A novel combination regimen of BET and FLT3 inhibition for FLT3-ITD acute myeloid leukemia

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 doi:10.3324/haematol.2020.247346

Received: January 16, 2020.

Accepted: November 9, 2020.

Pre-published: January 28, 2021.

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Supplementary Methods

Primary stromal culture

Culture flasks and plates for stromal culture were coated with 0.1% gelatin (Sigma Aldrich, St. Louis, MO) in PBS and incubated at 37ºC for 30 minutes prior to plating stromal cells. Total mononuclear cells were harvested from bone marrow aspirates from healthy donors and then plated onto gelatinized T75 flasks with FMBD1 medium (consisting of Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% horse serum (Sigma-Aldrich, St. Louis, MO), 10% FBS, 10^{-5} M hydrocortisone 21-hemisuccinate (Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin, and 0.1 mM β-mercaptoethanol).{Breems, 1994 #1569} After 72 hours of incubating the total mononuclear cells at 37ºC, non-stromal cells in suspension were removed and replaced with fresh FBMD1 medium. The attached stromal cells in the flasks were incubated at 37ºC until the cells had expanded into a confluent monolayer. Stromal cells were passaged when confluency was reached and re-plated at a 1:2 ratio on gelatinized T175 flasks.

Stromal and leukemic cell co-culture

Stromal cells were attached to cell culture plates at least 24 hours prior to co-culture. Stroma was detached from flasks using 0.25% trypsin-EDTA solution (Sigma Aldrich, St. Louis, MO) and re-suspended in fresh FBMD1 medium. Stromal cells were then added onto gelatinized cell culture plates at specific concentrations depending on the type of plate. For 96 well plates, stroma was plated at a concentration of 3,000 cells/well. For 12 well plates, stroma concentration was 30,000 cells/well. After 24 hours, stroma had attached and the FBMD1 medium was

removed. From this point forward, the co-cultures were incubated with RPMI with 10% FBS, penicillin/streptomycin, and L-glutamine.

RNA sequencing

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Messenger RNA (mRNA) was enriched using oligo(dT) beads and then randomly fragmented by adding fragmentation buffer. First-strand cDNA was synthesized using random hexamer primers followed by addition of a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I to initiate the second-strand synthesis. Double-strand cDNA molecules were end-repaired, followed by adenine ligation and then sequencing adaptor ligation. The double-stranded cDNA libraries were size-selected and then PCR-enriched. Libraries were sequenced on Illumina sequencers, generating an average of 30 million, cleaned, 150 base read pairs. The reads were aligned to GRCh38 using STAR; then aligned reads were counted on the gene-level using the rsem-calculate-expression function in the RSEM package.{Li, 2011 #1575;Dobin, 2013 #1574} The transcripts with count per million (CPM) value less than 1 in over 80% of all samples were filtered out. Counts were normalized with the TMM method (edgeR::calcNormFactors), heteroscedasticity removed (limma::voom), and fit to linear models (limma::lmfit); differential gene expression was tested with empirical Bayes (limma::eBayes).{Robinson, 2010 #1593;Ritchie, 2015 #1576} P values were adjusted globally using the topTable function (limma::topTable). Gene set enrichment analyses were performed using camera function (limma::camera). Upstream regulator analyses were performed using ingenuity pathway analysis software (IPA) (Qiagen, Hilden, Germany).

Quantitative PCR

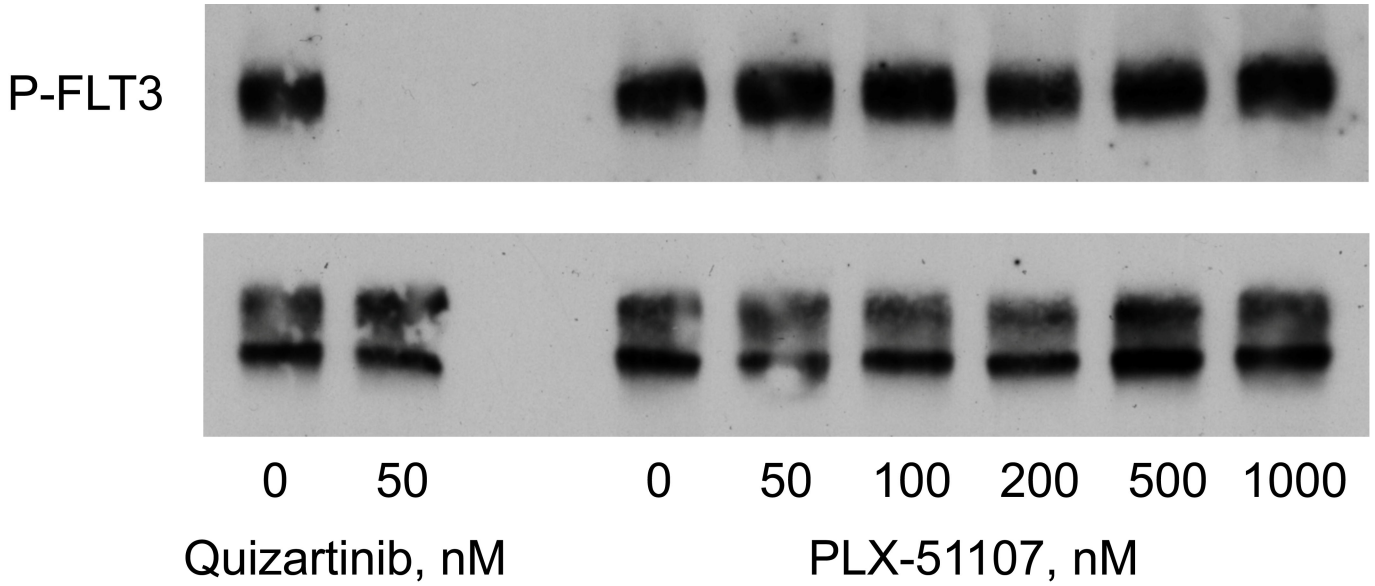
RNA was isolated from 10 million blasts per primary AML sample. The Taqman GAPDH primer (Hs02758991_g) was purchased from Thermo Fisher Scientific (Waltham, MA). The primers for MYC were designed accordingly:

Myc-F: 5' CTGGTGCTCCATGAGGAGA-3'

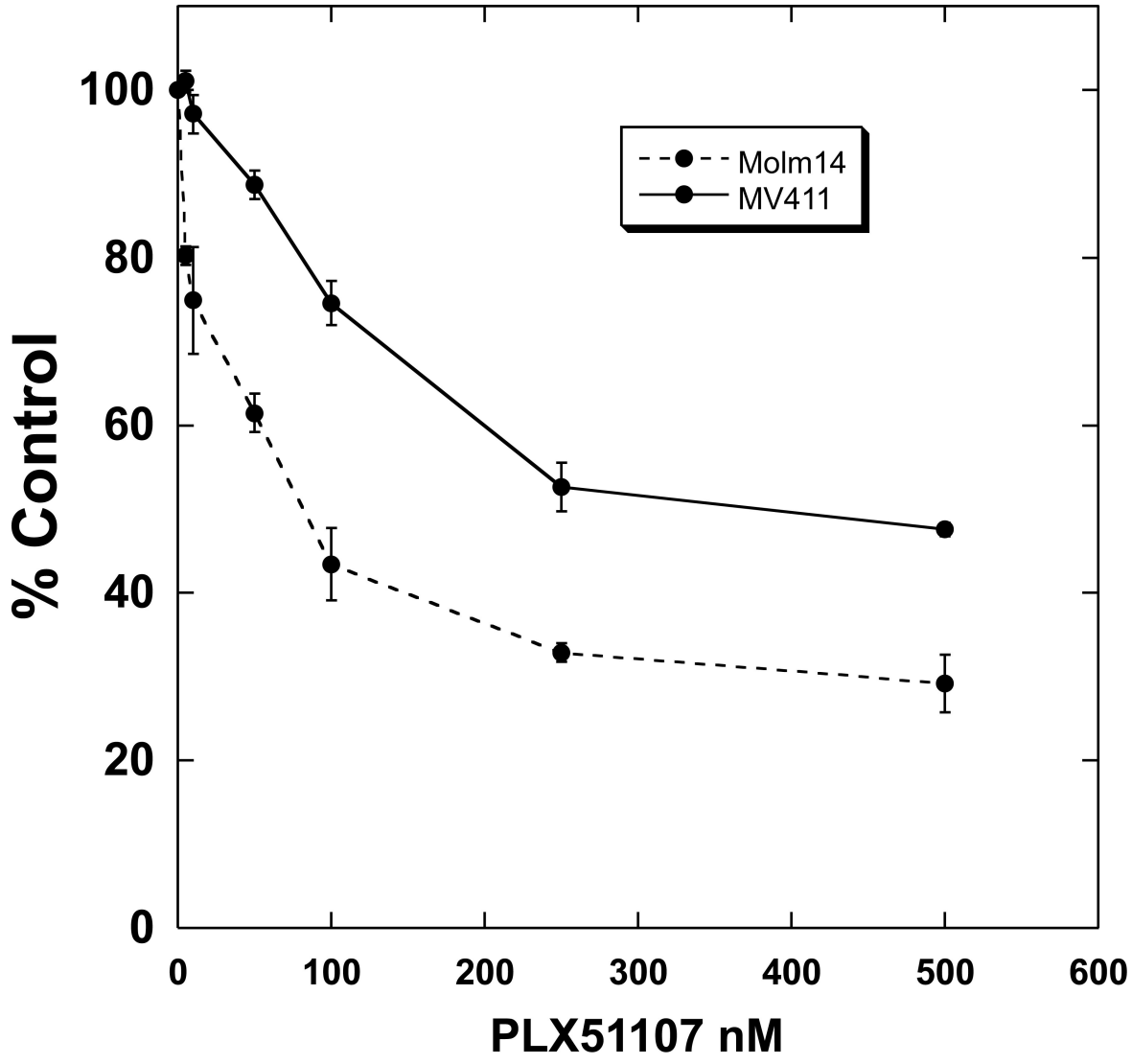
Myc-R: 5' CCTGCCTCTTTTCCACAGAA-3'

Xenograft studies

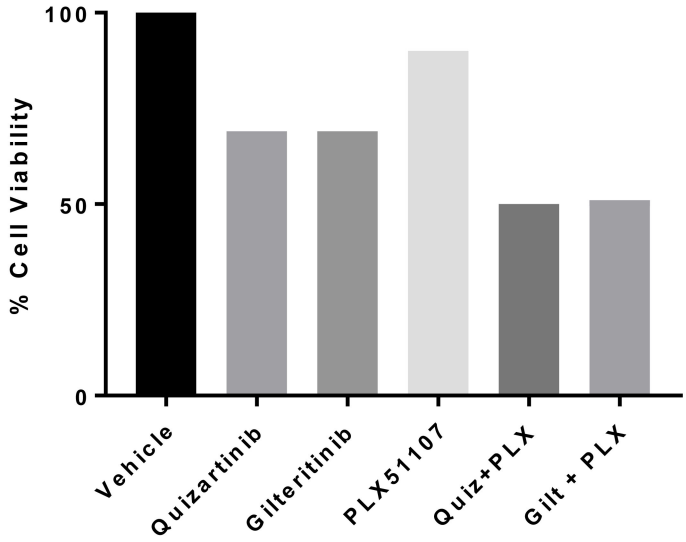
MV4-11 cells were implanted subcutaneously in the flank of male nude mice using matrigel as an adsorbing matrix. Tumor volumes and body weights were measured three times per week throughout the studies. Subcutaneous tumors were allowed to reach tumor volumes > 100 mm^3 and then randomized to different treatments. Tumors were measured with calipers and volumes were calculated with the formula Length x Width x Height /2. Once these tumors had been established to a size of > 100 mm^3, oral dosing with 1 or 5 mg/kg quizartinib, 10, 20 or 40 mg/kg PLX51107 or combination was initiated and continued for up to 25 days.



Supplemental Figure 1. PLX51107 does not inhibit FLT3 autophosphorylation. Molm14 cells were incubated with increasing concentrations of PLX51107 for 1 hour. Cells were then lysed, immunoprecipitated with anti-FLT3 antibody (S-18) and after electrophoresis and membrane transfer, blots were probed with anti-phosphotyrosine ("P-FLT3") and then stripped and re-probed with anti-FLT3. As a control, cells were exposed to 50 nM quizartinib and analyzed in the same manner.



Supplemental Figure 2. Cytotoxic effect of PLX51107 against FLT3-ITD cell lines. FLT3-ITD-mutated cell Molm14 and MV411 were incubated in triplicate wells for 48 hours with increasing concentrations of PLX51107. Viability was determined using the MTT assay, as described in Methods. Error bars depict standard deviation of 3 experiments.



Supplemental Figure 3. Quizartinib compared with gilteritinib in combination with PLX51107. Primary cells from a patient with relapsed FLT3-ITD AML were co-cultured with mesenchymal stromal cells and the effects of 50 nM quizartinib, 100 nM gilteritinib, 250 nM PLX51107, or the combinations, on cell viability were determined in triplicate wells using the MTT assay as described in Methods.