AKT signalling is required for ribosomal RNA synthesis and progression of Eμ-*Myc* B-cell lymphoma *in vivo*.

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Abbreviations: ribosomal DNA, rDNA; RNA polymerase I, Pol I; phosphate buffed saline, PBS; hematoxylin and eosin (H&E).

Keywords: AKT, MYC, lymphoma, oncogenesis, ribosome biogenesis

Abstract

The dysregulation of PI3K/AKT/mTORC1 signalling and/or hyperactivation of MYC are observed in a high proportion of human cancers and together they form a "super signalling" network mediating malignancy. A fundamental downstream action of this signalling network is the upregulation of ribosome biogenesis and subsequent alterations in the patterns of translation and increased protein synthesis, which are thought to be critical for AKT/MYC driven oncogenesis. We demonstrated that AKT and MYC can cooperate to drive ribosomal DNA (rDNA) transcription and ribosome biogenesis, with AKT being essential for rDNA transcription and *in vitro* survival of lymphoma cells isolated from a MYC driven model of B-cell lymphoma (Eμ-*Myc*). Here we show that the allosteric AKT inhibitor, MK-2206 rapidly and potently antagonises rDNA transcription in Eμ-*Myc* B-cell lymphomas *in vivo* and this is associated with rapid reduction in indicators of disease burden including spleen weight and the abundance of tumour cells both in the circulation and lymph nodes. Extended treatment of tumour-bearing mice with MK-2206 resulted in a significant delay in disease progression associated with increased B-cell lymphoma apoptosis. Our findings suggest that malignant diseases characterized by unrestrained ribosome biogenesis may be vulnerable to therapeutic strategies that target the PI3K/AKT/mTORC1/MYC growth control network.

Introduction

The PI3K/AKT/mTORC1 signalling hub plays an essential role in malignant transformation [1-5] and is dysregulated in many cancers with multiple components of this pathway being shown to act as oncogenes or tumour suppressors [3, 6-8]. Most studies examining the mechanism(s) by which this signalling hub contributes to malignancy have focused on the processes of pro-survival, cell cycle progression, angiogenesis and metabolic rate. However, it is becoming increasingly apparent that the downstream actions of this pathway to regulate ribosome biogenesis and translation are essential for its oncogenic effects [9-11]. The oncogene *MYC* is dysregulated in 15-20% of human malignancy and, like the PI3K/AKT/mTORC1 pathway, recent studies

have demonstrated that MYC plays a critical role in regulating rDNA transcription [12-15]. Indeed PI3K/AKT/mTORC1 and MYC signalling can cooperate, converging at a number of steps to establish master control of ribosome biogenesis, and thus protein synthesis [4, 5, 11]. The availability of sufficient functional ribosomes is a fundamental rate-limiting step for growth and proliferation in mammalian cells [16, 17] and diseases, such as cancer, that are associated with uncontrolled growth and proliferation are frequently characterized by increased ribosome synthesis [10]. Importantly for this study, increased ribosome synthesis and subsequent modulation of the efficiency of translation of key cell proliferation, growth, and survival proteins is an essential mechanism by which MYC promotes tumourigenesis [10, 18-22].

We recently demonstrated that RNA polymerase I (Pol I) transcription can be selectively targeted with the small molecule CX-5461 to treat *Myc*-driven B-cell lymphoma [22]. This model of spontaneous B-cell lymphoma is a prototypical example of tumour driven by uncontrolled cell growth as evidenced by the increased rates of rRNA and protein synthesis and larger size of B-cells from equivalent stages of development [22, 23]. Given the key role of AKT in MYC dependent rDNA transcription and ribosome biogenesis [11] we hypothesized that specific inhibition of AKT using the allosteric inhibitor MK-2206, currently in Phase I/II clinical trials [24], would antagonise MYC driven rRNA synthesis in the Eμ-MYC B-cell lymphoma model and delay lymphoma progression. MK-2206 (8-(4-(1-aminocyclobutyl)phenyl)-9-phenyl-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3(2H)-one) is a highly specific inhibitor of AKT, with low nanomolar IC_{50} values for all three isoforms of AKT (AKT1 – 8nM; AKT2 – 12nM; AKT3 – 65nM) and 100-fold selectivity for AKT over a large panel of other protein kinases [25]. By specifically targeting the pleckstrin homology domain of AKT isoforms, MK-2206 prevents recruitment of the kinase to the plasma membrane and subsequent activating phosphorylation events at threonine 308 and serine 473 residues [25]. MK-2206 at doses between 120mg/Kg and 480mg/Kg has demonstrated therapeutic properties in pre-clinical cancer models *in vivo* and robustly inhibited AKT activity and tumour cell growth in ovarian, lung, breast and

neuroblastoma cancer xenograft models [25-28]. In this study, MK-2206 rapidly and potently antagonised rDNA transcription, restored spleen weight to normal levels and reduced the number of tumour cells in the circulation and lymph nodes. Extended inhibition of AKT activity and rRNA synthesis with MK-2206 treatment of mice bearing Eμ-*Myc* B-cell lymphoma caused a significant delay in disease progression associated with increased tumor cell apoptosis.

Results

AKT signalling is required for rRNA gene transcription *in vivo.*

To establish the ability of MK-2206 to inhibit AKT activity and rDNA gene transcription *in vivo*, C57Bl/6 mice were transplanted intravenously with MSCV GFP Eμ-*Myc* B-cell lymphoma cells. Once disease was established (14 days post-injection) mice were administered with a single dose of vehicle (30% captisol: 0.1 mL/Kg) or 200mg/Kg MK-2206, which was established as the maximum tolerated dose of MK-2206 suitable for the extended treatment of these mice (Fig. S1**)**. Axillary lymph nodes were taken at 6, 24 and 48 hours post-treatment. Western analysis confirmed that a single dose of MK-2206 robustly inhibited AKT activity in the lymph nodes within 6 hours and inhibition was maintained at 24 hours, as measured by the abundance of phosphorylated AKT (p-AKT) at Serine 473 (Fig. 1A). In contrast, total AKT abundance was not altered (Fig. 1A). Furthermore, AKT inhibition was associated with suppression of 47S rDNA gene transcription, evident at 6 hours and statistically significant by 24 hours (45% decrease; Fig. 1B)

Acute administration of MK-2206 inhibits Eμ-*Myc* B-cell lymphoma progression.

Following a single dose of MK-2206 at 6, 24 and 48 hours the spleen, axillary lymph nodes and cardiac blood were examined for disease. An enlarged spleen is characteristic of Eμ-*Myc* B-cell lymphoma-bearing mice [29] and reflects disease burden. Spleen weight was significantly reduced 24 and 48 hours post-administration of MK-2206 (Fig. 2A). MK-2206 treatment also prevented the increase in GFP-expressing circulating lymphoma cells after 24 hours of treatment and this population was decreased at 48 hours compared

to time matched vehicle controls (Fig. 2B). MK-2206 treatment also prevented the typical expansion of the white blood cell population (Fig. 2C) and the increase in lymph node size observed in the control mice at 24 and 48 hours post- treatment (Fig. 2D).

MK-2206 treatment induces apoptosis of Eμ-*Myc* B-cell lymphoma cells *in vivo.*

We have previously demonstrated that inhibition of AKT activity leads to apoptosis of Eμ-*Myc* B-cell lymphoma cells *in vitro* [11]. To determine whether MK-2206-mediated delay of lymphoma progression *in vivo* also correlated with apoptosis, TUNEL staining was performed on fixed inguinal lymph nodes and spleens from mice treated with vehicle (control) or MK-2206 for 24 hours. MK-2206 robustly elevated the proportion of apoptotic (TUNEL positive) cells in the lymph nodes (Fig. 2E) and spleen (Fig. S2A), compared to the small proportion of apoptotic cells observed in the control mice which is consistent with the known pro-apoptotic effects of MYC over expression [30, 31] in the Eμ-*Myc* B-cell lymphoma model. This demonstrates that inhibition of AKT signaling promotes apoptosis of the lymphoma cells *in vivo,* which most likely accounts for the delay in lymphoma progression observed.

MK-2206 treatment prolongs survival and delays Eμ-*Myc* B-cell lymphoma progression *in vivo.*

To determine the efficacy of repeated dosing with MK-2206 on Eμ-*Myc* B-cell lymphoma progression, C57Bl/6 mice bearing GFP Eμ-*Myc* tumors were treated at day 8 post-tumor inoculation with either vehicle (30% captisol: 0.1mL/Kg) or MK-2206 (200mg/Kg) three times weekly over the course of the experiment. MK-2206 treatment significantly prolonged survival of mice relative to the control group (Fig. 3A) and, as was observed following single dose treatment (Fig. 2), this was associated with a significant reduction in both spleen weight (Fig. 3B) and the population of circulating tumour cells (Fig. 3C) compared to the control group. While MK-2206 treatment prolonged survival, the mice eventually succumbed to nodal and extra-nodal disease (Fig. 3D). The extended survival of these mice compared to the control group is consistent with our observation in the single dose experiment (Fig. 2) that

MK-2206 treatment can delay lymphoma progression. In addition, TUNEL staining showed robust increases in apoptosis in both the inguinal lymph nodes (Fig. 3E) and the spleens (Fig. S2B) of mice repeatedly dosed with MK-2206, compared to control mice. Furthermore, MK-2206 treatment also induced an increase in the apoptotic, sub G1 cell population in the axillary lymph nodes (Fig. S2C) as determined by FACS analysis. This data is consistent with the induction of apoptosis as the major therapeutic mechanism through which pharmacological AKT inhibition delays lymphoma progression and prolongs survival in this B-cell lymphoma model.

Discussion

Dysregulation of ribosome biogenesis is no longer a passive readout for malignant transformation but is now a realistic therapeutic target [22, 32, 33]. Our previous *in vitro* analyses characterised a critical role for AKT in the control of rDNA transcription at multiple levels including Pol I transcription elongation and/or processing of the rRNA, together with its control of mTORC1-dependent Pol I transcription initiation [11]. Consequently, inhibiting AKT activity reduces rRNA synthesis more rapidly and potently than the mTORC1 specific inhibitor rapamycin alone [11]. Importantly for the current study, this was most notable in MYC driven B-cell lymphoma cells *in vitro* where reduced AKT activity resulted in potent inhibition of rRNA synthesis and cell death, while mTORC1 inhibition had little effect on either [11]. Here we show that this reliance of rRNA synthesis and cell survival on AKT activity *in vitro* can be targeted in an *in vivo* model of MYC-driven B-cell lymphoma and is associated with induction of apoptosis of the lymphoma cells, delayed disease progression and thus prolonged survival. This therapeutic response is similar to the protection observed in neuroblastoma xenografts treated with MK-2206 [28]. When taken together with our previous study demonstrating selective apoptosis of Eμ-*Myc* B-cell lymphomas by inhibition of Pol I [22] these data raise the possibility that at least part of the mechanism by which AKT inhibition prolongs survival of these mice is through inhibition of Pol I transcription.

Inhibition of ribosome biogenesis may be an important determinant of the anti-cancer effects of PI3K/ AKT/mTORC1 pathway inhibitors, particularly in those driven by dysregulation of the critical cell growth regulatory pathways downstream of MYC, PI3K and RAS. For example, inhibition of mTORC1 with everolimus resulted in a similar robust improvement in overall survival in Eμ-*Myc* B-cell lymphoma (1.3 fold to 2-fold) [23] compared to MK-2206 (1.3-fold) however this was associated with elevated cellular senescence rather than apoptosis. Given the lack of effect of mTORC1 inhibition on rRNA synthesis in lymphoma cells [11], this difference in cellular response (senescence vs apoptosis) may be due to everolimus induced inhibition of the translation of specific mRNAs required to antagonize senescence [34].

Furthermore, it is possible that the ability of MK-2206 to target ribosome biogenesis and function at multiple steps, including long term inhibition of mTORC1 [11] may be an important element in its ability to drive cells down the apoptotic pathway. Consistent with this hypothesis, there is emerging evidence that targeting multiple members of signalling pathways or key cellular process may provide additive or even synergistic effects on cancer cell proliferation and/or survival. It is becoming clear that approaches which give a more robust inhibition of the pathway favor cytotoxic over cytostatic response [35]. For example, targeting multiple members of the PI3K pathway such as PI3K, mTOR and mTORC1 resulted in synergistic treatment of hepatocellular carcinoma [36]. Similarly, targeting AKT and mTORC1 in combination therapy of mice bearing neuroblastoma xenografts resulted in enhanced therapeutic efficacy [28] and combined inhibition of the critical growth control pathways PI3K/mTOR and RAS cooperate to inhibit ovarian cancer cell growth *in vitro* and *in vivo* [37] (Sheppard et al., unpublished).

While specific targeting of Pol I [22] and AKT signaling can prolong survival in the Eμ-*Myc* model of B-cell lymphoma, in both cases with time the mice succumb to resistant disease. This is a common outcome in response to the majority of cancer targeted therapies - despite profound improvements in survival by targeting oncogenes, resistance develops in many patients [38, 39]. Inhibition of AKT results in potent inhibition of rRNA synthesis by

targeting rDNA transcription initiation and elongation/processing [11]. It is thus possible that combinations of PI3K/AKT/mTOR and Pol I inhibitors may enhance tumour response by cooperative inhibition of ribosome biogenesis. In addition, it will be important to further define the mechanisms by which inhibition of PI3K/AKT antagonizes lymphoma cell survival as extra-ribosomal targeting of cell survival pathways may also lead to cooperative therapeutic effects. Indeed AKT plays a key role in promoting cell survival via a number of diverse mechanisms including the regulation of p53 by MDM2 as well as pro- and anti-apoptotic members of the Bcl-2 family of proteins such as the BH3-only protein BAD [40-43]. The disruption of these pathways may play a significant role in MK-2206 mediated apoptosis of MYC driven lymphoma cells and it will be important to test this hypothesis in future *in vivo* studies.

In conclusion, our findings raise the exciting possibility that malignant diseases driven by dysregulation of the key controllers of cell growth including the MYC, PIK3CA and RAS oncoproteins, may be vulnerable to therapeutic strategies that target AKT signalling.

Experimental Procedures

All animal experiments were performed according to protocols approved by the institutional animal experimentation ethics committee.

MK-2206 preparation

MK-2206 was a gift from MERCK. MK-2206 was dissolved using sonication (50 Hz) in 30% (w/v) captisol (CyDEX) prepared in sterile water.

Preparation and transplantation of Eμ-*Myc* B-cell lymphoma into C57Bl/6 recipient mice

Eμ-*Myc* B-cell lymphoma cells (clone 4242) derived from an Eμ-*Myc* lymphoma and transduced with a MSCV GFP construct were generated as described previously [22]. Cells were thawed, washed and resuspended in phosphate-buffed saline (PBS) prior to intravenous injection into recipient C57Bl/6 mice (~$2x10^5$ cells/mouse). Disease was monitored by determining white blood cell counts (WBCC) from peripheral whole blood collected into 10

mM EDTA and analysed by the Adiva120 Hematology System as described previously [22].

Acute dose and repeated-dosing therapy

Mice were randomized into two groups, vehicle control or MK-2206 treated. For the acute dose therapy 15 mice per group were treated with 30% captisol (0.1mL/Kg) or MK-2206 (200mg/Kg) by oral gavage and after 6, 24 or 48 hours the spleens, lymph nodes and circulating WBCC collected. For the repeated-dosing therapy mice were treated with 30% captisol (0.1mL/Kg) or MK-2206 (200mg/Kg) by oral gavage three times per week until an ethical end point was reached. At the end point the spleens, lymph nodes and circulating blood was collected.

Histology

Spleens and inguinal lymph nodes were fixed in 10% neutral buffered formalin, embedded in paraffin wax, serially sectioned and processed for Haematoxylin and Eosin (H&E) staining or TUNEL analysis (Millipore Apoptag Peroxidase In situ Apoptosis Kit S7100) as described previously [22]. Sections were analysed using the Olympic BX-51 at 40X magnification and quantitation of TUNEL staining performed with MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices).

Single cell suspension from axillary lymph nodes

Single cell suspensions were generated from the axillary lymph nodes by grinding the tissue, filtering the cells through a 0.7µm filter and washing with 2% fetal bovine serum in PBS. Cell number was determined using the Z2 Coulter Counter (Beckman Coulter) and a proportion of cells used for FACS analysis of GFP/B220 expression or propidium iodide (PI) staining, also for extraction of protein or RNA.

FACS analysis for GFP/B22O expression

White blood cells isolated using red blood cell lysis buffer (144 mM NH_4Cl, 17 mM Tris-HCl, pH7.65) and $1x10^6$ axillary lymph node cells were analysed for

GFP and B220+ (CD45R) surface marker expression using B220-APC (BD Pharmigen 553092) and FACS Canto II (BD Pharmigen). Results were analysed using FCSExpress software.

Western analysis

Protein was extracted from axillary lymph node cells using SDS-lysis buffer (0.5 mM EDTA, 20 mM HEPES, 2% (w/v) SDS, pH 7.9), boiling at 95°C, shearing with a 26G needle and then centrifuged at 13000rpm for 10min. Protein concentration was determined using the DC Protein Assay (BioRAD 500-0112) with bovine serum albumin (BSA) as a standard as per manufactures instructions. Equal concentrations of protein were separated by SDS-PAGE, transferred to PDVF membrane and immunoblotted with Phospho-AKT S473 (Cell Signalling (CS) #4058), AKT (CS#9272) or tubulin (Sigma Aldrich T9026) and the respective HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody (BioRAD). Protein abundance was visualized using Western Lighting Plus (Perkin Elmer) enhanced chemiluminescence kit and x-ray film (Kodak). Quantitation was performed using ImageQuant TL Software (GE Healthcare).

qRT-PCR analysis for rDNA transcription

RNA was extracted from axillary lymph node cells using the Bioline Isolate RNA Kit (BIO 520-44) with the addition of a ^{32}P labeled riboprobe to determine RNA recovery. cDNA was prepared from RNA equivalent to equal cell number using SuperScript III (Invitrogen) and random hexamer primers (Promega) as per manufacturer's instructions. The abundance of 47S rRNA 5' external transcribed spacer (5'ETS) was measured by quantitative real time polymerase chain reaction (qRT-PCR) using Applied Biosystems Fast SybrGreen master-mix and Applied Biosystems StepOne Plus qRTPCR machine as per manufactures instructions. The primer sequences used were – 5'ETS Forward: 5'CCAAGTGTTCATGCCACGTG3'; 5'ETS Reverse: 5'CGAGCGACTGCCACAAAAA3'.

Propidium Iodide Analysis of SubG1 Cell Population

Axillary lymph nodes (1x10^6 cells) from the repeated dosing experiment were fixed on ice with 95% ethanol, stained with 50µg/mL propidium iodide (PI) and

0.1mg/mL RNase A in PBS (MgCl2/CaCl2) supplemented with 5% (v/v) FBS. Samples were analysed on FACS Canto II (BD Pharmigen) and proportion of cells in the SubG1 region determined using FCSExpress software.

Statistical tests

The survival curve data was analysed using Mantel-Cox and Gehan-Breslow-Wilcoxin tests using GraphPad Prism Software. All the remaining data was assessed using a student t-test (two-tailed) using GraphPad Prism Software.

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Supporting information

Figure S1: Maximum tolerated dosage (MTD) of MK2206 is 200mg/Kg

Figure S2: MK-2206 induces apoptotic cell death in spleen and axillary lymph node.

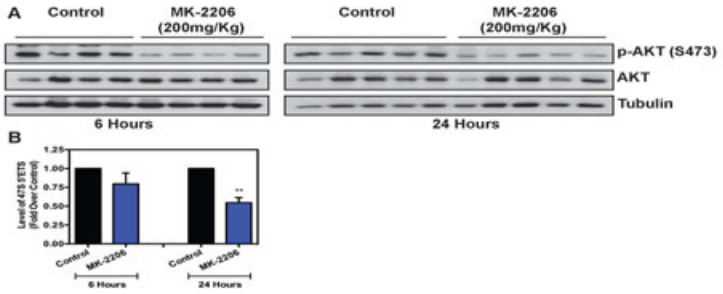

Figure 1: AKT signalling is required for rRNA gene transcription *in vivo*

Axillary lymph nodes were collected from recipient mice transplanted with Eμ-*Myc* B-lymphoma cells treated with vehicle (30% captisol) or MK-2206 (200mg/Kg) after 6 and 24 hours. A) Protein was extracted, separated by SDS-PAGE and subjected to Western analysis for phosphorylated AKT (p-AKT), total AKT and tubulin. Each sample is an individual mouse, n=4-5. Mean +/- SEM of p-AKT abundance normalized to tubulin for MK-2206; 0.341 +/- 0.046 (6 hours); 0.276 +/- 0.041 (24 hours) fold over control; both time-points ** $p<0.01$. B) RNA was extracted and rDNA gene transcription rates determined by qRT-PCR for 5'ETS. Graph represents mean +/- SEM, n=5, ** $p<0.01$.

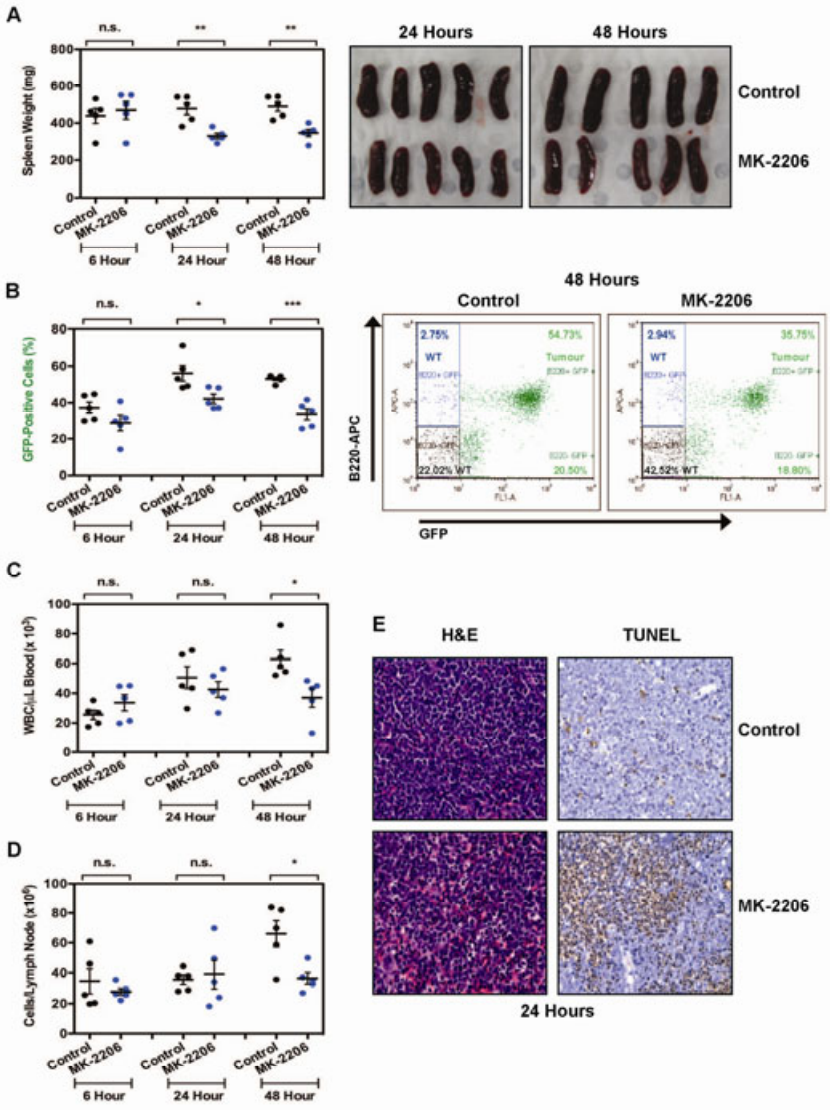

Figure 2: Acute administration of MK-2206 has therapeutic effect on Eμ-*Myc* lymphoma progression

Spleens, lymph nodes (axillary and inguinal) and cardiac blood were extracted from mice transplanted with Eμ-*Myc* B-lymphoma cells treated with vehicle (30% captisol) or MK-2206 (200mg/Kg) after 6, 24 and 48 hours. A) Spleen weight was determined and the results from n=5 graphed. B) White blood cells isolated from cardiac blood were analysed by FACS for GFP expression. Results were graphed and a representative FACS profile of a control and MK-2206 sample at 48 hours illustrated. C) White blood cell counts were determined using the Adiva120 Hematology System and results from n=5 graphed. D) Single cell suspensions were generated from axillary lymph nodes, cell number per lymph node was determined using the Z2 Coulter Counter and results from n=5 graphed. E) Inguinal lymph nodes (24 hour treatment) were fixed, embedded, serially sectioned and stained with haematoxylin plus eosin (H&E) or TUNEL. Representative sections shown of H&E (n=5) and TUNEL positive cells were quantified from 3 fields of view for n=1; Control 3.58% +/- SD 1.81 and MK-2206 22.94% +/- SD 10.65. All graphs represent mean +/- SEM, n=5, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

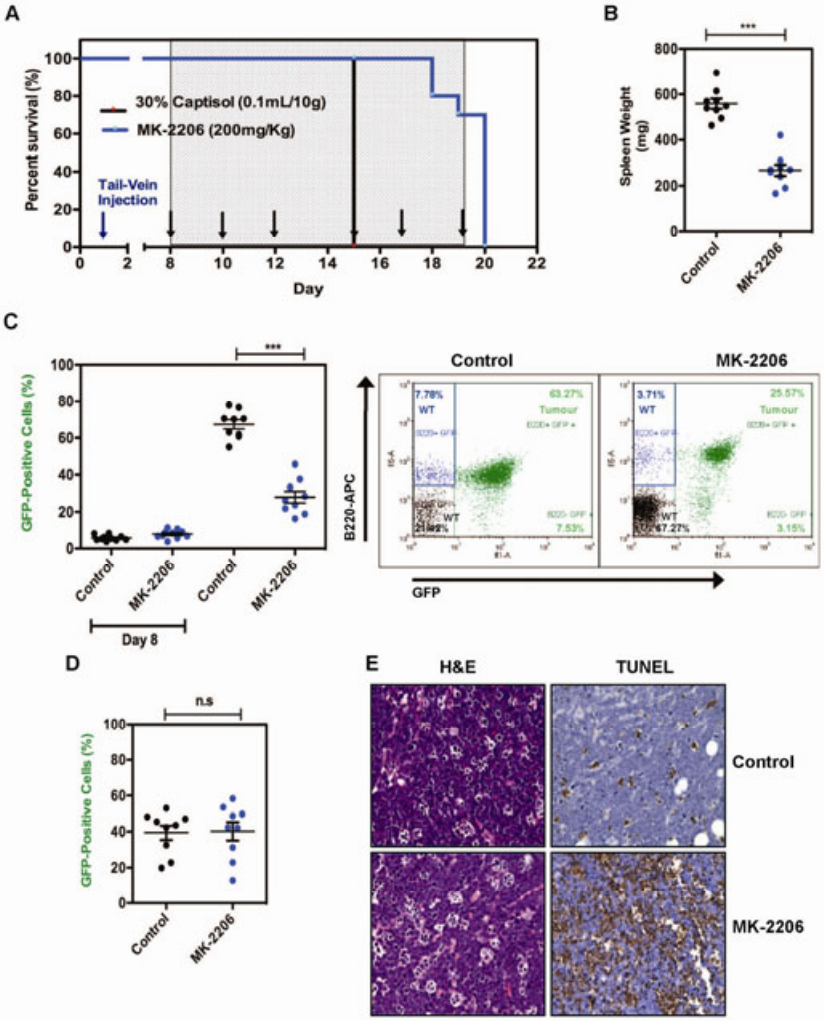

Figure 3: MK-2206 prolongs survival and delays Eμ-*Myc* lymphoma progression *in vivo*

A) Survival curve of mice transplanted with Eμ-*Myc* B-lymphoma cells dosed repeatedly with vehicle (30% captisol) or MK-2206 (200mg/Kg), n=10, $p<0.0001$. At an ethical end point spleen, lymph nodes (axillary and inguinal) and cardiac blood were collected. B) Spleen weight was determined and results of n=9 graphed. C) White blood cells were isolated from cardiac blood at day 8 (start of treatment) and at end-point and analysed by FACS for GFP expression. Results of n=9 were graphed and a representative FACS profile of a control and MK-2206 treated sample illustrated. D) Single cell suspensions were generated from axillary lymph nodes and analysed by FACS for GFP expression, the results from n=9 were graphed. E) Inguinal lymph nodes were fixed, embedded, serially sectioned and stained with haematoxylin plus eosin (H&E) or TUNEL. Representative sections shown of H&E (n=9) and TUNEL positive cells were quantified from n=4; Control 7.40% +/- SEM 3.02 and MK-2206 32.79% +/- SEM 2.42, *** $p<0.001$. All graphs represent mean +/- SEM, n=9, *** $p<0.001$, n.s. non-significant.

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