Pharmacological modulation of CXCR4 cooperates with BET bromodomain inhibition in diffuse large B-cell lymphoma

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SUPPLEMENTAL MATERIALS AND METHODS

Patient information

Patient cohort was constituted of 27 females and 25 males (median age 61.5 years) diagnosed between 2000 and 2011 with *de novo* DLBCL, homogeneously treated with rituximab-based chemotherapy (R-CT) regimens and with available follow-up data. All the tumors were classified as DLBCL according to the current WHO classification. After a median follow-up of 9.6 years for surviving patients, 15 patients had died, with a 5-year overall survival (OS) of 73% (95% confidence interval: 67.6%-79.2%). Median progression-free survival (PFS) was 2.75 years, with a 5 year PFS of 70.3% (95% confidence interval: 63.7%-76.9%). Patients with previous indolent lymphoma, immunodeficiency-associated lymphomas, post-transplant lymphoproliferative disorders, intravascular, central nervous system, primary effusion lymphomas and primary mediastinal lymphomas were excluded from the study.

Fluorescence-based cytokine arrays

Total protein extracts were obtained from five 15 μm thin slices for each frozen tumor tissue, using Raybiotech's lysis Buffer complemented with protease inhibitor and according to supplier's instructions. Hematoxylin and eosin staining was performed previously for each case to check tissue representativeness. Ten μg proteins were then quantified by Bradford protein assay (Bio-Rad) and subjected to a Human Cytokine Antibody (Array G Series 2000, Raybiotech) allowing the simultaneous analysis of 175 cytokines. Each cytokine was included in the array per duplicate. Incubation with arrayed antibody support, biotinylated primary antibodies and fluorescence-labeled streptavidin secondary antibodies was performed as per manufacturer´s instructions. Fluorescence detection and signal quantification were performed at 532 nm on an Axon GenePix ® laser scanner. Data were normalized using the 'vsn' package of Bioconductor.

Western blot antibodies

PVDF or nitrocellulose membranes were incubated with anti-phospho-Erk1/2, anti-Erk 1/2, anti-phospho-Akt, anti-Akt, anti-phospho-GSK3β, anti-MYC (Cell Signaling Technology), anti-CXCR4 (ProSci), anti-α-tubulin and β-actin (Sigma-Aldrich) antibodies, followed by species-matched secondary horseradish peroxidase (HRP)-labeled antibodies (Cell Signalling Technology).

CXCR4 activity assay

CXCR4-dependent modulation of intracellular cAMP was evaluated at DiscoveRx. Briefly, CHO-K1 cells were exposed for 1 hour to recombinant CXCL12 at the EC_{50} dose, with or without a 30 min pre-treatment with the different compounds, and intracellular cAMP was quantified with the HitHunter cAMP XS+ assay using forskolin as a positive control of GPCR inhibition. Chemiluminescent signal was detected on a Perkin Elmer Envision instrument. Percentage inhibition was calculated using the following formula: % Inhibition = 100% x (mean relative luminescence unit (RLU) of test sample - mean RLU of EC80 control) / (mean RLU of forskolinpositive control -mean RLU of EC80 control).

Docking study

The interaction mechanism with CXCR4 was predicted by molecular docking using Autodock software. IQS-01.01RS and AMD3100 molecular structures were generated and geometry optimized by using MOE software (Chemical Computing Group). CXCL12 and CXCR4 structures were obtained from the Protein Data Bank (accession numbers 2N55 and 3OE6 correspondingly). Blind docking was conducted including the whole receptor into the grid box. The best interaction conformation was found in the binding pocket.

RNA isolation and real-time PCR

Total RNA was extracted using TRIZOL (Thermo Fisher) following manufacturer's instructions. One microgram of RNA was retrotranscribed to complementary DNA using moloney murine leukemia virus reverse transcriptase (Thermo Fisher) and random hexamer primers (Roche). mRNA expression was analyzed in duplicate by quantitative real-time PCR on the Step one system by using predesigned Assay-on-Demand primers and probes (Thermo Fisher). The relative expression of each gene was quantified by the comparative cycle threshold method ($\Delta\Delta C_t$). β-actin was used as an endogenous control.

SUPPLEMENTAL TABLE

Supplemental Table S1.- Clinical features of DLBCL patients

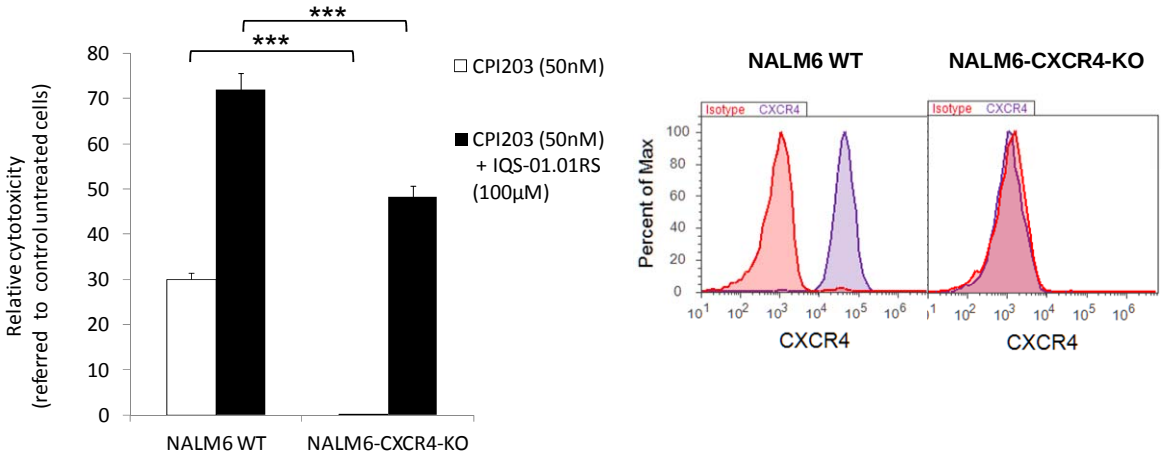
Main clinical features	N=52
Sex (Male/Female)	25/27
Median age (range)	61.5 (22-91)
Ann Arbor stage	
I-II	38.5 % (20)
III-IV	61.5 % (32)
Extranodal sites	
0,1	67.3% (35)
≥2	32.7% (17)
IPI score	
0-1: Low risk	30.8% (16)
2-3: Intermediate risk	34.6% (18)
4-5: High risk	28.8% (15)
data not available	5.8% (3)
DLBCL subtype	
GCB	51.9% (27)
non-GCB	40.4% (21)
n.d.	7.7% (4)
BM involvement at diagnosis	
positive	13.5% (7)
negative	78.8% (41)
n.d.	7.7% (4)
MVD	
high	53.8% (28)
low	25% (13)
n.d.	21.1% (11)
Treatment	
R-CHOP	88.5% (46)
R-CHOP-like	11.8% (6)
R-COP	3
R-trophosphamide	3

Abbreviations: IPI, International Prognostic Index; n.d., not determined; BM, bone marrow; MVD, microvascular density.

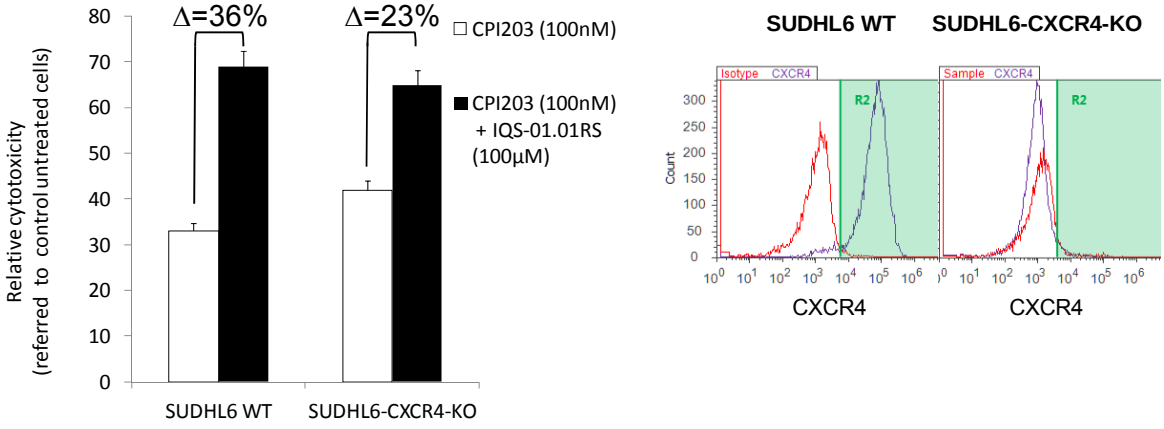
SUPPLEMENTAL FIGURES

Supplemental Figure S1

A



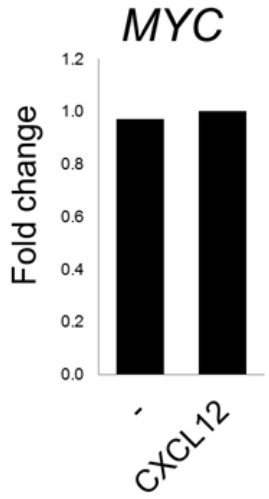
B



Supplemental Figure S1.- Genetic depletion of CXCR4 modulates CPI203/IQS-01.01RS drug interaction. A) A CRISPR/Cas9 gene editing tool was employed to generate a SUDHL-6-CXCR4-knocked out (KO) cell line, using a previously described methodology.[1] Membrane CXCR4 loss in KO cells was confirmed by flow cytometry (right panel). Parental and KO cell lines were treated for 48h with the indicated drugs

and cytotoxicity was evaluated by MTT assay using untreated cells as a reference (left panel). B) Parental and CXCR4-KO NALM6 cells [2] were treated and analyzed as in A).

Supplemental Figure S2



Supplemental Figure S2.- qPCR analysis of *CXCR4* mRNA levels in SUDHL6 stimulated or not for 1 hour with 200 ng/ml recombinant CXCL12. Unstimulated cells were used as a reference.

SUPPLEMENTAL REFERENCES

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2. Randhawa S, Cho BS, Ghosh D et al. Effects of pharmacological and genetic disruption of CXCR4 chemokine receptor function in B-cell acute lymphoblastic leukaemia. Br J Haematol. 2016;174(3)**:**425-436.