middle Column: Small intestine showing shortening of the villi indicating drug-related adverse events. Right Column: Colon showing disruption of the mucosa structure indicating drug-related adverse events **(B)** Graph showing average toxicity grade per treatment group **(C)** Correlation analysis- linear regression performed on overall toxicity grade vs. Treg count demonstrated correlation between parameters (R^2= 0.5, p=0.03 indicated significance). Representative of 2 independent experiments. *p<0.05, **p<0.005, ***p<0.0005.

Graphic summary

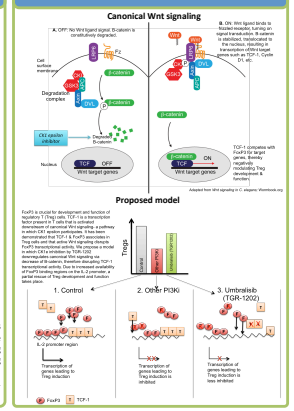

Figure 3. Effect of CK1e inhibition on normal human T cells

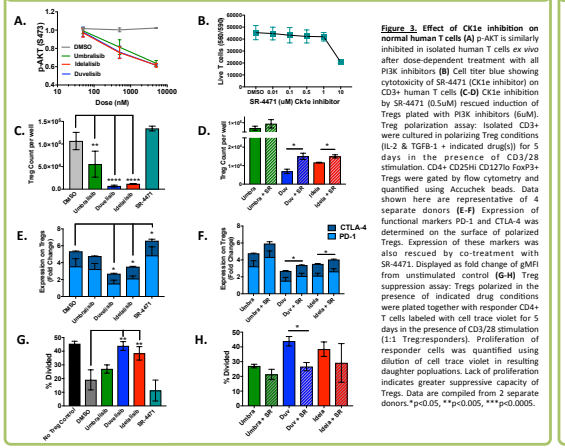

Figure 3. Effect of CK1e inhibition on normal human T cells (A) p-AKT is similarly inhibited in isolated human T cells *ex vivo* after dose-dependent treatment with all PI3K inhibitors **(B)** Cell titer blue showing cytotoxicity of SR-4471 (CK1e inhibitor) on CD3+ human T cells **(C-D)** CK1e inhibition by SR-4471 (0.5uM) rescued induction of Tregs plated with PI3K inhibitors (6uM). Treg polarization assay: Isolated CD3+ were cultured in polarizing Treg conditions (IL-2 & TGFb-1 + indicated drugs)) for 5 days in the presence of CD3/28 stimulation. CD4+ CD25hi CD127lo FoxP3+ Tregs were gated by flow cytometry and quantified using Accuchek beads. Data shown here are representative of 4 separate donors **(E-F)** Expression of functional markers PD-1 and CTLA-4 was determined on the surface of polarized Tregs. Expression of these markers was also rescued by co-treatment with SR-4471. Displayed as fold change of gMFI from unstimulated control **(G-H)** Treg suppression assay: Tregs polarized in the presence of indicated drug conditions were plated together with responder CD4+ T cells labeled with cell trace violet for 5 days in the presence of CD3/28 stimulation (1:1 Treg:responders). Proliferation of responder cells was quantified using dilution of cell trace violet in resulting daughter populations. Lack of proliferation indicates greater suppressive capacity of Tregs. Data are compiled from 2 separate donors.*p<0.05, **p<0.005, ***p<0.0005.

Figure 4. Effect of CK1e inhibition on murine CLL T cells

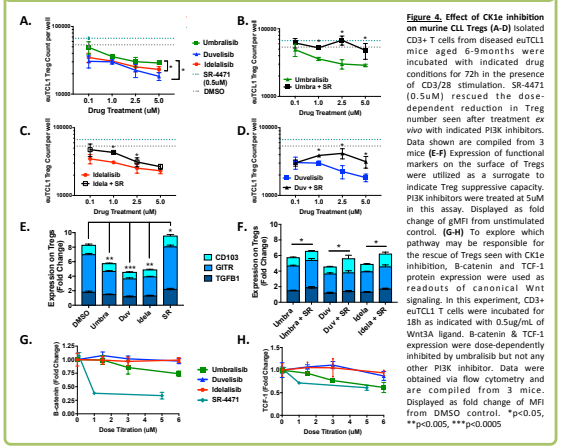

Figure 4. Effect of CK1e inhibition on murine CLL Tregs (A-D) Isolated CD3+ T cells from diseased euTCL1 mice aged 6-9months were incubated with indicated drug conditions for 72h in the presence of CD3/28 stimulation. SR-4471 (0.5uM) rescued the dose-dependent reduction in Treg number seen after treatment *ex vivo* with indicated PI3K inhibitors. Data shown are compiled from 3 mice **(E-F)** Expression of functional markers on the surface of Tregs were utilized as a surrogate to indicate Treg suppressive capacity. PI3K inhibitors were treated at 5uM in this assay. Displayed as fold change of gMFI from unstimulated control. **(G-H)** To explore which pathway may be responsible for the rescue of Tregs seen with CK1e inhibition, B-catenin and TCF-1 protein expression were used as readouts of canonical Wnt signaling. In this experiment, CD3+ euTCL1 T cells were incubated for 18h as indicated with 0.5ug/mL of Wnt3A ligand. B-catenin & TCF-1 expression were dose-dependently inhibited by umbralisib but not any other PI3K inhibitor. Data were obtained via flow cytometry and are compiled from 3 mice. Displayed as fold change of MFI from DMSO control. *p<0.05, **p<0.005, ***p<0.0005

CONCLUSIONS

- **Umbralisib (TGR-1202) oral treatment induced less incidence of toxicity in CLL mice compared to other PI3K inhibitors**
- **Peripheral Treg number associated with incidence of toxicity in CLL mice treated with PI3K inhibitors**
- **Umbralisib displayed less anti-Treg effects in a dose-dependent manner compared to other PI3K inhibitors in normal and murine CLL T cells**
- **Umbralisib uniquely inhibited CK1e in euTCL1 T cells dose-dependently**
- **CK1e inhibition by umbralisib may offer an explanation for less anti-Treg effects.**

REFERENCES

- Van Loosdregt et al. Canonical Wnt signaling negatively modulates regulatory T cell function. Immunity. 2013 Aug;39(2):298-310.
- Deng et al. Silencing c-Myc translation as a therapeutic strategy through targeting PI3Kd and CK1e in hematological malignancies. Blood. 2017 Jan 5;129(1):88-99.
- Okkenhaug, K., & Vanhaesebroeck, B. (n.d.). PI3K in lymphocyte development, differentiation and activation. Nature Reviews Immunology, 317-330.
- Herman SE, Gordon AL, Wagner AJ, et al. Phosphatidylinositol 3-kinase-δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. Blood. 2010;116(12):2078-88.
- Winkler DG, Faia KL, DiNitto JP, et al. PI3K-δ and PI3K-γ inhibition by IPI-145 abrogates immune responses and suppresses activity in autoimmune and inflammatory disease models. Chem Biol. 2013;20(11):1364-74.

CONFLICT OF INTEREST

Miskin: TG Therapeutics (Employment & Equity Ownership); Maryanski TG Therapeutics (Employment & Equity Ownership); Sahakian, Pinilla declare no conflict of interest.

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