Differential Regulation of Human T cells by TGR-1202, A Novel PI3Kδ Inhibitor

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ABSTRACT

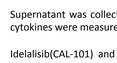
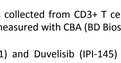
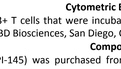
TGR-1202 is a novel, next-generation PI3Kδ inhibitor presenting significant structural and pharmacological differences from prior small-molecule PI3Kδ inhibitors. TGR-1202 has high clinical efficacy in treatment of B cell malignancies with a substantially differentiated adverse event profile compared to previous PI3Kδ inhibitors, specifically concerning hepatotoxicity or colitis which have been minimal or non-existent. It has been postulated that these effects may be due to T cell immune-mediated mechanisms. ***We hypothesized that TGR-1202 preserves the function of the regulatory T cell (Treg) population, translating to decreased immune-mediated side effects after treatment.*** Here, we aimed to compare effects of clinically available PI3Kδ inhibitors on T cells with an emphasis on Tregs in a cohort of healthy donors.

First, we observed comparable dose dependent increases in cytotoxicity beginning at 25uM following treatment of isolated T cell populations with Idelalisib, Duvelisib, or TGR-1202. At this dose apoptosis was induced between 48 and 72h. *Second*, all inhibitors reduced cytokine production in CD3+ T cells upon stimulation. Particularly, reduction of Th2 cytokines, IL-10 and IL-4, was less pronounced after TGR-1202 treatment, indicating relative conservation of T cell response. All inhibitors lowered mRNA expression of T-bet (Th1), GATA-3 (Th2) and FoxP3 (Treg), however FoxP3 levels were consistently higher in TGR-1202 treated T cells.

Third, we detected normal CD4:CD8 ratio and unaffected proliferative capacity of CD4+ and CD8+ subsets after drug treatment. *Finally*, all inhibitors decreased total percent of Tregs following stimulation (CD4+ $CD25^{HI}$ FoxP3+) accompanied by decreased expression of co-inhibitory molecules CTLA-4 and PD-1 on the Tregs. Interestingly, TGR-1202 significantly preserved the percent of Tregs closer to normal as well as surface expression of CTLA-4 and PD-1 on Tregs, indicating greater retention of immune checkpoint blockade and suppressive phenotype.

We report herein that TGR-1202 affects human T cells differently than Idelalisib and Duvelisib. TGR-1202 sustains IL-10 production, FoxP3 mRNA expression, and maintains Treg percentage and expression of immune checkpoint molecules, suggesting relative preservation of numbers and function of Tregs. Data presented begin to provide novel insight into immune mediated cellular mechanisms responsible for lack of side effects in clinical trials of TGR-1202. *In vivo* models to further characterize effects on the T cell compartment are ongoing.

BACKGROUND

TGR-1202	Idelalisib	IPI-145
		

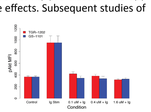
Fold-selectivity				
Isoform	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ
TGR-1202	>10000	>50	>48	1
idelalisib[1]	>300	>200	>40	1
duvelisib[2]	>640	>34	>11	1

[1]Flinn et al. 2009; [2]Porter et al. 2012

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 (p110δ) expressing isoform of PI3K is restricted to hematopoietic cell types; therefore p110δ represents a viable target for the treatment of B-cell malignancies with little cytotoxicity in other cell types. However, drugs targeting p110δ may have potential off-target effects in other immune cell types. For example, potential off-target effects in the T-cell compartment may have important implications for immunosuppressive or immunostimulatory mechanisms which may contribute to the progression, or elimination of disease as well as immune-mediated toxicities in patients.

Idelalisib (CAL-101) and **Duvelisib** (IPI-145) are two novel, orally available PI3K inhibitors in clinical development that show selectivity for **p110δ** and **p110δ/γ** respectively. 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 (i.e. 17p and 11q deletions). In the phase 1 study of single-agent Idelalisib in 54 R/R CLL patients who were heavily pre-treated, therapy was well-tolerated generally, but 15% of participants discontinued therapy due to adverse effects. Subsequent studies of idelalisib have displayed even higher rates of discontinuations due to AEs.

TGR-1202 is a potent and selective inhibitor of **p110δ**, which has a markedly different structure from Idealisib and Duvelisib. TGR-1202 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, equipotent to idelalisib (Friedman et al, ASH 2012). TGR-1202 has shown promising activity in B cell lymphomas, with a 94% nodal response rate observed in CLL patients treated with TGR-1202 monotherapy with, thus far, significantly fewer severe adverse effects compared to other PI3Kδ inhibitors, especially with respect to hepatotoxicity, colitis, and opportunistic infections such has pneumonia and pneumonitis.



Friedman et al, ASH 2012: Akt is phosphorylated after cross-linking of the BCR by anti-Ig ("Ig Stim"). The addition of TGR-1202 or GS-1101 returns Akt phosphorylation to baseline at concentrations between 0.1 to 1.6 μM.

OBJECTIVE

In this series of studies we sought to compare the effects of clinically available PI3Kδ inhibitors Idelalisib, TGR-1202 and Duvelisib on T cells with an emphasis on regulatory T cells in a cohort of healthy donors.

MATERIALS AND METHODS

Magnetic Cell Purification

EasySep Human T-cell enrichment (StemCell Tech.) was 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 Immunocult™ Human CD3/CD28 T cell Activator (StemCell Tech.)

Viability

Isolated normal human CD3+ T cells were plated in 96 well flat bottom plate at 100 000 cells/well in 200uL complete media with CAL-101, TGR-1202, IPI-145 0-50uM. Viability was measured after 24hrs with Cell Titer blue assay (Promega, Madison, WI)

Flow Cytometry Immunophenotyping

Flow cytometric analysis was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-CD3, -CD4, -CD8, -CD25, -IL-7R, -CD279 (PD-1), -CTLA-4, -FOXP3, Annexin V, -CD45RA, -CD62L Becton Dickinson, San Jose, CA, and eBiosciences, San Diego, CA) and the vitality dye Live Dead Yellow (LifeTech. Inc.). Data was acquired on an LSRII cytometer (Beckman Coulter), and analyzed with FlowJo software (Tree Star, Ashland, OR). Absolute cell numbers calculated using AccuCheck Counting beads (Invitrogen, Fredrick, MD).

Proliferation & Suppression

Isolated CD4+ or CD8+ T cells were stained with CellTrace Violet (CTV) according to manufacturer's protocol and incubated for 3-5 days with or without enriched regulatory T cells (CD4+ CD127low CD49d-, StemCell Tech). Dilution of CTV on live cells was measured on an LSRII cytometer (Beckman Coulter), and analyzed with FlowJo software (Tree Star, Ashland, OR).

qRT-PCR

Total RNA was isolated from all samples using Trizol (LifeTech. Inc.) with manufacturer supplied protocols. cDNA was generated using iScript (Bio Rad). IQ Syber Green Supermix (Bio Rad) was utilized in all qRT-PCR reactions.

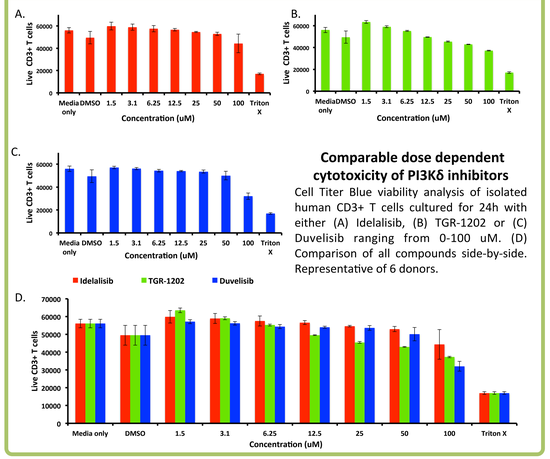
Cytometric Bead Array

Supernatant was collected from CD3+ T cells that were incubated with drug or vehicle at 10uM for 24 hours, and Th1/Th2 cytokines were measured with CBA (BD Biosciences, San Diego, CA) according to manufacturer's protocol.

Compounds

Idelalisib(CAL-101) and Duvelisib (IPI-145) was purchased from SelleckChem, Houston TX. TGR-1202 was supplied by TG Therapeutics, Inc.

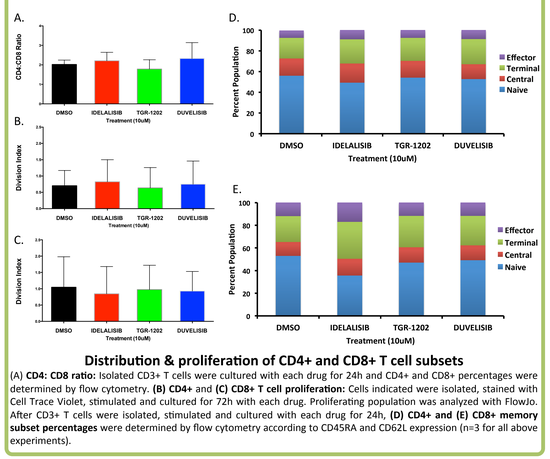
FIGURE 1



Comparable dose dependent cytotoxicity of PI3Kδ inhibitors

Cell Titer Blue viability analysis of isolated human CD3+ T cells cultured for 24h with either (A) Idelalisib, (B) TGR-1202 or (C) Duvelisib ranging from 0-100 uM. (D) Comparison of all compounds side-by-side. Representative of 6 donors.

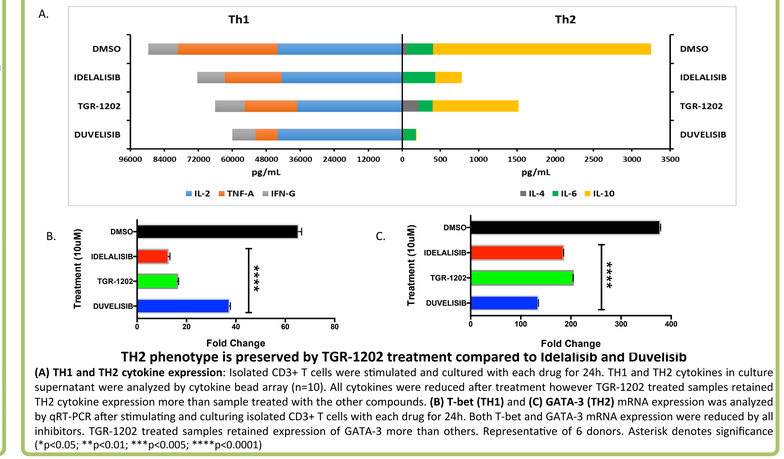
FIGURE 2



Distribution & proliferation of CD4+ and CD8+ T cell subsets

(A) **CD4: CD8 ratio:** Isolated CD3+ T cells were cultured with each drug for 24h and CD4+ and CD8+ percentages were determined by flow cytometry. **(B) CD4+** and **(C) CD8+ T cell proliferation:** Cells indicated were isolated, stained with Cell Trace Violet, stimulated and cultured for 72h with each drug. Proliferating population was analyzed with FlowJo. After CD3+ T cells were isolated, stimulated and cultured with each drug for 24h, **(D) CD4+ and (E) CD8+ memory subset percentages** were determined by flow cytometry according to CD45RA and CD62L expression (n=3 for all above experiments).

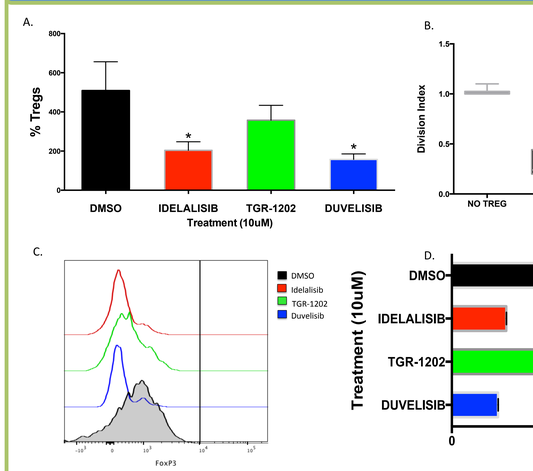
FIGURE 3



TH2 phenotype is preserved by TGR-1202 treatment compared to Idelalisib and Duvelisib

(A) TH1 and TH2 cytokine expression: Isolated CD3+ T cells were stimulated and cultured with each drug for 24h. TH1 and TH2 cytokines in culture supernatant were analyzed by cytokine bead array (n=10). All cytokines were reduced after treatment however TGR-1202 treated samples retained TH2 cytokine expression more than sample treated with the other compounds. **(B) T-bet (TH1)** and **(C) GATA-3 (TH2)** mRNA expression was analyzed by qRT-PCR after stimulating and culturing isolated CD3+ T cells with each drug for 24h. Both T-bet and GATA-3 mRNA expression were reduced by all inhibitors. TGR-1202 treated samples retained expression of GATA-3 more than others. Representative of 6 donors. Asterisk denotes significance (*p<0.05; **p<0.01; ***p<0.005; ****p<0.0001)

FIGURE 4



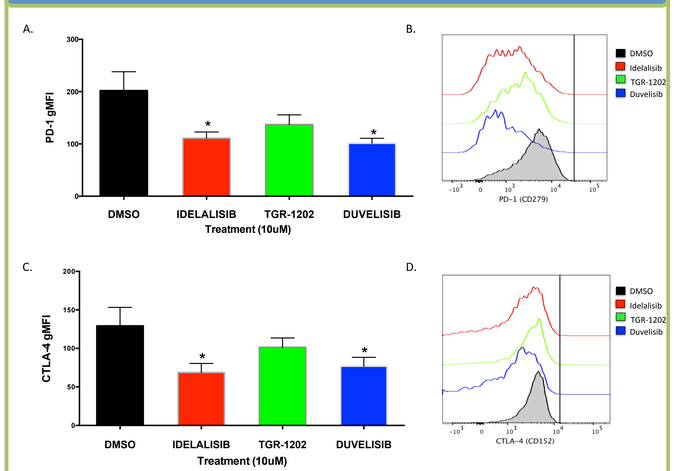
Treatment (10uM)	FoxP3 MFI
DMSO	1123
IDELALISIB	433
TGR-1202	753
DUVELISIB	371

Protein Expression (as MFI)

TGR-1202 reduces regulatory T cell number and diminishes function to a lesser degree compared to other PI3Kδ inhibitors

(A) Percentage of regulatory T cells (Tregs): Isolated CD3+ T cells were stimulated and cultured for 24h with each drug and regulatory T cell percentage was determined by flow cytometry (CD4+ CD25+ FoxP3+ IL-7RLO; n=10) **(B) Tregs treated with TGR-1202 retain their suppressive capacity compared to Tregs treated with Idelalisib and Duvelisib**: Isolated regulatory T cells were treated for 24h then cultured with CD4+ responders stained with Cell Trace Violet. After 5 days Cell Trace Violet dilution was measured to determine proliferation of responder cells. Less proliferation indicated greater suppressive capacity of Tregs (n=6) **(C) Histogram showing FoxP3 expression** on CD4+ CD25+ T cells (representative of 10 donors) **(D) FoxP3 mRNA expression**: Isolated CD3+ T cells were stimulated and cultured for 24h with each drug and FoxP3 mRNA expressed was determined by qRT-PCR (representative of 6 donors) **(E) Heat-map table showing FoxP3 mean fluorescence intensity on CD4+CD25+ T cells** after culture with each drug for 24h (representative of 10 donors). Asterisk denotes significance (*p<0.05; **p<0.01; ***p<0.005; ****p<0.0001).

FIGURE 5



Treatment (10uM)	MFI	
	PD-1	CTLA-4
DMSO	4116	3777
IDELALISIB	1275	2447
TGR-1202	1920	3145
DUVELISIB	784	1774

Protein Expression (as MFI)

TGR-1202 exhibits less effect on reduction of checkpoint molecules compared to other PI3Kδ inhibitors (A) PD-1 expression on Tregs (CD4+ CD25+ FoxP3+ IL-7RLO) was measured by flow cytometry after incubation of CD3+ T cells with each drug for 24h with stimulation (n=10). **(B)** Histogram showing representative mean fluorescence intensity for **PD-1 expression** on Tregs. **(C) CTLA-4 expression on Tregs** (CD4+ CD25+ FoxP3+ IL-7RLO) was measured by flow cytometry after incubation of CD3+ T cells with each drug for 24h with stimulation. **(D)** Histogram showing representative mean fluorescence intensity for **PD-1 expression** on Tregs. **(E) Heat-map table showing mean fluorescence intensity for PD-1 and CTLA-4 expression** on Treg. (representative of 10 donors). Asterisk denotes significance (*p<0.05; **p<0.01; ***p<0.005; ****p<0.0001)

CONCLUSIONS

1. **TGR-1202 exhibits dose dependent cytotoxicity against human T cells beginning 25uM comparable to other PI3Kδ inhibitors Idelalisib and Duvelisib.**
2. **Distribution and proliferative capacity of CD4+ and CD8+ populations and memory populations are not altered by any PI3Kδ inhibition**
3. **TH1 and TH2 cytokines and transcription factors are reduced after all PI3K inhibition but TGR-1202 allows conservation of TH2 cytokine expression and GATA-3 mRNA compared to other PI3Kδ inhibitors Idelalisib and Duvelisib.**
4. **Regulatory T cell population number and function is reduced overall after PI3K inhibition but to a lesser degree in TGR-1202 treated samples.**
5. **TGR-1202 does not robustly reduce the expression of PD-1 and CTLA-4 on Tregs, suggesting that suppressive phenotype is maintained to a greater extent compared to Idelalisib and Duvelisib.**
6. **These studies may begin to elucidate mechanisms responsible for lack of immune-mediated adverse events in patients treated with TGR-1202.**

REFERENCES

1) Okkenhaug, K., & Vanhaesebroeck, B. (n.d.). PI3K in lymphocyte development, differentiation and activation. *Nature Reviews Immunology*, 317-330.

2) 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.

3) 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.

4) Fooksman DR, Shaikh SR, Boyle S, Edidin M. Cutting edge: phosphatidylinositol 4,5-bisphosphate concentration at the APC side of the immunological synapse is required for effector T cell function. J Immunol. 2009;182(9):5179-82.

5) Dong, S., Guinn, D., Dubovsky, J. A., Zhong, Y., Lehman, A., Kutok, J., . . . Johnson, A. J. (2014). IPI-145 antagonizes intrinsic and extrinsic survival signals in chronic lymphocytic leukemia cells. *Blood, 124*(24), 3583-3586. doi:10.1182/blood-2014-07-587279

6) Falchi, L., Baron, J. M., Orlikowski, C. A., & Ferrajoli, A. (2016). BCR Signaling Inhibitors: an Overview of Toxicities Associated with Ibrutinib and Idelalisib in Patients with Chronic Lymphocytic Leukemia. *Mediterr J Hematol Infect Dis, 8*(1), e2016011. doi:10.4084/MJHID.2016.011

7) Woyach, J. A., Johnson, A. J., & Byrd, J. C. (2012). The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood, 120*(6), 1175-1184. doi:10.1182/blood-2012-02-362624

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CONFLICT OF INTEREST

Conflict of Interest disclosure: HM and DM: TG Therapeutics, Inc.; Employment and equity ownership.
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