The Bruton tyrosine kinase inhibitor CC-292 shows activity in mantle cell lymphoma and synergizes with lenalidomide and NIK inhibitors depending on nuclear factor-κB mutational status

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Supplemental Information

Materials and Methods

Cell lines and patient samples

Primary tumor cells from 11 MCL patients (see clinical characteristics in Table 1), diagnosed according to the World Health Organization (WHO) classification criteria were used.[1] Patient´s informed consents were granted following the guidelines of the Hospital Clínic Ethics Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from peripheral blood by gradient centrifugation on Ficoll (GE Healthcare, Little Chalfont, UK) and used fresh or cryopreserved in liquid nitrogen in RPMI 1640 containing 10% DMSO (Sigma-Aldrich, St Louis, MO, US) and 60% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and maintained within the Hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). MCL cell lines REC-1 and MINO were obtained from ATCC (LGC Standards, Barcelona, Spain). UPN-1, MAVER-1 and Z138 were kindly provided by Dr A. Turhan (Institut Gustave Roussy, Villejuif, France) (2004), Dr A. Zamo (University of Verona, Verona, Italy) (2008) and Dr E. Ortega-Paino (Lund University, Lund, Sweden) (2006), respectively. MCL primary samples and cell lines were cultured in RPMI 1640 or DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin (Thermo Fisher Scientific) and were maintained in a humidified atmosphere at 37ºC containing 5% CO_2. Normocin (100 µg/mL) (InvivoGen, San Diego, USA) was added to the cell line cultures to prevent Mycoplasma contamination in cell lines, which were routinely tested for Mycoplasma infection by PCR. The identity of all cell lines was verified by using AmpFISTR identifier kit (Thermo Fisher Scientific, Waltham, MA, USA). The mesenchymal stromal cell line StromaNKtert was obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and cultured as described previously.[2]

Treatments

The BTK inhibitor CC-292 and lenalidomide were kindly provided by Celgene (San Diego, CA, USA). Two NIK inhibitors [AM-0216 and AM-0561] and an isomeric control of AM-0216 [AM-0650] were kindly provided by Amgen (Seattle, WA, USA). AM-0216 inhibits NIK with a Ki of 2 nM. AM-0650 is the enantiomer of AM-0216, with a Ki of 290 nM against NIK. AM-0561 is a more potent active analog with a Ki of 0.3 nM.[3]

Cell proliferation assay and apoptosis quantification

MCL cells ($5x10^4$) were treated with CC-292, lenalidomide or NIK inhibitors for the times indicated and 0.5 mg/mL MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, St Louis, MO, USA) was added for 2–6 additional hrs before

spectrophotometric measurement. Each measurement was made in triplicate. Values were represented using untreated control cells as reference. Apoptosis induction was evaluated by flow cytometry in an Attune acoustic focusing cytometer (Thermo Fisher Scientific) after staining MCL cells with Annexin V-FITC (BD-Pharmingen, Franklin Lakes, New Jersey, USA) and co-stained with CD19-PE (BD-Pharmingen) in the case of primary cells.

Gene expression profiling (GEP) and metadata analysis

Total RNA was isolated from MCL cells using the TRIzol reagent (Thermo Fisher Scientific) followed by a cleaning step using the RNAeasy kit (Qiagen, Hilden, Germany). RNA integrity was examined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only high quality RNA was then retrotranscribed to cDNA (Qiagen) and hybridized on HGU219 microarray (Affymetrix, Santa Clara, CA, USA). All samples were simultaneously run in a GeneTITAN platform (Affymetrix). Principal component analysis (PCA) was done with Partek Genomics Suite. For the identification of CC-292 modulated pathways, Gene Set enrichment Analysis (GSEA) v2.0 (Broad Institute) was performed using experimentally derived custom gene sets[4,5] and the Human NF-κB Signaling pathway and Targets from PCR Array gene list (SABiosciences, Qiagen). A two class analysis with 1000 permutations of gene sets and a weighted metric was used. Bonferroni correction for multiple testing was applied and only gene sets with FDR ≤0.10 and a normalized enrichment score (NES) of $\geq$1.5 were considered significant. The leading edge genes were displayed using Cluster (v2.11) and TreeView (v1.6) softwares (Eisen Laboratory, Berkeley, CA, USA). Microarray data were deposited in the NCBI´s Gene Expression Omnibus and is accessible through GEO series accession number GSE94328.

Western blot assays

MCL cells were lysed in 1% Triton or RIPA buffer as indicated, containing protease and phosphatase inhibitors. Protein lysates were resolved by SDS-PAGE electrophoresis as described previously,[6] developed with enhanced chemiluminescence substrate (SuperSignal, Thermo Fisher Scientific) and visualized on a LAS4000 Fujifilm device (Fujifilm, Tokyo, Japan). BTK, phospho-BTK (Y223), ERK, phospho-ERK (Thr202/Tyr204), p52/100, and phospho-IκB (Ser32) antibodies were from Cell Signaling Technology (Danvers, MA, USA); IRF4 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and α-tubulin and β-actin from Sigma-Aldrich.

Flow cytometry

Cells were pretreated with 1μM CC-292 at 37ºC for 1h and subsequently stimulated with 10μg/ml anti-IgM (Jackson Immunoresearch Laboratories, Grove, PA, USA) for 24h. Cellular activation was evaluated by co-staining of MCL cells with CD69-PC7/CD86-FITC, including

CD19-PE and Annexin V-Pacific Blue in the case of primary cells, followed by cytofluorimetric evaluation in an Attune cytometer (Thermo Fisher Scientific).

Migration assay

SDF-1α/CXCL12-induced migration was evaluated using 24-well chemotaxis chambers containing 5 μm pore size inserts (Corning, NY, USA) and coated with 1 μg/ml VCAM-1. The lower chamber contained 200ng/ml CXCL12. The cells were pretreated with 1μM CC-292 at 37ºC for 1h and deposited in the upper compartment allowing them to migrate for 3h at 37ºC and enumerated by flow cytometry.

Co-culture experiments

StromaNKtert cells were seeded on day 0. MCL cell lines or primary cells were added on the following day at 1:5 ratio (stromaNKtert:MCL) and cultured in the presence or absence of the drug. After 3 or 6 days viable MCL cells were counted by flow cytometry after CD19-PE/Annexin V-FITC staining.

ELISA cytokine quantification

CCL3 and CCL4 levels were assessed in duplicate using ELISA kits (eBioscience, San Diego, US) in supernatants harvested from cells that had been pretreated with 1μM CC-292 at 37ºC for 1h and subsequently stimulated with 10μg/ml of anti-IgM for 24h.

Statistical analysis

Statistical comparisons were performed as Student's paired t-test and non-parametric unpaired t-test with the use of Graphpad Prism 4.0 software.

Supplemental Table 1: MCL patient characteristics

Study label	Gender[1]	Sample type[2]	%tumor cells[3]	MCL variant[4]	Stage[5]	MIPI[6]	*BIRC3* mutation[7]	11q loss/UPD[7]	*TP53* mutation[7]	*17p* loss/UPD[7]
MCL#1	M	PB	83	C	IV-B	HR	1	1	0	1
MCL#2	M	PB	85	SC	IV	HR	0	0	0	0
MCL#3	M	PB	83	B	IV-B	ND	ND	0	ND	1
MCL#4	F	PB	97	SC	IV	HR	0	0	1	1
MCL#5	M	LN	71	C	IV-A	IR	1	1	0	0
MCL#6	M	PB	94	B	IV-A	HR	0	0	0	0
MCL#7	M	PB	69	B	IV-B	HR	1	1	0	0
MCL#8	F	PB	78	B	IV-A	HR	ND	0	1	1
MCL#9	M	PB	84	SC	IV-A	HR	ND	0	0	0
MCL#10	M	PB	86	C	IV	HR	1	1	0	0
MCL#11	M	PB	96	C	IV	HR	0	0	1	1

[1]M: male, F: female. [2] PB: peripheral blood, LN: lymph node. [3]Percentage of tumor cells assessed by flow cytometry based on CD19 CD5 positive cells. [4] C: Classic; B: Blastoid, SC: Small Cells. [5] Defined by Ann Arbor stage system.[6] MIPI: MCL International Prognostic Index: high-risk (HR), intermediate risk (IR)[7]. [7] 1: alteration present, 0: alteration absent, ND: not done. Whole exome sequencing and genomic data from Bea S, PNAS 2013.[8]

Supplemental figure legends

Figure S1. CC-292 induces marginal apoptosis. Viability (Annexin-V$^+$) of MCL cell lines treated with CC-292 at the indicated doses for three days. Results are the mean ± SEM of three independent experiments.

Figure S2. Activation of alternative NF-κB pathway in primary MCL cases. Constitutive expression of p52/100 in primary MCL cases was assessed by western blot using β-actin as loading control.

Figure S3. CC-292 inhibits BCR-induced cellular activation and secretion of CCL3 and CCL4 chemokines. A) The expression of CD69 and CD86 on UPN-1 and MAVER-1 cells was compared between cells pre-treated or not with 1μM CC-292 under anti-IgM stimulation (10μg/ml) for 24h. The bars in this graph represent mean ± SD expression relative to stimulated control of 3 independent experiments. B) Expression of CD69 and CD86 of anti-IgM-stimulated CD19$^+$CD5$^+$ cells from 4 MCL primary samples, either pre-treated or not with 1μM CC-292 for 24h. C) CCL3 and CCL4 protein secretion was evaluated by ELISA in the culture supernatant of UPN-1, MAVER-1 and MCL#5, in the absence (control) or upon of BCR stimulation (anti-IgM 10μg/ml) for 24 hr. Bars correspond to the mean ± SD from duplicates of the concentrations in pg/ml.

Figure S4. CC-292 interferes with CXCL12-induced migration. A) UPN-1, Z138 and four primary samples (MCL#1, MCL#2, MCL#10 and MCL#11) were pretreated with 1μM CC-292 or with vehicle (DMSO) for 1h. Then, cells were challenged to migration towards a CXCL12 gradient through a VCAM-coated chemotaxis chamber for 3h. The mean ± SD of primary samples and cell lines migrations referred to the CXCL12-stimulated control, are displayed. B) UPN-1, Z138 and MCL#10 cells were pre-incubated with vehicle or 1μM CC-292 for 1hr followed by stimulation with CXCL12 for 5min. pERK and ERK expression were assessed by western blot, using α-tubulin as a loading control.

Supplemental figures

Figure S1

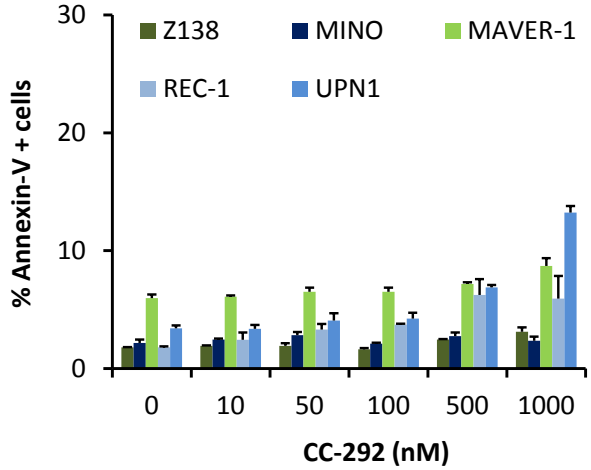


Figure S2

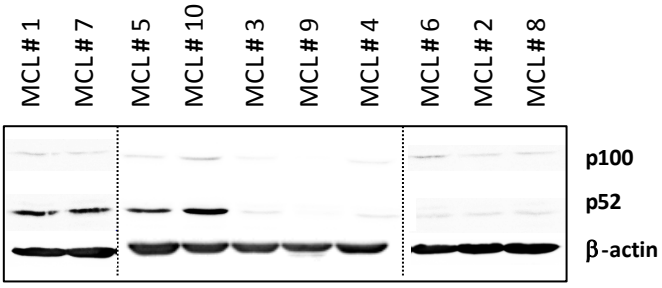


Figure S3

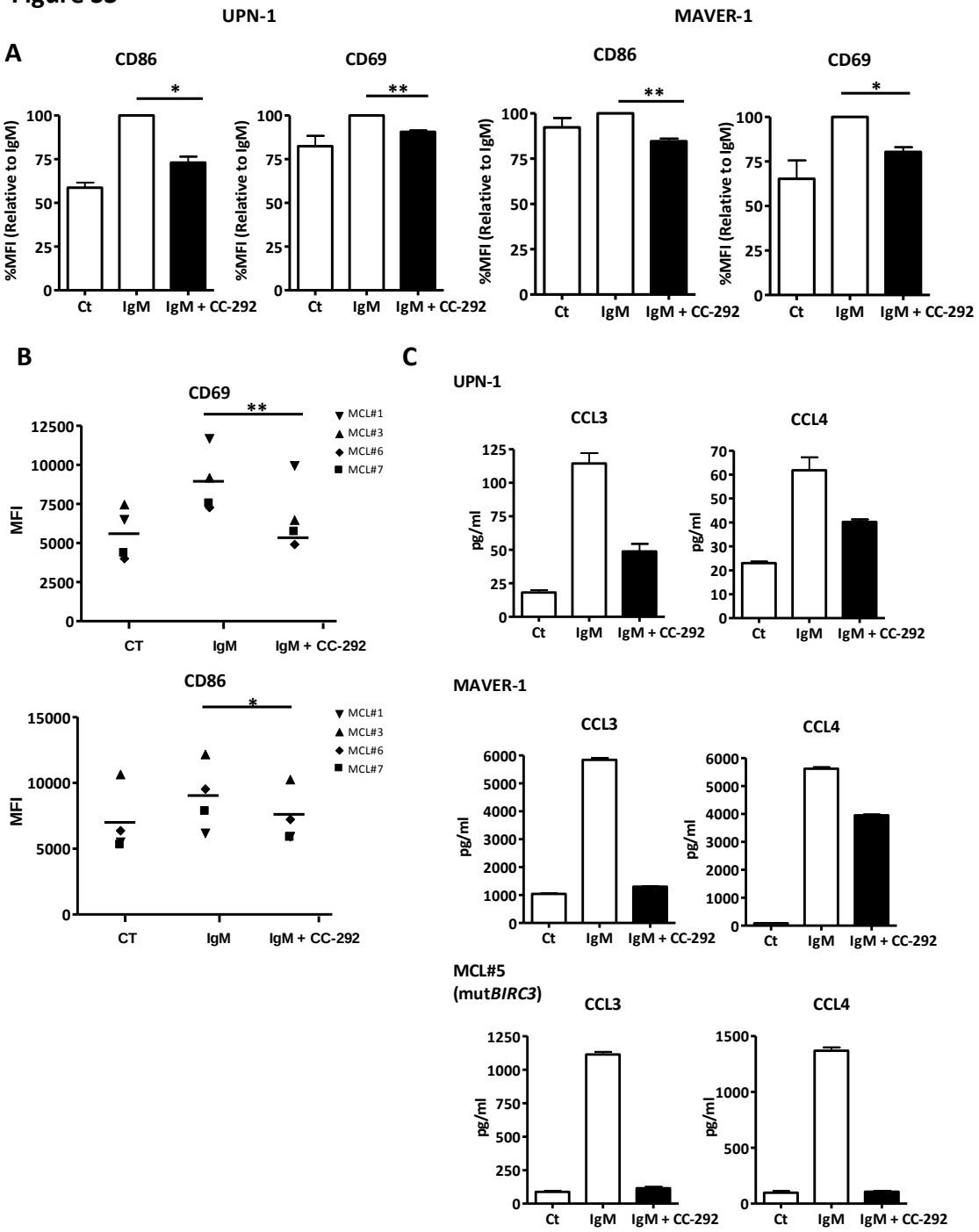
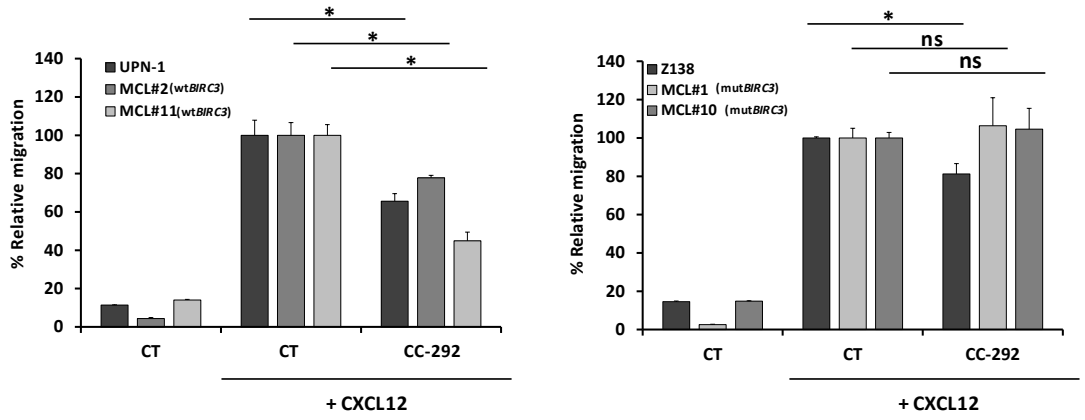
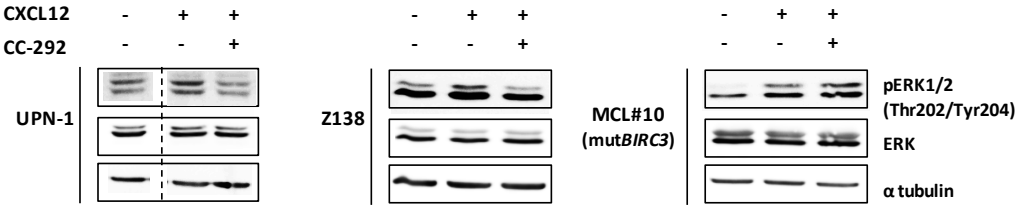

UPN-1
MAVER-1
A
CD86
CD69
CD86
CD69
%MFI (Relative to IgM)
Ct
IgM
IgM + CC-292
B
CD69
CD86
MFI
MCL#1
MCL#3
MCL#6
MCL#7
CT
IgM
IgM + CC-292
C
UPN-1
MAVER-1
MCL#5
(mutBIRC3)
CCL3
CCL4
pg/ml

Figure S4

A


UPN-1
MCL#2(wtBIRC3)
MCL#11(wtBIRC3)
% Relative migration
140
120
100
80
60
40
20
0
*
*
*
CT
CT
CC-292
+ CXCL12
Z138
MCL#1 (mutBIRC3)
MCL#10 (mutBIRC3)
% Relative migration
ns
ns
CT
CT
CC-292
+ CXCL12

B


CXCL12 - + + - + + - + +
CC-292 - - + - - + - - +
UPN-1
Z138
MCL#10 (mutBIRC3)
pERK1/2 (Thr202/Tyr204)
ERK
α tubulin

Supplemental references

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