In vitro and *in vivo* activity of a new small-molecule inhibitor of HDAC6 in mantle cell lymphoma

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Inventory of Supplemental Data

Perez-Salvia M et al. In vitro and in vivo activity of a new small-molecule inhibitor of HDAC6 in mantle cell lymphoma.

Supplementary Methods

In vitro inhibitory activity of histone deacetylase

50 μM of substrate peptide (see 'substrate peptides' section below) and an optimal concentration of the corresponding enzyme (see 'enzymes' section below) in the assay buffer and 1% final concentration of DMSO were incubated in the presence of gradient concentrations of inhibitors (10-dose IC50 mode with 3-fold serial dilution) at 30°C for 2 h. The reactions were carried out in a 96-well microplate for fluorometry in a 50 μl reaction volume. After the deacetylation reaction, Fluor-de-Lys-Developer (BioMol Cat. # KI-105) was added to each well to digest the deacetylated substrate, thus producing the fluorescent signal. The reaction was allowed to proceed for 45 minutes at 30°C with 5% CO_2; then the fluorescent signal was measured with an excitation wavelength at 360 nm and an emission wavelength at 460 nm in a microplate-reading fluorometer (GeminiXS; Molecular Devices, Sunnyvale, CA). A curve of Deacetylated Standard (Biomol, Cat. # KI-142; made from 100 μM with 1:2 dilution and 10-doses, 6 μl) allowed the conversion of fluorescent signal into micromoles of deacetylated product. All experiments were performed in triplicate. IC50 was calculated by fitting the experimental data to a dose-response curve. DMSO was used as negative control;

Substrate peptides: All HDAC assays were performed using acetylated AMC-labeled peptide substrate:

- Substrate for isoforms HDAC1, 2, 3, 4, 5, 6, 7, 9, 10 and 11 assays: Acetylated fluorogenic peptide from p53 residues 379-382 (RHKKAc) (BioMol Cat. # KI-104).
- Substrate for HDAC8 assays: Acetylated fluorogenic peptide from p53 residues 379-382 (RHKAcKAc) (BioMol Cat. # KI-178).

Assay buffer: 50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ (supplement with 1 mg/ml BSA for dilution) (BioMol Cat. # KI-143).

Enzymes:

- HDAC1 assay: 75 nM Human HDAC1 (GenBank Accession No. NM_004964): Full length with C-terminal GST tag, MW= 79.9 kDa, expressed by baculovirus expression system in Sf9 cells (BioMol Cat. # SE-456).
- HDAC2 assay: 5 nM Human HDAC2 (GenBank Accession No. Q92769): Full length with C-terminal His tag, MW= 60 kDa, expressed by baculovirus expression system in Sf9 cells (BioMol Cat. # SE-500).

- HDAC3 assay: 2.3 nM Human HDAC3/NcoR2 (GenBank Accession No. NM_003883 for HDAC3, GenBank Accession No.NM_006312 for NcoR2): Complex of human HDAC3, full length with C-terminal His tag, MW= 49.7 kDa, and human NCOR2, N-terminal GST tag, MW= 39 kDa, co-expressed in baculovirus expression system (BioMol Cat. # SE-507).
- HDAC4 assay: 266 nM Human HDAC4 (GenBank Accession No. NM_006037): Amino acids 627-1085 with N-terminal GST tag, MW= 75.2 kDa, expressed in baculovirus expression system (BioMol, Hamburg, Germany).
- HDAC5 assay: 588 nM Human HDAC5 (GenBank Accession No. NM_001015053): Full length with Nterminal GST tag, MW= 150 kDa, expressed by baculovirus expression system in Sf9 cells (BioMol, Hamburg, Germany).
- HDAC6 assay: 13 nM Human HDAC6 (GenBank Accession No. BC069243): Full length with N-terminal GST tag, MW= 159 kDa, expressed by baculovirus expression system in Sf9 cells (BioMol Cat. # SE-508).
- HDAC7 assay: 962 nM Human HDAC7 (GenBank Accession No. AY302468): Amino acids 518-end with N-terminal GST tag, MW= 78 kDa, expressed in baculovirus expression system (BioMol, Hamburg, Germany).
- HDAC8 assay: 119 nM Human HDAC8 (GenBank Accession No. NM018486): Full length, MW=42 kDa, expressed in an E. coli expression system (BioMol Cat. # SE-145).
- HDAC9 assay: 986 nM Human HDAC9 (GenBank Accession No. NM178423): Amino acids 604-1066 with C-terminal His tag, MW= 50.7 kDa, expressed in baculovirus expression system (BioMol, Hamburg, Germany).
- HDAC10 assay: 781 nM Human HDAC10 (GenBank Accession No. NM_032019): Amino acids 1-631 with Nterminal GST tag, MW= 96 kDa, expressed by baculovirus expression system in Sf9 cells (BioMol Cat. # SE-559).
- HDAC11 assay: 781 nM Human HDAC11 (GenBank Accession No. NM_BC009676) with N-terminal GST tag, MW= 66 kDa, expressed in baculovirus expression system (BioMol Cat. # SE-560).

Cell lines and primary samples

The following 48 cell lines were used in the study: Breast cancer (BT474, MCF-7, MDA-MB-231, SK-BR-3), Prostate cancer (DU145, LNCaP, PC-3), Colorectal cancer (Colo205, DLD-1, HCT-116, HT-29, LoVo, SW620), Lung cancer (A549, Calu-6, NCI-H226, NCI-H460, SK-MES-1), Glioblastoma (U87MG), Fibrosarcoma (HT-1080), Pancreatic cancer (MIAPACA-2, Bx-PC-3, PANC-1), Kidney cancer (786-O), Liver cancer (Hep3B, HepG2, SK-HEP-1), Osteosarcoma (143b), Melanoma (A375, SK-

MEL-5), Ovarian cancer (SK-OV-3, OVCAR3), Chronic Myeloid Leukemia (K562), Orthotopic Multiple Myeloma (RPMI-8226), Hodgkin's lymphoma (KMH2, L428 and HD-MY-Z), Burkitt's lymphoma (Ramos and Jiyoye), Follicular Lymphoma (WSU-NHL, SC-1 and SU-DHL-6) and Mantle Cell Lymphoma (MINO, REC-1, IRM-2, HBL-2, Z-138 and JEKO-1). All cancer cell lines were obtained from the American Type Culture Collection (ATCC) or the Leibniz-Institut DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ), except IRM-2 and HBL-2 that were provided by Miguel A. Piris (Pathology Department, Fundación Jiménez Díaz, Madrid, Spain). IRM-2 and HBL-2 have been widely used as MCL cell line models[1-6] and a detailed description can be found at Cellosaurus - a knowledge resource on cell lines (https://web.expasy.org/cellosaurus). Cells were cultured at 37ºC with 5% CO_2 in the appropriate growth medium. For primary tissues, we obtained buffy coats from anonymous healthy donors through the Catalan Blood and Tissue Bank (CBTB). The CBTB follows the principles of the World Medical Association (WMA) Declaration of Helsinki. Before providing the first blood sample, all donors received detailed oral and written information and signed consent form at the CBTB. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient (Amersham, Buckinghamshire, UK) centrifugation. Lymphatic node cells from two MCL patients were obtained following informed consent and approval of the Institutional Review Boards of the Josep Carreras Institute and the Catalan Institute of Oncology. Cells were cultured in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37ºC with 5% CO2.

Dose-response assays

For dose-response assays, cell lines were seeded at a density of 5000 to 20000 cells/well in 96-well plates. The optimal number of cells for each experiment was determined to ensure that each one was in growth phase at the assay endpoint. After overnight incubation, experimental culture medium containing increasing concentrations of QTX125, Tubastatin A, Tubacin or ACY1215 was added into each well. Cell viability was determined by MTS or MTT assays at 72h or 96 h after treatment, respectively. MTS assay was performed following the manufacturer's protocol CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay MTS (Promega). Briefly, a mixture of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS was freshly prepared in solution, added to each well and incubated for 4 hours at 37ºC in a humidified, 5% CO_2 atmosphere. Absorbance was read at 490 nm using a spectrophotometer. Alternatively,

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reagent was added and incubated for 3 h, after which the cells were lysed for 16 h with MTT lysis buffer (50% N-N dimethylformamide, 20% sodium dodecyl sulfate, 2.5% glacial acetic acid, 2.1% 1N HCl, at pH 4.7). Plates were measured at 560 nm using a spectrophotometer. Cell viability was also determined by cell counting with the Trypan Blue exclusion method.

Immunoblotting assays

Total protein from cells was extracted with Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glicerol, 2% SDS, 0.01% Bromophenol Blue, 5% β-mercaptoethanol). Specific antibodies against target proteins were used: α-Tubulin (ab52866, Abcam), Acetylated Tubulin (T6793, Sigma Aldrich), Caspase-9 (133109, Santa Cruz), Caspase-8 (9746, Cell Signaling), Caspase-3 (9668, Cell Signaling), Cleaved Caspase-3 (9661, Cell Signaling), PARP (556362, BD Pharmingen™), CD20 (ab78237, Abcam), and β-Actin-HRP (A3854, Sigma Aldrich).

Detection of Apoptosis by Flow Citometry

Cells were treated with QTX125 at 25nM, 50nM, 100nM and 500 nM or with vehicle for 24 and 48h hours. Annexin V-Alexa Fluor® 488/PI staining was used for the quantification of early and late apoptotic cells following manufacturer's instructions (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI for Flow Cytometry, V13245 Invitrogen). Briefly, cells were stained with Alexa Fluor® 488 Annexin V and PI for 15 min and were examined by flow cytometry (FACS Canto, BD Biosciences) using 488 nM excitation with 530/30 and 575/26 bandpass filters. A minimum of 10,000 cells was analysed per sample. Quantification and illustration as dot plot was done using FACSDiva Software. Experiments were performed in triplicate. PBMCs from healthy donors (n=4) or cells from MCL patients (n=2) were treated for 48h with increasing concentrations of QTX125. Cells were then washed with PBS. PBMCs from healthy donors were incubated with (APC)-conjugated anti-CD19, and APC-eFluor® 780- conjugated anti-CD3 for 20 min in the dark and then incubated with Annexin V-Alexa Fluor® 488 and 7-amino-actinomicina D (7AAD) in annexin-binding buffer for 15 minutes. Cells from MCL patients were incubated with Annexin V-Alexa Fluor® 488 and 7-amino-actinomicina D (7AAD) in annexin-binding buffer for 15 minutes. Cells were then examined by flow cytometry (FACS Canto II, BD Biosciences). Quantification was done using FACSDiva Software. The results of viability presented correspond to the AnnexinV/7AAD negative cells.

Mouse xenografts

REC-1 and MINO cells were harvested during exponential growth and re-suspended in cold phosphate buffered saline (PBS) at a concentration of 1 x 10^8 cells/mL. Female CB.17 SCID mice (CRL: NU(NCr)-Foxn1nu, Charles River) were used for the study. All mouse experiments were approved and performed in accordance with the guidelines of the Institutional Animal Care Committee of the Bellvitge Biomedical Research Institute. Xenografts were initiated by subcutaneously implanting a 0.1 mL suspension of 1 x 10^7 REC-1 or MINO tumor cells in 50% MatrigelTM (BD Biosciences) into the right flank of each test animal and tumors were monitored as their volumes approached the target range of 100 to 500 mm^3. Tumors were measured in two dimensions using calipers, and volume was calculated using the formula: V (mm^3)=(L × W^2)/2. After tumor implantation (Day 1 of study) the animals were sorted into the different groups (n=8 for REC-1 and n=10 for MINO) according to the treatment plan summarized below. QTX125 was dissolved at a final concentration of 7.5 mg/mL in a 10%DMSO : 35% PEG400 in DI water vehicle. This dosing solution provided a 60 mg/kg dose in an 8 mL/kg dosing volume. The used in vivo dose of QTX125 (60 mg/Kg) for MCL xenografts is similar to the one used in the initial studies of ACY-1215 (50 mg/Kg) for multiple myeloma xenografts,[7] the HDAC6 inhibitor that it is undergoing clinical trials in multiple myeloma patients. Cyclophosphamide was dissolved in saline to a final stock concentration of 20 mg/mL, and further diluted to a dosing solution of 10 mg/mL which provided a 100 mg/kg dose in a dosing volume of 10mL/kg. All treatments were administered intraperitoneally (i.p.) at the dosing volumes above and scaled to the body weight of the animal.

Treatment plan for REC-1

Group 1 received vehicle daily for 28 days (qd x 28) and served as the control.

Group 2 received cyclophosphamide at 100 mg/kg daily dosing for 5 days (qd x 5).

Group 3 received QXT125 at 60 mg/kg on a schedule of five days on/two days off for four weeks (5/2 x 4).

Group 4 received QXT125 at 60 mg/kg daily dosing for 28 days (qd x 28).

Tumors were measured using calipers twice per week.

Treatment plan for MINO

Group 1 received vehicle daily until we stopped for ethical reasons because tumor burden was no longer justifiable (15 days).

Group 2 received QXT125 at 60 mg/kg on a schedule of five days on/two days off for four weeks (5/2 x 4).

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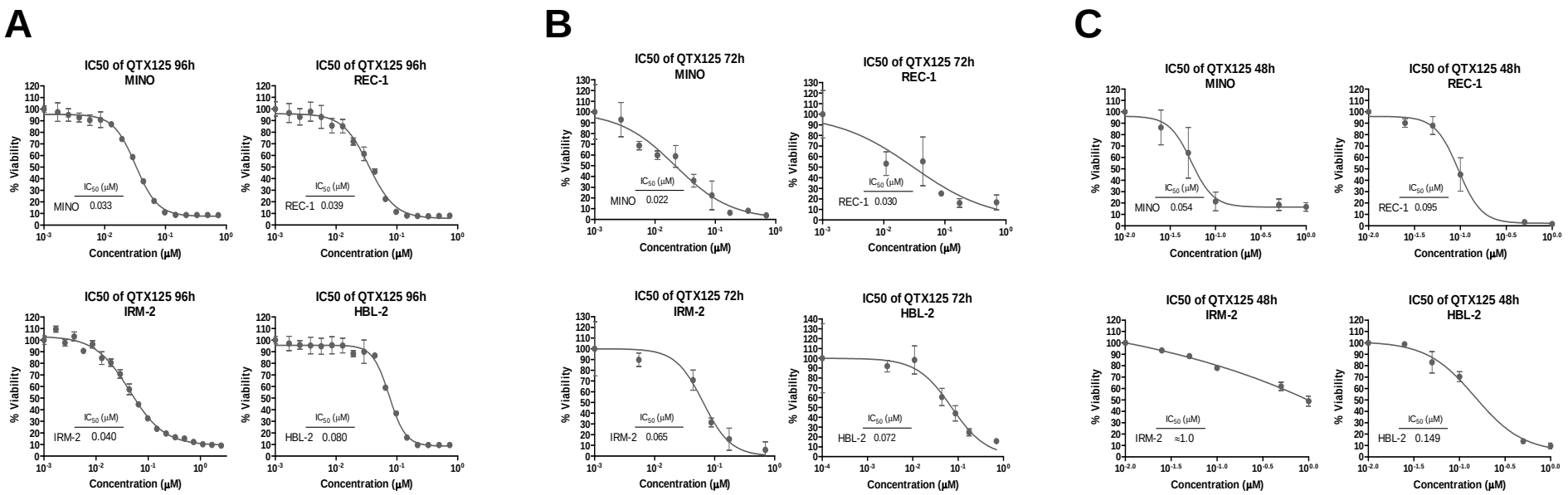
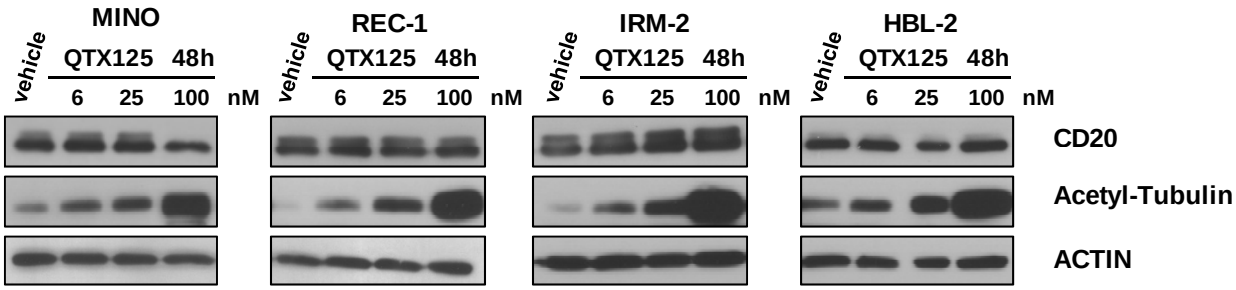
Figure S1



Supplementary Figure S1. Effect of QTX125 in cell viability in the MCL cell lines MINO, REC-1, IRM-2 and HBL-2 determined by the MTT assay (**A**), cell counting with the Trypan Blue exclusion method (**B**), and flow cytometry where cell viability is expressed as the percentage of AnnexinV/PI negative cells (C). IC50 values are shown for each cell line.

Figure S2



Supplementary Figure S2. Western-blot for CD20 expression in the MCL cell lines MINO, REC-1, IRM-2 and HBL-2 upon the use of QTX125. Acetylated-Tubulin is shown as a marker of HDAC6 inhibition upon QTX125 use. Actin is shown as a loading control.

Supplementary Table S1. Immunophenotype performed by flow cytometry of the two mantle cell lymphoma cases.

Antigens	MCL Primary Patients	
	AA3319	**AA9683**
CD3	8%	6%
CD4	4%	4%
CD8	3%	3%
CD19	92%	95%
CD45	99%	100%
Light Chain Kappa	0%	0%
Light Chain Lambda	92%	94%
CD5	94%	98%
CD19/CD5	92%	95%
CD10	1%	1%
CD19/CD10	0%	0%
CD11c	1%	1%
CD19/CD11c	0%	0%
CD20	93%	94%
CD22	91%	91%
CD23	10%	2%
CD38	87%	53%
CD19/CD38	80%	50%
CD43	90%	94%
CD19/CD43	83%	90%
CD79b	91%	88%
CD103	0%	2%
CD200	2%	1%
FMC7	79%	34%
Bcl2	Positive	Not determined
Ki67	20 %	Not determined
Myc	Negative	Not determined
Bcl6	Negative	Not determined