Joanna Savvidou *et al. Medical Research Archives* vol 7 issue 12.

Medical Research Archives

RESEARCH ARTICLE

Overcoming Innate Resistance to a Beta-Catenin Inhibitor-Tegavivint- by Manipulating Autophagy in Multiple Myeloma

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ABSTRACT

Multiple myeloma (MM) is an incurable haematological malignancy with patients eventually exhausting all available therapeutic modalities and succumbing to the disease. Various lines of evidence have demonstrated that the Wnt/β-catenin pathway is frequently up-regulated in advanced MM. Tegavivint (BC2059), an inhibitor of the Wnt/β-catenin pathway, that we have previously shown to have anti-MM activity *in vitro, ex vivo* and *in vivo*, is currently in clinical trials for the treatment of solid tumours. In this study, we further demonstrate the dose dependent pro-apoptotic activity of BC2059, including activity against the innately resistant human MM cell line (HMCL) LP1. We show that BC2059 causes down-regulation of β-catenin and it's down-stream target survivin, in parallel with reactive oxygen species (ROS) generation. Coincident with this, BC2059 induces autophagy with a rise in LC3II and fall in p62. Moreover, chemical inhibition of autophagy was highly synergistic with BC2059 in all HMCL tested, with evidence of pro-apoptotic potentiation of BC2059 activity, with enhanced generation of active caspases 8, 9 and 3. Consistent with this, BC2059 treatment of LP1 in combination with knock down of the autophagy related protein Atg5 markedly enhanced cell killing. In conclusion, our results demonstrate that resistance to BC2059 can be partially attributed to the induction of cytoprotective autophagy and that inhibition of autophagy enhances BC2059-induced MM cell killing. The combination of Wnt/β-catenin pathway and autophagic inhibition represents a novel therapeutic strategy for advanced MM that warrants further evaluation.

Introduction

Multiple myeloma (MM) is a clonal plasma cell dyscrasia affecting approximately 100,000 people every year worldwide[1]. It is the second most common haematologic malignancy in the developed world and accounts for 1-2% of all malignancies[2]. Although MM is still considered a single disease, in reality it is a collection of cytogenetically distinct plasma cell malignancies[3]. For the past 15 years introduction of new therapies has significantly improved the overall survival of MM patients. However, majority of patients develop refractoriness to available therapeutic modalities and succumb to the disease, highlighting the need for novel therapeutic interventions.

The Wnt pathway is a highly evolutionary conserved intercellular communication route that regulates processes such as proliferation, differentiation, migration, fate determination and cell polarity[4]. In mature organisms the canonical Wnt signalling pathway modulates the balance between stemness and differentiation in several stem cell niches, including haemopoiesis within the bone marrow[5]. Canonical Wnt pathway revolves around the stabilisation and localisation of β-catenin[6]. Upon activation by Wnt ligands, β-catenin escapes phosphorylation and proteasomal degradation, migrates into the nucleus where it binds to the DNA-bound T cell factor/lymphoid enhancer factor (TCL/LEF) complex initiating transcription of Wnt target genes such as *CD44, CCND1 (encoding cyclin D1), AXIN, c-MYC, BIRC5 (encoding survivin)*[7].

The role of Wnt canonical pathway dysregulation in malignant transformation has long been established[7]. The best studied paradigm is colorectal carcinoma (CRC) where up-regulation of the pathway leads to constitutive, ligand-independent signalling[8]. With regards to MM, several groups, including ours, have shown expression of active, non-phosphorylated nuclear β-catenin in patients derived myeloma cells and human myeloma cell lines (HMCL) unlike healthy plasma cells[9-14]. BC2059 (Tegavivint), an AV-65 derivative, is an anthracene dioxime compound that has been shown to interrupt the Wnt/β-catenin pathway by disrupting transducin β-like protein 1 (TBL1) interaction with the coactivator molecule β-catenin[15]. It has demonstrated anti-tumour activity in AML[15], post-myeloproliferative neoplasm secondary AML[16], MM[14] and chemotherapy resistant or metastatic osteosarcoma[17] whereas, it is in clinical trial for desmoid tumours (*NCT03459469).*

Despite the central role of Wnt canonical pathway in several tissues homeostasis (skin, gut, haematopoietic) BC2059 is well tolerated *in vivo*[14-17]. Nevertheless, there is an unmet need to augment its anti-tumour effect with decreasing, if possible, its potential on-target toxicity. Autophagy is an evolutionarily conserved catabolic process. It is thought to play an important pro-survival role in cell homeostasis, mainly during periods of starvation or metabolic stress. (Macro) autophagy (hereafter referred as autophagy) involves the formation of double membrane vesicles, the autophagosomes, which engulf proteins and organelles. These autophagosomes fuse with lysosomes and their contents are degraded by lysosomal proteases[18]. Recent studies have shown that tumour resistance to anticancer therapies can

be partially attributed to the up-regulation of autophagy[19]. At the same time, a growing body of evidence implies that chemotherapy-induced autophagy can partially mediate the cytotoxic action of these drugs. Therefore, induction of autophagy by chemotherapeutic agents and its impact on tumour cell survival is considered to be drug, extent of induction, duration and cellular context specific[20]. In this study we investigate the role of autophagy in innate resistance of HMCL to BC2059 and its potential therapeutic implications.

Materials and Methods
HMCL, culture conditions and chemicals
U266 and NCI-H929 were obtained from the American Type Culture Collection (ATCC). LP1 cell line was from Deutshe SammLung von Mikroorgaanismen und Zellculturen. These cells were authenticated by the supplier using cytogenetics, DNA typing, immunophenotyping, and cell line speciation. Cell lines are screened every 2 months for mycoplasma contamination by VenorGeM Mycoplasma Detection Kit. The HMCLs were authenticated on December 2016 by CellBank Australia by the use of short tandem repeat profiling, in line with the standard ANSI/ATCC ASN-0002-2011, and matched publicly available data. HMCLs were grown and treated at densities of 2.0×10^5 cells/mL in RPMI1640 media (Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS (Lonza) and 2 mmol/L L-glutamine (Gibco, Invitrogen). All cells were cultured in a humidified incubator at 37°C with 5% CO_2 and used until 20th passage. All HMCL were passaged 24h before the experimental setup to ensure high viability

(>80%). Cells with poorer viability were not included in the experiments.

BC2059 was provided by BetaCat Pharmaceuticals/Iterion Therapeutics and resuspended in DMSO. Bortezomib was purchased by assaymatrix, Melbourne, Australia. Chloroquine (CQ), 3-methyl alanine (3-MA) from Sigma and Bafilomycin A1 from BioAustralis .N-acetyl cysteine (NAC) (Sigma) was made fresh before each experiment and pH adjusted to 7.2-7.8.

Cell proliferation and viability
Cell proliferation of the treated HMCL was determined by Trypan blue staining and hemocytometer counts, and the degree of cell death was assessed by FACS with propidium iodine (PI) staining at 24hr.

Flow cytometry studies
For a-caspase 3 intracellular staining, cells were treated with BC2059 for 24hr and then harvested. Cells were then washed and fixed in 3.8% PFA on ice for 15 minutes before washed with cold PBS, centrifuged and supernatant discarded. Cells were then resuspended in 50μl of 1/20 a-caspase 3 antibody (FITC-active-caspase 3, 559341, BD Biosciences) in 0.1% saponin in 1% FBS/PBS permeabilisation buffer, for 30 minutes in the dark at RT. Cells were then washed twice with PBS, resuspended in 100μl of 2% FBS/PBS for immediate FACS acquisition (FC).

For a-caspase 8 and 9 intracellular staining, we used active-caspase 8 and 9 staining kits (FITC-IETD-FMK, ab65614 and FITC-LEHD-FMK, ab65615, abcam respectively). Cells were treated with chemicals for 24hr. 1μl of anti-a-caspase 8 or 9 antibodies were

added in to the well plates and incubated for 1h at 37^0C incubator with 5% CO_2. Cells were then collected, washed with Wash Buffer twice and then resuspended in 100μl of Wash Buffer and immediately acquired by FC.

For p62 intracellular staining, cells were treated with BC2059 for 24hr and then harvested, washed and fixed with 3.8% PFA on ice for 15 minutes before cells were washed with cold PBS, centrifuged and supernatant discarded. Then cells were permeabilised with 0.1% Tween-20/ PBS for 20 minutes. The cells were then incubated in 1×PBS/ 5% normal goat serum/ 3% FBS to block non-specific protein-protein interactions followed by Alexa Fluor-488 SQSTM1/p62 antibody (ab185015, abcam) (1/500 dilution) for 30 minutes at RT. Isotype control antibody was rabbit IgG Alexa Fluor 488 used at the same concentration and conditions as the primary antibody. For positive control, cells were treated with 500nM of Rapamycin (autophagy inducer).

For survivin intracellular staining, cells were treated with BC2059 for 24hr and then harvested, washed and stained with viability stain (Fixable Viability Dye eFluor 660, 65-0864, Invitrogen) according to the manufacturer's protocol. Cells were then washed, fixed (as above) permealised with 0.2% Triton-X/PBS and then stained with survivin antibody (survivin PE conjugated antibody, IC6472P, R&D systems) or isotype control for 60min. Survivin median fluorescence intensity (MFI) was estimated on the viability stain negative (live) cells.

Electron Microscopy

After 6h incubation, with or without CQ, NCI-H929 cells were fixed in 2.5% glutaraldehyde/ 0.1M Na-Cacodylate buffer for 120 min at room temperature. Cells were postfixed in 1% OsO_4 at room temperature for 60 min, dehydrated through graded ethanol solutions, and embedded in Epon (Procure 812, 2–dodecenylsuccinic acid anhydride [DDSA], araldite, benzyldimethylamine [BDMA]). Cells were visualised with Hitachi TEM H7500. (Bars represent 5μm).

Reactive Oxygen Species (ROS) Assay

Cellular ROS was measured using a detection assay kit (DCFDA/H2DCFDA-Cellular ROS Assay kit,ab113851, abcam) according to the manufacturer's protocol. In brief, 2′,7′-dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell, was added to LP1 cells for 30min. Cells were then washed with Buffer, resuspended in Buffer Solution/10% FBS and transferred in a black-well 96-well plate (CORNING, Costar Assay Plate 96 well). After 1h BC2059 was added at increasing concentrations and fluorescence (495nm/529nm) measured with a microplate reader (FLUOstar OPTIMA) at 1hr, 3hr, 5hr and 6hr. Results are exressed as percentile changes of ROS generation compared to the UT population at 1hr post staining.

Western blotting

Western blotting was performed as described previously[14]. After membranes were blocked with 5% BSA, 0.1% Tween-20/PBS they were incubated first with anti-β-catenin

(9562, Cell Signaling), anti-LC3B (2775, Cell Signaling) or anti-tubulin (loading control) (T9026, Sigma-Aldrich), were then incubated with secondary horseradish peroxidase–tagged antibody (Dako). The HRP secondary antibodies were detected with Pierce ECL chemiluminescence (Thermo Fischer Scientific) as per manufacturer's instructions. Chemiluminescent membranes were then exposed to film 9AGFA, Scoresby) and developed using a 100-plus film developer (All-Pro Imaging). Experiments were performed in triplicate.

Transient ATG5 knock-down

For ATG5 knockdown, 500uL of 10^5/mL LP1 cells were plated into a 24-well plate and transfection was carried out with Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the protocol provided by the manufacturer. For ATG5 knockdown, we used ON-TARGETplus Human ATG5 siRNA at concentrations recommended by the manufacturer, whereas Silencer Negative Control No. 2 siRNA (Thermo Fisher Scientific) was used as a negative control, at the same concentration. Six hours after the transfection cells were treated with BC2059 200nM and then harvested after 24hr. Cell death was monitored by PI staining with FACS.

Combination indices and statistical analysis

Combination indices were calculated with CalcuSyn software (CI < 1 defines synergism. Statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software, Inc.).

Results

BC2059 induces apoptotic cell death in HMCL

In previous reports we have demonstrated the anti-proliferative and cytotoxic effect of BC2059 in HMCL [14-16]. The majority of HMCL tested demonstrated IC_{50} ranging from 40nM to 200nM (173nM for NCI-H929, 186nM for U266) whereas LP1 was found to be relatively resistant with IC_{50} of 320nM (supplementary figure 1). We further validated the pro-apoptotic effect of BC2059 by monitoring the levels of active-caspase 8 (extrinsic apoptotic pathway), active-caspase 9 (intrinsic apoptotic pathway) and active-caspase 3 (effector caspase) by flow cytometry (FC) in HMCL with intermediate (U266, NCI-H929) or high IC_{50} (LP1) treated with the drug for 24hr. All 3 HMCL tested showed a dose dependent increase of both active-caspase 8 and 9 (figure 1a and supplementary figure 2a-b). For LP1 active-caspase 8 and 9 positive cells increased 4.0±1.8 and 5.3±1.7 times respectively relative to the untreated population at IC_{50}. The effector active-caspase 3 positive cells were 1.9±0.14 times increased relative to the untreated population at LD_{50} for LP1, proportional to the cytotoxic effect of the drug, monitored by PI staining (figure 1a and supplementary figure 2a-b).
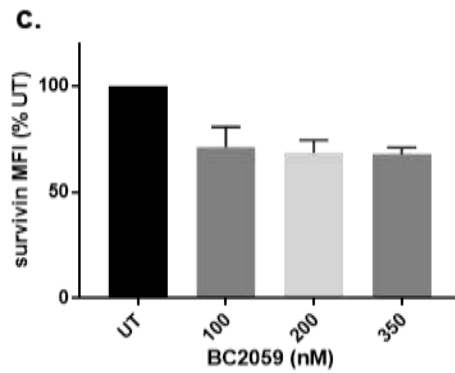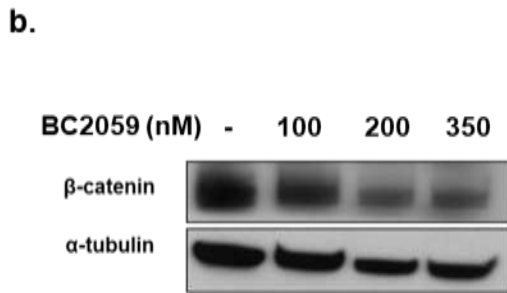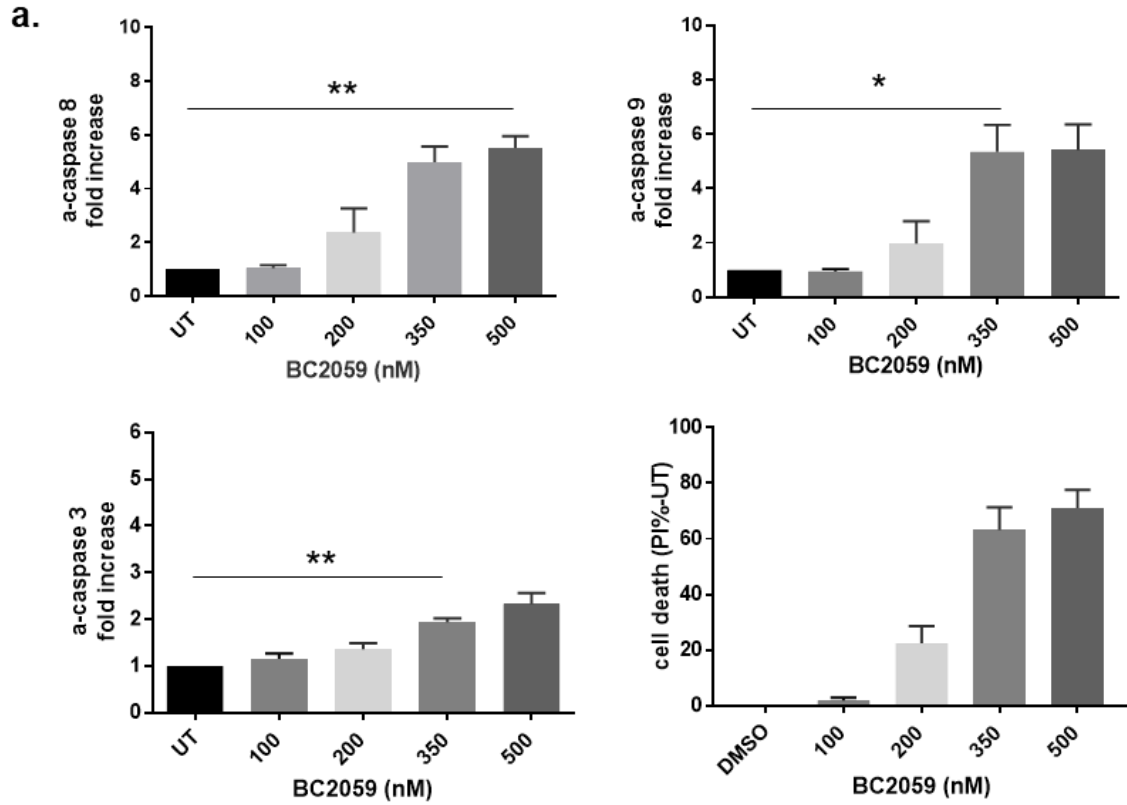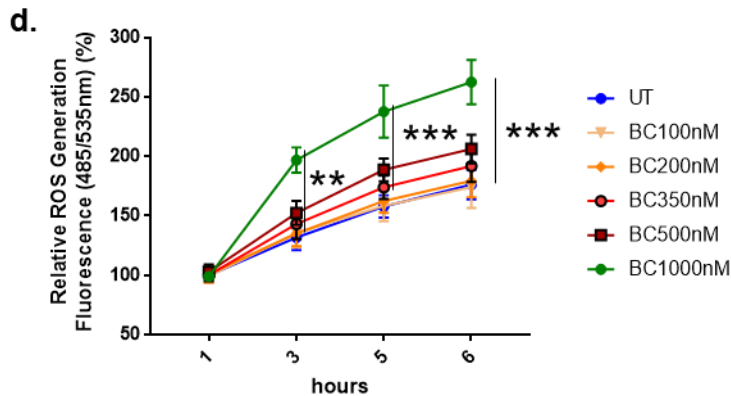
Figure 1. **BC2059 activates both the intrinsic and extrinsic apoptotic pathways, induces down-regulation of β-catenin and down-stream target survivin and stimulates ROS generation in a dose-dependent manner. (a)** LP1 cells were treated with BC2059 at increasing concentrations for 24hr. Active-caspases were monitored by FC and their increase is expressed as fold increase compared with the untreated (UT) population (n=3, mean± se). p values for the caspases increase were calculated using one sample t-test, p values< 0.05 are considered statistically significant. Cell death was monitored by PI staining and FC and expressed as increase percentage compared to the UT population (n=3, mean± se). **(b)** BC2059 treatment of LP1 cells for 24h causes reduction of total β-catenin protein expression. Loading control: α-tubulin. Representative blot of n=3. **(c)** LP1 cells were treated with BC2059 at increasing concentrations for 24hr. Intracellular expression of survivin was monitored by FC and expressed as percentage of MFI, where control MFI (untreated cells) was considered equal to 100%. **(d)** LP1 cells were stained with DCFDA and ROS generation was monitored after the addition (t=1hr) of increasing concentrations of BC2059 at different time points (n=4, mean± se). Results are expressed as percentile changes of ROS generation compared to the UT population at 1hr post staining (100%).

In accordance with our previous data, BC2059 induced down-regulation of β-catenin in LP1 at doses ≥ 200nM (figure 1b) while inducing a moderate decrease of intracellular expression of β-catenin's down-stream target BIRC5/survivin as monitored by FC on live cells (figure 1c). A drop of median fluorescence intensity (MFI) relative to the untreated population at IC_{50} was observed (32%±5.4). Likewise, BC2059 induced generation of reactive oxygen species (ROS) in a time and dose-dependent manner (figure 1d). The increase of ROS was significant with the addition of 1μM BC2059 just 2h after drug addition. Nevertheless, ROS generation did not prove to be cytotoxic since addition of ROS scavenger N-acetyl cysteine (NAC) at 10mM and 20mM, 3h before BC2059 did not exert protection against BC2059 cytotoxicity at 24h (supplementary figure 3).

HMCL depend on basal autophagy for proliferation

Induction of autophagy has been demonstrated in healthy long-lived plasma cells[21] and myeloma MM cells[22] possible through the mechanism of enabling MM cells to sustain the stress of antibody production. To quantify the autophagic activity of HMCL at baseline we assessed the autophagic flux in 4 HMCL 24hr after the addition of chloroquine (CQ), a late autophagy inhibitor. All 4 HMCL showed intense autophagic fluxes at baseline (figure

2a). In concordance electron microscopy confirmed high autophagic flux of NCI-H929 after the administration of CQ for 6h by the appearance of large autophagosomes in the majority of cells (figure 2b).

Interestingly, the majority of HMCL tested showed moderate dependence on the autophagic pathway for proliferation (figure 2c), with a less pronounced cytotoxic effect (figure 2d). Furthermore, induction of autophagy in NCI-H929, the most autophagy-dependent HMCL for proliferation, with rapamycin (mTOR inhibitor-autophagy inducer) had a pro-proliferative effect (figure 2e).
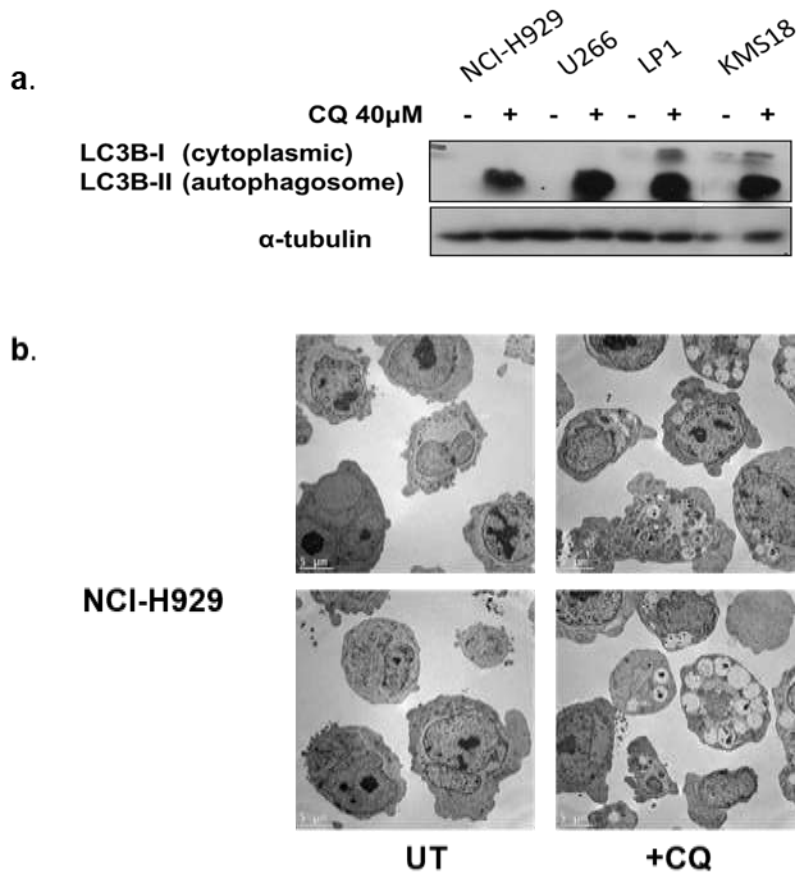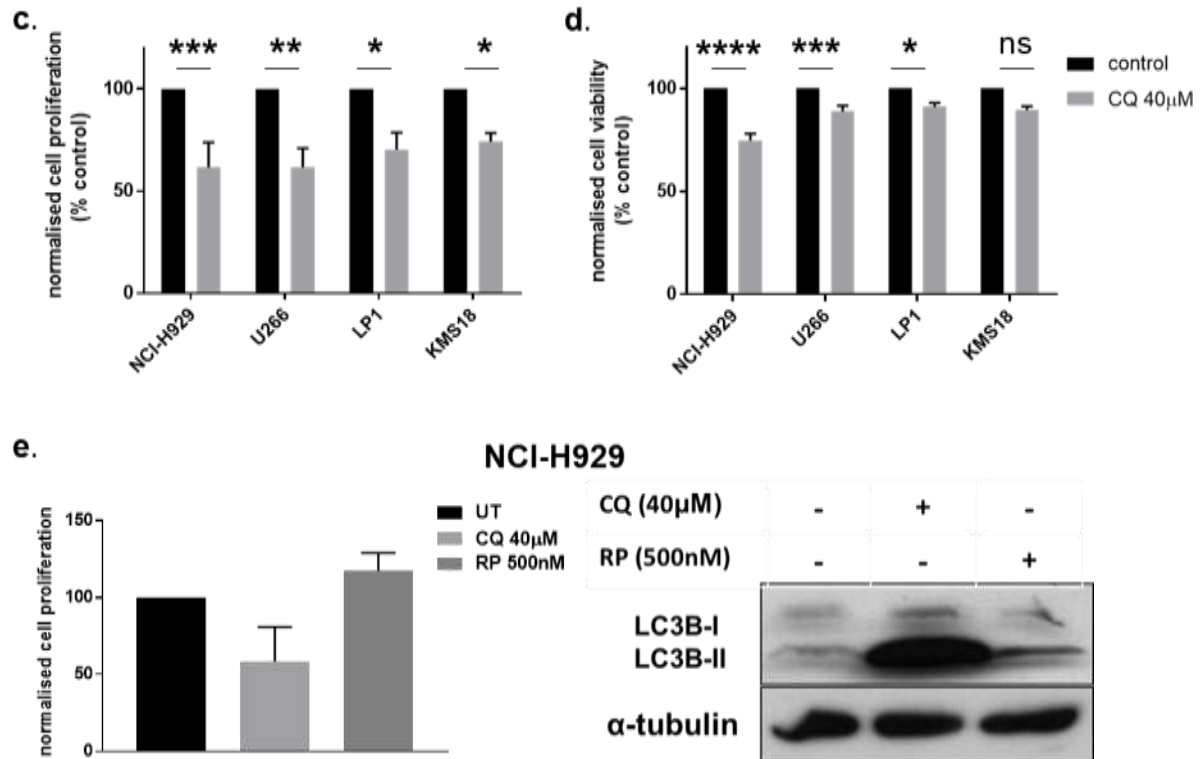
Figure 2. **HMCL are characterized by high basal autophagic flux.** (**a**) Immunoblot comparing the levels of endogenous lipid conjugated LC3B-II to unconjugated LC3B-I in 4 HMCL, in the absence or presence of CQ at 24hr. (**b**) NCI-H929 cells were left UT or treated with 40µM CQ for 6h. Representative EM images demonstrate large number of autophagosomes upon addition of CQ in the majority of cells ((Bars represent 5µm). (**c**) Inhibition of autophagy in HMCL by CQ has a cytostatic effect. 4 HMCL were treated with 40µM of CQ or left untreated. At 24hr viable cells were determined by haemocytometer counts and cell death measured by FC detection of PI positive cells. (**d**) Effect of inhibition and stimulation of autophagy on absolute cell numbers and LC3II/LC3I ratio upon treatment of NCI-H929 with CQ (40 µM) or Rapamycin (500nM), respectively, at 24hr.

BC2059 augments autophagic flux in HMCL

To assess the effect of BC2059 on the autophagic flux, LC3B conversion and p62 expressions were measured using WB and FC, respectively. At 24hr BC2059 was able to induce autophagy as indicated by an increase in LC3B conversion and a concomitant decrease of p62 in all 3 HMCL (NCI-H929, U266, LP1) tested (figure 3a). Rapamycin was used in NCI-H929 as a positive control for the decrease of p62 monitored by FC.

Neither melphalan at 50µM nor bortezomib at 5nM induced autophagy in NCI-H929 cells (figure 3b), although they cause a comparable drop in cell numbers at 24h as that induced by 200nM BC2059, confirming that autophagy induction in MM is not a non-specific response to every pro-cell death chemical insult.
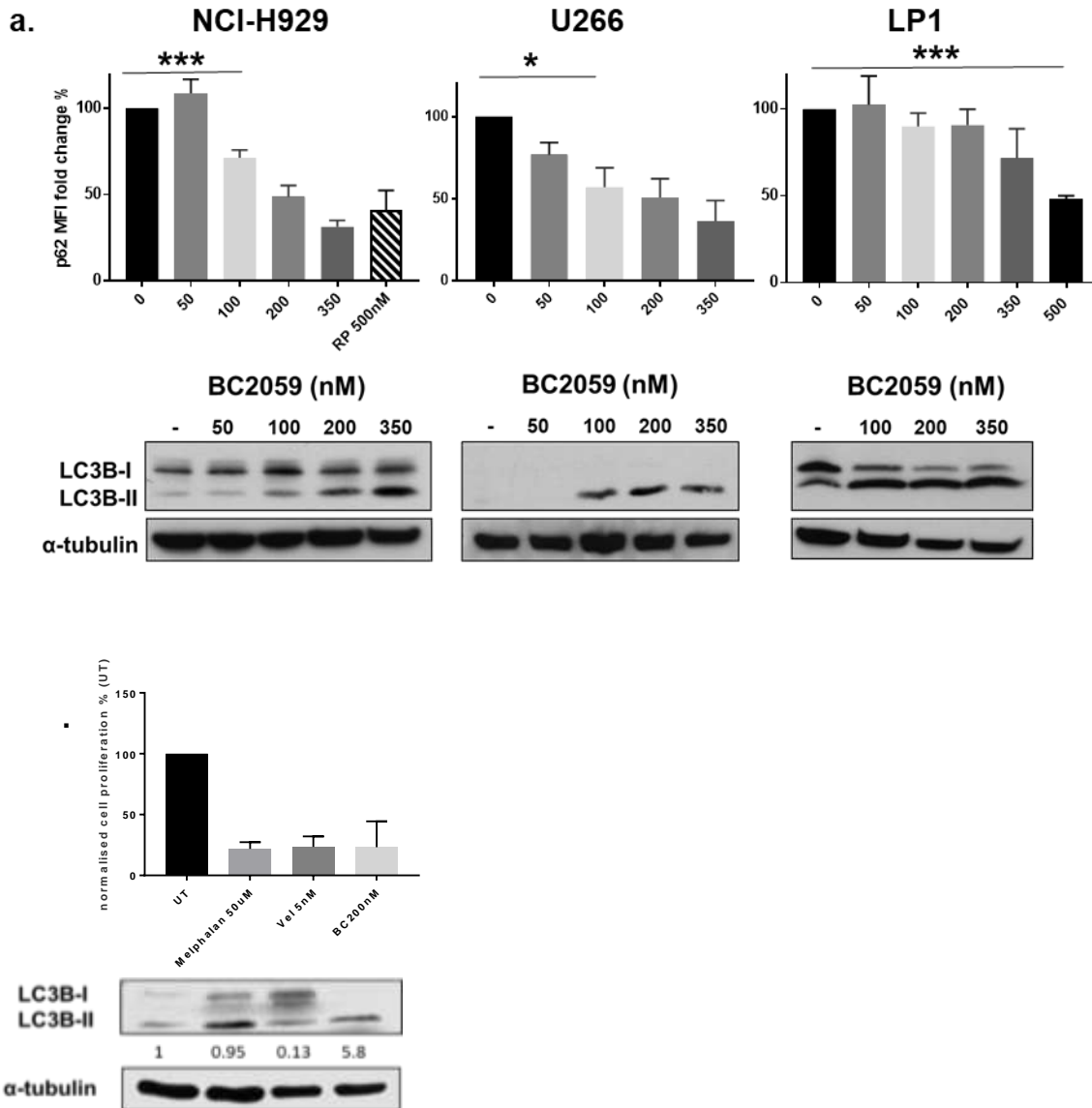
Figure 3: **BC2059 augments autophagic flux in HMCL. (a)** NCI-H929, U266 and LP1 were treated with increasing concentrations of BC2059 for 24hr. Intracellular p62 was quantified with FC (upper panels) and expressed as percentage of MFI, where control MFI (untreated cells) was considered 100%. Rapamycin treatment was used as a positive control for autophagy stimulation (n=3-5, mean± se). LC3I conversion to LC3II at the corresponding dosage was monitored by WB (lower panels) (representative immunoblots, n=3). **(b)** Reduction in cell numbers at 24hr following treatment of NCI-H929 with melphalan, bortezomib or BC2059 (upper panel) and LC3I and LC3II levels of the corresponding treatments (lower panel). Numbers indicate the levels of LC3II/LC3I ratio measured by densitometry, where α-tubulin is used as loading control and LC3II/LC3I of the control (untreated cells) is considered 1.

Inhibition of autophagy induces synergistic killing of HMCL with BC2059

To suppress autophagy we used chloriquine (CQ), a late stage autophagy inhibitor, which as a weak base, accumulates in acidic vesicles such as lysosomes and disrupts vesicular acidification, preventing further fusion of autophagosomes with lysosomes[23].

The combination of BC2059 with CQ had a significant synergistic effect against all 3 HMCL tested (U266, NCI H929, LP1) (figure 4a), with combination indices (CIs) ranging from 0.8 to 0.3. Furthermore, inhibition of autophagy reduced the LD_{50} of

BC2059 for all 3 HMCL, but most importantly in LP1 (resistant cell line), where CQ addition caused a drop of LD_{50} to half, overcoming the innate resistance of the cells to the drug (figure 4b).
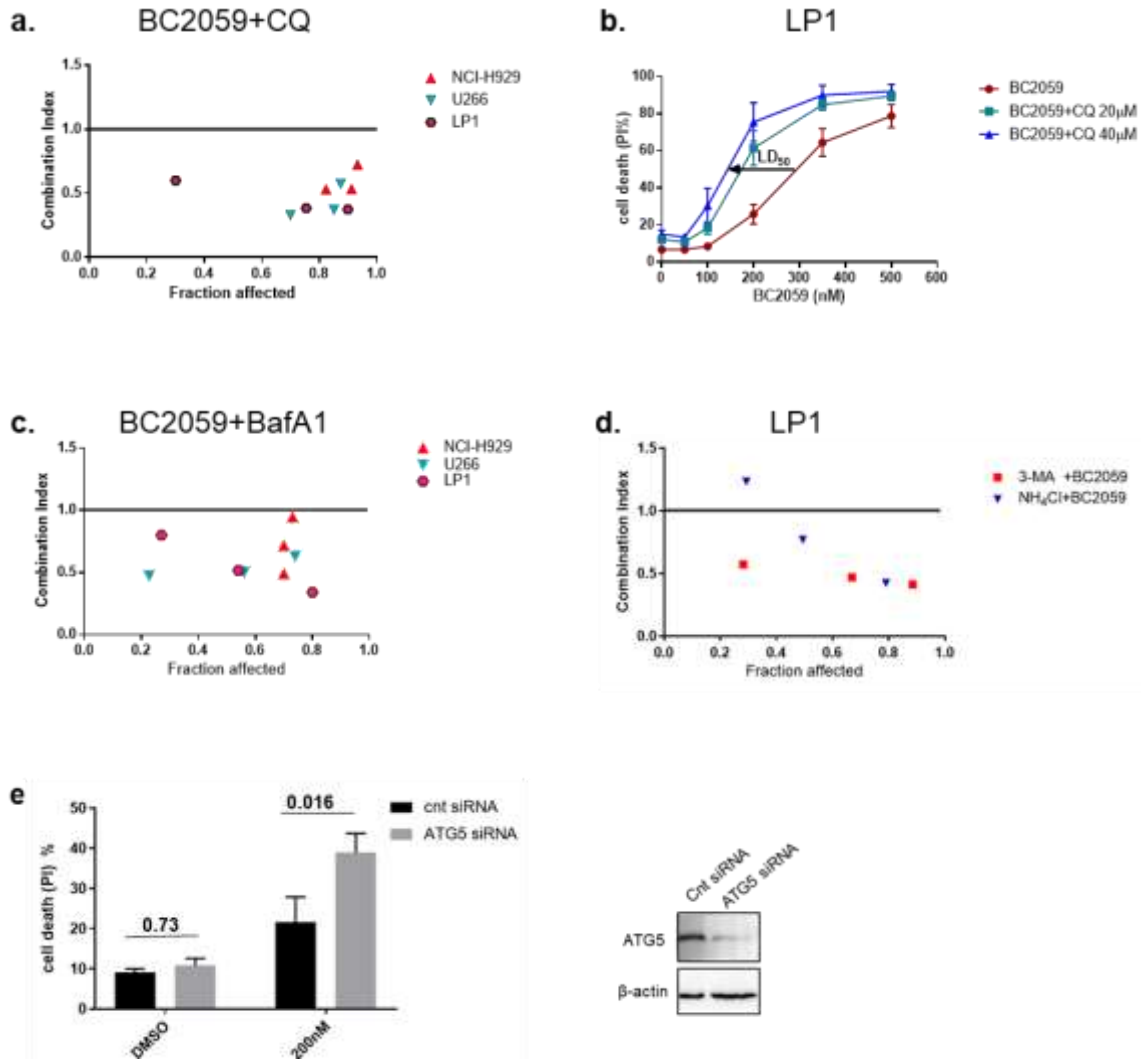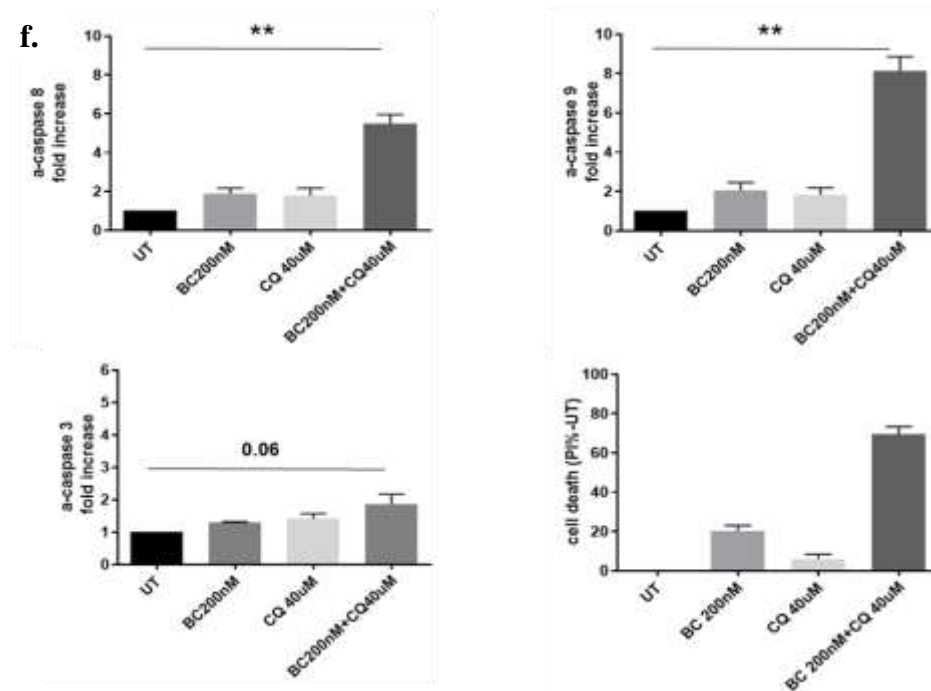
Figure 4: **Chemical inhibition of autophagy enhances the pro-cell death effect of BC2059 in HMCL. (a)** NCI-H929, U266 and LP1 were treated with BC2059 (100nM-200nM-350nM) with or without the addition of CQ (40µM-1hr prior to the addition of BC2059-) for 24hr, and cell death was monitored using FC and PI staining. The level of synergy was expressed as the CI (calculated with Calcusyn software from the concentration-effect curves of the mean of three independent experiments) and plotted against the fraction affected. **(b)** Concentration-effect curves for LP1 treated with BC2059 for 24h alone or after the addition of CQ at 20µM or 40µM (n=3±se). **(c)** NCI-H929, U266 and LP1 were treated with BC2059 (100nM-200nM-350nM) with or without the addition of Bafilomycin A1 (100nM-1hr prior to the addition of BC2059-) for 24hr, and the CI were calculated and plotted against the fraction affected (n=3). **(d)** LP1 cells were treated with BC2059 (100nM-200nM-350nM) with the addition or not of autophagy inhibitors 3-MA and NH_4Cl (10mM and 50mM respectively) and the CI were calculated and plotted against the fraction affected (n=3). **(e)** ATG5 knockdown was performed in LP1 HMCL, 6hr after transfection cells were treated with 200nM of BC2059 for 24hr. Cell death was monitored by PI^+ staining (*n* = 3 ±se). ATG5 protein level was evaluated 24 hours after transfection by immunoblotting. ATG5 siRNA transfected cell lysates were compared with the negative control siRNA lysate. b-Actin: loading control. **(f)** LP1 cells were treated with BC2059 (200nM), CQ (40µM) or both drugs (CQ 60 min prior to BC2059) for 24hr. Active caspase-8,-9 and-3 were measured with FC and the results expressed as fold increase compared to untreated (UT) cells (n=3, ±se). Cell death was monitored by PI staining

 Although CQ is the only clinically available inhibitor of autophagy it lacks specificity, thus we tested the effect of other chemical inhibitors of autophagy when combined with BC2059. All 3 HMCL cells were treated with bafilomycin A1, a specific inhibitor of V-ATPase that inhibits the acidification of lysosomes and endosomes thus preventing maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes. Combination therapy of BC2059 with bafilomycin A1 proved to be synergistic in all 3 HMCL tested (figure 4c), with CIs less than 1. Furthermore, when LP1 cells were treated with either NH_4Cl or 3-MA, a

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lysosomal lumen alkaliser and a PI3K inhibitor, respectively, the combination proved to be synergistic (figure 4d).

Additionally, knock-down of the autophagy related protein ATG5 in combination with 200nM of BC2059 for 24h augmented the cytotoxic effect of BC2059 when compared with the negative control (control siRNA) (figure 4e). Collectively these results showed that BC2059 induces pro-survival autophagy, which when inhibited sensitizes the cells to the drug.

Inhibition of autophagy augments the pro-apoptotic effect of BC2059

Although autophagy and apoptosis undoubtedly represent distinct cellular processes with fundamentally different biochemical and morphological features, the protein networks that control their regulation and execution are highly interconnected[24]. From the clinical point of view, we were interested to evaluate the changes in the apoptotic pathways, both intrinsic and extrinsic, upon chemical inhibition of the pro-survival autophagy induced by BC2059. LP1, U266 and NCI-H929 were treated with BC2059 (200nM and 100nM respectively), CQ (40μM) or the combination of the two drugs (CQ treatment 60min before BC2059) and the levels of activated caspases 8, 9 and 3 were measured with FC whereas cell death was evaluated by PI staining and FC. In all 3 HMCL the addition of CQ caused a significant increase of activation of both the intrinsic and extrinsic apoptotic pathways, which led to synergistic cell death (figure 4f and supplementary figure 4).

Discussion

MM is a highly heterogeneous and incurable disease. Substantial evidence suggests that the Wnt/canonical pathway contributes to both disease progression and drug resistance, irrespective of the genetic background[6,25], confirming the pathway as an attractive potential therapeutic target. Similarly, autophagy is shown to promote cancerous cells proliferation and resistance to their apoptosis, counteracting the effect of stressful microenvironment conditions[26]. Recently, autophagy inhibitors have been used in different cancer models, in combination with chemotherapy in order to augment the cytotoxic effect of the chemotherapy. Thus, it is hypothesized that pro-survival autophagy may represent a major obstacle to successful chemotherapy, representing an additional appealing therapeutic target.

In accordance with our previous work we showed that BC2059 exerts cytotoxic effect in HMCL in a dose-dependent manner, mainly by activation of the apoptotic cascade, both extrinsic and intrinsic (figure 1a). Even in HMCL with relative resistance to the drug, BC2059 was able to down-regulate Wnt-canonical key player, β-catenin and one of its down-stream targets, survivin (figure 1c). Survivin is a small protein which functions as an apoptosis inhibitor. It is known to be overexpressed in myeloma cell lines and its expression in primary myeloma cells has been associated with poor prognosis, disease progression, and drug resistance[27]. Initiation of the apoptotic cascade could be partially attributed to the significant down-regulation of survivin by the addition of BC2059 as early as 24hr. On

the other hand, survivin down-regulation, chemical or genetic, has been shown to induce autophagy[28], possibly by interfering with its interactions with LC3 and beclin1[29,30]. This interactions could partially explain the ability of BC2059 to further induce the autophagic flux in HMCL in a dose dependent and drug-specific manner (figure 3a,b). This induction seems to be an adaptive pro-survival mechanism in order for the cells to withstand the chemotherapeutic stress. Thus, chemical or genetic inhibition of autophagy was able to significantly increase the cytotoxic effect of the drug, even in the most resistant HMCL (LP1). This synergistic effect was mainly achieved by releasing the inhibitory impact of autophagy on the apoptotic activation cascade (figure 4).

Additionally, we were able to show that higher doses of BC2059 induced ROS generation just 2hr after drug addition. It was previously reported that loss of β-catenin, augments the production of ROS after irradiation or chemical injury, demonstrating that the Wnt pathway plays a role in the regulation of oxidative stress[31]. Nevertheless, ROS generation did not exert cytotoxic effect on HMCL, since addition of a ROS scavenger had no cytoprotective effect (supplementary figure 4). On the contrary, ROS removal augmented the cytotoxic effect of the drug. As previously described, ROS levels have been shown to regulate autophagy induction in the cells. Furthermore, several anti-cancer drugs have been shown to activate autophagy through ROS induction, leading in turn to drug resistance[32].

In various solid tumours and in MM patients it has already been demonstrated that hydroxychloroquine (HCQ) in combination with other chemotherapeutic agents, including bortezomib, is both feasible and well tolerated[33,34]. It is interesting to note that there are currently 66 trials examining the effect of hydroxychloroquine (HCQ) in cancer. Regarding MM, 3 clinical trials have used HCQ in combination with standard chemotherapy, studying the possible benefits of autophagy inhibition. Some of the major challenges in the use of HCQ against cancer, is the definition of the necessary dose, its tolerability, as well as the identification of a reliable biomarker of its activity. Collectively, and in accordance with previous studies, our results demonstrate an alternative approach that may represent a new paradigm for enhancing the activity of established or novel anti-MM regimens.

Conflict of interest
The authors have no conflict of interest to declare.

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Supplementary materials
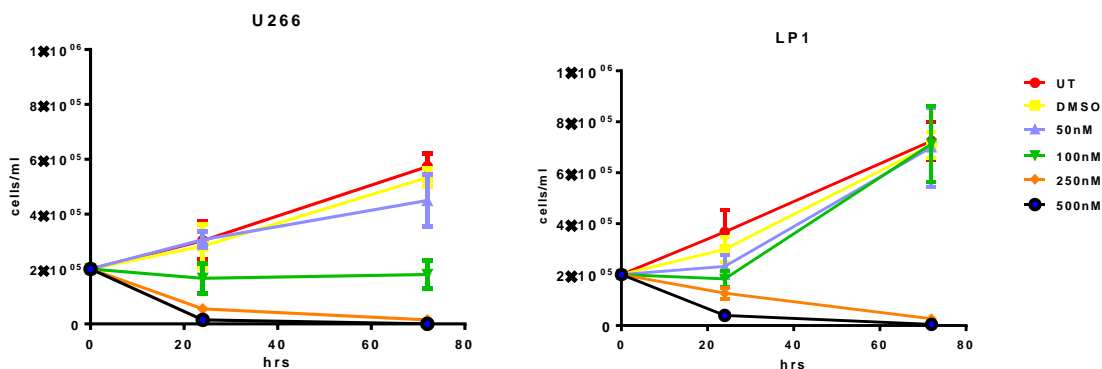
Supplementary figure 1: Absolute cell numbers of viable U266 and LP1 cells were determined by haemocytometer counts of HMCL cultured alone (UT), with BC2059 (50, 100, 250, 500nM) or with vehicle (n=3± se).
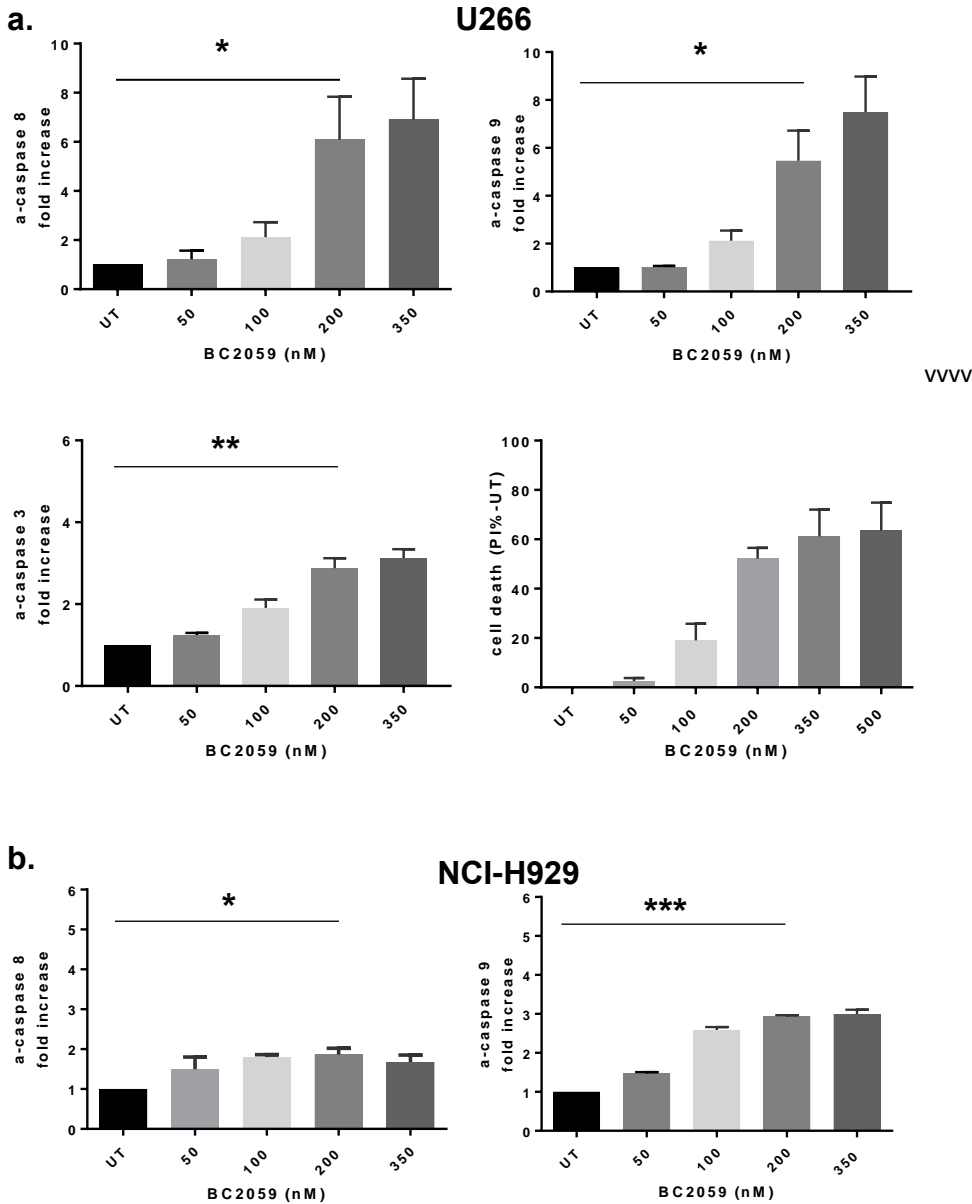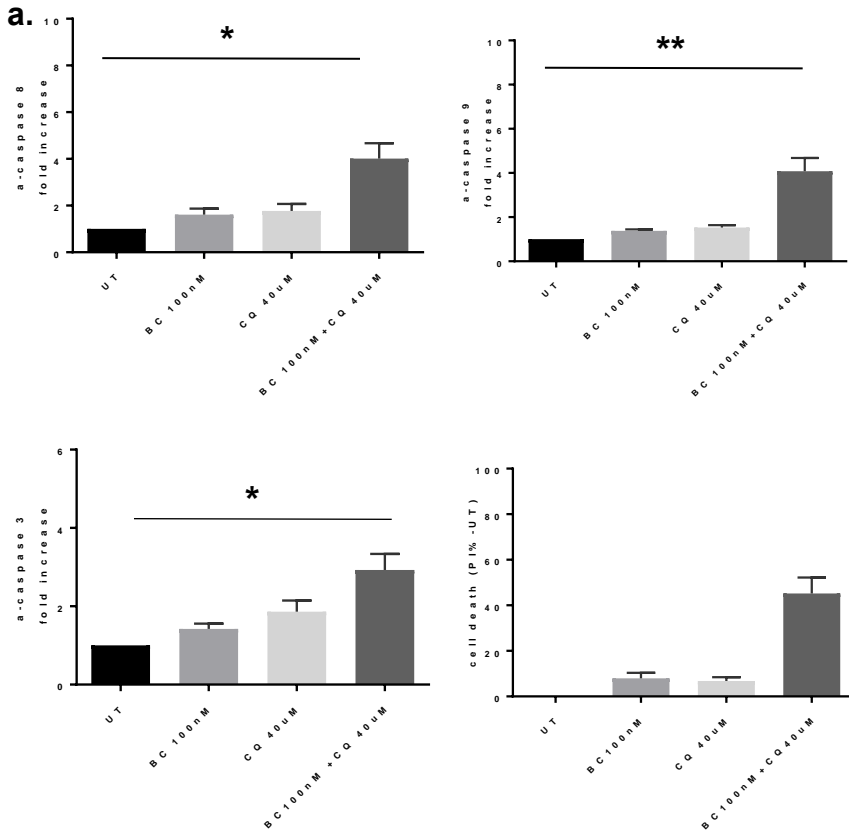
Supplementary figure 2: U266 and NCI-H929 cells were treated with BC2059 at increasing concentrations for 24hr. Active-caspases were monitored by FC and their increase is expressed as fold increase compared with the UT population (n=3, mean± se). p values for the caspases increase were calculated using one sample t-test, p values< 0.05 are considered statistically significant. Cell death was monitored by PI staining and FC and expressed as increase % compared to the UT population (n=3, mean± se).
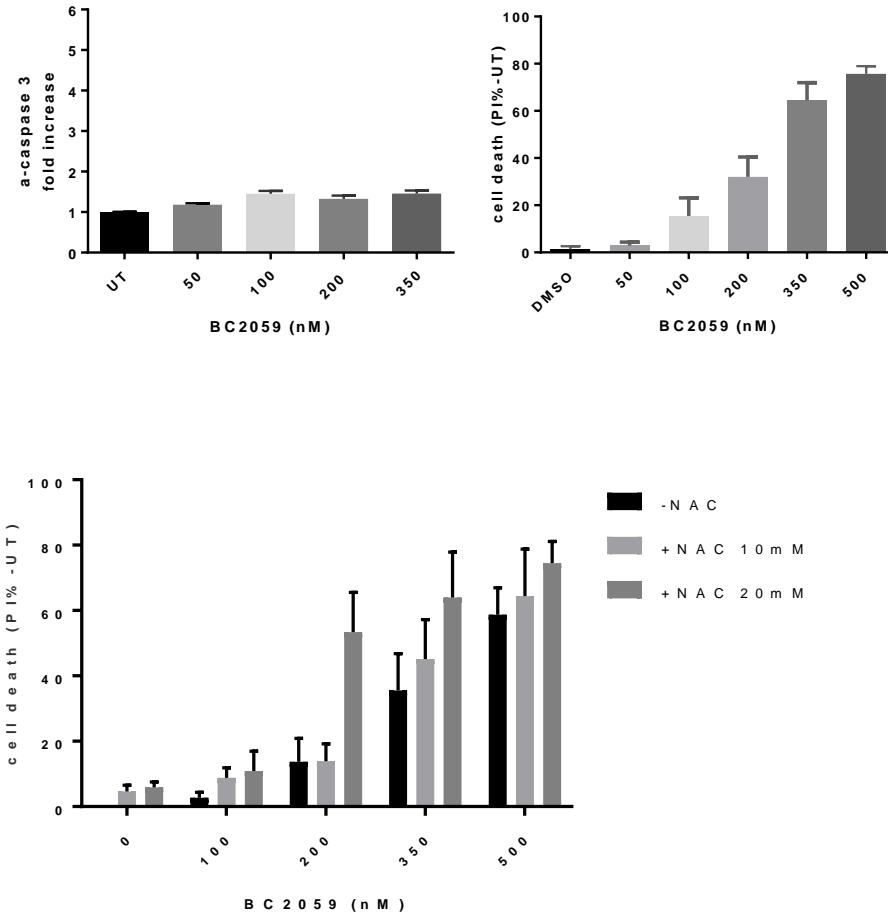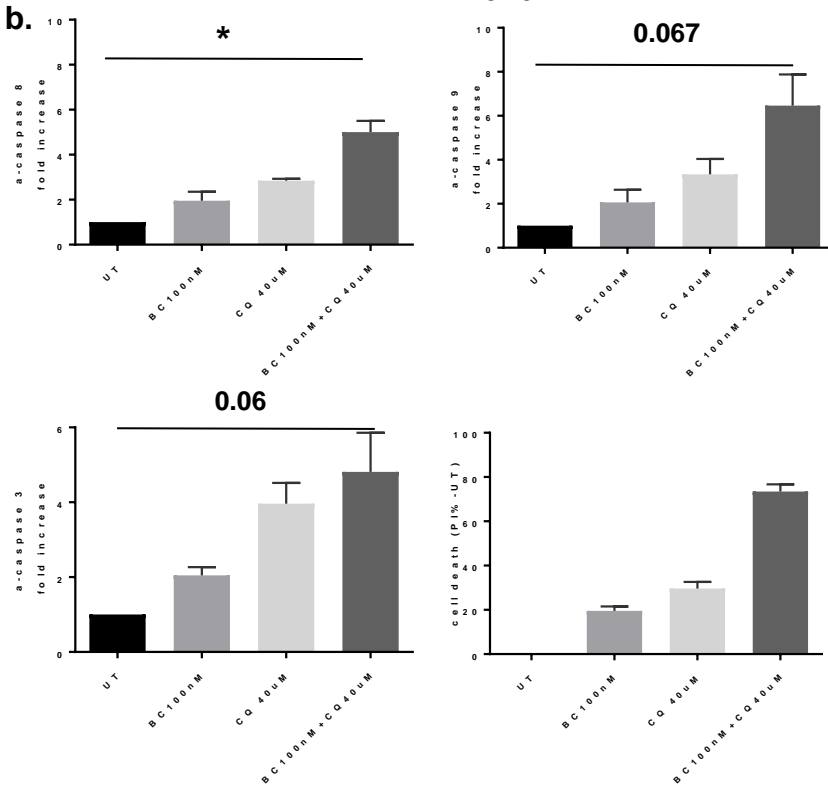
Supplementary figure 3: LP1 HMCL were treated with BC2059 at increasing concentrations for 24hr in the absence or presence of NAC, which was added 3hr in advance. Cell death was monitored by PI staining and FC and expressed as increase % compared to the UT population (n=3, mean± se).

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U266

NCI-H929

Supplementary figure 4: U266 (a) and NCI-H929 cells were treated with BC2059 (100nM), CQ (40µM) or both drugs (CQ 60 min prior to BC2059) for 24hr. Active caspase-8,-9 and-3 were measured with FC and the results expressed as fold increase compared to untreated (UT) cells (n=3-5, ±se). Cell death was monitored by PI staining.

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