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Inhibition of a new AXL isoform (AXL3) induces apoptosis of mantle cell lymphoma cells

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Abstract:
Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin lymphoma having a poor overall survival that is in need for the development of new therapeutics. In this study, we report the identification and expression of a new isoform splice variant of the tyrosine kinase receptor AXL in MCL cells. This new AXL isoform, called AXL3, lacks the ligand-binding domain of the commonly described AXL splice variants and is constitutively activated in MCL cells. Interestingly, functional characterization of AXL3, using CRISPRi, revealed that only the knockdown of this isoform leads to apoptosis of MCL cells. Importantly, pharmacological inhibition of AXL activity resulted in a significant decrease in the activation of well-known pro-proliferative and survival pathways activated in MCL cells (i.e.b-catenin, AKT, and NF-kB). Therapeutically, pre-clinical studies using a xenograft mouse model of MCL indicated that bemcentinib is more effective than ibrutinib in reducing the tumour burden and to increase the overall survival. Our study highlights the importance of a previously unidentified AXL splice variant in cancer and the potential of bemcentinib as a targeted therapy for MCL.

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Figure 1

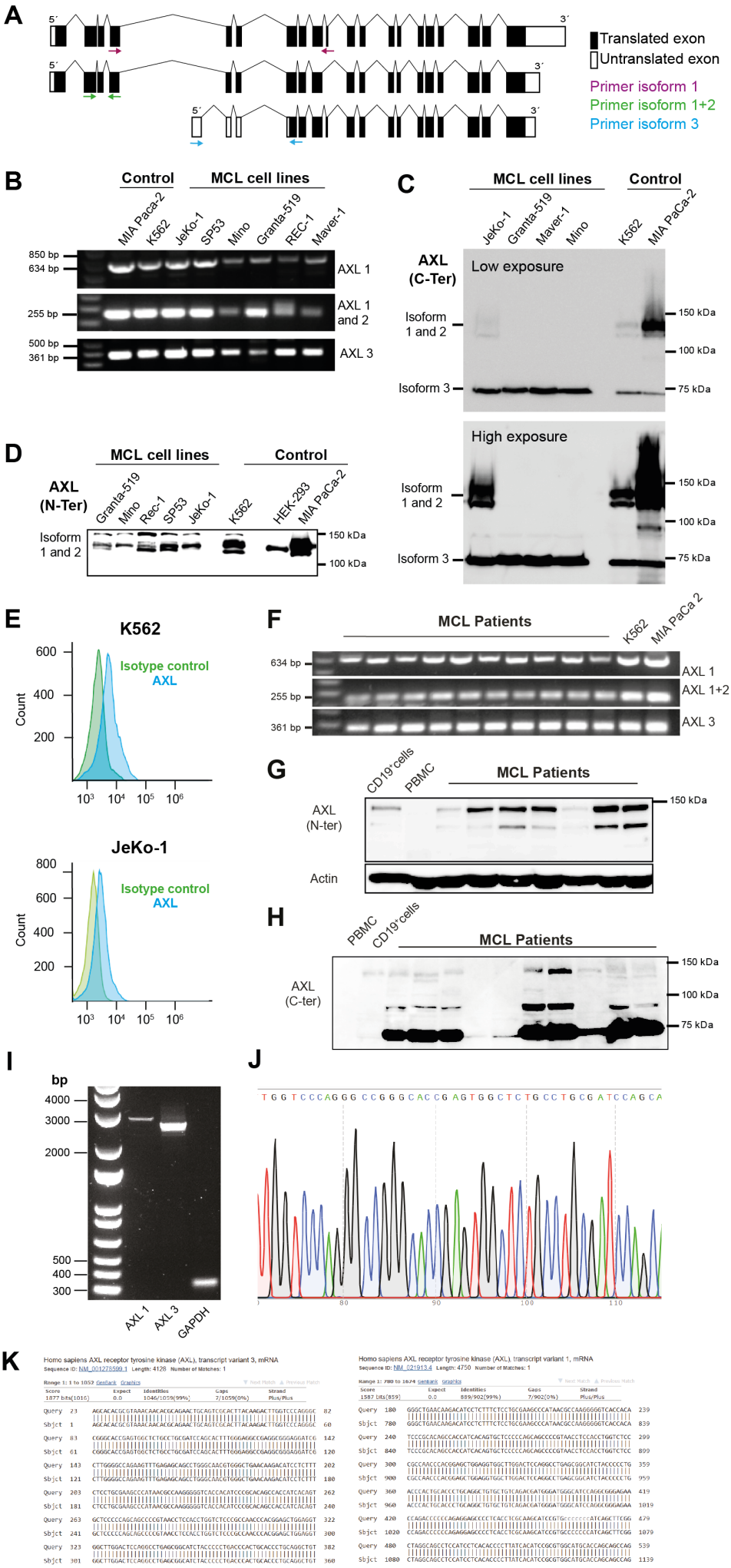


Figure 2

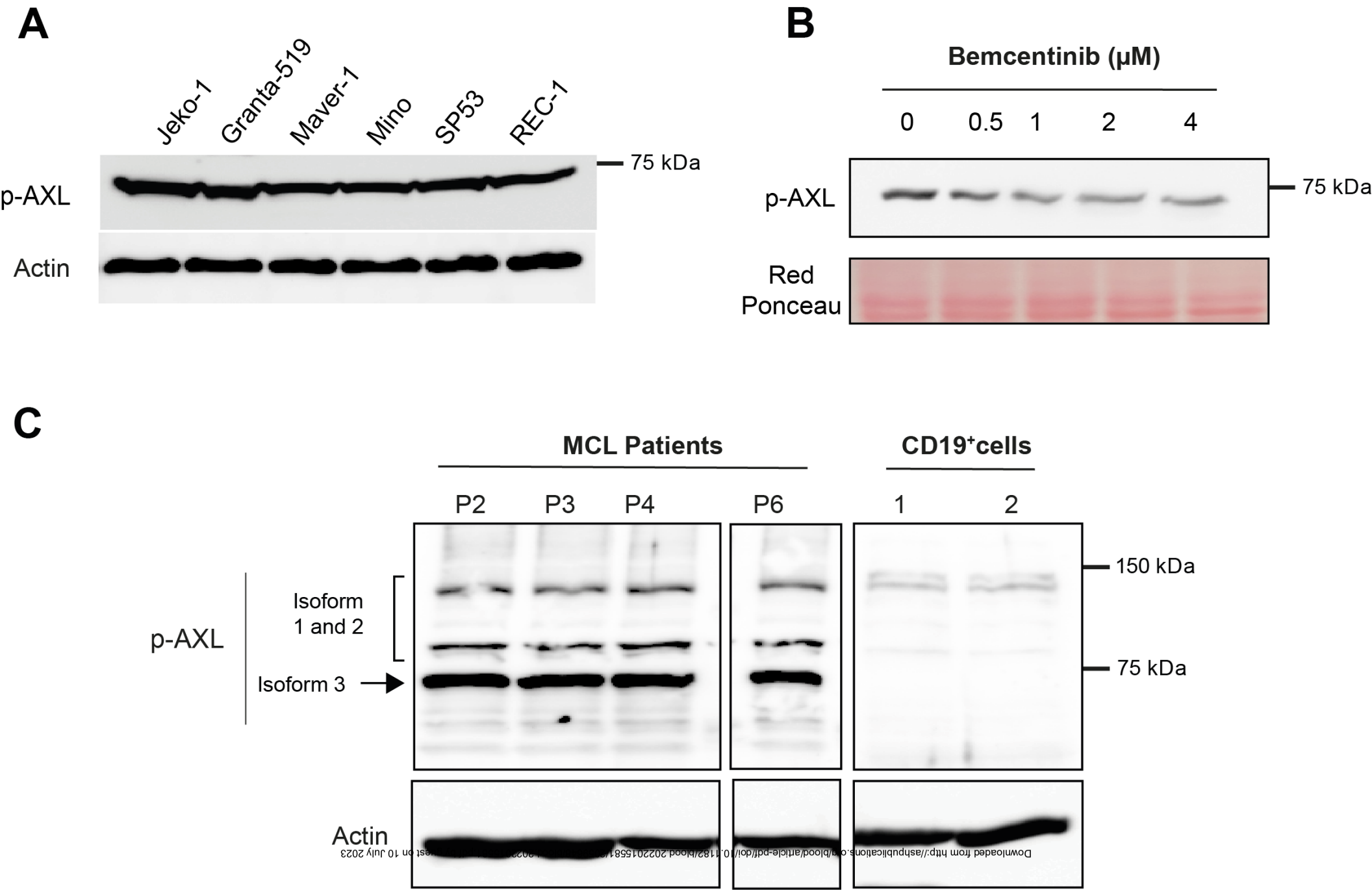
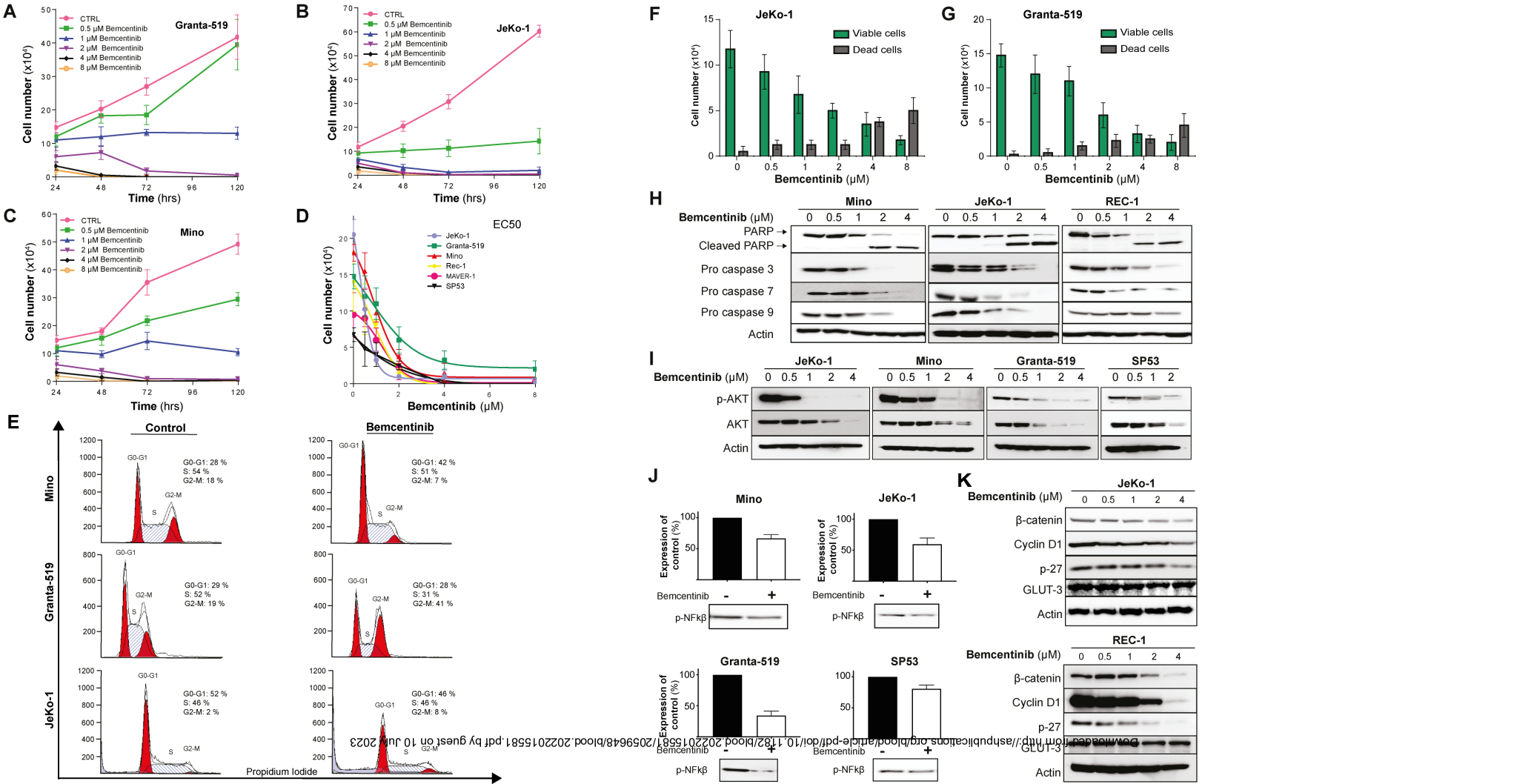


Figure 3



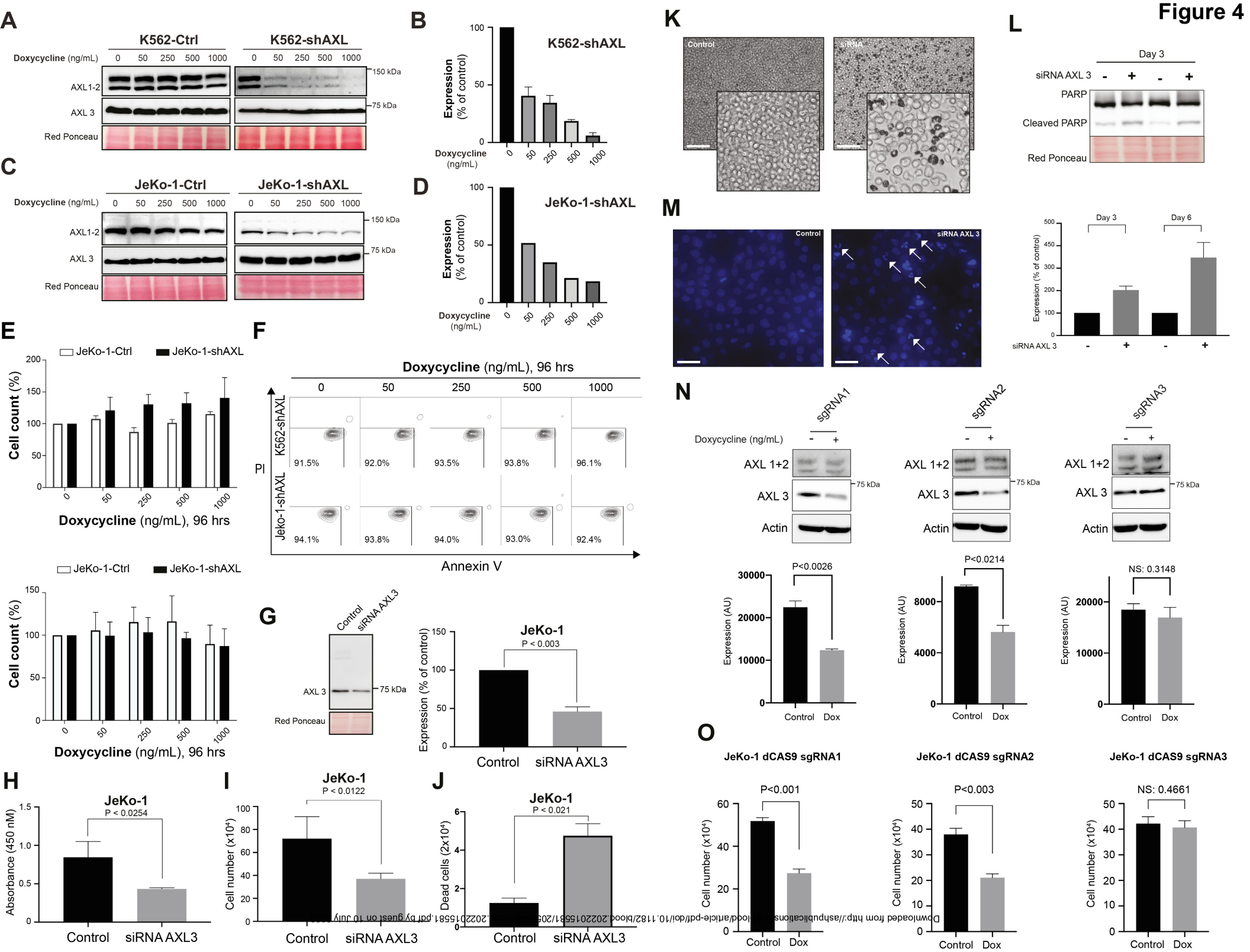


Figure 5

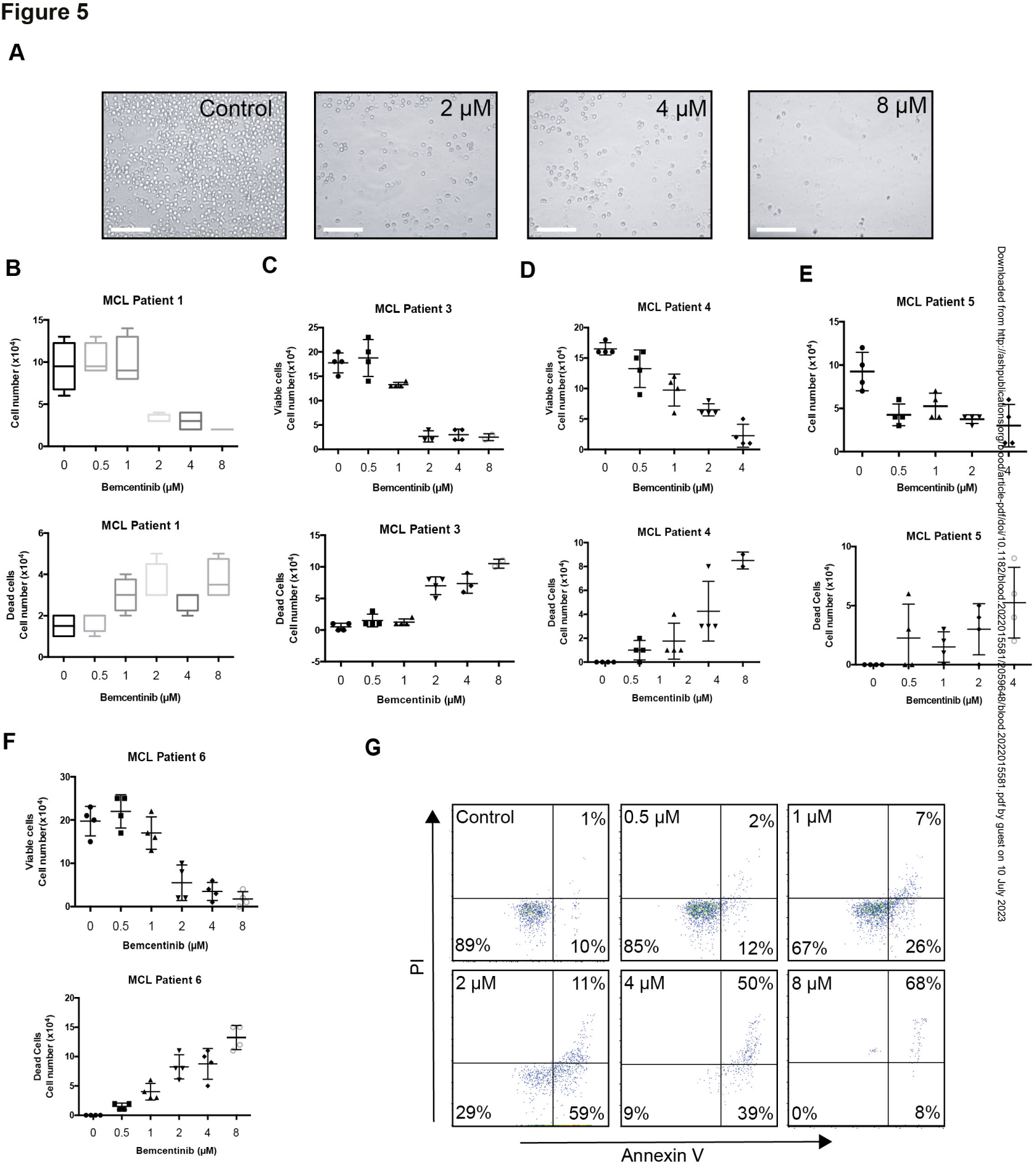
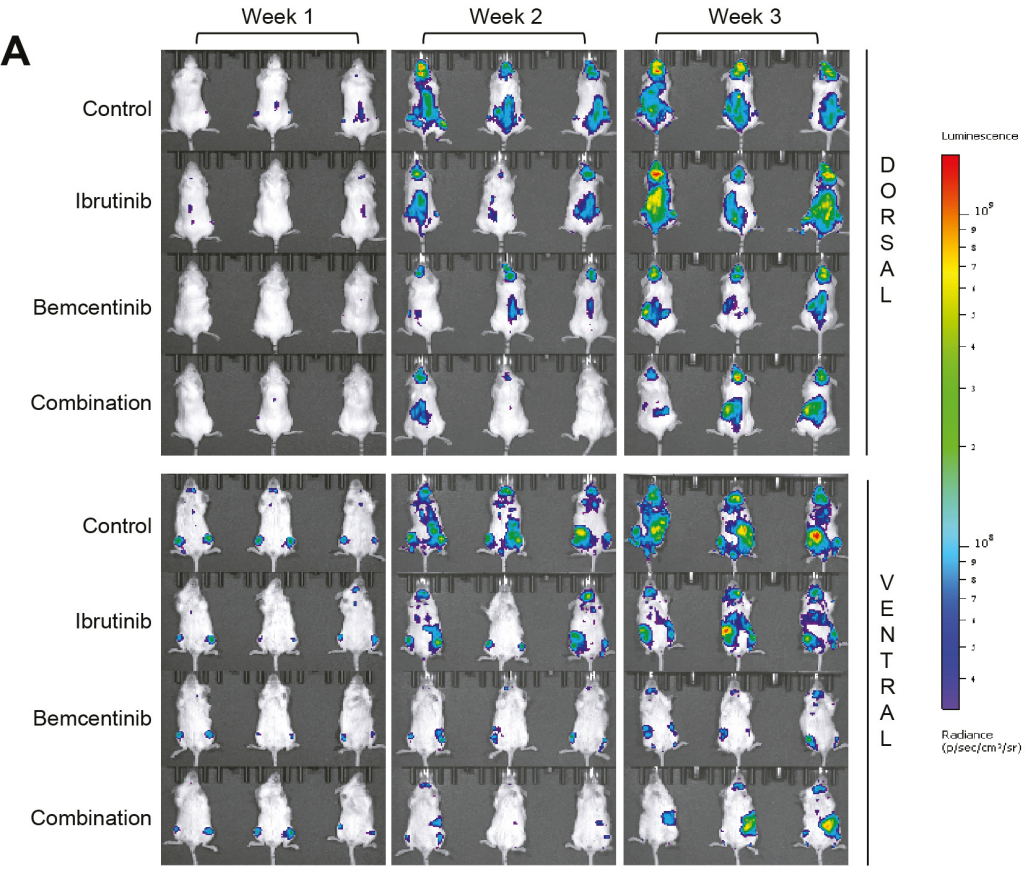
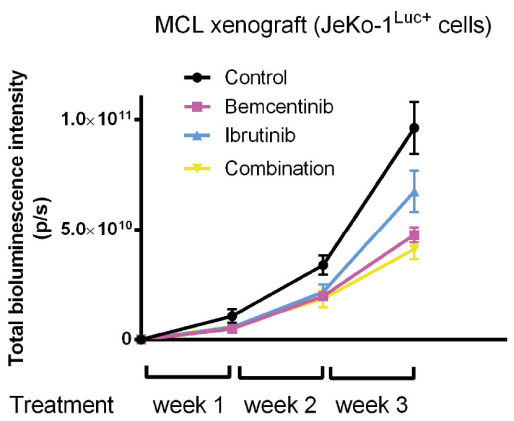
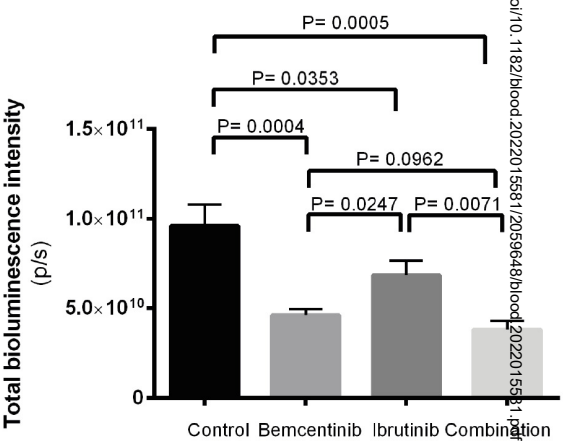
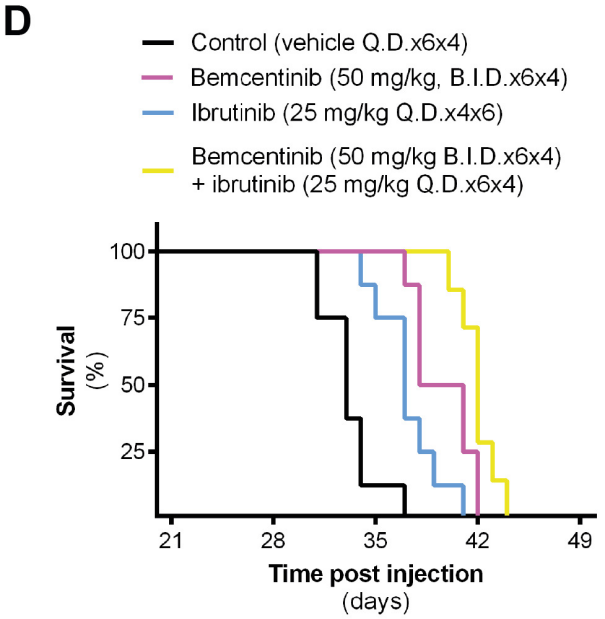


Figure 6









Control vs Bemcentinib P=0.001
Control vs Ibrutinib P=0.0207
Control vs Combination P=0.0001
Bemcentinib vs Ibrutinib P=0.0347
Combination vs Bemcentinib P= 0.0361
Combination vs Ibrutinib P= 0.0006

Inhibition of a new AXL isoform (AXL3) induces apoptosis of mantle cell lymphoma cells

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***Data sharing statement**:* Supplemental Data and Methods can be found in a supplement material available with the online version of this article. For original data, please contact the corresponding author. Sequencing data can be found under accession numbers:
Sequence 1 : GenBank OR103210

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Abstract

Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin lymphoma having a poor overall survival that is in need for the development of new therapeutics. In this study, we report the identification and expression of a new isoform splice variant of the tyrosine kinase receptor AXL in MCL cells. This new AXL isoform, called AXL3, lacks the ligand-binding domain of the commonly described AXL splice variants and is constitutively activated in MCL cells. Interestingly, functional characterization of AXL3, using CRISPRi, revealed that only the knockdown of this isoform leads to apoptosis of MCL cells. Importantly, pharmacological inhibition of AXL activity resulted in a significant decrease in the activation of well-known pro-proliferative and survival pathways activated in MCL cells (i.e.β-catenin, AKT, and NF-κB). Therapeutically, pre-clinical studies using a xenograft mouse model of MCL indicated that bemcentinib is more effective than ibrutinib in reducing the tumour burden and to increase the overall survival. Our study highlights the importance of a previously unidentified AXL splice variant in cancer and the potential of bemcentinib as a targeted therapy for MCL.

Key Points

- The newly identified AXL3 isoform is aberrantly expressed and represents a new biomarker and therapeutic target for MCL.

In a pre-clinical model of MCL, inhibition of AXL tyrosine kinase, using bemcentinib, shows superior activity than ibrutinib.

Introduction

Mantle cell lymphoma (MCL) is an aggressive type of B-cell non-Hodgkin's lymphoma (NHL) that originates from mature B-cells. MCL represents between 5 to 7 % of all NHL [1-5]. Clinical management of MCL remains a significant challenge. While current therapeutic options include several regimens of conventional chemotherapy, most patients will relapse, highlighting the need for more efficient molecularly targeted therapeutics specific for this disease. One of the characteristic features of MCL is the recurrent chromosomal translocation, t(11;14)(q13;q32) that brings the cyclin D1 gene under the control of the enhancer of the immunoglobulin heavy chain gene (IgH), leading to the overexpression of the cyclin D1 protein which in turn leads to a deregulation of the G_1/S phase transition in the cell cycle [6]. Cyclin D1 abnormal overexpression of was initially thought to be the primary driver of the disease. However, it was demonstrated that additional defects in many other cellular processes, such as those involved in cell survival and proliferation (WNT/β-catenin [7,8], NF-κB [9,10], AKT [11], IL22RA1 [12], apoptosis, DNA repair [13]), in addition to contributions from the tumour microenvironment [14,15] are also required. More recently, the aberrant expression of the transcription factor SOX11 has been identified to be involved in the regulation of MCL cell growth [16,17]. Additionally, BCR expression, which is crucial for normal B-cell function, has been demonstrated to be constitutively activated in MCL development [18]. Importantly, ibrutinib monotherapy targeting the tyrosine kinase BTK, which is constitutively activated in MCL, has been shown to achieve an overall response rate (ORR) of 68 % (*n* = 111) in refractory/relapsed MCL patients in phase II clinical trial [19]. The median duration of response was around 17.5 months [19,20]. Clinical studies evaluating ibrutinib as a single agent [21] or in combination with other agents, such as rituximab, resulted in an ORR of 87 % (*n* = 50). More recently, emerging new therapies were investigated in MCL[22,23]. Next generation of BTK inhibitors Acalabrutinib, Zanubrutinib and pirtobrutinib have all been evaluated in MCL, with Pirtobrutinib demonstrating encouraging results in refractory B- cell malignancies, including MCL[24]. On the of immunotherapy front, CAR T cells targeting CD19 have demonstrated durable remission in relapse and refractory MCL patients [25]. Although these results highlight the potential of molecularly targeted approaches and immunotherapy (CAR T/BiTes),

those treatments are non-curative and resultingly, novel therapeutic modalities are still urgently required for this disease [20,26,27].

The receptor tyrosine kinase (RTK) AXL belongs to the TAM family of receptors [28,29]. This small family of RTKs shares the same overall structure and together they regulate and contribute to a wide array of signalling pathways leading to cellular responses such as survival, proliferation, and migration [30,31]. According to The Human Protein Atlas[32], AXL is ubiquitously expressed in the human body but not detected in lymphoid tissue. Its expression in normal B-cells is assumed to be absent at the mRNA and protein level [33,34]. The vitamin K-dependent protein growth arrest-specific protein 6 (Gas6) [35] is the principal ligand of AXL. The AXL receptor can also be activated through ligand-independent homodimerization and heterodimerization with a non-TAM receptor, such as EGFR [36]. AXL signalling is transduced through PI3K-AKT-mTOR, MEK-ERK, NF-κB and JAK/STAT activation [37]. Interestingly, *Axl* was first discovered and cloned from chronic myeloid leukaemia cells following blastic transformation where it was identified as a transforming gene [28,29]. AXL was described to have oncogenic activity in a variety of solid tumours (breast and lung cancer, melanoma and glioma) [28,31,34,37], and several forms of leukaemia (acute myeloid leukaemia, chronic lymphocytic leukaemia) [33,38]. AXL expression was associated with poor prognosis and outcome in AML [38] and has been demonstrated to be a driver of invasiveness, migration and of therapeutic resistance in cancer cells [37]. AXL has also been linked to the epithelial-to-mesenchymal transition (EMT) in breast cancer and non-small cell lung carcinoma (NSCLC) [37,39,40]. Interestingly, although AXL expression was investigated in most haematological malignancies [29], it has never been examined in MCL.

In this study, we investigate whether AXL could constitute a therapeutic target in MCL. We first demonstrate that AXL is expressed and activated in MCL. We then provide evidence for the existence of a third AXL isoform and its role in the survival of MCL cells. Moreover, we demonstrate the efficacy of bemcentinib, as a single agent or in combination, in a newly developed MCL xenograft model providing a translational basis for clinical implementation of AXL inhibition in MCL as a novel therapeutic strategy.

Methods

The study was performed in accordance with the Declaration of Helsinki, and all samples, were collected following written informed consent. All patients were above 16 years of age. The biobank and the clinical protocols were approved by the ethical committee at the Haukeland University Hospital, Bergen, Norway (Ethical approval REK Vest 2012/2245) and Avicenne Hospital HUPSSD, Paris, France.

Details of procedures can be found in the Supplemental Methods section.

Cell culture and patient samples

The MCL cell lines (JeKo-1, SP53, REC-1, Mino, Granta-519) previously described in the following study [7] were used. The MCL cell line Maver-1 was a gift from Dr. Alberto Zamò (University of Verona, Italy). The CML cell line K562, and the ductal pancreatic adenocarcinoma cell line MIA-PaCa-2 were purchased from DSMZ (Braunschweig, Germany), while the Phoenix-AMPHO cells were kindly provided by BergenBio AS (Bergen, Norway).

Establishment of an inducible AXL shRNA knockdown system

To investigate the role of AXL in MCL, a short hairpin RNA (shRNA) conditional knockdown approach was employed. The CML cell line K562 was used as a positive control. To transduce JeKo-1 and K562 cells with the vector of interest, retrovirus was made using Phoenix-AMPHO cells.

Generation of inducible sgRNA CRISPRi cell lines

All plasmids used in the CRISPRi experiment were bought from Addgene (Watertown, MA, USA) unless otherwise stated. JeKo-1 cells were first transduced with Lenti-dCas9-KRAB-blast (plasmid #89567). dCas9 positivity was done by western blotting like previously described using an anti-Cas9 monoclonal antibody (Prod nr. 10C11-A12, ThermoFisher Scientific). Positive cells were then transduced with a FgHtUTG (plasmid #70183) plasmid cloned with sgRNA sequences targeting AXL3. The cells were named JeKo-1 dCas9 sgRNA1, JeKo-1 dCas9 sgRNA2 and JeKo-1 dCas9 sgRNA3.

sgRNA design and cloning into the inducible sgRNA expression vector

The CRISPRi sgRNA sequences were designed using the designer tool CRISPR-ERA Version 1.2: using an input of 1500 bp upstream of TSS and 1500 bp downstream of the TSS of AXL3 transcript (http://www.ncbi.nlm.nih.gov/).

***In vivo* evaluation of bemcentinib in an MCL mouse xenograft model**

The lentiviruses, RediFect Red-FLuc-GFP, were purchased from PerkinElmer Inc. (Waltham, MA, USA) and used to transduce the JeKo-1 cells and generate the JeKo-1^{Luc+} cells that contain GFP and Luciferase reporter. All the animal experimentations were approved by the Norwegian Animal Research Authority and performed according to the European Convention for the protection of vertebrates used for scientific purposes.

Results

AXL is expressed in MCL cell lines and primary MCL cells

To investigate the presence of AXL in MCL, we first demonstrated the expression of AXL transcript using RT-PCR. Primers were specifically designed to amplify AXL isoform 1 and 3 based on the exon structure released in public database (NCBI) (**Figure 1A, Figure S1A**). However, since the AXL isoform 2 differs only by a 27 bp internal exon, the primer set named AXL1-2 is also able to amplify AXL isoform 1 and 2. RNA extracted from five MCL cell lines was used, and the results are illustrated in **Figure 1B**. AXL mRNA was detectable in the MCL cell lines examined. MIA-PaCa-2 and K562 cells served as positive controls. We detected AXL protein expression in MCL cell lines using western blotting. As AXL is a heavily glycosylated protein, we used two different antibodies with distinct binding sites to avoid false negative detection of AXL. One antibody targeting the N-terminal part of the protein (labelled N-ter) and the other one recognizes the C-terminal part of the AXL protein (labelled C-ter). As shown in **Figure 1C**, the C-terminal antibody can recognize AXL at its expected molecular weight in MIA-PaCa-2 cells (around 98 kDa), as well as the glycosylated form at 120-140 kDa. However, in all MCL cells, this antibody recognizes a band corresponding to the expected size of AXL3 (around 70 kDa). This isoform can also be detected in K562 and MIA-PaCa-2 but at a lower level of expression than in MCL cells. In contrast, the AXL N-ter antibody can identify AXL expression at a size of around 120-140 kDa in all the cell lines tested (**Figure 1D**). Flow cytometry was used to evaluate the expression of AXL in MCL cell lines. As demonstrated in **Figure 1E**, the Jeko-1 MCL cell line showed an increased signal in comparison to the isotype control. K562 cells served as the positive control in this experiment. We then assessed the expression of AXL in primary MCL patient cells at both mRNA and protein level. As shown in **Figure 1F**, the different AXL transcripts can be detected in MCL patient samples. Furthermore, using either the AXL N-ter or C-ter antibodies, AXL protein expression can be detected in MCL primary cells (**Figure 1G and H)** and is overexpressed in comparison to PBMCs or $CD19^+$ cells. To confirm that MCL expresses the AXL3 transcript we cloned the full cDNA transcript including the 5´and 3´UTR parts from JeKo-1 and K562 cells (Ensemble database: putative AXL-203 transcript ID: ENST00000593513.1, 2557 base pair). As shown in **Figure 1I**, the amplification product using the primer to clone the AXL3

transcript (**Supplemental Figure 1B**) led to the amplification of products at the expected size. The full product was cloned, and the alignment of the sequencing result confirmed the presence and identity of the AXL3 transcript with the predicted exon structure (**Figure 1J and K**). We also cloned part of the AXL1 messenger excluding a portion of the untranslated 3` messenger part. The full amplification product was also clone and confirmed the existence of AXL1 transcript in MCL cells (Ensemble database: AXL-201 transcript ID: ENST00000301178.9, 4717 base pair).

AXL is constitutively activated in MCL cell lines and primary MCL cells

To investigate the level of AXL activation in MCL cell lines and MCL primary cells, we examined its phosphorylation status by western-blot using an antibody specific for phosphorylated AXL (p-AXL). As shown in **Figure 2A**, AXL phosphorylation can be detected in all MCL cell lines with bands corresponding to the expected molecular weight of AXL3. To confirm the specificity of the detected AXL phosphorylation, the MCL cell line JeKo-1 was treated with increasing doses of the AXL inhibitor, bemcentinib. As described in **Figure 2B**, the AXL inhibitor induced a significant decrease in the phosphorylation status of AXL. AXL phosphorylation was also evaluated in MCL primary patient cells (**Figure 2C)**, demonstrating AXL phosphorylation. Both in MCL cell lines and patient cells, the strongest AXL phosphorylation was detected for the band corresponding to the third isoform of AXL. $CD19^+$ cells demonstrated some weak p-AXL staining for AXL isoform 1-2 in comparison to MCL cells (**Figure 2C**). We also evaluated the expression of GAS6, the ligand of AXL, in MCL cells. As shown in **Figure S2A,** the GAS6 transcript can be detected in all MCL cell lines. However, no GAS6 protein expression can be detected in the supernatant of MCL cell culture medium (**Figure S2B**). Together, these observations indicate that the newly described AXL3 protein is the major AXL isoform activated in MCL cells, and this is supported by the observation that the known ligand of AXL is not expressed at the protein level in MCL cell lines, and no p-AXL1 and 2 isoforms can be detected.

Pharmacologic inhibition of AXL activity by bemcentinib induces significant cell-growth inhibition and apoptosis of MCL cell lines

To determine the biological importance of AXL in MCL cells, we inhibited AXL activation in MCL cell lines using the most potent pharmacological inhibitor described

so far, bemcentinib [41]. To show that bemcentinib can bind AXL in vivo we used the cellular thermal shift assay (CETSA) (**Figure S3**). To determine the EC_{50} of bemcentinib on MCL cell lines, the MCL cells were treated with a wide range of drug concentrations. As shown in **Figure 3**, bemcentinib induced a significant reduction in the number of cells in a time and dose-dependent manner (**Figure 3A, B and C**). At 48 hours post-treatment, the EC_{50} values were in the range of 1 to 2 µM for most of the MCL cell lines (**Figure 3D**) with SP53 being the most sensitive cell line and Maver-1 the least sensitive (**Figure 3D**). As illustrated in **Figure 3E**, cell cycle analysis revealed that bemcentinib induces cell cycle arrest either in the G1 phase, for the Mino cells, or at the G2 phase for the Granta-519 and JeKo-1 MCL cells. Next, we investigated whether bemcentinib induced apoptosis in MCL cells. As shown in **Figure 3F and G**, bemcentinib treatment of various MCL cell lines for 48 hours leads to an increase in trypan blue positive cells. Moreover, a dose-dependent cleavage of PARP, as well as proteolytic activation of pro-caspases 3, 7 and 9 can be observed after bemcentinib treatment (**Figure 3H**). We also found that increasing concentrations of bemcentinib induced a dose-dependent decrease in AKT (**Figure 3I**) and NF-κB activation (**Figure 3J**). Furthermore, we also evaluated the biological effect of bemcentinib on other pathways known to be dysregulated in MCL. As shown in **Figure 3K**, bemcentinib induced a decrease in the expression of cyclin D1, p27 and of β-catenin. Taken together, these results demonstrate that major signalling pathways involved in the survival and proliferation of MCL cells are inhibited by the inhibition of AXL activation.

Knockdown of the AXL 3 isoform induces apoptosis of MCL cells

To further investigate the role of AXL in MCL, we used a conditional shRNA approach. The MCL cell line JeKo-1 and the CML cell line K562 were transduced with the inducible AXL shRNA or control shRNA vector containing the mCherry reporter vector. As shown in **Figure S4A and B**, an mCherry fluorescence signal, indicating the presence of the inducible construct, is observed in the different transduced cell lines. AXL knockdown was induced by adding different concentrations of doxycycline (ranging from 0 to 1000 nM; **Figure 4A and C**). The knockdown efficiency was analysed by Western blotting, and a 90% decrease of AXL1 and 2 can be observed in the two different models (**Figure 4B and D and S4C**

and D). In contrast, no decrease in AXL protein was observed in the control cells (**Figure 4A and C - Left panel**). Interestingly, the level of AXL3 expression is not effected by the increased concentrations of doxycycline in the K562 transduced cell line. Similarly, a decrease in protein expression of AXL1 and AXL2 can be observed in JeKo-1-shAXL cells after addition of doxycycline. However, as seen in the K562 cells, AXL3 expression remains unaltered. To evaluate the effect of decreased AXL expression on cell growth and viability we used the trypan blue assay. Surprisingly, no statistical difference in total cell number was observed between the different doxycycline concentrations in any of the transduced cell lines (**Figure 4E**). Moreover, the control and the shRNA-AXL cell lines present no statistical difference in total cell number after 96 hrs of doxycycline treatment. Annexin V/PI flow cytometry was performed to further evaluate the impact of AXL1 and AXL2 knockdown on cell viability. After 96 hrs of doxycycline treatment, cells were stained and run on the flow cytometer and no statistical differences in the number of apoptotic cells were observed for the different concentrations of doxycycline used (**Figure 4F**). All cell lines were approximately 90% viable after 96 hrs exposure to 1000 nM doxycycline. To further evaluate the biological significance of AXL in MCL, we investigated the role of AXL3 on MCL biology. AXL3 expression was downregulated using a siRNA approach and we assessed the effects on cell growth and apoptosis on MCL cells. As shown in **Figure 4G**, siRNA specifically targeting AXL3 (**Figure S5A**) induced a substantial reduction in AXL3 protein expression of approximately 50% within 72 hours of transfection. Blockade of AXL3 expression using siRNA significantly decreased cell growth by approximately 50% at 5 days post-transfection as determined by WST-1 assay (**Figure 4H**) and cell count (**Figure 4I, Figure S5B**). Moreover, bright-field optical microscope imaging revealed the presence of dead cells in cells transfected with the AXL3 siRNA (**Figure 4J and K**). As shown in **Figure 4L**, inhibition of AXL3 expression by siRNA in JeKo-1 cells induced the cleavage of PARP. Moreover, Hoechst staining (**Figure 4M**) clearly demonstrated formation of apoptotic bodies with condensed and fragmented nuclei. These results underline that, in contrast to the AXL1 and 2 isoforms, only the specific downregulation of AXL3 protein expression has an impact on MCL cell growth and viability. Since the design of specific AXL3 siRNA needed to target the 5´ UTR sequence of the AXL3 mRNA and prevented us to find multiple suitable effective siRNA sequences, we decided to validate our siRNA observations using an CRISPRi

approach. To this end, JeKo-1 cells were made positive for dCAS9 expression (**Figure S6 A, B and C**) and we designed three sgRNAs targeting the AXL3 promoter (**Figure S6D**). As shown in **Figure 4N (Left and middle panel)**, two of the sgRNAs targeting the AXL3 lead to a decrease in AXL3 protein expression. This was accompanied by a decrease in the cell proliferation (**Figure 4O**). In contrast, no effect on cell proliferation was observed in control cells (**Figure S6E)** or cells carrying the sgRNA that failed to decrease AXL3 expression (**Figure 4N and O - right panel**).

Bemcentinib induces cells death of primary MCL cells *in vitro*

To further evaluate the efficacy of bemcentinib on MCL cells, we treated primary MCL cells with various concentrations of bemcentinib (**Figure 5A)**. Bemcentinib induced a significant dose-dependent reduction in cell growth, as well as a decrease in cell viability, as determined by trypan blue staining (**Figure 5B, C, D, E and F**). This result is further supported by the Annexin V/PI staining which shows an increase in cell death after treatment with bemcentinib (**Figure 5G**). As observed for the MCL cell lines, the EC_{50} of bemcentinib is around 1.5 μM in MCL primary cells (**Figure 5B, C, D, E and F**). Moreover, we also demonstrate synergy between bemcentinib and ibrutinib in vitro (**Supplemental Figure 7**). Overall, these results indicate that MCL primary cells are sensitive to bemcentinib *in vitro*.

Bemcentinib shows superior efficacy than ibrutinib to decrease MCL tumour burden and enhances the overall survival

To investigate whether pharmacological targeting of AXL could potentiality benefit patients, we used our newly developed orthotopic JeKo-1 xenograft mouse model of MCL (**Figure S8A-F**). The *in vivo* efficacy of bemcentinib, ibrutinib and the combination of the two drugs was evaluated. As shown in **Figure 6A,** the disease develops in the bone marrow, spleen, femur and brain. BLI revealed that at week 3 the control group had the highest average total bioluminescence signal, followed by the ibrutinib treatment group and the bemcentinib treatment group, respectively (**Figure 6B and C**). The bemcentinib treatment group demonstrated significantly lower total bioluminescence signal compared to the control group (p = 0,00004; unpaired Student's t-test), while the ibrutinib treatment group also showed statistically significant decrease in total bioluminescence in comparison to the control

group (p = 0.0353; unpaired student's t-test). In addition, the total bioluminescence signal of the bemcentinib treatment group was significantly lower than the ibrutinib treatment group (p = 0.0247; student's unpaired t-test). The total bioluminescence of the combination group was also significantly lower than the control (p = 0.0005; unpaired student's t-test) and ibrutinib group (p = 0.0071; unpaired student's t-test). These results demonstrate that bemcentinib shows efficacy in a pre-clinical xenograft MCL mouse model. The combination of bemcentinib and ibrutinib demonstrates the highest overall survival (**Figure 6D**).

Discussion

AXL is a tyrosine kinase receptor involved in various biological processes such as stimulation of cell proliferation and regulation of the innate immune response [42] . Its role in the oncogenesis of several forms of solid cancers, including pancreatic [43], ovarian [44] and breast carcinoma [45], has been widely demonstrated. AXL expression has also been investigated in various forms of haematological malignancies. Neubauer *et al*. [34] found that the AXL transcript can be detected in several forms of leukaemia, including AML [38,46] and CLL[33]. However, AXL expression has never been reported in mantle cell lymphoma. In the present work, we demonstrate that AXL is expressed at the mRNA and protein level in MCL cell lines and patient cells. Interestingly, we have also demonstrated for the first time the existence of a third AXL isoform in MCL cells. To confirm the existence of the AXL3 mRNA we cloned the full transcript (**Figure 1**). Sequencing confirms that the exon structure of this messenger corresponds to the predicted AXL3 isoform. Despite the isoform being predicted and described in the Ensembl database, to our knowledge, no previous reports have cloned, identified, or studied the isoform in human cells. In contrast to the two well-characterized AXL1 and 2 isoforms, the third AXL protein presents a singular extracellular structure. The transcript encodes for a protein of 626 a.a., which lacks the two Ig-like domains but retains the two-fibronectin type 3-like (FNIII) repeats. This configuration makes this isoform a peculiar AXL receptor as there is only one other type of tyrosine kinase family that possesses only extracellular fibronectin type 3 domains, the ROS tyrosine kinase family. Unlike AXL isoform 1 and 2, GAS-6 will not bind and activate the AXL3 isoform. Instead, the ligand of the FNIII domain remains to be formally identified. Nevertheless, some studies seem to indicate that the fibronectin III domain can bind to integrin proteins [47,48], indicating that AXL activation can be triggered by cell-cell contact or by interaction with the extracellular microenvironment. Moreover, we cannot exclude that this third isoform can also be activated by a ligand-independent mechanism or by heterodimerization with another receptor as it was previously reported for AXL1 and 2 [49]. The AXL expression/activation pattern seems complex in MCL. We were able to detect GAS-6 transcript in most MCL cell lines (Supplemental Figure S2), indicating that an autocrine activation cannot be ruled-out in MCL. These novel insights into the expression and activation of AXL isoforms may significantly impact therapeutic

strategies. Pharmaceutical attempts to block AXL activation by preventing GAS-6 binding may be by-passed in cells expressing the AXL3 isoform. Splice variant switching, obtained by genetic mutation or other mechanisms, is now a well-established mechanism used by cancer cells to gain proliferation advantage or resistance to chemotherapy treatments [50-52].

To further evaluate the impact of AXL inhibition in MCL cells we have used a conditional knockdown shRNA approach to decrease the expression of AXL. The shRNA construct was previously characterized to be effective in reducing AXL expression [39]. Interestingly, using this approach we were able to effectively shut-down AXL1 and 2 protein expression in the AXL shRNA JeKo-1 and AXL shRNA K562 cells. In both cell lines, the AXL3 protein expression was unaffected. Despite the ability of bemcentinib to induce cell death of wild-type JeKo-1 and K562 cells, neither cell proliferation, nor viability was affected by the knockdown of AXL1 and AXL2. (**Figure 4**). These results diverge from the reported findings of Ben-Batalla *et al*.[38], that describe that silencing of AXL1-2 in CML cells resulted in a reduction of cell viability[38]. However, the authors have not evaluated the effect of the AXL knockdown on the AXL3 protein expression that may be responsible for the observed effect on cell viability. Nevertheless, our results are in accordance with Dufies *et al*. [53] and Gioia *et al*. [54], who have shown that AXL1-2 knockdown doesn't induce cell apoptosis in CML. Moreover, a study performed in the squamous cell carcinoma line (MET1) also reported that knockdown of AXL1-2 did not affect cell proliferation [55] nor induce apoptosis. As shown in **Figure 1**, the AXL3 isoform, at the protein level, seems to be considerably more highly expressed than AXL 1 and 2 in MCL cell lines, as well as in the CML cell line K562. The observation that AXL3 protein cannot be downregulated in our conditional shRNA system may be explained by the structure of the AXL3 mRNA. In fact, this isoform presents a long 5´UTR sequence that may be involved in the stabilization of the messenger. Moreover, it is also well-established that mRNAs can be regulated by post-secondary modifications, like N6-adenosine methylation, that can control mRNA decay as reported for SOX2 or KLF4 mRNA [56]. Therefore, it is possible to consider that different AXL mRNAs may have different lifetimes and may be more or less sensitive to a particular RNAi sequence (shRNA or siRNA) used to induce the degradation of the AXL mRNA. To further evaluate the function of AXL3, we have custom designed a specific siRNA

targeting only AXL isoform 3. As shown in **Figure 4G**, with this approach we were able to decrease AXL3 protein expression that was observable at 72 hours (**Figure 4G**). As shown in **Figure 4H, I, K, L and M,** the downregulation of AXL3 expression leads to the reduction of growth and apoptosis of MCL cells. To further characterize the function of AXL3, we took advantage of the fact that this isoform uses a different promoter than the previously described AXL isoforms, to selectively block the transcription of AXL3 by a CRISPRi approach. As illustrated in **Figure 4N**, induction of the sgRNA targeting the *Axl3* promoter leads to a decrease of AXL3 protein expression as well as to a significant decrease in cell proliferation. Interestingly, the time course of expression of AXL3 decrease was slower in the CRISPRi experiment than in the siRNA approach. However, this result is not unexpected as CRISPRi affects transcription rather than RNA degradation and are in accordance with previous reports using CRISPRi [57,58]. By using the CETSA, we were able to show that bemcentinib can bind the AXL3 isoform. Altogether these results indicate that the inhibition of the expression or activity of AXL3 is responsible for the growth inhibition and apoptosis of MCL cells.

Our *in vitro* data reveal that bemcentinib can reduce the proliferation and induces apoptosis of cells that were not killed by standard dose of ibrutinib (i.e. Granta-519, **Figure 3A and 3D)**. This observation is of interest as it underlines that ibrutinib resistance can be overcome by blocking AXL activation. One-third of patients treated with ibrutinib alone relapse within 2 years of treatment [26]. It may therefore be valuable to investigate if a co-treatment of MCL cells with an AXL and a next generation of BTK inhibitor, like pirtobrutinib could be favourable. To support this concept, our study also provide evidence that inhibition of AXL activity, in conjunction with the BTK inhibitor ibrutinib, increases the overall survival of MCL-carrying mice in comparison to mice treated with either ibrutinib or bemcentinib alone. Moreover, it was previously shown that co-treatment can be beneficial for MCL patients with other drugs[59,60]. In this context, the development of new therapeutic modalities targeting simultaneously several vulnerabilities identified in MCL should be of interest for the development of multimodal therapy for the long-term remission or cure of MCL patient.

It is interesting to note that the AXL3 isoform can also be detected in other haematological (i.e. chronic myelogenous leukaemia) and solid (i.e. Pancreatic

ductal adenocarcinoma) malignancies, suggesting that AXL3 may also have broader implications for the aetiology of several cancer forms. In particular, the development of therapeutic antibodies targeting AXL in pancreatic, or breast cancer, have so far demonstrated a limited application. This may be explained, in part, by the inability of those antibodies to recognize and bind the new AXL3 isoform permitting tumour cell evasion by this therapeutic strategy.

In conclusion, we show that MCL cells express AXL and that it contributes to the biology of MCL by promoting cell growth and survival characteristics. We also discovered that MCL cells express a third AXL isoform that this is responsible for the cell growth and survival effect observed in MCL. Moreover, we also demonstrate, in a xenograft mouse model of MCL, that bemcentinib, an AXL inhibitor that got fast-track designation in Non-Small Cell Lung cancer and currently in clinical trials in acute myeloid leukaemia, may hold promise in treating MCL as a single agent or in combination with BTK inhibitors and/or immunotherapy modalities (CAR T/BiTes).

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Authorship Contributions

P.G., M.E.G., and E.M.C. designed the experiments, P.G., M.E.G., S.B., J.H., I.K., M.M.S, C.L., Z.F. and M.P. performed all the experimental procedures, P.G., M.E.G., I.K. and E.M.C. analysed the data. L.H., B.P. and F.B.M. provided MCL patients. P.G., M.E.G., I.K. and E.M.C. wrote the paper.

Declaration of Conflicts of Interest

The authors declare no competing interests and none of the authors have shares or received grants or financial support from BerGenBio AS.

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655 2494.

656 **Figures Legends**

657

658 **Figure 1. MCL cells overexpress a new AXL isoform**.

659 (**A**) Schematic representation of AXL mRNA structure. The different set of primers
660 used for AXL mRNA amplification (RT-PCR) are indicated in purple (specific
661 amplification of isoform 1), green (amplification of isoform 1 and 2), and blue
662 (specific amplification of isoform 3). The new AXL 3 isoform (Bottom) is missing the
663 first four coding exons of the AXL isoform 1 and 2. (**B**) Relative mRNA expression of
664 the different AXL transcripts investigated by RT-PCR in MCL cell lines. All the
665 different AXL mRNA isoforms can be detected in MCL cells. MIA-PaCa-2 and K562
666 were used as positive controls for AXL amplification. (**C**) Detection of AXL protein
667 expression in MCL using an antibody targeting the N-terminal or (**D**) C-terminal
668 domain of AXL. The C-terminal antibody is able to recognize all AXL isoforms. The
669 AXL N-terminal antibody recognizes the isoform 1 and 2 but not the AXL3 isoform
670 since the antibody epitope is not present in AXL3. K562 and MIA-PaCa-2 served as
671 positive controls. (**E**) AXL can be detected at the cell surface of MCL cells by flow
672 cytometry staining using the AXL N-ter antibody. K562 served as a positive control.
673 Experiments were performed in triplicate. (**F**) AXL mRNA expression was
674 investigated by RT-PCR in primary MCL cells. All the AXL isoforms were expressed
675 in MCL primary cells. K562 and MIA-PaCa-2 served as positive controls for the AXL
676 1 and 2. (**G**) and (**H**) AXL is expressed at the protein level in MCL cells. AXL is
677 overexpressed in MCL cells in comparison to PBMCs or $CD19^+$ B-cells. (**I**) AXL 3 full
678 transcript was cloned and sequenced (**J** and **K**) and corresponds to the predicted
679 AXL3 isoform.

680

681 **Figure 2. AXL is constitutively activated in MCL cell lines and patient cells.**

(**A**) Western blot studies demonstrated the phosphorylation of AXL in all MCL cell lines. (**B**) JeKo-1 cells were treated with bemcentinib at concentrations ranging from 0,5 to 4 μM. Phosphorylation of AXL was evaluated using a p-AXL antibody. bemcentinib treatment reduces the level of AXL phosphorylation in MCL cells. (**C**) AXL phosphorylation status was evaluated in MCL patient cells by western blot. The AXL phosphorylation was detectable in all MCL patient cells. The results also indicated that AXL3 is the isoform predominantly activated in primary MCL cells. In contrast, $CD19^+$ B-cells from healthy individuals present a weak AXL activation and the complete absence of AXL3 phosphorylation. Experiments were performed in triplicate.

Figure 3. Bemcentinib inhibits cell growth and induces cell cycle arrest and apoptosis of MCL cells.

(**A, B, C and D**) MCL cell lines were treated with the AXL inhibitor bemcentinib in concentrations ranging from 0 to 8 μM. The number of viable cells, as determined by trypan blue exclusion, decreased in a time and dose-dependent manner. Experiments were performed in triplicates and the means +/- standard deviations are shown. (**E**) Cell cycle analysis performed by flow cytometry revealed that AXL activity inhibition induces a cell cycle arrest of MCL cells. Triplicate experiments were performed and a representative result for each MCL cell lines is shown. (**F and G**) The JeKo-1 and Granta-519 MCL cell lines were treated with different concentrations of bemcentinib. The number of dead cells was assessed by trypan blue exclusion. Bemcentinib treatment induced cell death in a dose-dependant manner. The experiments were performed in triplicates and the means +/- standard deviations are shown. (**H**) Apoptosis was monitored by caspase activation in MCL cells (Mino, JeKo-1 and REC-1) treated with bemcentinib. Inhibition of AXL activity triggered apoptosis as revealed by PARP cleavage and caspase 3, 7 and 9 activations. (**J**) AXL inhibition by bemcentinib decreased NF-kβ activation in MCL cell lines. NF-kβ activation was evaluated by western blot using a phopho-antibody. Quantification of NF-kβ activation is shown for each MCL cell lines. The experiments were performed in triplicates and the means +/- standard deviations are shown. (**K**) The expression of β-catenin, Cyclin D1, p-27 and GLUT-3 was investigated by western blot in JeKo-1

715 and REC-1 MCL cells. Bemcentinib treatment induced a dose-dependent decrease
716 in β-catenin, Cyclin D1 and p-27 expression. No decrease in GLUT3 was observed.
717 The experiments were performed in triplicates and a representative blot is shown for
718 each cell line.
719
720 **Figure 4. AXL 3 knockdown induce apoptosis of MCL cells.**
721 (**A**) K562 and (**C**) JeKo-1 cells were transduced with a doxycycline inducible shAXL
722 construct to block AXL expression. K562 and JeKo-1 control vector and shAXL cells
723 were treated with an increasing dose of doxycycline for 96 hours. (**B** and **D**)
724 Quantification of AXL expression by western blot after doxycycline treatment. (**E**)
725 Quantification of cell number after doxycycline induction of shAXL. No effect on cell
726 proliferation was observed for the control and shAXL cells. Experiments were
727 performed in triplicate and the means +/- standard deviations are shown. (**F**)
728 Apoptosis on JeKo-1 shAXL or K562 shAXL cells was evaluated by annexin V/PI
729 staining after 96 hours of doxycycline treatment. Experiments were performed in
730 triplicates, and a representative flow chart is shown. (**G**) JeKo-1 cells were
731 transfected with AXL3 siRNA and AXL protein expression was assessed by western
732 blot (left). Quantification studies (right) showed that AXL expression was decreased
733 in MCL after treatment with AXL siRNA as compared to cells treated with scrambled
734 siRNA ($p<0.003$). Experiments were performed in triplicates and the means +/-
735 standard deviations are shown. (**H**) Treatment of the JeKo-1 MCL cell line with
736 AXL siRNA significantly decreased the number of cells in comparison to cells treated
737 with scrambled siRNA, as assessed by using the MTS assay ($p<0.0254$) and (**I**) cell
738 counting, the differences were statistically significant ($p<0.0122$). (**J**) JeKo-1 cells
739 were treated with scrambled or AXL3 siRNA and cell death was determined by
740 trypan blue assay ($p<0.021$). (**K**) The morphological changes were examined by light
741 microscopy. Scale bar= 100 μM. Cell shrinkage and formation of apoptotic bodies
742 were considered as apoptotic cells (Magnification×200). (**L**) Using western blot,
743 cleaved PARP was detectable in JeKo-1 cells treated with AXL3 siRNA.
744 Quantification of PARP cleavage after AXL3 siRNA treatment (bottom). (**M**)
745 Nucleolar morphological changes were observed under a fluorescence microscope
746 after Hoechst-33342 staining. Scale bar= 20 μM. Condensed or fragmented nuclei
747 were considered as apoptotic cells (×400). Arrows indicate apoptotic cells. (**N**) AXL3

expression was downregulated using a CRISPRi method. sgRNAs targeting the AXL3 promoter were design to be induced by doxycycline treatment. Effect on AXL3 protein expression was monitored by western blot. Quantification of AXL3 expression in comparison to control cells. Experiments were performed in triplicates and the means +/- standard deviations are shown. (**O**) Effect of AXL3 CRISPRi on cell proliferation was determined by cell counting. Experiments were performed in triplicates and the means +/- standard deviations are shown.

Figure 5. Bemcentinib induces apoptosis of MCL primary cells. (A) MCL patient cells were treated with DMSO or various concentrations of bemcentinib (0 to 8 μM) for 24 hours. Relevant microscopy pictures revealed the presence of apoptotic cells and a reduction of the cell populations. Scale bar= 100 μM. (**B**, **C**, **D**, **E** and **F**) Cell proliferation and cell death were measured by cell counting and trypan blue staining. Experiments were performed 4 times and the mean and distribution are shown. (**G**) Cell death was also investigated by flow cytometry using Annexin V/PI staining in MCL patient cells. Bemcentinib induced apoptosis in primary MCL cells *in vitro*.

Figure 6. Inhibition of AXL by Bemcentinib reduces tumour burden and increases survival in an MCL xenograft mouse model.
(**A**) Female NSG mice were injected i.v. with 5×10^5 JeKo-1^{Luc+} cells. The mice were imaged weekly, both dorsally and ventrally (10 minutes after i.p. injection with 150 mg/kg of 25 mg/mL D-luciferin). The mice were distributed in the following groups control (vehicle only q.d), bemcentinib treatment (50 mg/kg b.i.d.), ibrutinib treatment (25 mg/kg q.d.), and combination treatment (ibrutinib + bemcentinib) based on total bioluminescence. Following the assignment, there was no statistical difference between the groups ($p > 0.99$; one-way ANOVA test). JeKo-1^{Luc+} xenografts were treated for 4 weeks with vehicle q.d. (control), ibrutinib (25 mg/kg, q.d.), bemcentinib (50 mg/kg, b.i.d.) or the combination of ibrutinib and bemcentinib (same dose as single treatment) (n= 6). (**B**) BLI was assessed weekly to monitor disease progression. (**C**) Quantification of BLI signal from the different treatment groups week 3 (n= 6). Bemcentinib significantly reduced the BLI signal in comparison to the control group ($p<0.0004$) or to Ibrutinib single treatment ($p<0.0247$) (**D**) Kaplan-Meier

781 plots showing the effect of the different treatments on the survival of mice
782 xenografted with MCL cells (*n* = 8).