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Supplementary Information

Pharmacological profiling and cytotoxicity assays

CLL cells were isolated from peripheral blood samples that were obtained pre and post acalabrutinib therapy. These lymphocytes were suspended in medium and were incubated with either DMSO, or with indicated concentrations of venetoclax, bendamustine, carfilzomib, fludarabine, duvelisib, acalabrutinib, and ACP-319 (PI3K delta inhibitor) for 24 hrs. Concentrations of individual drugs were selected based on plasma pharmacology of the agents. Higher concentrations were used for drugs that bind to plasma proteins (acalabrutinib, duvelisib, ACP-319). After incubations, cells were stained with Annexin V and propidium iodide and counted using flow cytometry, as described.(1)

BTK Target Occupancy ELISA

Blood samples were collected pre-treatment on day 1, day 2, and day 8 and 4 h after dosing on day 1 and day 8. PBMC pellets for all time points were lysed in 100 µL (for 5 x 10^6 cells) ice cold lysis buffer (50 mM Trizma Hydrochloride, 250 mM sucrose, 5 mM MgCl2, 1 mM DTT, 0.05% digitonin, 1 X protease inhibitor cocktail) and samples were prepared in triplicate (1.5 x 10^6 cells). For each time point, the cell lysate was incubated for 1 hour in the presence or absence of acalabrutinib (1 µM). The incubation of the cell lysate with an excess of acalabrutinib is used to correct for background signal not related to free BTK. After the incubation with or without acalabrutinib, the cell lysates were incubated with the BTK target occupancy probe ACP-4016 for 1 hour before being plated (5 x 10^5 cells/well) onto an anti-BTK antibody coated plate. Two hour incubation was followed by a 1 hour incubation with

streptavidin horseradish peroxidase (strep-HRP) to allow binding between strep HRP and the probe ACP-4016. Substrate that reacts with the bound strep-HRP was added to allow for measurement of luminescence.

The percentage of BTK protein bound by acalabrutinib was calculated using the difference in the luminescence signal of the samples incubated with or without exogenous acalabrutinib at a saturating concentration (1 µM), and is expressed as a percentage of the luminescence signal in the predose sample. As such, the signal of the predose sample represents 100% free BTK (0% occupied BTK), while the predose sample incubated with 1µM exogenous acalabrutinib represents 0% free BTK (100% occupied BTK). Only subjects with a signal to noise ≥5 (dynamic range) for the Day 1 pre sample were included in the data analysis, because lower dynamic ranges result in high assay variance.

Preclinical studies in the TCL1-192 mouse model

All mouse studies were approved by The University of Texas MD Anderson Cancer Center's Institutional Animal care and Use Committee. The CLL TCL1-192 adoptive transfer mouse model was previously described (2).

In the first experiment (**Figure 7**), on day 0, 40 SCID mice (Taconic Biosciences, Inc. Hudson, NY) received 5.10^6 TCL-192 leukemic cells by retro-orbital injection. Following injection of leukemic cells, the mice were separated randomly in 4 groups (n=10 per group) and stated to be treated on day 19 and day 26 as indicated in schema. Venetoclax (100 mg/kg formulated in 2-phosal 50 propylene glycol, polyethylene glycol 400 and ethanol (60:30:10)) and acalabrutinib (15mg/kg formulated in 0.5% methylcellulose) were administrated daily by oral gavage.

Peripheral white blood cell differential counts were assessed in each group to evaluate leukemic progression before and during treatment. Mice were followed for survival except that at day 35, 3 mice from each groups were sacrificed for histopathology analysis.

In the second experiment (**Figure 8**), on day 0, 90 SCID mice received 5.10^6 TCL-192 leukemic cells by retro-orbital injection and then were separated randomly in 6 groups (n=15 per group). Treatment started at day 7 and/or day 14 with the designated agents as indicated in schema. Treatment scheduling and dosage were similar to that indicated for the first experiment. Mice were followed for survival except that at day 35, 5 mice from each groups were sacrificed for necropsy analysis.

References used for Supplementary Information

1. Balakrishnan K, Burger JA, Fu M, Doifode T, Wierda WG, Gandhi V. Regulation of Mcl-1 expression in context to bone marrow stromal microenvironment in chronic lymphocytic leukemia. Neoplasia. 2014;16(12):1036-46.

2. Chen SS, Batliwalla F, Holodick NE, Yan XJ, Yancopoulos S, Croce CM, et al. Autoantigen can promote progression to a more aggressive TCL1 leukemia by selecting variants with enhanced B-cell receptor signaling. Proc Natl Acad Sci U S A. 2013;110(16):E1500-7.