Beta-catenin inhibitor BC2059 is efficacious as monotherapy or in combination with proteasome inhibitor bortezomib in multiple myeloma

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Running title: Pre-clinical efficacy of beta-catenin inhibitor in myeloma.

Keywords: multiple myeloma, therapy, beta-catenin, synergism, in-vivo efficacy.

Abbreviation List: AML acute myeloid leukaemia, ASCT autologous stem cell transplant, APC adenomatous polyposis coli, °C degrees Celsius, BMMC bone marrow mononuclear cells, BMSC bone marrow stromal cells, CK1 casein kinase 1, CLL chronic lymphocytic leukaemia, CM conditioned media, Dvl Dishevelled, FITC Fluorescein isothiocyanate, Fzd Frizzled receptors, GSK3 glycogen synthetase kinase 3, HCC hepatocellular carcinoma, HMCL human myeloma cell lines, LRP6 low-density lipoprotein receptor related protein 6, MM multiple myeloma, cPARP cleaved poly(ADP-ribose) polymerase, PBMC peripheral

1

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blood mononuclear cells, SMRT-N-CoR retinoid and thyroid hormone receptor -nuclear receptor co-receptor, SUMOylation small ubiquitin-like modifier, TCF/LEF T-cell factor/lymphoid enhancer factor, TBC total beta-catenin, TBL1/TBLR1 transducin β-like protein and transducing β-like related protein, TBP antiTATA binding protein.

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This work was supported by the Monash University, Melbourne, Victoria, Australia and Australian Centre for Blood Diseases, Monash University/Alfred Hospital, Melbourne, Victoria, Australia.

Word count: 6,070

Figures: 6
Abstract

Currently available treatment options are unlikely to be curative for the majority of multiple myeloma (MM) patients, emphasizing a continuing role for the introduction of investigational agents that can overcome drug resistance. The canonical Wnt/beta-catenin signalling pathway, essential for self-renewal, growth and survival, has been found to be dysregulated in MM, particularly in advanced stages of disease. This provides the rationale for evaluating the novel beta-catenin inhibitor BC2059 as monotherapy and in combination with proteasome inhibitors in vitro and in vivo. Here we show nuclear localisation of beta-catenin in human myeloma cell lines (HMCL), consistent with activation of the canonical Wnt pathway. BC2059 attenuates beta-catenin levels, in both the cytoplasm and the nucleus, reducing the transcriptional activity of the TCF4/LEF complex and the expression of its target gene axin2. Treatment of HMCL with BC2059 inhibits proliferation and induces apoptosis in a dose-dependent manner. This is also observed in HMCL-stromal cell co-cultures, mitigating the protective effect afforded by the stroma. Similarly, BC2059 induces apoptosis in primary MM samples in vitro, causing minimal apoptosis on healthy peripheral blood mononuclear cells (PBMC). Furthermore it synergizes with the proteasome inhibitor bortezomib both in HMCL and primary MM samples. Finally, in xenograft models of human myelomatosis, BC2059 delays tumour growth and prolongs survival with minor on-target side effects. Collectively these results demonstrate the efficacy of targeting the Wnt/beta-catenin pathway with BC2059 both in vitro and in vivo, at clinically achievable doses. These findings support further clinical evaluation of BC2059 for the treatment of MM.
Introduction

Multiple myeloma (MM) is an incurable neoplastic plasma-cell disorder, accounting for approximately 1% of neoplastic diseases and 13% of haematologic cancers (1). Despite the improvement in progression-free and overall survival of MM patients after the introduction of high-dose chemotherapy and stem-cell rescue (autologous stem cell transplant (ASCT)) and more effective pharmacotherapies, the available treatment regimens are not curative (2). Thus, it is important to continue to identify and validate new therapeutic agents that target alternative pathways, which contribute to the pathogenesis and progression of MM.

The canonical Wnt signalling pathway modulates the balance between stemness and differentiation in several adult stem cell niches, including haemopoiesis within the bone marrow (3). Furthermore, it is commonly dysregulated in a range of solid tumours including colon, liver and pancreas carcinoma (4) and haematological malignancies including acute myeloid leukaemia (AML) (4,5), chronic myeloid leukemia (6), chronic lymphocytic leukaemia (CLL) (7), and MM (8). In the absence of Wnt ligands, cytoplasmic beta-catenin, which is the key player of the Wnt pathway, is constantly degraded by the Axin complex (comprised of axin, tumour suppressor adenomatous polyposis coli [APC], casein kinase 1 [CK1], and glycogen synthetase kinase 3 [GSK3]). The amino terminal of beta-catenin, upon phosphorylation by CK1 and GSK3, is subsequently recognised by an E3 ubiquitin ligase subunit, ubiquitinated and degraded by the proteasome. Thus beta-catenin is inhibited from reaching the nucleus where the Wnt target genes remain repressed under the influence of the DNA-bound T-cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. Conversely, upon binding of Wnt ligand to the seven-pass transmembrane Frizzled receptor (Fzd) and its coreceptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close...
relative LRP5, Dishevelled protein (Dvl) phosphorylates LRP6 leading to the recruitment of the Axin complex to the cell membrane. As such, beta-catenin remains unphosphorylated, accumulates in the cytoplasm and then translocates into the nucleus, where it binds to the TCL/LEF complex initiating transcription of Wnt target genes (9).

Human myeloma cell lines (HMCL) and primary MM cells have been shown to frequently express active, non-phosphorylated nuclear beta-catenin, whereas in normal B cell subpopulations (naïve, germinal center, and memory B cells) and plasma cells the non-phosphorylated form of beta-catenin is undetectable (8,10). Additionally, immunohistochemical study of MM bone marrow biopsies for nuclear expression of beta-catenin has revealed strong beta-catenin staining in the malignant plasma cells of 34% of MM patients, where its presence was significantly correlated with more advanced disease stage (11). Furthermore, Wnt pathway genes (both upstream and downstream of beta-catenin) have been found to be up-regulated in MM primary tumours when compared with normal plasma cells (10). However, in contrast to other malignancies such as colon carcinoma or hepatocellular carcinoma (HCC) where the up-regulation of the Wnt-canonical pathway is due to mutations involving key components of the pathway, e.g. APC, in MM no such mutations have been found, moreover, further activation of the pathway can be promoted by exposure to activators such as LiCl or Wnt3a, implying the existence of an intact, functional signalling pathway (10,12). Consistent with this, evidence suggests that the aberrant activation of the Wnt canonical pathway may be secondary to the expression of Wnt ligands by both MM and bone marrow stromal cells (BMSC) (8,10) frequently in parallel with co-expression of various Fz receptors and co-receptor LRP6 (12), and/or the hypermethylation of soluble Wnt inhibitor promoters (11,13). Furthermore, recent data indicate that beta-catenin can be further stabilized in MM, especially in the nuclear compartment, by the process of SUMOylation (small ubiquitin-like modifier) (14). SUMOylation is known to be
aberrantly activated in MM (15), and genetic inhibition of the process is able to increase the destruction of β-catenin by the ubiquitin-proteasome system (14). Overall, the available data confirming the up-regulation of the Wnt canonical pathway in MM, and its potential role in the proliferation, survival and drug resistance of MM cells, make it an attractive therapeutic target.

BC2059 (an anthracene-9,10-dione dioxime compound: 2-((3R,5S)-3,5dimethylpiperidin-1ylsulfonyl)-7-((3S,5R)-3,5-dimethylpiperidin-1-ylsulfonyl) (supplementary figure S1), is a novel Wnt/beta-catenin pathway inhibitor that has been shown to disrupt the binding of beta-catenin to Transducin β-like protein 1 (TBL1) and its related protein (TBLR1), facilitating its destruction (16). TBL1 and TBLR1 were initially identified as members of the co-repressor silencing mediator for retinoid and thyroid hormone receptor (SMRT)-nuclear receptor co-receptor (N-CoR) complex (17). Upon ligand binding, TBL1-TBLR1 mediate the exchange of the nuclear receptor co-repressors, SMRT-N-CoR, for co-activators. Additionally, TBL1-TBLR1 play a specific role in the recruitment of beta-catenin to the Wnt target-gene promoter. In the absence of Wnt ligands the promoter region of the Wnt target genes is hypoacetylated due to the presence of the repressor complex (TCF, TLE1-HDAC1) whereas upon activation beta-catenin enters the nucleus, binds to TBL1-TBLR1, which preferentially binds to the hypoacetylated histones, thus localising beta-catenin to the region of the Wnt target-gene promoter. Subsequently, beta-catenin interacts with TCF, dissociating TLE1-HDAC1, thus inducing hyperacetylation which leads to the recruitment of co-activators and the initiation of transcription (18). Interestingly, SUMOylation of TBL1-TBLR1 can further potentiate interaction and transcriptional activation of beta-catenin- TBL1-TBLR1 complex (19) and TBL1, besides acting as a transcriptional co-activator of beta-catenin also protects beta-catenin from degradation (20). In this study, we show that BC2059, has significant anti-
proliferative and pro-apoptotic effects on HMCL and primary MM cells and can mitigate the protective/pro-proliferative effect of stromal cells (SC), Wnt3a or conditioned media (CM) derived from MM patient bone marrow stroma. The drug was confirmed to decrease beta-catenin protein levels and the expression of down-stream target genes, furthermore, BC2059 was shown to synergise with low doses of proteasomal inhibitors in killing MM cells and was effective in a murine xenograft model of human MM, thus providing a rational for the further evaluation of the drug in the treatment of MM.

**Materials and methods**

BC2059 (2-((3R,5S)-3,5dimethylpiperdin-1ylsulfonyl)-7-((3S,5R)-3,5-dimethylpiperdin-1-ylsulfonyl) (16) was provided by BetaCat Pharmaceuticals, Maryland, USA. Investigations were conducted in accordance with the Declaration of Helsinki and acknowledged national and international guidelines and was approved by the Alfred Hospital review board.

**Cell lines and culture conditions**

HMCL U266, NCI H929, and RPMI 8226 were obtained from the American Type Culture Collection (ATCC). OPM2 and LP1 cell lines were from Deutshe SammLung von Mikroorgaanismen und Zellculturen. These cells were authenticated by the supplier using cytogenetics, DNA typing, immunophenotyping, and cell line speciation. Commercially available cell lines are purchased every 2 to 3 years. ANBL6, OCI-My1, and XG-1 were kind gifts from Dr. Frits Van Rhee (Winthrop P. Rockefeller Cancer Institute) in 2008, whereas KMS11, KMS12 BM, KMS12 PE and KMS18 were kind gifts from Kawasaki Medical School (Japan) in 2008. The authors have confirmed that the cell lines are plasma cells by CD138, CD38, and CD45 by flow cytometry. HMCL were grown and treated at densities between 2.0 and 2.5 x10^5 cells/ml in RPMI-1640 media (Gibco, Invitrogen, Mulgrave,
Victoria, Australia) supplemented with 10% heat-inactivated foetal bovine serum (Lonza, Mt Waverley, Victoria, Australia) and 2mM L-glutamine (Gibco, Invitrogen). IL-6 dependent cell lines were cultured with 2–5 ng/ml IL-6 as required. All cells were cultured in a humidified incubator at 37°C with 5% CO₂ and used until 20th passage. All HMCL were passaged 24h before the experimental setup to ensure high viability. Cell lines are screened every 2 months for mycoplasma contamination by VenorGeM Mycoplasma Detection Kit. The HMCL were authenticated on December 2016 by CellBank Australia (NSW, Australia) by the use of Short Tandem Repeat (STR) profiling, in line with the standard ANSI/ATCC ASN-0002-2011, and matched publicly available data.

**Immunoblotting and cell fractionation**

HMCL were fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (ThermoScientific) as per manufacturer’s instructions. Protein concentration was quantified using the DC Protein Assay (Bio-RAD, Gladesville, New South Wales, Australia). Subsequently, 20µg of each protein lysate was separated by 6% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF (Immobilon-P, Millipore) using the Bio-Rad wet transfer system. Membranes were blocked with 5% BSA 0.1% Tween-20/PBS for 60 minutes (min) then incubated with rabbit polyclonal anti-beta-catenin (Cell Signaling Technology), mouse monoclonal anti-ɑ-tubulin (Sigma-Aldrich) or mouse monoclonal antiTATA binding protein antibody (Abcam) overnight at 4°C or for 2h at room temperature. The blots were washed three times for 15min with 0.1% Tween-20/PBS, then incubated with secondary horseradish peroxidise tagged antibody (swine anti-rabbit Ig HRP or rabbit anti-mouse Ig HRP (Dako, Campbellfield, Victoria, Australia) for 1 or 2 hours (h) at room temperature before washing as above. Blots were visualised with Supersignal west pico ECL reagents (Pierce, ThermoFisher Scientific).
For beta-catenin protein level measurement, KMS18 cells were treated with BC2059 at 2 different doses (IC$_{50}$, 1.5×IC$_{50}$), then at 16h the cells were lysed with RIPA lysis buffer as described (21) or fractionated as per manufacturer’s instructions, and 70µg or 30µg of protein lysate were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF or nitrocellulose respectively (Hybond ECL, Amersham Biosciences). NCI H929 and U266 cells were treated with escalating doses of BC2059 for 20h and lysed with RIPA lysis buffer. 70µg of protein lysate were separated by 4-20% gradient precast gel (Min-Protean TGX™ gels, Bio-Rad) and blotted into PVDF. For beta-catenin levels in HS5 stromal cells, 70µg of KMS18 and HS5 protein lysate were separated by 6 % SDS-polyacrylamide gel electrophoresis. Membranes were blocked with 5% BSA 0.1% Tween-20/PBS or Odyssey Blocking Buffer (LI-COR Biosciences) and incubated first with rabbit polyclonal anti-beta-catenin, mouse monoclonal anti-ɑ-tubulin or mouse monoclonal anti-TATA binding protein antibody, and then with secondary as above or with goat anti-rabbit (IRDy 800CW, LI-COR Biosciences) or goat anti-mouse (IRDy 680RD, IRDy800CW LI-COR Biosciences) according to the manufacturer’s instructions. Total lysates blots were visualised with Supersignal west pico ECL reagents (Pierce, ThermoFisher Scientific), whereas fractionated lysates blots were visualised on an Odyssey Infrared Imaging system and densitometry was performed using the Image Studio software (Odyssey). Results were normalised to loading controls (ɑ-tubulin or β-actin for the whole cell lysates and cytoplasm fractions and TATA binding protein for the nuclear fractions). Experiments were performed in triplicate.

**Proliferation and viability assays**

Proliferation was measured using the Celltiter 96 AQeous one solution cell proliferation assay (Promega, South Sydney, New South Wales, Australia) on a panel of 12 HMCL. Cells
were cultured at $2.0 \times 10^5$ cells/ml in 100µl fresh media in 96-well plates for 24h and 72h with escalating concentrations of BC2059 (50-10000nM). 20µl of MTS reagent was added for the final 4h of treatment and the plates were read at 490nm using a Fluostar Optima plate reader (BMG Labtech, Mornington, Victoria, Australia). Parallely, cell viability of the treated HMCL was determined by trypan blue staining and haemocytometer counts and the degree of cell death was assessed by FACS with propidium iodine (PI) staining. To study the pro-apoptotic effect of BC2059, HMCL were treated with increasing doses of the drug for 24h and the cells were stained with FITC-annexinV antibody (Molecular Probes by Life technologies, Eugene, Oregon, USA) for 30 min in the dark at RT, washed, resuspended in annexin buffer and acquired by FACS. Analysed data represents the fold increase of annexinV positive cells divided by the positive annexinV cells of the untreated sample. For the detection of cleaved poly(ADP-ribose) polymerase (cPARP) U266 HMCL were treated with BC2059 for 24 h. Cells were collected and fixed in 2% para formaldehyde (PFA) for 20 min at 4°C. Cells were then stained with FITC-cPARP (BD Biosciences) antibody in permealisation buffer (PBS/0.1% saponin) for 30 min in the dark at RT, washed, resuspended in PBS and acquired by FACS. Analysed data represents the fold increase of cPARP positive cells divided by the positive cPARP cells of the untreated sample. For the synergy experiments, 6 HMCL were treated with BC2059 in combination with bortezomib for 24h before being harvested, resuspended in FACS Buffer (0.5% heat-inactivated foetal bovine serum in PBS) supplemented with 62.5ng/ml PI (Sigma-Aldrich), and analysed immediately by FACS. The proportion of PI-positive cells was quantitated by subtracting the background death of untreated cells. Cell death induced by single drug-treatment was compared to the combination treatment and combination indices (CI) calculated using the CalcuSyn software package (Biosoft, Cambridge, UK). All experiments were performed at least in triplicate.
Co-culture assays

HMCL in isolation or in the presence of the immortalised stromal cell line HS5 were seeded at 2×10^5 cells/ml in 24-well plates and incubated for 4h in a humidified incubator at 37°C with 5% CO2 then varying concentrations of BC2059 were added and the cells incubated for a further 20h. Cells were trypsinised (TrypLE™ Express Enzyme, phenol red, Gibco Life Technologies), washed with PBS and stained with CD10 FITC (Beckman Coulter) for 30min at 4°C. Unbound antibody was washed off and the cells resuspended in FACS Buffer (0.5% heat-inactivated foetal bovine serum in PBS) supplemented with 62.5ng/ml PI (Sigma-Aldrich) and analysed immediately by FACS. The proportion of PI-positive CD10+ (HS5) and CD10- (HMCL) cells was quantitated by subtracting the background death of untreated cells. For bone marrow stromal cell (BMSC) conditioned media (CM) preparation, bone marrow mononuclear cells from 3 MM patients were isolated from heparinised aspirates by Ficoll gradient, pooled, resuspended in 10% FBS DMEM media and incubated for 24h after which the supernatant was removed and the adherent cells then grown in 10% FBS until 80% confluence was achieved. The medium was then removed and cells were incubated for another 48h in serum-free DMEM. The CM1 was then centrifuged and stored at -80°C for later use. The experiment was repeated with 3 additional MM patients’ derived BMSC CM (CM2). Subsequently, NCI H929 were starved overnight, resuspended in serum-free medium (SFM) or CM at 2×10^5 cells/ml and incubated for 4h. BC2059 was then added and the cells incubated for a further 16h. Viable cell numbers were then determined by trypan blue staining and haemocytometer count. Similarly, for Wnt3a and Wnt5a stimulation, NCI H929 were starved overnight, resuspended at 2×10^5 cells/ml in SFM and treated with 100nM of rhWnt3a or 100nM or 200nM of rhWnt5a (both R&D systems). After 4h BC2059 was added and cells were incubated for a further 16h and viable cell numbers enumerated with trypan blue staining and haemocytometer count. All experiments were performed in triplicate.
Primary samples

Primary MM samples were obtained from relapsed and refractory MM patients, following written informed consent with approval from the Alfred Hospital Research and Ethics Committee. Bone marrow mononuclear cells (BMMC) were isolated with Ficoll-Paque Plus (Amersham Biosciences, Rydalmere, New South Wales, Australia), washed in PBS and red blood cells were lysed with NH₄Cl solution (8.29g/L ammonium chloride, 0.037 g/L ethylene diamine tetra-acetic acid, 1 g/L potassium bicarbonate). Cells were then washed with PBS, quantitated by haemocytometer and subsequently cultured in RPMI-1640 media supplemented with 10% heat-inactivated foetal bovine serum and 2mM L-glutamine for 24h. The percentage of MM cells was quantified by CD45 and CD38 staining by FACS. The next day cells were plated at 2.5×10⁵ MM cells/mL and treated with BC2059 (250-1000nM) alone or in combination with bortezomib (10-40nM) for 24h and 72h. Drug-induced MM-specific cell apoptosis was then compared with untreated controls by staining for CD45 FITC (BD, North Ryde, New South Wales, Australia), CD38 PerCP-Cy5.5 (BD) and Apo 2.7 PE (Immunotech Beckman Coulter, Mt Waverley, Victoria, Australia) followed by FACS analysis.

Super TOP-/FOP-FLASH Wnt reporter

KMS11 cells (1×10⁵) were transfected with Super 8xTOP-FLASH or Super 8xFOP-FLASH Wnt reporter plasmids (a gift from Randall Moon, Addgene, plasmid 12456 and 12457) containing wild-type or mutant TCF DNA binding sites by using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions and co-transfected with the pRL-TK vector (Promega, Madison, WI, USA) as an internal control reporter vector. Cells were then treated with rhWnt3a for 8h before treatment
with BC2059 for an additional 12h. Reporter activity was then assessed by using the Dual Luciferase Assay System (Promega, Madison, WI, USA). Results were normalised to Renilla values for each sample. The reporter assay results represent the average of two independent transfection experiments.

**Quantitative real-time PCR (qRT-PCR)**

The primer sequences for the axin 2 and surviving qRT-PCR were:

- forward GCCGATTGCTGAGAGGAACTG
- reverse AAAGTTTTGGTATCCTTCAGGTTTCAT,
- forward AGAACTGGCCCTTCTGGAGG
- reverse CTTTTTATGTTCCTCTATGGGGTC.

KMS18 cells were treated with BC2059 for 16h and NCI H929 and U266 for 20h. Total RNA from untreated and treated cells was prepared using QIAGEN RNeasy mini kit (QIAGEN, Germantown, MD, USA) and any residual genomic DNA was removed utilizing the Turbo-DNase I kit (AMBION, Austin, TX, USA). Reverse transcription was performed on 1000 ng of total RNA with 100 U of Superscript III reverse transcriptase (Life Technologies) and random hexamers (Life Technologies, 2.5 μM final concentration), according to manufacturers guidelines. The reaction mixture consisted of 8 μl of PowerSYBR PCR Master Mix (Life Technologies) with 500 nM of each forward (F) and reverse (R) primers for target gene and 2 μl of diluted template cDNA. PCR was performed with a LightCycler® 480 Real-Time PCR machine (Roche) at 95 °C for 10 min, with 45 cycles of amplification 95 °C for 15 s, 62 °C for 30 s, 72 °C for 30 s. All experiments were performed in triplicate and each test sample was run in duplicate. For sample loading normalization, β-actin (ACTB) (encoding β-actin) was used. Amplified products were all verified by melting curves analysis. Data were analyzed using SDS software version 2.3 (Life Technologies) and
copy number of target genes was determined by the comparative threshold cycle method (ΔΔCT) using the Pfaffl method. Data are presented as mean±S.E.M.

**Beta-catenin knock down**

For beta-catenin knock-down, 2ml of 2x10⁵/ml KMS18 cells were plated into a 12-well plate at day 0 (KMS18 doubling time :36 h). At day 1, media were replaced by 1ml of Opti-MEM (Gibco Opti-MEM I Reduced Serum Medium, Fischer Scientific), and transfection was carried out with Lipofectamine RNAmax (ThermoFisher Scientific) according to the protocol provided by the company. For beta-catenin knock down we used SignalSilence beta-catenin siRNA I and beta-catenin siRNA II (Cell Signaling Technology) at concentrations recommended by the company, whereas Silencer Negative Control No. 1 siRNA (ThermoFisher Scientific) was used as a negative control, at the same concentration. Six hours after the transfection, 1ml of fresh media (RPMI-10% FCS) was added in every well. At day 2 and 3, cells were treated with BC2059 (50nM, 100nM, 150nM) and then harvested at day 4 (72h after transfection). Cell death was monitored by PI staining with FACS, whereas untreated cells were collected for beta-catenin protein level measurement by immunoblotting. Cells were lysed with RIPA lysis buffer, and 70µg of protein was separated by 6% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF as already described. β-actin (mouse mAb HRP conjugate Cell Signaling Technology) was used as loading control.

**In vivo studies**

Approval for the murine studies was obtained from the Animal Ethics Committee of the Alfred Hospital, Melbourne, Australia (E/1376/2031/M). Adult age-matched Cg-Prkdscid II2rgtm1Wjl/SzJ mice (Jackson Laboratories, US) were injected (intravenously i.v.) with 1×10⁶ U266 HMCL, carrying the FUL2-TGvector (a generous gift from Dr. Marco Herold, WEHI, Melbourne, Australia) (with luciferase2 and GFP under the constitutively active
ubiquitin and IRES promoter respectively). At day 21 a limited course of treatment was commenced after confirmation of established measurable disease by bioluminescence. Control mice (n=4) received 17% Solutol® HS 15 (Sigma-Aldrich), whereas treated mice received 5mg/kg (n=4) or 10mg/kg BC2059 (n=5) twice a week i.v. for 3 consecutive weeks (6 doses in total). Tumour burden was monitored on a weekly basis by in vivo imaging, from the 2nd week of the experiment (day 18) until the first mice reached scientific end-points. Briefly, mice were anaesthetised, injected intraperitoneally (i.p.) with 125mg/kg luciferin, and imaged with the Lumina III XR system (Perkin Elmer). Acquisition and analysis was performed with the Living Image system. Peripheral blood counts were evaluated sequentially during the course of the experiment (Hemavet®, Drew Scientific, Inc.). Upon reaching scientific end-points (e.g. hind limp paralysis, >20% weight loss) mice were humanely euthanised and tissues (skin and colon) collected. Tissues were formalin-fixed and embedded in paraffin, sectioned, and stained with H&E and beta-catenin antibody. Images were taken with an Olympus BX51 microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Targeting the dysregulated Wnt/canonical pathway by BC2059 induces apoptosis in HMCL

We first evaluated beta-catenin expression pattern in a panel of 12 genetically heterogeneous HMCL and confirmed the presence of nuclear beta catenin in all cases (Figure 1A),
signifying the dysregulation of the canonical pathway in MM. The presence of two closely situated bands on immunoblotting implied the presence of both an unphosphorylated (lower migrating band) and phosphorylated beta-catenin (higher migrating band) moieties. The anti-proliferative impact of BC2059 was confirmed by MTS assay (Figure 1B) demonstrating that by 24h all 12 HMCL treated with 500nM BC2059 had less than 15% metabolic activity. The extent of the inhibitory effect of BC2059 was then recapitulated by quantitation of viable cell number with trypan blue staining in two HMCL (NCI H929 and KMS18) showing marked reductions in viable HMCL numbers at both 24hr and 72hr following treatment (Figure 1C). We then demonstrated the cytotoxic effect of BC2059 in all 12 HMCL. Cell death was evident at both 24h (Figure 1D) and 72h (supplementary Figure 2) following treatment with BC2059 with an LD50 of <250nM demonstrated for 11 of 12 HMCL. The pro-apoptotic effect of the drug was validated by the measurement of annexinV staining on three HMCL with different sensitivities to the drug (NCI H929 and U266: intermediate sensitivity, LP1: resistant). Escalating doses of BC2059 were able to increase the proportion of annexinV expressing cells in every cell line tested (Figure 1E). Furthermore, in U266, BC2059 was able to upregulate the expression of cPARP (Figure 1F), a known marker of apoptosis, downstream of active caspase 3, confirming the pro-apoptotic effect of the drug. BC2059 had minimal toxicity when tested against normal PBMC, with median cell death at 1000nM at 24h of only 5% (Figure 1G).

**BC2059 mitigates the protective anti-apoptotic and pro-proliferative effect of both BMSC and exogenous Wnt3a.**

MM benefits from bone marrow microenvironmental signals that promote survival, proliferation and drug resistance. Of specific relevance to the present study BMSC have been
found to secrete Wnt ligands that likely contribute to the observed dysregulation of the Wnt/beta-catenin pathway in MM (5). We therefore evaluated the pro-necrotic effect of BC2059 on NCI H929 and KMS18 cultured with HS5. Baseline cell death of HMCL was similar in both conditions (alone or co-culture) for both cell lines tested (NCI H929 alone: 13%±2.68, coculture: 16%±1.34, KMS18 alone: 11.13±0.4, co-culture: 11.7%±1.1). At low doses of the drug stromal cells significantly protected HMCL from cytotoxicity, whereas addition of BC2059 at doses equal to IC₈₀ mitigated the pro-survival effect of the stromal cells, with >50% cell death achieved in both cases (Figure 2A). Cell death of the stromal cells by the drug did not exceed 14.8% at the highest doses (500nM) both when cultured alone and in the co-culture system (Figure 2B). BC2059’s lack of cytotoxicity on HS5 stromal cells could be attributed to the undetectable levels of beta-catenin protein in these cells (figure 2C), when compared with a HMCL (KMS18). Similarly, the growth advantage of NCI H929 when cultured with CM derived from MM-patients (CM1 and CM2) (Figure 2D) or rhWnt3a (Figure 2E) was inhibited by BC2059 in a dose dependent fashion. Interestingly, exposure to rhWnt5a ligand, a known activator of the non-canonical pathway, under the same conditions had no proliferative effect on NCI H929 (Figure 2F).

**BC2059 induces apoptosis of primary MM cells and synergises with bortezomib.**

To assess the potential of BC2059 for future combination treatment approaches HMCL were treated with BC2059 and the proteasome inhibitor bortezomib at a range of doses and the presence of synergy calculated using CalcuSyn software (CI <1 defines synergism). For 6 of 7 HMCL, the combination was synergistic or additive with doses ranging from IC₂₀-IC₄₀ for BC2059 and 4-12nM for bortezomib. This included the HMCL most resistant to single agent BC2059, LP1 (IC₅₀: 320nM). Only KMS18, which was exquisitely sensitive to single agent
BC2059 (IC$_{50}$:73nM), could synergy not be demonstrated (Figure 3A). The effect of BC2059 on ex-vivo primary MM cells was similarly evaluated both as a single agent and in combination with bortezomib. Primary MM tumour cells derived from 11 patients with relapsed and/or refractory MM were treated for 72h, after which the CD38+CD45- MM cell population was assessed for apoptosis by flow cytometry (Figure 3B). The median cell death at 1000nM was 50.05%, ranging from 17% to 90% with 42% of samples demonstrating an LD$_{50}$ of <1000nM to single agent BC2059. The combination of BC2059 and bortezomib was tested against 2 primary MM tumour samples (Figure 3C). In both cases BC2059 synergised with bortezomib with synergy quotients (SQ) ranging from 1.3 to 8.2 (where SQ>1 defines synergism). Interestingly, one of the tumours that was refractory to bortezomib both in vivo (patient was bortezomib refractory) and in vitro demonstrated marked synergy with the combination of BC2059 and bortezomib with an SQ=8.2.

**BC2059 blocks beta-catenin/TCF transcriptional activity, promotes the destruction of beta-catenin protein and shows specificity for beta-catenin.**

To evaluate the capacity of BC2059 to block beta-catenin/TCF transcriptional activity in MM, we transfected KMS11 with Super TOP-FLASH (wild-type TCF/LEF) and Super FOP-FLASH (mutant TCF/LEF) Wnt reporter plasmids. Upon treatment of KMS11 cells with rhWnt3a, contemporaneous exposure to BC2059 (IC$_{50}$:215nM) resulted in a statistically significant dose-dependent reduction in beta-catenin specific transcriptional activity (Figure 4A). A desired characteristic of potential Wnt inhibitors will be their ability to block transcriptional activity without interfering with the destruction of beta-catenin in the cytoplasm by the destruction complex. Therefore, we tested the effect of BC2059 on beta-catenin protein levels utilising the BC2059-sensitive cell line KMS18. KMS18 cells (2×10$^5$ per ml) were treated for 16h with 2 doses of BC2059 (IC$_{50}$ and 1.5×IC$_{50}$, IC$_{50}$: 73nM) and
reduction of the cell number was monitored (Figure 4B). Similarly, KMS18 cells were treated with the same doses and the level of whole-cell, cytoplasmic and nuclear beta-catenin levels was then quantified following immunoblotting and densitometry (Figure 4C-D). Treatment with BC2059 at 1.5×IC50 resulted in a decrease in whole-cell beta-catenin to almost 50% of baseline (0.54±0.16) and a >50% reduction in nuclear beta-catenin (total beta-catenin [TBC]) (reduced to 0.48±0.03 of baseline). This was associated with a marked reduction in transcription of the downstream beta-catenin transcriptional target axin 2 (0.22±0.16 of baseline) (Figure 4E). A similar reduction of whole-cell beta-catenin protein was monitored in two additional HMCL tested (NCI H929, U266, with IC50: 173nM and 186nM respectively) upon treatment with escalating doses of BC2059 (Figure 5A), causing significant reduction in transcription of beta-catenin target genes axin 2 (Figure 5B upper panel) and survivin at 1.5×IC50 (Figure 5B lower panel).

Knock down of beta-catenin in the sensitive cell line KMS18 for 72h, was able to diminish the cytotoxic effect of the drug (figure 5C left panel), whereas immunoblotting of the UT cell lysates at the same time point confirmed decreased expression of the protein (Figure 5C right panel).

**BC2059 demonstrates anti-MM activity in vivo and has minimal toxicities.**

We then evaluated the in vivo effect of BC2059 utilising a xenograft murine model of systemic human myelomatosis. NSG mice were transplanted (i.v.) with U266 HMCL stably expressing GFP and luciferase2. After 3 weeks, when disease was established in all cases, the mice were treated with vehicle alone, 5mg/kg BC2059 or 10 mg/kg BC2059 twice weekly for three consecutive weeks (d21-d39, 6 doses in total). Tumour burden was monitored on a weekly basis with in vivo bioluminescence imaging after i.p. injection of luciferin, and the median flux (photons/sec) of dorsal and ventral views for each mouse was sequentially monitored.
documented. Upon the development of signs of disease the mice were euthanised and tissues (skin and colon) collected for histopathologic studies. The mice treated with 5mg/kg showed a significant delay of tumour growth compared to vehicle only after the termination of treatment (p=0.0085 and p=0.0078 in day 46 and day 53 respectively), whereas mice treated with 10mg/kg of BC2059 demonstrated a significantly slower rate of tumour growth since the beginning of treatment (with the exception of day 39) (p=0.014, p=0.016, p=0.0038 and p=0.0075 in day 25, 32, 46, 53 respectively) (Figure 6A). Moreover, mice treated with the higher dose of BC2059 (10mg/kg) even with a total of only 6 doses of BC2059 demonstrated superior survival (p=0.024) when compared to the vehicle cohort (Figure 6B). Importantly, treatment with BC2059 did not result in either weight loss or peripheral blood cytopenias (Figure 6C) when compared to vehicle-treated mice.

Finally, in view of the importance of the Wnt/beta-catenin pathway on the homeostasis of adult skin and intestine we performed immunohistochemical studies of the dorsal skin and colon harvested at the time of euthanasia. Interestingly, although we did not notice any macroscopic skin changes, the biopsies from treated mice showed dose dependent stalling of the hair cycle process after involution with variable recycling of hair occurring. At 5mg/kg the process was patchy, whereas at 10mg/kg the process was more diffuse with concurrent fibrosis and loss of the dermal adipocyte tissue (Figure 6D), consistent with an on-target cutaneous effect of BC2059 due to down-regulation of beta-catenin expression in hair follicle cells (Figure 6E). In contrast, no significant histological changes were seen in the gastrointestinal tract of the drug treated mice (supplementary Figure 3).

**Discussion**
The aetiology of a range of diverse malignancies, including hematologic neoplasms, may be attributable, at least in part, to Wnt canonical pathway dysregulation (4,22-24). Furthermore, while beta-catenin expression is normally lost in healthy differentiated B-lineage plasma cells, its expression, and transcriptional activity in conjunction with TCF is maintained through a yet unknown mechanism in some MM cells (8,10). The presence of high levels of nuclear beta-catenin (active form) in all of the HMCL that we tested is consistent with the latter and implies that up-regulation of the Wnt pathway may be a common, albeit probably late (11), event in the progression of MM irrespective of the underlying genetic make-up of the disease. We demonstrated, in accordance with previous published data (8,12), that the Wnt canonical pathway in MM can be further stimulated with the addition of Wnt3a (Figure 2E), promoting the proliferation of the MM cells and is consistent with an intact Wnt pathway. Furthermore, our finding that enhanced activation of the Wnt pathway confers further growth and survival advantage to MM is in accordance with previously published data (8,10), and provides a strong basis to evaluate blockade of the Wnt pathway as a potential therapeutic modality for the treatment of MM.

We demonstrated that BC2059 exposure reduced nuclear beta-catenin protein levels in a dose and time dependent manner and specifically impaired beta-catenin/TCF transcriptional activity without any evidence of interference, based on the reduction in the overall cellular level of beta-catenin, of beta-catenin binding to its destruction complex. According to previously published data (16), the drug interferes with the interaction of beta-catenin with TBL1-TBLR1, which are uniformly expressed in HMCL (supplementary Figure S4) and which have a dual role of facilitating the transcriptional activity of the former (18), as well as protecting it from Siah-1 mediated degradation both in the nucleus and cytoplasm (20). Thus, BC2059 not only decreases the transcription of beta-catenin target genes, but also decreases...
the levels of beta-catenin in both cellular compartments. Additionally, BC2059 initiation of apoptosis (Figure 1E, 1F) and activation of caspases in HMCL further facilitates the destruction of beta-catenin (25). The potent anti-proliferative effect of BC2059 on a range of diverse HMCL after a single dose was a clear demonstration of the impact of this capacity in inhibiting the Wnt pathway. However, as the Wnt canonical pathway is essential for the homeostasis of a variety of tissues, such as skin and hair follicles, the intestinal mucosa and the haematopoietic system, via maintenance of their stem cell pool, strategies to utilise the minimally effective doses of Wnt/beta-catenin inhibitors in the clinic are required. The proliferation and viability of the HMCL were significantly inhibited in a time- and dose dependent manner, with IC$_{50}$ ranging from 40 to 250nM, corresponding to likely clinically achievable concentrations. Knowing that tumor-microenvironment interaction can confer drug resistance to MM and following the recommendation that in-vitro screening of anti-MM agents should incorporate assays including the presence of non-malignant accessory cells (2), we next evaluated the effect of BC2059 on HMCL when cultured with non-MM stromal cells. For all the HMCL tested, BC2059 at IC$_{80}$ was able to mitigate the protective effect provided by the stroma. This activity was then recapitulated with the demonstration that approximately 50% of primary MM tumours in an autologous bone marrow co-culture displayed >50% cell death with <1µM of BC2059 when used as a single agent. However, BC2059 showed minimal effect on PBMC, which are negative for active nuclear beta-catenin (Figure 1G), and on HS5 stromal cells (Figure 2B, 2C), proving specificity of the drug. Furthermore, partial decrease of the protein by siRNA knock-down was able to decrease the cytotoxic effect of the drug (Figure 5C). Due to the complex and context specific mechanisms that govern the Wnt/beta-catenin pathway and the possible compensatory routes for persistent beta-catenin/TCF4-regulated transcription, primarily lowering beta-catenin levels is not, as might be expected, enough to fully abrogate the effect of the drug. This is further supported by the
absence of a direct correlation between total and/or nuclear level of beta-catenin and HMCL IC₅₀ to BC2059. Finally, based on previous data demonstrating the existence of possible paracrine stimulation of MM cells within the BM microenvironment mediated by secreted Wnt ligands, we tested the effect of BC2059 in the presence of MM patient derived stromal cell CM and rhWnt3a. In both instances BC2059 was able to abrogate, in a dose dependent manner, the proliferative stimulus provided to the MM cells. In contrast, exposure to rhWt5a provided no proliferative benefit to the HMCL thus demonstrating the pathway specificity (canonical versus non-canonical) of Wnt dysregulation in promoting MM growth and survival. Collectively, considering the complexity and tissue-specificity of the Wnt-canonical pathway, these results demonstrate specificity for BC2059, albeit minor off-target effects cannot be totally excluded.

Beta-catenin up-regulation has been correlated with bortezomib resistance providing a clear rationale for the combination of BC2059 with bortezomib to enhance the anti-MM effect with the least possible toxicity (26). Accordingly, the addition of bortezomib to BC2059 proved to be synergistic against the HMCL and the primary MM cells tested, moreover, in one case, absolute in vivo resistance to bortezomib confirmed in the clinical setting, was partly overcome ex vivo by the addition of BC2059. Taken together these results suggest that bortezomib resistance, that may in part be attributable to beta-catenin up-regulation, may be overcome by the addition of BC2059.

Finally, the use of BC2059 in vivo prolonged the survival of MM-bearing mice, with only a brief therapeutic exposure (6 doses totally). Furthermore, the effect of BC2059 impacted the growth kinetics of the disease in an ongoing fashion even after the termination of therapy, with slower tumour growth especially for the 10mg/kg treated mice through until the
termination of imaging when compared to control animals (figure 6B). One of the biggest challenges in the development of Wnt/beta-catenin pathway inhibitors is to diminish the possible on-target effects on other Wnt-dependent healthy tissues. In this context we evaluated treated animals for clinical and histological evidence of the on-target effects of BC2059. MM-bearing mice objectively tolerated the treatment well, with no major hair-loss or differences in body weight between the cohorts, the latter consistent with functional integrity of the gastrointestinal track. Furthermore, colon biopsies did not reveal any major changes attributable to Wnt/beta-catenin inhibition. In contrast, skin biopsies demonstrated dose dependent dysregulation of normal hair cycling with a concomitant reduction in adipose tissue (hypodermis) and increased thickness of the dermis. It has been already shown (27) that inhibition of the epidermal Wnt/beta-catenin signalling reduces adipocyte differentiation in the adult mouse dermis. This negatively affects the synchronised adipocyte differentiation and hair growth cycle, which follow an oscillating pattern throughout adult life. Our findings recapitulated this. Importantly, however, BC2059 treatment did not have any deleterious effect on haematopoiesis as evidenced by the absence of any emergent cytopenias in the treated groups. Although pathological bone fractures were not noticed on the treated mice, knowing the importance of the pathway for the bone homeostasis (28-30) further investigation is currently underway.

Inhibition of dysregulated Wnt/beta-catenin signalling at different levels has been demonstrated to inhibit the growth and survival of MM cells (8,10,21,31-35). However, available evidence would suggest that targeting the transcriptional activity of beta-catenin with BC2059, may be able to overcome the context specific variability and possible compensatory mechanisms that mitigates the efficacy of alternative approaches. Collectively, our results demonstrate that BC2059 has significant anti-MM effects against genetically
heterogeneous HMCL and primary MM tumours in a range of in vitro and in vivo models, synergises with bortezomib and displays clear evidence of on-target effects in vivo, warranting further evaluation of BC2059 as a potential novel anti-MM therapeutic.

Acknowledgments

Monash Histology Platform
AMREP Flow Cytometry Core Facility

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Figure Legends

Figure 1. Wnt canonical pathway is active in HMCL, whereas its inhibition with BC2059 induces arrest of proliferation and apoptotic cell death, with minimal effect on healthy peripheral blood mononuclear cells. (A) Nuclear (N) and cytoplasmic (C) fractions of HMCL. Loading control, α-tubulin-cytoplasmic, anti-TATA binding protein (TBP)-nuclear. (B) BC2059 inhibits HMCL proliferation in a dose-dependent manner. 12 HMCL were cultured for 24 and 72h untreated (UT), or with BC2059 (50, 100, 500, 1000nM). Cell proliferation was then determined by MTS assay (24h data shown, n=3-6, mean ± s.e.) (C) Absolute cell numbers of viable cells were determined by haemocytometer counts of HMCL cultured alone (UT), with BC2059 (50, 100, 250, 500nM) or with vehicle. BC2059 greatly decreased the proliferation of the 12 HMCL over 72h (NCI H929 and KMS18 data shown, n=3, ± s.e.). (D) BC2059 induces cell death in HMCL. Proportion of PI+ cells after BC2059 treatment for 24h. Data shown is the mean of three to four independent experiments ± s.e. (E) BC2059 induces apoptosis in HMCL. Annexin V positive cells after BC2059 treatment for 24h, in three HMCL (n=3, ± s.e.). (F) BC2059 induces cleavage of PARP. cPARP positive cells were determined by FACS, after treatment of U266
HMCL with BC2059 for 24h (n=3, ± s.e.). (G) BC2059 has minimal toxicity on healthy peripheral blood mononuclear cells.

**Figure 2. Treatment of HMCL with BC2059 mitigates the anti-apoptotic and pro-proliferative effect of the stroma and Wnt-canonical pathway stimulation.** (A) 2 HMCL (KMS18, NCI H929) were cultured either alone or with stromal cells (HS5) and treated with BC2059 for 24h, up to IC$_{80}$ and cell death of HMCL calculated (n=3, ± s.e., results analysed using a two-way ANOVA with Boferroni post-test **** = p < 0.0001, ** = p < 0.01, ns = p > 0.05). (B) HS5 stromal cells cell death was calculated in the coculture experiment (n=3, ± s.e.). (C) beta-catenin expression in HS5 stromal cells compared with that in a HMCL (KMS18). Loading control: β-actin. The blot shown is the representative of two biological replicates. (D) NCI H929 were culture in SFM, CM1 or CM2 4h before the addition of BC2059 (50, 100, 150, 200, 250, 350nM) and 16h later viable cell numbers were determined by haemocytometer counts (n=3, ± s.e, results were analysed using a two-way ANOVA with Boferroni post-test ****= p < 0.0001, * = p < 0.05, ns = p > 0.05). (E) Similarly, the pro-proliferative effect of the addition of rhWnt3a (100nM) on NCI H929, in terms of viable cell numbers was overcome by the addition of BC2059 (50, 100, 150, 200, 250, 350nM) for 16h (n=3, ± s.e., results were analysed using a two-way ANOVA with Boferroni post-test ****= p < 0.0001, ***=p < 0.001, ns=p > 0.05) (F) In contrast the addition of rhWnt5a (100nM and 200nM) on NCI H929 did not demonstrate a pro-proliferative effect (n=3, ± s.e., results were analysed using a two-way ANOVA with Bonferroni post-test).

**Figure 3. BC2059 synergises with bortezomib against HMCL and induces apoptosis of primary MM samples as a single agent and in combination with bortezomib.** (A)
BC2059 (IC_{20}-IC_{40}) in combination with bortezomib (4-12nM) demonstrated synergism against HMCL (n=3). Synergy was measured using the Combination Index (CI) calculated by CalcuSyn software, where values less than 1 represent synergism. CI was plotted against the fraction of the HMCL killed with the various used doses after 24h. (B) Proportion of apoptotic (Apo 2.7 +) CD38+ CD45- primary MM cells in an autologous bone marrow coculture assay after 72h of BC2059 treatment with 1μM and 5 μM (n=11). (C) Proportion of apoptotic (Apo 2.7 +) CD38+ CD45- primary MM cells after 24h BC2059 and/or bortezomib treatment (n=2).

Figure 4. BC2059 inhibits the activity of the beta-catenin/TCF transcriptional complex and facilitates beta-catenin destruction. (A) KMS11 endogenous reporter activity is enhanced on addition of rhWnt3a but blocked by BC2059. RLU = relative light units. (n=2, mean ± s.e., transfected cells stimulated with rhWnt3a and treated with BC2059 were analysed using one-way ANOVA with Tukey post-test). (B) BC2059 causes apoptosis of KMS18 cells at 16h at doses equal to IC_{50} and 1.5xIC_{50}. 2x10^5 KMS18 cells were left untreated or treated with BC2059 (IC_{50}, 1.5xIC_{50}, IC_{50}:73nM) and 16h later absolute cell numbers of viable cells were determined by haemocytometer counts (n=4). (C) Immunoblotting of whole cell lysates from untreated and treated KMS18 cells and densitometric analysis showed a significant reduction of total beta-catenin protein expression at 1.5xIC_{50} (n=4). Loading control: α-tubulin. (D) BC2059 treatment of KMS18 cells for 16h promotes the destruction of the nuclear (active) beta-catenin (total beta-catenin [TBC]) and to a lesser extent that of the cytoplasmic (inactive) beta-catenin (total beta-catenin [TBC]). Immunoblot of cytoplasmic and nuclear fractions of KMS18 treated cells and densitometric analysis of beta-catenin levels (n=3). Loading controls: α-tubulin, cytoplasmic, TATA-binding protein (TBP), nuclear. (E) Decreased beta-catenin protein expression correlates with
down regulation of the down-stream target gene axin 2. KMS18 cells (n = 3) treated for 16h with 1.5xIC$_{50}$.

**Figure 5. BC2059 inhibits the transcription of down-stream target genes of beta-catenin, and shows specificity for beta-catenin.** (A) BC2059 treatment of NCI H929 (IC$_{50}$:173nM) and U266 (IC$_{50}$:186nM) cells for 20h promotes the decrease of total beta-catenin protein. (B) NCI H929 and U266 cells treated with 1.5xIC$_{50}$ of BC2059 for 20h show decreased transcription of beta-catenin target genes axin 2 (upper panel) and survivin mRNA (lower panel). (C) beta-catenin knock down was performed in KMS18 HMCL, and the cells were treated for two consecutive days with escalating doses of BC2059. Cell death was monitored by PI+ staining, 72h after transfection (n=3 ±s.e.) (left panel). beta-catenin protein level was evaluated 72h after transfection by immunoblotting. beta-catenin siRNA I and siRNA II transfected cell lysates were compared to the negative control siRNA lysate (scramble). β-actin: loading control.

**Figure 6. In vivo efficacy of BC2059.** (A) Tumour growth was monitored weekly by in vivo bioluminescence imaging. The median flux (photons/sec) of dorsal and ventral views for each cohort is documented. Multiple t-test (between vehicle and 5mg/kg or vehicle and 10mg/kg for each time-point) was used for statistical analysis without assuming SD, and statistical significance was determined using the Holm-Sidak method, with alpha=5.000%. At the last bioluminescence measurement (day 53) tumour burden for both 5mg/kg and 10mg/kg cohorts was significantly lower than the vehicle (p=0.0078 and p=0.0075 respectively) (B) NSG human-MM-bearing mice were treated with 5 or 10mg/kg of BC2059 or vehicle alone. Kaplan-Maier survival curves of the three cohorts are shown (log-rank test for trend performed, p=0.023). (C) BC2059 treatment did not affect the body weight (BW) loss and the
haematopoiesis of the MM-bearing mice. Two-way Anova was used for statistical analysis and no significant difference in BW loss was found between the groups throughout the progress of the disease. At the time of euthanasia blood was collected and complete cell count performed. One-way Anova was used for statistical analysis and no significant difference was found between the cohorts. (D) H&E staining of mice dorsal skin revealed the effects of BC2059 in skin homeostasis, with a dose dependent decrease of the hypodermis (black arrow), dysregulation of the normal hair growth cycle and increase of the dermis (black line). (E) Immunohistochemical staining of mice dorsal skin with beta-catenin antibody.
2A

![Graph showing cell death in KMS18 and NCI H929 cells cultured alone or in co-culture.](image)

2B

![Graph showing cell death in HS5 cells cultured with different concentrations of a compound.](image)

2C

![Western blot images showing changes in the levels of β-catenin, γ-catenin, and plakoglobin in HS5 cells.](image)

2D

![Graph showing cell number in SFM, CM 1, and CM 2 conditions.](image)

2E

![Graph showing cell number in SFM and SFM + rWnt3a conditions.](image)

2F

![Graph showing cell number in SFM, rWnt5a (100nM), and rWnt5a (200nM) conditions.](image)
6A

Total Flux (photons/sec)

- Vehicle
- 5mg/kg
- 10mg/kg

days

6B

Percent survival

- Vehicle
- 5 mg/kg
- 10mg/kg

p=0.0236

Days

6C

% of BW loss

Start of treatment
End of treatment
another cycle

6D

WBC (K/L)

PLT (K/L)

Hb (g/dL)

- Vehicle
- 5mg/kg
- 10mg/kg

6E

vehicle
5mg/kg
5mg/kg
10mg/kg

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Molecular Cancer Therapeutics

Beta-catenin inhibitor BC2059 is efficacious as monotherapy or in combination with proteasome inhibitor bortezomib in multiple myeloma

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Mol Cancer Ther Published OnlineFirst May 12, 2017.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-16-0624

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