Double autophagy stimulation using chemotherapy and mTOR inhibition combined with hydroxychloroquine for autophagy modulation in patients with relapsed or refractory multiple myeloma

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Supplementary File

The study protocol was approved by institutional review boards and ethics committees, and conducted in accordance with the Declaration of Helsinki, the guidelines of the International Conference on Harmonization for Good Clinical Practice, and all applicable federal and local regulations. All patients provided written informed consent before treatment initiation.

Eligibility criteria: Eastern Cooperative Oncology Group performance score ≤ 2, adequate organ and marrow function absolute neutrophil count ≥ 1000/ uL, platelet count ≥ 50,000/uL, creatinine ≤ 2.0mg/dL, total bilirubin ≤ 2.0mg/dL, AST/ ALT ≤ 2X the upper limit of normal, fasting glucose ≤ 200mg/dL. Key exclusion criteria were known macular degenerative disease or retinopathy, conditions requiring treatment with hydroxychloroquine, uncontrolled concurrent illness, standard anti-myeloma therapy within 14 days, investigational therapy within 28 days, steroids within 7 days, radiation within 14 days, or concomitant medications that inhibit CYP 3A within 72 hours.

Treatment Regimen: Patients received cyclophosphamide 300 mg/m^2 daily continuous intravenous infusion and dexamethasone 40 mg daily by mouth for 4 days while hospitalized, rapamycin by mouth from cycle 1 day minus 2 (12 mg loading dose followed by 4 mg daily for 5 more days) and (phase I trial)/ OR (pilot trial) HCQ by mouth from cycle 1 day 5 onwards *(Figure 2A main manuscript).* The hydroxychloroquine dose on the pilot study was 800mg, and there were 4 dose levels on the phase 1 study (400mg, 600mg, 800mg, 1200mg daily). Cycles were repeated every 28 days, with a 14 day allowance to delay subsequent cycles due to toxicities. Dose modifications for toxicities were allowed for any of the four pharmacologic agents, and if more than one drug was possibly responsible, multiple dose modifications were allowable.
Supportive care measures included intravenous fluids, acyclovir and fluconazole prophylaxis. Prophylactic levofloxacin was given if the patient became neutropenic (ANC <500). Pegfilgrastim 6 mg was given 24 to 48 hours after completion of cyclophosphamide.

Safety assessments: These included the characterization of dose limiting toxicities (DLT), grade 3 and 4 treatment emergent adverse events (TEAEs) and serious adverse events (SAEs), using the Common Terminology Criteria for Adverse Events version 4.0.
Dose limiting toxicity definition: The following expected events secondary to cyclophosphamide were excluded: thrombocytopenia during the 1st cycle in the absence of clinically significant bleeding if the platelet count returns to >50,000/uL by day 1 of the second cycle, neutropenia that improved within 10 days to an ANC >1000/uL, lymphopenia or anemia of any grade, nausea and vomiting responsive to medical therapy, grade 3 neutropenic fever or infection during neutropenia, tumor lysis syndrome, metabolic abnormalities that are correctable to grade 1 or baseline within 1 week or elevated transaminases, total or direct bilirubin or alkaline phosphatase. Ophthalmology examinations were performed at baseline and repeated one year later as per the American Academy of Ophthalmology recommended screening for retinal toxicity in patients receiving HCQ.(1)

Pharmacodynamic and pharmacokinetic assessments: The tertiary exploratory pharmacokinetic (PK) endpoint used whole blood samples for HCQ trough levels obtained between day 21 and 28 of cycle 1, and serum rapamycin trough levels obtained on day 1 and 5 of cycle 1. Rapamycin quantification was measured in whole blood samples using an immunoassay run on an architect I-2000 SR analyzer at the institution laboratory. HCQ levels were measured using minimum and maximum whole blood levels of HCQ, using high

performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). (2) Whole blood was stored at -80 degrees Celsius and the assay performed in a batch by the bioanalytical shared resource/pharmacokinetics core at OHSU.

The tertiary exploratory pharmacodynamic (PD) endpoints used bone marrow derived CD138-positive plasma cells to allow detection of changes in the autophagy and mTOR pathways in myeloma cells prior to any chemotherapy, after exposure to cyclophosphamide, dexamethasone and rapamycin, and after approximately 28 days of exposure to HCQ. *(Figure 2A main manuscript)* A total of 10 subjects had 3 complete sets of bone marrow aspirates. Purified plasma cells were isolated from bone marrow aspirates using a Ficoll (GE Healthcare Biosciences) gradient and CD138 microbeads (Miltenyi Biotec 130-093-062; 545). Sections of 60–90 nm were placed onto 200 mesh grids, then stained with uranyl acetate and lead citrate, and examined with a FEI Techni 12 electron microscope at 80 Kv. Digital images were acquired using an Active Vu- M 16 Mpixel camera (Advanced Microscopy Techniques, Danvers, MA). Three independent assessors (of which 2 were blinded to timepoint and subject identification) counted AVs in electron micrographs of 25 cells from each sample as previously described (3). Confirmatory immunoblotting for microtubule-associated protein 1 light chain 3 (MAP1 LC3A/LC3, Cell Signaling Technology, rabbit 12741) was performed in some samples. LC3A is a cytoplasmic protein that is conjugated to the surface of AVs during their formation; the cytoplasmic form migrates as LC3-I on gel electrophoresis whereas the AV-conjugated form migrates as LC3-II, thus the LC3-II/LC3-I ratio is expected to correlate with the number and size of accumulated AVs. Bone marrow biopsies were fixed in Zanker's formalin, embedded in paraffin blocks, and sectioned. Sections were stained for CD138 and phospho-rS6 Ser235/236 ((D57.2.2E) XP® Rabbit mAb, Cell Signaling Technology). IgG control antibodies were used for negative controls. For this analysis, 7 paired samples were of sufficient quality for analysis. The tertiary exploratory endpoint to describe next generation sequencing used 'Genetrails, a multiplexed next generation DNA sequencing panel that was developed and optimized for Clinical Laboratory Improvement Amendments (CLIA) approved clinical testing by the OHSU Knight Diagnostic Laboratories. This test is designed to detect alterations in a panel of genes, which are known to play a role in cancer growth. Each specimen is examined microscopically by a pathologist and genomic DNA is extracted and purified from dissected, tumor-rich areas. Mutations are screened by massively parallel sequencing using a combination of multiplexed PCR (AmpliSeq primers) and emulsion PCR, followed by semiconductor-based sequencing on an Ion Torrent platform. The standard panel in use at the time of this study covers target exons and flanking intronic sequences for 35 genes including AKT1, PIK3CA, AKT2, PIK3R1, AKT3, HRAS, PTEN, ALK, KDR, RAC1, BRAF, KIT, RB1, CDK4, KRAS, RET, CDKN2A, MAP2K1, STK11/ LKB1, DDR2, MET TP53, EGFR NF1, TSC1, ERBB2, NOTCH1, TSC2, FGFR1, NRAS, VHL, FGFR3, NTRK2, GNA11 and NTRK3. This analysis was performed in 11 subjects.

Statistical methods: Overall response rate (ORR) and clinical benefit rate (ORR + minor response) were tabulated with a 2-sided 95% exact confidence interval (CI).
Secondary endpoint: to assess response utilized the IMWG uniform response criteria (4, 5) in the following categories: complete response (CR); partial response (PR); very good partial response (VGPR); stable disease (SD) and disease progression (PD). As per the consensus recommendations on clinical trial reporting, the minor response (MR) category was used as per the European Group for Blood and Bone Marrow Transplant (EBMT) (6, 7).
Tertiary exploratory endpoints included pharmacokinetic (PK) analysis of hydroxychloroquine and rapamycin trough levels in whole blood and pharmacodynamic (PD) analysis of autophagy and mTOR pathways in purified bone marrow plasma cells at

specified timepoints during treatment. Time to event endpoints including duration of response, progression free and overall survival were evaluated using Kaplan Meier methodology. Repeated measures ANOVA with cohorts as a between group factor and time point as within group factor was used to compare difference in mean autophagic vesicle (AV) counts between time points. Using Baysesian Information Criteria (BIC) compound symmetry (CS) was chosen as an optimal covariance structure within subject correlation.

Pilot study results: Rapamycin-treated subjects received 2, 3, and 4 cycles of therapy, achieving a PD, MR, and SD respectively, and stopped therapy due to disease progression or insufficient response (2 patients). No DLTs and 1 serious adverse event (grade 4 pseudomonas bacteremia following a hand injury) occurred on the rapamycin arm. Hydroxychloroquine treated subjects received 5, 2 and 1 cycles of therapy, achieving a PR, SD and PD respectively and discontinued therapy due to disease progression and diarrhea (2 patients). One DLT occurred, grade 3 diarrhea and nausea, refractory to supportive measures.

Phase 1 study adverse events: In general, hematologic toxicity was more likely related to myeloma disease progression than to treatment related toxicity with grade 3 or 4 neutropenia occurring in 50% and lymphopenia and thrombocytopenia in 100% of subjects. Clinically significant bacterial infections occurred in 2 subjects in cohort 4 (hydroxychloroquine 1200 mg), resulting in osteomyelitis in 1 subject who discontinued therapy due to refractory disease and thrombocytopenia after one cycle and cellulitis with staphylococcus aureus bacteremia in a 2nd subject who was able to continue therapy after treatment with antibiotics. Diarrhea was frequent in the higher dose cohorts but generally mild, with grade 3 diarrhea occurring in 2 patients. Dose reductions for toxicity were required in 11 patients: 4 hydroxychloroquine (for diarrhea, rash, acute kidney injury, and prolonged QTc), 1 rapamycin (for transaminitis), 3 cyclophosphamide (for thrombocytopenia, hyponatremia, and acute kidney injury), and 3 dexamethasone (2 for edema, and 1 for agitation). Dosing delays due to AEs occurred in 4 patients, for thrombocytopenia, clostridium difficile infection, acute renal failure and an upper respiratory tract infection. Discontinuations due to toxicity occurred in 4 subjects for diarrhea, thrombocytopenia (n=2), osteomyelitis, and congestive heart failure (SAE, possibly related to study drugs during cycle 7). No ocular toxicities occurred on this study.

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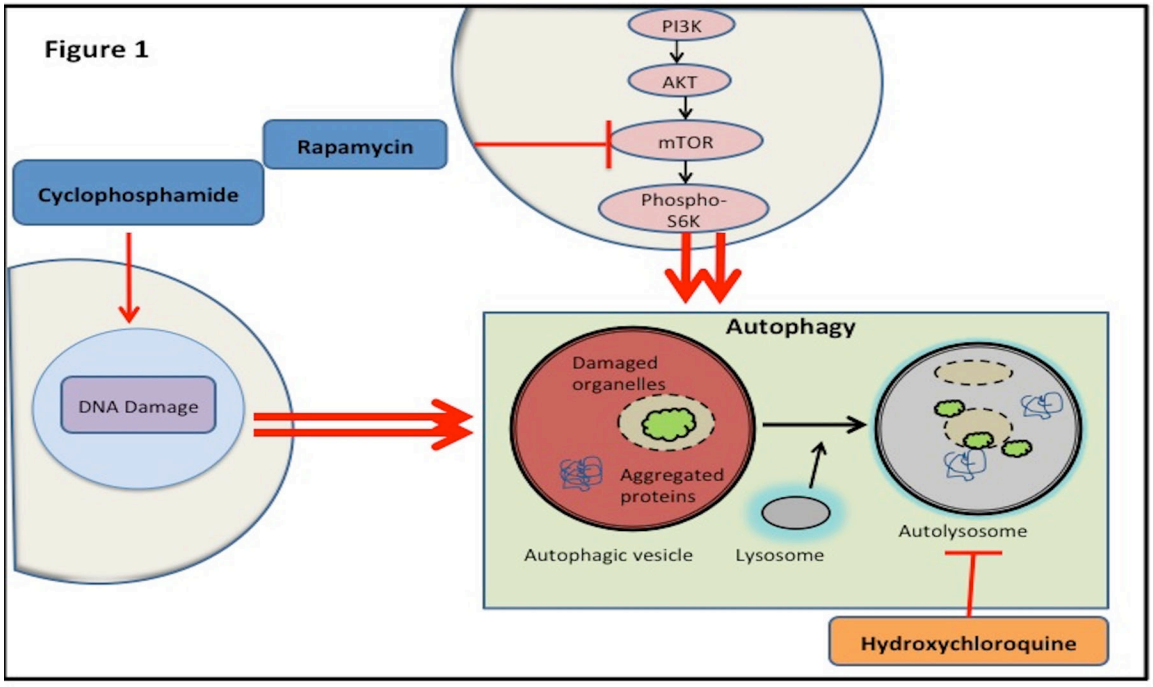
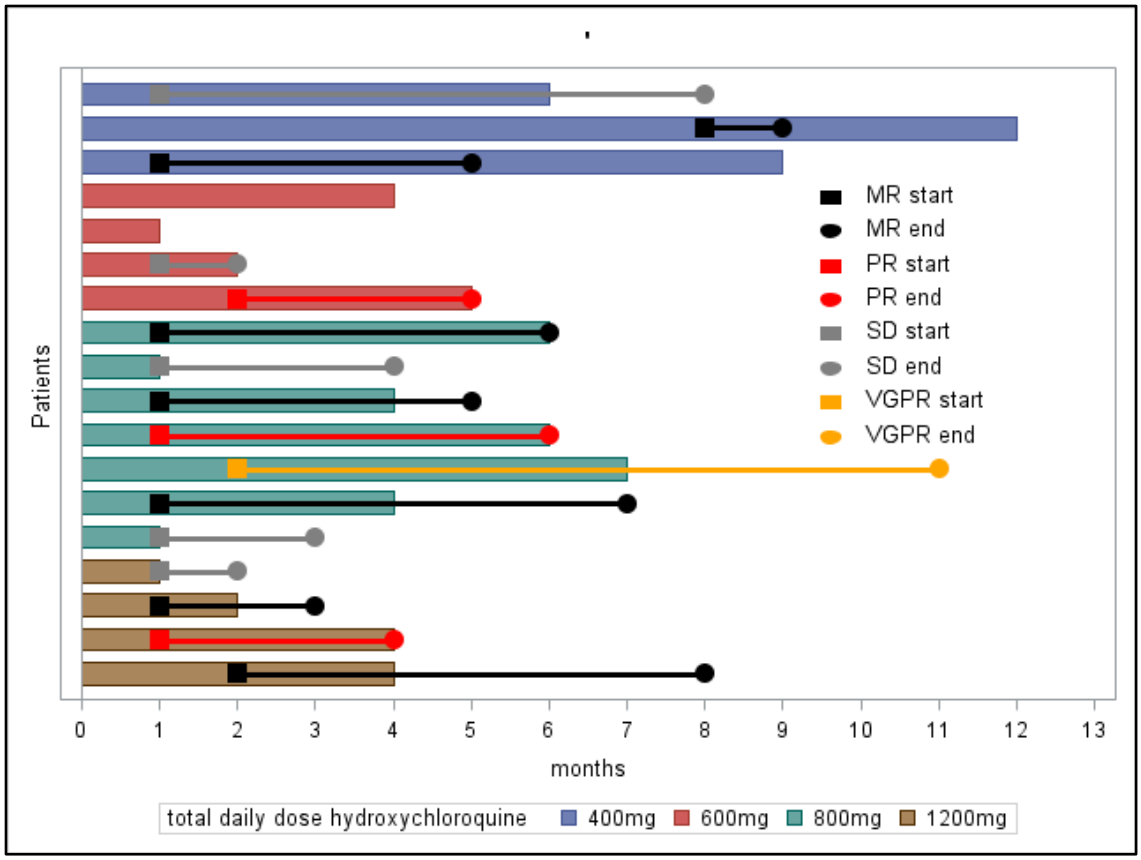
Table 1					
HCQ dose cohort	All subjects (n = 18)	400mg (n=3)	600mg (n=4)	800mg (n=7)	1200mg (n=4)
Anemia					
Grade 3	13	3	3	4	3
Grade 4	1	0	0	0	1
Neutropenia					
Grade 3	4	1	0	1	2
Grade 4	5	0	0	4	1
Lymphopenia					
Grade 3	17	2	4	7	4
Grade 4	11	2	1	5	3
Thrombocytopenia					
Grade 3	14	2	3	6	3
Grade 4	6	1	2	1	2
Neutropenic fever					
Grade 3	2	0	0	1	1
Bacterial infection					
Grade 3	2	0	0	0	2
Transaminase elevation					
Grade 3	2	1	1	0	0
Diarrhea					
Grade 3	2	0	0	1	1
Clostridium difficile colitis					
Grade 3	2	1	0	0	1
Fatigue					
Grade 3	4	0	2	1	2
Hypokalemia/ hyperkalemia					
Grade 3	5	2/1	0	3	0
Grade 4	1	0	0	1	0
Hyponatremia					
Grade 3	2	1	1	0	0
Hypophosphatemia or hypomagnesemia					
Grade 3	8	3	1	3/1	1
Hyperglycemia					
Grade 3	3	1	0		2
Prolonged QTc					
Grade 3	1	1	0	0	0
Congestive heart failure					
Grade 3	1	0	0	1	0
Syncope					
Grade 3	1	1	0	0	0
Acute renal failure					
Grade 3	1	0	0	0	1

Table 1 supplementary. Grade 3 and 4 adverse events occurring in patients on the phase 1 trial. HCQ = hydroxychloroquine

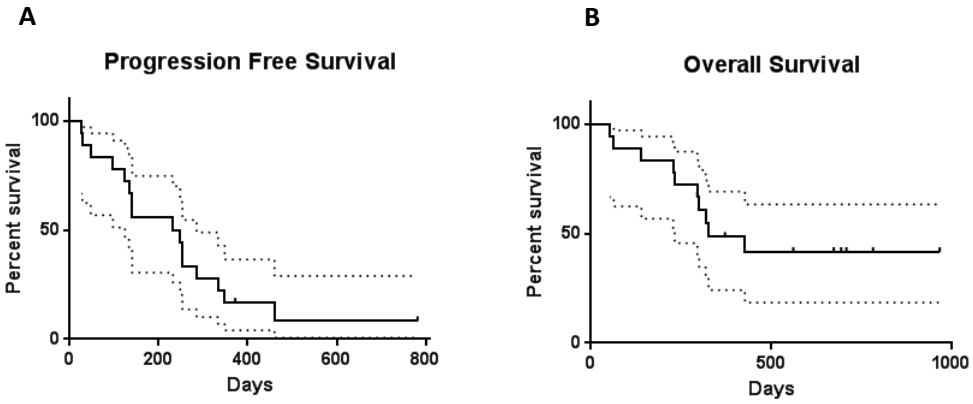


Supplementary figure 1. Pathway interactions between kinase signaling, protein metabolism and autophagy. The serine- threonine kinase mTOR plays an important role in regulating growth and proliferation in both normal and tumor cells. Activated mTOR engages anabolic pathways and inappropriate activation of this signaling cascade is found in most cancers. Therapeutic stressors, mTOR inhibitors, rapamycin, and DNA damaging alkylating agent, cyclophosphamide, lead to double stimulation of the autophagy pathway (red arrows). Autophagy serves a cytoprotective mechanism by clearing damaged organelles, aggregated proteins and recycling these to sustain survival of the cancer cell. Hydroxychloroquine inhibits the autophagy pathway at the autolysosome formation phase. Arrows: activation; flat lines: inhibition, PI3K: phosphotidyl inositol-3 kinase; mTOR: mechanistic target of rapamycin; HCQ: hydroxychloroquine.



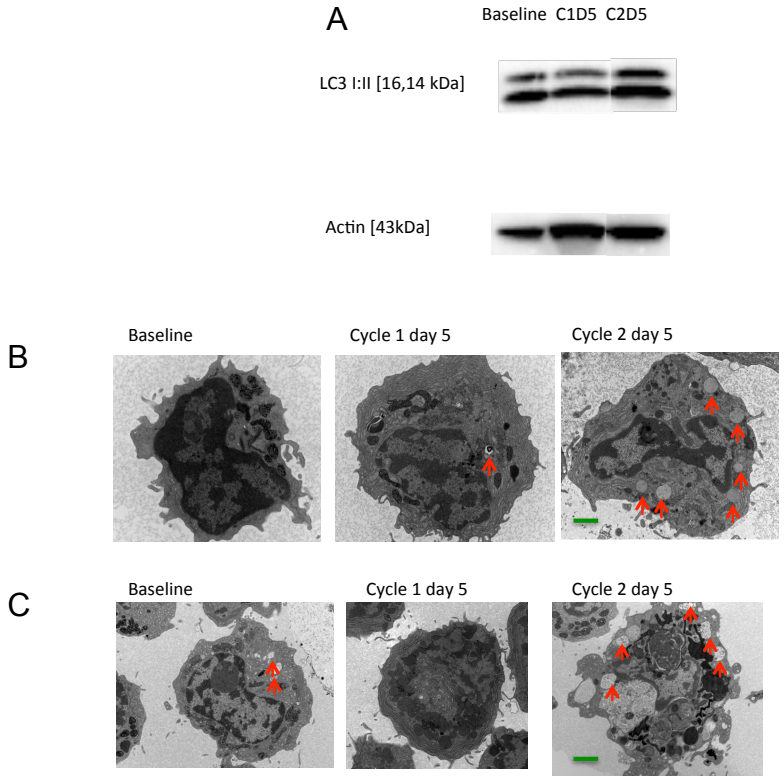
Supplementary figure 2. Duration of therapy and response using the International Myeloma Working Group Criteria: partial (PR); very good partial response (VGPR); stable disease (SD) categories and minor response (MR) per the European Group for Blood and Bone Marrow Transplant in 18 patients receiving cyclophosphamide, dexamethasone, rapamycin, and hydroxychloroquine in the phase 1 study. Solid bars represent duration of study therapy for individual patients, with bar color indicating the cohort by daily hydroxychloroquine dose. The colored squares show the beginning of the best response, and the colored circles show the time of disease progression. Solid colored lines represent the duration of response. Three subjects continued on therapy beyond biochemical progression due to limited treatment options

Supplementary figure 3



Supplementary figure 3. Kaplan-Meier progression free and overall survival curves of 18 patients receiving cyclophosphamide, dexamethasone, rapamycin, and hydroxychloroquine on the phase 1 study. Median PFS was 8.6 months (95% CI, days 124 – 285). Median overall survival was 11.3 months (95% CI, days 232 – not estimable).

Supplementary figure 4



Supplementary figure 4. Pharmacodynamic analysis of autophagy pathway in vivo patient samples. A) Western blot showing an increased LC 3 II:I ratio cycle 2 day 5 (subject in cohort 4). B and C) Electron Micrographs from subjects at baseline; after 6 days of rapamycin plus 4 days of cyclophosphamide and dexamethasone on cycle 1 day 5; and on cycle 2 day 5 after HCQ 800mg daily has been given for 28 days. Electron micrograph, direct magnification 4800X, green scale bar: 1 m. Red arrows indicate autophagic vesicles (AV). B) Subject in cohort 3. C) Subject in cohort 1.