Silencing c-Myc Translation as a Therapeutic Strategy through Targeting PI3K

Delta and CK1 Epsilon in Hematological Malignancies

Short title: Targeting PI3K and CK1 in Myc Driven Lymphoma

Changchun Deng¹,²,³,* , Mark R. Lipstein²#, Luigi Scotto², Xavier O. Jirau Serrano², Michael A. Mangone², Shirong Li³, Jeremie Vendome⁴, Yun Hao⁵, Xiaoming Xu², Shixian Deng², Ronald B. Realubit⁶, Nicholas P. Tatonetti⁵, Charles Karan⁶, Suzanne Lentzsch³, David A. Fruman⁷, Barry Honig⁴, Donald W. Landry², and Owen A. O'Connor¹,²

¹Center for Lymphoid Malignancies, ²Division of Experimental Therapeutics, ³Division of Hematology & Oncology, Department of Medicine; ⁴Department of Systems Biology and Howard Hughes Medical Institute, ⁵Department of Biomedical Informatics, ⁶Joint Centers for Systems Biology-Columbia Genome Center, Columbia University Medical Center, New York, NY, USA; ⁷Department of Molecular Biology & Biochemistry, University of California Irvine, Irvine, CA, USA

#New address:
Mark R. Lipstein:
Division of Medical Sciences
Harvard Medical School
260 Longwood Avenue
Boston, MA 02115

Corresponding Author:
Changchun Deng, M.D., Ph.D.
Assistant Professor of Medicine & Experimental Therapeutics
Center for Lymphoid Malignancies
Columbia University Medical Center
51 West 51st Street, 2nd Floor
New York, NY 10019
Tel: (212) 326-5720/305-7926
Email: cd2448@columbia.edu

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KEY POINTS

- A novel PI3Kδ inhibitor TGR-1202 synergizes with the proteasome inhibitor carfilzomib by silencing c-Myc in preclinical models of lymphoma.

- The unique activity of TGR-1202 as a single agent and in combination with carfilzomib is driven by an unexpected activity targeting CK1ε.
ABSTRACT

Phosphoinositide 3-kinase (PI3K) and the proteasome pathway are both involved in activating the mechanistic target of rapamycin (mTOR). Because mTOR signaling is required for initiation of mRNA translation, we hypothesized that co-targeting the PI3K and proteasome pathways might synergistically inhibit translation of c-Myc. We found that a novel PI3K delta isoform inhibitor TGR-1202, but not the approved PI3Kδ inhibitor idelalisib, was highly synergistic with the proteasome inhibitor carfilzomib in lymphoma, leukemia, and myeloma cell lines and primary lymphoma and leukemia cells. TGR-1202 and carfilzomib (TC) synergistically inhibited phosphorylation of the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), leading to suppression of c-Myc translation and silencing of c-Myc dependent transcription. The synergistic cytotoxicity of TC was rescued by overexpression of eIF4E or c-Myc. TGR-1202, but not other PI3Kδ inhibitors, inhibited casein kinase-1 (CK1) epsilon. Targeting CK1ε using a selective chemical inhibitor or shRNA complements the effects of idelalisib, as a single agent or in combination with carfilzomib, in repressing phosphorylation of 4E-BP1 and the protein level of c-Myc. These results suggest that TGR-1202 is a dual PI3Kδ/CK1ε inhibitor, which may in part explain the clinical activity of TGR-1202 in aggressive lymphoma not found with idelalisib. Targeting CK1ε should become an integral part of therapeutic strategies targeting translation of oncogenes such as c-Myc.
INTRODUCTION

c-Myc is a master transcription factor and one of the most frequently altered genes across a vast array of human cancers including diffuse large B-cell lymphoma (DLBCL)\(^1,2\), and is thus an attractive therapeutic target\(^3\). However, no direct inhibitor of c-Myc has been successfully developed for the treatment of any cancer. The c-Myc protein has a short half-life of less than 30 minutes\(^4\), and the complex secondary structures in the 5’ untranslated region (UTR) of MYC mRNA make its translation highly dependent on the eukaryotic translation initiation factor 4F (eIF4F)\(^5,6\). eIF4F exists as a complex comprised of the eIF4E, eIF4A, and eIF4G subunits. eIF4E can be sequestered by 4E-BP1, which acts as a “brake” for initiation of mRNA translation\(^7\). Hyper-phosphorylation of 4E-BP1, caused by upstream signals such as mTORC1, leads to release of eIF4E from 4E-BP1, assembly of the eIF4F complex, and robust mRNA translation\(^8-10\). In keeping with these data, mTORC1 and dual mTORC1/mTORC2 inhibitors have been found to cause varied degrees of inhibition of 4E-BP1 phosphorylation and translation initiation for tumor promoting genes\(^11-17\). However, the therapeutic effects of mTOR inhibition in c-Myc driven cancer remain poorly understood.

A number of mTORC1 inhibitors have been approved for renal cell cancer, but they demonstrate limited activity in other cancers including DLBCL. The dual mTORC1/mTORC2 inhibitor MLN0128 was recently reported to exhibit no activity in lymphoma\(^14\). These results suggest that most cancers, including lymphoma, likely employ multiple signaling pathways to ensure robust translation, and therefore are able to bypass translational downregulation caused by mTOR inhibitors. As an example,
CK1 epsilon (CK1ε) activates mRNA translation through phosphorylating 4E-BP1 at residues distinct from those responsive to mTOR \(^{18}\). PI3K is also involved in phosphorylating 4E-BP1 independently of mTORC1 \(^{19}\). Furthermore, there is emerging evidence that the proteasome system is involved in the activation of mTORC1 \(^{20,21}\), presumably through regulating the intracellular pool of amino acids \(^{22,23}\). Collectively these data suggest that phosphorylation of 4E-BP1 orchestrates multiple upstream signals required for optimal translation initiation for dynamic proteins in high demand, such as c-Myc. We hypothesized that modulating 4E-BP1 using a multi-targeting approach against PI3K, the proteasome, and CK1ε could be an effective strategy for silencing of c-Myc translation in aggressive c-Myc dependent lymphoma.
METHODS

Additional methods are described in Supplemental Data.

Cell culture and reagents

The cell lines were obtained from ATCC and grown in Iscove Modified Dulbecco Medium with 10% FCS. Carfilzomib, bortezomib, and idelalisib were purchased from Selleck. TGR-1202 was provided by TG Therapeutics.

Cytotoxicity assay

Cytotoxicity was performed on cultured cells using the Cell Titer Glo assay, as previously described ²⁴.

Statistics

*In vitro* studies in cell lines were repeated twice, and those in primary patient samples and mice were done once. All cytotoxicity studies were done with triple replicas. The mean and the standard error of the mean (SEM) were graphed or charted. Synergy was measured by Excess over Bliss values ²⁵. For the *in vivo* studies mice were randomized to different treatment cohorts. Statistical analysis of difference in tumor volume and tumor weight among the groups was evaluated using a one-way ANOVA followed by individual comparisons using LSD (equal variance assumed). All significance testing was done at the $P$ less than .05 level, protecting the family-wise error rate.
RESULTS

_TGR-1202 is a novel selective PI3Kδ inhibitor_

TGR-1202 has the core structure required for targeting PI3Kδ, as circled in Figure 1A. In a cell-free kinase assay based on detection of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), TGR-1202 potently inhibited PI3Kδ with half maximal effective concentration (EC50) of 22.2 nanomolar (nM) (Figure 1B). The EC50 values of TGR-1202 against the other isoforms of PI3K were substantially higher (Figure S1A), confirming TGR-1202 is a PI3Kδ inhibitor with a selectivity comparable to idelalisib, which is a first-in-class approved PI3Kδ inhibitor. Next, human lymphoma and leukemia cell lines known for constitutively activated AKT were treated with TGR-1202 or the vehicle control for 4 hours. TGR-1202 inhibited phosphorylated AKT at Ser473 in a concentration dependent manner (Figure 1C). At 1 micromolar (μM) TGR-1202 reduced the phosphorylation of AKT by 43-87% in these cell lines. In a subcutaneous xenograft model of T-cell acute lymphoblastic leukemia (T-ALL) in NOD/SCID mice using the MOLT-4 cell line, daily oral treatment with TGR-1202 at 150 mg/kg significantly shrank the tumors by day 25 (p < 0.001) (Figure 1D). In select lymphoma cells lines representing DLBCL (LY7), mantle cell lymphoma (MCL, Z-138), and cutaneous T-cell lymphoma (CTCL, H9), TGR-1202 was more active than idelalisib inhibiting the viability of lymphoma cells, based on measurement of ATP in metabolically active and viable cells (Figure 1E). In other lymphoma cell lines, however, TGR-1202 and idelalisib demonstrated a comparably modest anti-lymphoma activity (Figure S1B).
**TGR-1202 and carfilzomib synergistically kill blood cancer cells through disrupting the 4E-BP1-eIF4F-c-Myc axis**

Since PI3Kδ inhibitors as single agents demonstrated only limited activity in models of aggressive lymphoma, we explored whether PI3Kδ inhibitors (TGR-1202 “TG” and idelalisib “Ide”) might synergize with proteasome inhibitors (carfilzomib “Cfz” and bortezomib “Bz”) through synergistic repression of mTOR signaling. Four combination pairs were studied in the DLBCL cell line LY10, including TG + Cfz “TC”, Ide + Cfz “IC”, TG + Bz “TB”, and Ide + Bz “IB”. We adopted the Bliss additivism model \(^{25}\), which has been successfully employed by NCI-DREAM Drug Sensitivity Prediction Challenge, to predict drug : drug synergy. The model first calculates the expected inhibition for two drugs that are assumed to be additive, which is then used to calculate the Excess Over Bliss (EOB) value as an index of synergy. TC was clearly the most synergistic among the 4 combinations as shown by highly positive EOB values in the DLBCL cell line LY10 (**Figure 2A** and **Figure S2A**) and 11 other lymphoma cell lines representing DLBCL (LY1, SUDHL-4, SUDHL-2, and LY7), MCL (Jeko-1, Z138), T-ALL (PF-382, P12), CTCL (H9 & HH), and multiple myeloma (MM) (MM.1s) (**Figure 2B** and **Figure S2B-2C**). TC was highly synergistic in primary lymphoma and leukemia cells isolated fresh from 4 patients with relapsed small lymphocytic lymphoma (SLL), treatment naïve chronic lymphocytic leukemia (CLL), treatment naïve blastoid MCL with p53 deletion, and marginal zone lymphoma (MZL) with chromosome 17p deletion (**Figure 2C** and **Figure S2C**). The TC combination synergistically induced apoptosis, as measured by the cleavage of Poly (ADP-ribose) polymerase (PARP) and activation of caspase 3/7, in those cell lines and primary lymphoma and leukemia cells, while the IB and IC
combinations were less active by these assays (Figure 2D and Figure S2D). TC was not toxic to normal peripheral mononuclear cells (PBMC) cells (Figure S2F).

Next we investigated the above combinations and single agents for their differential effects on mTOR signaling, focusing on the mTORC1 substrate 4E-BP1 and a further downstream event, namely translation of c-Myc. The highly synergistic TC combination, but not IB or single agents, potently inhibited phosphorylation of 4E-BP1 and the protein level of c-Myc in cell lines representing DLBCL (LY10 and LY7), T-ALL (PF382), MM (H929), as well as primary SLL and CLL cells (Figure 3).

The reduction of the c-Myc protein level by TC in the above cell lines and primary lymphoma cells was unlikely due to increased degradation of c-Myc since carfilzomib is a potent proteasome inhibitor, but rather could be due to inhibition of transcription or translation. While the IB, TB, and IC combinations moderately reduced the protein level of c-Myc, TC markedly inhibited the expression of c-Myc in the LY10 cells (Figure 4A). Since none of the above combinations decreased the mRNA level of c-Myc in LY10 or LY7 (Figure 4B), TC most likely reduced c-Myc protein level by inhibiting its translation.

To further prove this hypothesis, we designed a bi-cistronic luciferase reporter (Figure 4C). Translation of renilla luciferase (LucR) is regulated by the 5' UTR of c-MYC and is dependent on eIF4F. In contrast, translation of firefly luciferase (LucF) is not eIF4F dependent as it has the Polio virus internal ribosome entry site (IRES). The relative efficiency of cap dependent translation downstream of the 5' UTR of c-MYC is measured by the ratio of LucR/LucF. The plasmid did transduce LY7 efficiently, but did
not transfect LY10 and a number of other DLBCL cell lines. Figure 4D demonstrates that TC was significantly more potent than IB in LY7 in reducing the R/F Luc ratio (p = 0.0013), confirming c-Myc translation as a mechanistic target of TC.

To confirm that c-Myc downregulation is a mechanistic target of the TC combination, we subcloned the MYC open reading frame without the 5’UTR, into the pcDNA3.1(+)IRES GFP plasmid. As a result translation of exogenous c-Myc is expected to be less dependent on IF4F and less prone to inhibition by 4E-BP1. As shown in Figure 4E, the c-Myc containing plasmid (M+) mildly increased the level of c-Myc protein compared to an empty vector (EV) in the LY7 DLBCL cells not exposed to the TC treatment. When treated with TC the level of the endogenous c-Myc protein was potently reduced in the cells transduced with the empty vector. In contrast, the level of c-Myc remained at a high level in the cells with the Myc+ plasmid, confirming that lack of 5’UTR of MYC in the plasmid conveyed resistance to translational downregulation of MYC by TC. Figure 4F demonstrates that LY7 cells with the Myc+ plasmid was significantly more resistant than those cells with the empty vector to TC. To investigate whether sequestering of eIF4E by hypo-phosphorylated 4E-BP1 was a potential mechanism of action by TC, we subcloned the cDNA of eIF4E into the pCDH-GFP vector. For poorly understood reasons the eIF4E plasmid was toxic to the DLBCL cell line LY7. We therefore stably transduced this plasmid and a control empty vector into the myeloma cell line H929, which has a well characterized chromosomal translocation involving c-Myc. Figure 4G demonstrated that in H929 cells untreated with TC the eIF4E plasmid produced slightly more eIF4E and c-Myc than the empty vector. In H929 cells treated with TC, the
eIF4E plasmid produced substantially more eIF4E and c-Myc proteins than the empty vector. Cells transfected with the eIF4E plasmid were significantly more resistant to TC than cells with the empty vector (Figure 4H). Collectively, these results indicate that the mechanism of the remarkable synergism observed with the TC combination is due to inhibition of 4E-BP1 phosphorylation and consequently downregulation of c-Myc translation.
TGR-1202 and carfilzomib in combination specifically silence the c-Myc transcription program

To investigate the effects of TC on the c-Myc transcription program, gene expression profiling (GEP) was performed by RNA-seq in the DLBCL LY10 cells treated by the vehicle control, TGR-1202, idelalisib, carfilzomib, bortezomib, and the 4 combinations including TC, TB, IC, and IB for 24h. We calculated fold change for each gene and the p-value in the combinations versus each of the single drug exposures. Next, we combined the 2 p-values to calculate a combined Z score, which were used to rank list the genes according to their up- or down-regulation at the transcriptional level by the combinations. We then performed gene set enrichment analysis (GSEA) to evaluate the enrichment of c-Myc target genes using the annotated gene sets in the Molecular Signatures Database (MSigDB). Figure 5A demonstrates that in the LY10 cells treated by TC the Running Enrichment Score (RES) of 4 “canonical” Myc target gene sets (GS52, GS70, GS29, and GS32) reached their peak RES scores at the end of the gene list ranked from most upregulated to most downregulated, with normalized enrichment score (NES) < 0 and false discovery rate (FDR) q-value = 0. In contrast, most unrelated gene sets have higher NES and/or FDR values in the TC treated sample (Figure S3A). We independently validated that 2 of the c-Myc target genes, eIF4B and E2F1, were markedly reduced by the TC combination but not by any single agents or the IB combination in the DLBCL cells LY10 and LY7 (Figure 5B). In agreement, E2F gene sets (GS43, GS38, and GS22) were also enriched among the downregulated genes (NES < 0 and FDR q-val = 0) by TC more than any other combination treatments (Figure S3B). We conducted GSEA on all the Myc and E2F target gene sets, and
found that the number of c-Myc and E2F1 gene sets downregulated by TC was significantly higher than those affected by IB (p = 0.014 for Myc and p < 0.00001 for E2F) (Figure 5C, and Table S1-2). Collectively, the above results demonstrate that TGR-1202 and carfilzomib in combination synergistically and selectively silence the c-Myc and E2F transcription programs.
**TGR-1202 demonstrates activity targeting CK1ε**

To understand why TGR-1202 was consistently superior to idelalisib when combined with carfilzomib, we compared the activity of TGR-1202 with two other PI3Kδ inhibitors, idelalisib and duvelisib/IPI-145, on a panel of 365 wild-type protein kinases using the kinome profiling platform from Reaction Biology (Malvern, PA). The PI3Kδ inhibitors were not active against this panel of protein kinases with one exception: at 1 μM TGR-1202 inhibited 60% of the activity of CK1ε, which was not observed with idelalisib or duvelisib (**Figure 6A** and **Table S3**).

Remarkably, TGR-1202 and the selective CK1ε inhibitor PF4800567 share a similar overall architecture with a central pyrazolopyrimidine amine (CPA) moiety substituted at the same positions 7 and 9 (**Figure 6B**). A previous X-ray crystallography study of PF4800567 has confirmed that the CPA moiety plays a key role in the binding of the inhibitor to CK1ε as it establishes two hydrogen bonds with the hinge region of CK1ε (**Figure S4A**). In this orientation, the chlorobenzen moiety of PF4800567 (substituted at position 7), occupies a hydrophobic pocket deeper in the protein (**Figure 6C**). *In silico* docking of TGR-1202 into the ATP pocket of CK1ε resulted in top scoring (best docking score -9.3) binding modes very consistent with that of PF4800567, with the CPA moiety superposing very well and establishing the exact same hydrogen bonds (**Figure 6C-D**). Importantly, the favorable docking scores obtained for these virtual binding modes reveal that the hydrophobic pocket reached by the chlorobenzen moiety for PF4800567 can favorably accommodate the somewhat larger corresponding moiety
in TGR-1202. In contrast, while idelalisib contains an adenine moiety that is reminiscent of the CPA moiety shared by PF4800567 and TGR-1202, the potential hydrogen bond donors and acceptors are distributed very differently (Figure S4B). Consistently, in silico docking of Idelasilib fails to identify high-scoring binding modes (best docking score -3.8) into the CK1ε ATP pocket.

Based on the above structural insight we synthesized two TGR-1202 analogs, CUX-03173 and CUX-03166 that differ by one extra methyl group on CUX-03166 (Figure S4C). Consistent with its high chemical similarity with TGR-1202, in silico docking of CUX-03173 results in a top-binding pose very close to that of TGR-1202 (Figure S4D-E). In contrast, in silico docking of CUX-03166 in CK1ε results in poses with significantly worse docking scores than CUX-03173 and TGR-1202, as the floor of the ATP-binding pocket leaves insufficient room for the methyl group on CUX-03166.

We experimentally determined the CK1ε inhibiting activity of the above compounds using the ADP-Glo™ Kinase Assay kit and recombinant CK1ε expressed by baculovirus in Sf9 insect cells. PF4800567 was highly potent against CK1ε with an EC50 of 7.4 nanomolar (nM) (Figure 6E and Figure S4F), consistent with the previous report 29. TGR-1202 was active against CK1ε, with an IC50 value of 6.0 μM. The IC50 for CUX-03173 was 9.4 μM. In contrast, idelalisib or CUX-03166 did not reach 50% inhibition even at 50 μM. Next we directly tested the effects of TGR-1202 on intracellular CK1ε by examining its effect on the autophosphorylation of CK1ε carboxyl-terminus regulatory
domain. Autophosphorylation is inhibited or stimulated by CK1ε inhibitors or phosphatase inhibitors such as calyculin A, respectively. In the negative control not treated with any of the tested kinase inhibitors, calyculin A produced time dependent autophosphorylation of CK1ε, as shown by up-shifting and dimming of the CK1ε band in the DLBCL cell line LY7 (Figure 6F). In samples treated by PF4800567 (1 μM), TGR-1202 and CUX-03173 (10-25 μM) the up-shifting of the CK1ε band was delayed and reduced (Figure 6F and Figure S4G). Idelalisib at 25 μM remained inactive by this assay (Figure S4G). These results demonstrate that among the studied PI3Kδ inhibitors TGR-1202 is uniquely characterized with structural features suitable for targeting CK1ε in lymphoma cells.
Targeting of CK1ε is required for effective inhibition of 4E-BP1 phosphorylation and c-Myc translation.

To establish that targeting CK1ε is required for optimal treatment of c-Myc dependent lymphoma, we first compared the effects of the dual PI3Kδ/CK1ε inhibitor TGR-1202, selective PI3Kδ inhibitor idelalisib, and selective CK1ε inhibitor PF4800567 on c-Myc and 4E-BP1. Figure 7A demonstrates that at the concentrations ranging from 15-50 μM, TGR-1202 was more potent than idelalisib and PF4800567 at repressing the expression of c-Myc in the DLBCL cell line LY7. Importantly, lymphoma cells transduced with the c-Myc overexpressing plasmid described in Figure 4E were significantly more resistant to TGR-1202 than the cells with the empty vector (Figure 7B and Figure S5A). To determine whether the reduction of c-Myc protein level by TGR-1202 was due to downregulated c-Myc translation, we performed the reporter assay as described in Figure 4C-D. The ratio of LucR/LucF was reduced by more than 50% by TGR-1202 at 15 μM but not by idelalisib or PF4800567 (p < 0.001) (Figure 7C). Interestingly, combining the selective PI3Kδ inhibitor idelalisib and selective CK1ε inhibitor PF4800567, both at 25 μM, reproduced the potent inhibition of 4E-BP1 phosphorylation and c-Myc protein level caused by the dual PI3Kδ/CK1ε inhibitor TGR-1202 at 25 μM in LY7 cells (Figure 7D). These results indicate that co-targeting PI3Kδ and CK1ε produces synergistic inhibition of 4E-BP1 phosphorylation and Myc translation.

Next we investigated whether knockdown of CK1ε could phenocopy TGR-1202 in the combination of idelalisib and carfilzomib, which was shown to be significantly less
synergistic than TC (Figures 2A & 4A). CK1ε-targeting shRNA reduced the protein level of CK1ε by more than 80%, but had no effect on c-myc, in the DLBCL cell line LY7 (Figure 7E AND Figure S5B). In LY7 cells with wild-type CK1ε, the combination of idelalisib and carfilzomib produced only mild inhibition of phosphorylation of 4E-BP1, as evidenced by a partial downward mobility shift of 4E-BP1. In contrast, in the cells with CK1ε knockdown, the combination of idelalisib and carfilzomib effectively inhibited phosphorylation of 4E-BP1, demonstrated by a complete mobility shift of 4E-BP1 and a substantial decrease in phosphorylated 4E-BP1 at T70. These results indicate that targeting CK1ε complements PI3Kδ inhibition in the setting of combination therapy with carfilzomib.
**TGR-1202 is active in treating DLBCL**

In a phase I clinical trial of TGR-1202 as a single agent (#NCT01767766) partial response was observed in 3 of 14 patients with DLBCL ([Figure 7F-G, and Figure S6A-B](#)). In contrast, earlier studies of idelalisib did not observe any responses in 9 patients with DLBCL \(^{34}\). Furthermore, TGR-1202 was associated with very limited (2%) grade 3/4 diarrhea, compared to 13% seen with idelalisib in patients with similar clinical parameters \(^{35}\). These results suggest that dual targeting of PI3Kδ and CK1ε by TGR-1202 may explain in part the preliminary activity of TGR-1202 in treating DLBCL and its distinctly favorable adverse event profile.
DISCUSSION

For many years c-Myc has been recognized as an “undruggable” target. More recently BRD4 inhibitors have shown promise as a c-Myc targeting therapy \textsuperscript{36}, with a few of them having entered phase I clinical studies. However, their safety and efficacy remain to be proven. In the current work we aimed to explore new therapeutic strategies for targeting c-Myc that could be rapidly validated in clinical studies. To that end we have demonstrated that the combination of TGR-1202 and carfilzomib, inhibitors of PI3K\(\delta\) and proteasome respectively, potently disrupts the 4E-BP1-eIF4F-c-Myc axis, leading to anti-tumor effects of TGR-1202 and carfilzomib in cell lines and primary cells representing broad subtypes of B- and T-cell lymphoma, leukemia, and multiple myeloma. The unique synergism of TGR-1202 and carfilzomib is driven in part by the unique pharmacologic activities of TGR-1202 reported in the current study.

Similar to the FDA approved drug idelalisib \textsuperscript{26}, TGR-1202 selectively inhibits PI3K\(\delta\). Interestingly, our results indicate that TGR-1202 is more effective than idelalisib in disrupting the 4E-BP1-eIF4F-c-Myc axis and inducing cell death in lymphoma cells, both as a single agent and in combination with proteasome inhibitors. In agreement with these preclinical data, TGR-1202 has demonstrated clinical activity in DLBCL, whereas other PI3K\(\delta\) inhibitors failed \textsuperscript{35}. TGR-1202 possesses the unique capability to inhibit CK1\(\varepsilon\) (Figure 6), which distinguishes it from idelalisib. Collectively, these results support the hypothesis that co-targeting multiple regulators of 4E-BP1, including the PI3K-AKT-mTOR pathway and CK1\(\varepsilon\) (as discussed below), is required for optimal suppression of translation initiation.
Previous studies in breast cancer cell lines and mouse xenografts have demonstrated that CK1ε plays an important role in promoting cancer cell proliferation by regulating phosphorylation of 4E-BP1 and initiation of mRNA translation. However, no CK1ε inhibitor is currently available for clinical study. The selective CK1ε inhibitor PF4800567 was very potent in the cell free kinase assay, with an EC50 of 7.4 nM (Figure 6E). Surprisingly, very high concentrations of PF4800567, in the range of 25-50 μM, were required to inhibit phosphorylation of 4E-BP1 (Figure 7). The reason of such discrepancy is not understood. Interestingly autophosphorylation of CK1ε at its C-terminus inhibits the kinase activity of CK1ε. Conversely, it is possible that by potently inhibiting autophosphorylation PF4800567 may somehow self-limit the inhibition of CK1ε.

*In silico* docking studies demonstrate that TGR-1202 binds the ATP pocket of CK1ε very well (Figure 6C-D). However, we do not yet have insights into how TGR-1202 interacts with CK1ε outside the ATP pocket, which may be relevant to the relatively low potency of TGR-1202 with an EC50 of 6.0 μM in the cell free kinase assay. Nevertheless, the drug TGR-1202 effectively inhibited autophosphorylation of CK1ε and phosphorylation of 4E-BP1 at 15 μM in lymphoma cells (Figure 6-7). We speculate that the two structural modules of TGR-1202, one for targeting PI3Kδ and one for CK1ε, may be actually advantageous for blocking the kinase domain of CK1ε. When administered at 1200 mg once daily in the phase I clinical study (#NCT01767766), TGR-1202 is very well tolerated and produced a maximal plasma concentration (Cmax) of 7700 ng/ml and
steady state concentration of 5200 ng/ml \(^{37}\), corresponding to 13.5 and 9.1 \(\mu M\). These results suggest that TGR-1202 is a first-in-class dual PI3K\(\delta\)/CK1\(\epsilon\) inhibitor that may be adequate for targeting CK1\(\epsilon\) in patients, especially in combination with carfilzomib.

Our results have a few clinical ramifications. The structural insights gained from TGR-1202 and CUX-03173 using \textit{in silico} docking and from future crystallography studies may allow for further optimization of TGR-1202 and development of next-generation CK1\(\epsilon\) inhibitors for cancer treatment. The combination of TGR-1202 and carfilzomib may represent a promising treatment for select patients with lymphoma that is characterized by c-Myc overexpression due to deregulated translation of c-Myc. A phase I/II study (NCT02867618) evaluating this regimen has been approved by the Institutional Review Board and is open to accrual. As TGR-1202 is not associated with frequent colitis and pneumonitis \(^{38}\), which have been reported for idelasib \(^{35}\), TGR-1202 will likely be well tolerated in combination with carfilzomib. Correlative studies of CK1\(\epsilon\), mTOR, 4E-BP1, and c-Myc in tumor samples before and after treatment are expected to provide insights into prognostic and predictive biomarkers for the treatment. Finally, if the combination of TGR-1202 and carfilzomib or TGR-1202 as a single agent is able to effectively turn off c-Myc, potentially these treatments can be administered briefly and immediately before standard chemotherapy regimens as a strategy to enhance the cure potential and response rates of chemotherapy.
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AUTHOR CONTRIBUTIONS

CD, MML, LS, XOJS, MAM, SL, JV, YH, XX, RBR designed and performed the experiments, and interpreted the results. NPT, CK, SL, DAF, BH, DWL, and OAO designed the experiments and provided advice. CD wrote the manuscript, DAF, DWL and OAO edited the manuscript.
CONFLICT OF INTEREST DISCLOSURES

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FIGURE LEGENDS

Figure 1. The PI3Kδ inhibitor TGR-1202 is active in lymphoma models. (A) The structural formulae of TGR-1202 and Idelalisib with the active quinazolinone moieties circled. (B) Cell-free in vitro kinase assay of PI3Kδ in the presence of TGR-1202. (C) Cell-based assay measuring inhibition of S473 p-AKT in leukemia and lymphoma cell lines treated for 4h. (D) Response of the subcutaneous xenograft model of T-ALL to 3 treatments, including vehicle control and TGR-1202 (150 mg/kg) over 25 days. The xenograft was derived from the MOLT-4 cell line in NOD/SCID mice. P values were < 0.001 between the treatment and control groups on day 25. (E) LY7, Z-138, and H9 cells were treated by idelalisib and TGR-1202 for 24h (LY7) and 48h (Z-138 and H9) then viability was measured by Cell Titer Glo.

Figure 2. TGR-1202 and carfilzomib synergistically inhibit survival of lymphoma and leukemia cell lines and primary cells. The following drugs were studied: TG: TGR-1202, Ide: idelalisib, Bz: bortezomib, Cfz: carfilzomib, IB: Ide + Bz, TC: TG + Cfz. (A) Excess over Bliss (EOB) values calculated for 4 combinations of treatment in the DLBCL cell line LY10. Cells were treated for 24h with the indicated drugs and concentrations as single agents and in combinations. Viable cells were quantitated by the Cell-Titer Glo assay (Promega). EOB values above 0 indicate synergy. (B) Cell lines representing different hematological malignancies were treated for 48h. The y- and x- axes indicated the observed and expected percentage of inhibition, respectively. The expected inhibition was calculated using the Bliss model. The diagonal line indicates the line of additivism. Synergy was demonstrated by observed inhibition in excess of the
expected inhibition. (C) Primary lymphoma and leukemia cells were isolated by Ficoll
gradient separation from three patients with SLL, CLL, MCL respectively. The SLL cells
were from pleural fluid, and the CLL and MCL cells were from peripheral blood. The
upper panel depicts the results combining Ide and Bz, and the lower panel TG and Cfz.
Ide and TG were given at 2.5, 5, and 7.5 μM, and their effects on viability were
presented by the unconnected “x” markers. All the other treatments were as indicated
on the graphs. Viability was determined after 48h of treatment. (D) LY10 and LY7 cell
lines were treated as indicated for 24h and processed for Western blot. Neg: Negative
control. For LY7, TG and Ide were at 3 μM, and Bz and Cfz at 5 nM; for LY10, TG and
Ide were at 3 μM, and Bz and Cfz at 2 nM.

Figure 3. **TGR-1202 and carfilzomib synergistically disrupt the 4E-BP1-eIF4F-c-Myc axis.** Cell lines, including LY10, LY7, PF382, and H929, and primary cancer cells
from patients with SLL and CLL were treated by TG (TGR-1202), Ide (idelalisib), Cfz
(carfilzomib), Bz (bortezomib), and their combinations as indicated for 24h. IB: Ide+ Bz,
TC: TG+Cfz, IC: Ide+TG. In the H929 cell line, TC-1 and TC-2 indicate TGR at 5 μM in
combination with carfilzomib at 6 and 7 nM, respectively. Cell were harvested and
processed for Western blot analysis using the antibodies against 4E-BP1, c-Myc, beta-
actin, and GAPDH.

Figure 4. **TGR-1202 and carfilzomib synergistically inhibits translation of c-Myc in
lymphoma and myeloma cell lines.** The following drugs were used: TG: TGR-1202,
Ide: idelalisib, Bz: bortezomib, Cfz: carfilzomib, IB: Ide + Bz, TB: TG + Bz, IC: Ide + Cfz,
TC: TG + Cfz. **(A-B)** Levels of c-Myc protein (A) and mRNA (B) in LY10 and LY7 cells treated as indicated for 24h. For LY10, TG and Ide were at 3 μM and Bz and Cfz at 2 nM; For LY7, TG and Ide were at 3 μM and Bz and Cfz at 5 nM. **(C)** Schema of a bicistronic luciferase reporter for the translation of c-Myc. UTR: untranslated region of c-Myc, IRES: internal ribosome entry site of polio virus, Luc-R: renilla luciferase, Luc-F: firefly luciferase. **(D)** Results of the luciferase assay using the bicistronic reporter from (C). LY7 stably expressing the reporter was treated as indicated for 24h. IB-1 and IB-2: Ide 3 μM and 5 μM respectively plus Bz 5 nM; TC-1 and TC-2: TG 3 μM and 5 μM respectively plus Cfz 5 nM. R/F luc ratio from the treatment groups was calculated as a percentage of the untreated control, and represents the efficiency of eIF4F cap-dependent translation regulated at the endogenous 5’UTR of c-Myc. The difference between the TC2 and IB2 treatments was statistically significant, with ** indicating the p-value of 0.0013. **(E-F)** LY7 cells stably transfected with a c-Myc expressing plasmid (M+) or an empty vector (EV) were treated for 24hr as indicated. The cDNA of c-Myc does not contain the endogenous 5’UTR. TC-1 and TC-2: TG 3 μM and 5 μM respectively plus Cfz 5 nM. Cells were then process for Western blot (E) or Cell Titer Glo to determine viability (F). The difference of viability between the M+ and EV samples was statistically significant, with ** indicating the p-value < 0.001. **(G-H)** The Myeloma cell line H929 was stably transduced with an eIF4E-overexpressing plasmid (eIF4E) by lentiviral transduction, or with the corresponding empty vector (EV). These cells and the untransduced control (No TDX) cells were treated for 24hr and assessed by Western blot (G) and Cell-Titer Glo (H). The difference between the eIF4E and EV samples was statistically significant, with ** indicating the p-value < 0.001.
Figure 5. TGR-1202 and carfilzomib inhibits c-Myc dependent gene transcription. (A) Gene set enrichment analysis of c-Myc target genes in LY10 cells treated by the TC combination. GS52: Schuhmacher_Myc_Targets; GS70: Dang_Regulated_By_Myc, GS29: Schlosser_Myc_Targets; GS32: Kim_Myc_Targets. The X-axis represents the listed genes ranked from most upregulated to most downregulated. The Y-axis indicates running enrichment scores (RES). All the 4 gene sets reached their peak RES score at the end of ranked gene list, indicating that the drug combination of TG and Cfz exhibits a negative effect on the transcription of c-Myc targets. (B) LY10 and LY7 cells were treated as indicated for 24h then processed for Western blot against two known targets of c-Myc, namely eIF4B and E2F1. (C) Chi² test was performed to compare Myc and E2F1 gene sets enriched and not-enriched for transcriptional downregulation in cells treated by the TC and IB drug combinations.

Figure 6. TGR-1202 acts as an inhibitor of CK1ε. (A) Excerpt of kinome profiling data, showing the activity of various casein kinases when treated by three PI3Kδ inhibitors at the same condition of 1 μM, including TGR-1202 (TG), idelalisib (Ide), and duvelisib (Duv). The details are listed in Supplemental Table 3. (B) Structural formulae of PF-4800567 and TGR-1202 with the central pyrazolopyrimidine amine moiety circled, and central ring atoms numbered. The arrows denote the positions involved as hydrogen bonds donor (amine group) and acceptor (position 1). (C) Comparison of the co-crystallization of PF-4800567 and CK1ε to in silico docking of TGR-1202 in the active site binding pocket. (D) Interaction map of TGR-1202 with the active site amino acids of CK1ε with figure legend at the bottom. (E) Cell-free kinase activity assay of
CK1ε measuring dose-activity curves of PF-4800567, TGR-1202, Idelalisib, CUX-03173, and CUX-03166. EC50 and $R^2$ values are listed in Supplemental Figure 4F. (F) Cell-based autophosphorylation assay of CK1ε C-terminus. LY7 Cells were pre-treated for 1h with the indicated drugs and then treated with the PP2A inhibitor Calyculin-A (Caly A) for 0, 15, 30, and 60 minutes when lysates were collected and Western blots were performed. PF: PF4800567, TG: TGR-1202, CUX: CUX-03173.

**Figure 7.** Targeting of CK1ε is required for inhibition of 4E-BP1 phosphorylation, silencing of c-Myc translation, and likely clinical activity in aggressive lymphoma.

The following drugs were used: Ide: idelalisib, TG: TGR-1202, PF: PF4800567, Cfz: carfilzomib. (A) Western blot analysis of LY7 cells treated with vehicle control (c-), and Ide, TG, and PF at 15, 25, 50 μM for 24h. (B) LY7 cells stably transfected with a c-Myc overexpressing plasmid (M+) or an empty vector (EV) were treated at the indicated concentrations of TG for 24hr, then processed for Cell Titer Glo to determine viability. The parental LY7 cell without plasmid transfection (NoP) served as an additional control. The protein levels of c-Myc in these samples were demonstrated in Supplemental Figure 5A. (C) LY7 cells stably expressing the bi-cistronic reporter described in Figure 4C were treated with the indicated drugs for 24h. R/F luc ratios from the treatment groups were calculated as a percentage of the untreated control, and represented the efficiency of eIF4F cap-dependent translation regulated at the endogenous 5’UTR of c-Myc. (D) Western blot analysis of LY7 cells treated by various singles agents and combinations for 24h. The numbers in the combinations indicated the drug concentrations in μM. (E) Effects of CK1ε knockdown on the combination of
Ide+Cfz. LY7 cells stably expressing the CK1ε targeting shRNA (shRNA+) or the parental untransduced control cells (shRNA-) were treated as indicated for 24h and assessed by Western blot. (F) Upper panel: Response to TGR-1202 as a single agent in all 14 patients with DLBCL enrolled in a phase I clinical study. The results of idelalisib were extracted from the publication by Westin (Clin Lymphoma Myeloma Leuk 2014). Lower panel: Frequency of diarrhea in all 81 patients taking TGR-1202 in the phase I study. The results of idelalisib were extracted from publication by Gopal (N Engl J Med. 2014). (G) Pre- and post-treatment images of X-ray computed tomography from two DLBCL patients treated with TGR-1202.
Figure 1

(A) TGR-1202

(B) % Inhibition

EC\textsubscript{50} = 22.2

(C) Phospho-AKT (Ser473)

(D) MOLT-4 Xenograft

(E) Viability (%)

LY7 (DLBCL)

Z-138 (MCL)

H9 (CTCL)

Treatment (\mu M)

Phospho-AKT (Ser473)
Figure 2

(A) 

(B) 

(C) 

(D) 

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Running Enrichment Score (RES)

GS52: Schuhmacher
NES q-val
-7.70 0.00

GS70: Dang
NES q-val
-6.56 0.00

GS29: Schlosser
NES q-val
-5.98 0.00

GS32: Kim
NES q-val
-6.38 0.00

(B) Control TG-3uM Ide-3uM Bz-2nM Ctz-2nM IB TC

eIF4B
E2F1
C-Myc
B-Actin

(C) Myc gene set

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E2F gene set

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Figure 6

**Central pyrazolo-pyrimidine moiety**

(C) PF48000557 (X-Ray)
TGR-1202 (In silico docking)

(D)

(E)

(F)

![Image](image_url)
Figure 7

(A) Western blots showing c-Myc and B-Actin levels in different conditions.

(B) Graph showing viability (% of control) for different concentrations of TG and Ide.

(C) Graph showing translation of c-Myc (% of control) for different concentrations of Ide and TG.

(D) Western blots showing 4EBP1, *S65, *T70, c-Myc, and B-Actin levels under different conditions.

(E) Table showing the effects of Ide and Cbz on 4EBP1 and shRNA.

(F) Table showing the response in DLBCL and Diarrhea for different conditions.

(G) CT scans showing patient #008 and patient #014 before and after treatment.
Silencing c-Myc translation as a therapeutic strategy through targeting PI3K delta and CK1 epsilon in hematological malignancies

Changchun Deng, Mark R. Lipstein, Luigi Scotto, Xavier O. Jirau Serrano, Michael A. Mangone, Shirong Li, Jeremie Vendome, Yun Hao, Xiaoming Xu, Shi-Xian Deng, Ronald B. Realubit, Nicholas P. Tatonetti, Charles Karan, Suzanne Lentzsch, David A. Fruman, Barry Honig, Donald W. Landry and Owen A. O'Connor