

## Original Article

# Monepantel antitumor activity is mediated through inhibition of major cell cycle and tumor growth signaling pathways

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Received March 1, 2021; Accepted April 24, 2021; Epub June 15, 2021; Published June 30, 2021

**Abstract:** In women, epithelial ovarian cancer is the leading cause of gynaecological malignancy-related deaths. Development of resistance to standard platinum and taxane based chemotherapy and recurrence of the disease necessitate development of novel drugs to halt disease progression. An established concept is to target molecular and signaling pathways that substantially contribute to development of drug resistance and disease progression. We have previously shown that, monepantel (MPL) a novel small molecule acetonitrile derivative is highly effective in suppressing growth, proliferation and colony formation of ovarian cancer cells. These effects are achieved through inhibition of the mTOR/p70S6K pathway in cancer cells. The present study was conducted to find in vivo corroboration and explore the effect of MPL on other growth stimulating putative signaling pathways. Here, female nude mice with subcutaneous OVCAR-3 xenografts were treated with 25 and 50 mg/kg doses of MPL administered (IP) three times weekly for 2 weeks. At the doses employed, MPL was modestly effective at suppressing tumor growth, but highly effective in inhibiting, mTOR, P70S6K and 4EBP1. There were also modest reductions in tumor cyclin D1 and retinoblastoma protein expression. Furthermore, it was found that MPL treatment causes down-regulation of IGF-1R, and c-MYC thus unveiling new dimensions to the growing antitumor actions of this potential anticancer drug. MPL treatment led to reduced tumor volume and weights without causing any detectable side effects. Coupled with the recent human safety data published on this molecule, expanded future trials are highly anticipated.

**Keywords:** Ovarian cancer, monepantel, tumor signaling, mTOR, IGF-1R, c-MYC

## Introduction

Despite significant advancement in the treatment of epithelial ovarian cancer (EOC), the disease still remains the leading cause of death due to gynaecologic malignancy [1]. The American Cancer Society estimates 21,750 new ovarian cancer diagnosis and 13,940 in the United States in 2020 [2]. Additionally, the causes of the disease still remain incompletely understood [3]. Together with p53, the PI3K/mTOR/p70S6K signaling are the most frequently altered pathways in a variety of cancers including ovarian cancer [4, 5]. Molecular aberrations in the phosphoinositide-3-kinase system including, PI3K, AKT and mTOR signaling pathway is seen in a high proportion of ovarian cancers. PI3K and AKT both diverge at mTOR

[6], the system which activates p70S6K, ribosomal protein S6 and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1). A component of the 40S ribosomal subunit, the S6 is required for the synthesis of polypeptides at the ribosome whereas, 4EBP1 regulates the translation of growth-promoting genes. On the basis both S6 and 4EBP1 are essential for cellular protein synthesis [7, 8]. These molecules are essential for the critical role played by mTOR in cellular growth, differentiation, apoptosis/autophagy, migration, invasion and the angiogenic properties of the tumor cells. In fact, the PI3K/Akt/mTOR signaling pathway may even be considered as one of the master regulators of cancer [9, 10] contributing to tumor growth, chemoresistance and metastasis.

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Advanced stage at presentation coupled with the high risk of relapse contribute overtly to the overall lethality of ovarian cancer [11]. Additionally the disease is highly heterogeneous in nature well characterized by the multiple histological subtypes [12]. Therefore, despite high initial response rates in many patients receiving chemotherapy post diagnosis, most patients with advanced ovarian cancer ultimately develop resistance to the first-line chemo drugs and end up with recurrent disease. To continue disease management, development of new therapeutics with novel mechanism of actions are therefore an absolute clinical necessity.

Monepantel is an anthelmintic drug that has been approved for the treatment of nematode infections in various farm animals [13, 14]. Efficacy in several drug resistant species, novel mode of action together with a unique safety profile are some of its outstanding properties. In nematodes, MPL interacts with ligand-gated ion channels present at the neuromuscular synapse thus interfering with signal transduction. The interaction leads to deregulation in nematode muscle contraction and eventual paralysis [13, 15]. The vast and highly detailed toxicology studies conducted as part of the drug approval procedure have all shown the safety of the drug in various rodents, and is extremely very well tolerated in farm animals including sheep, cattle, lambs and ewes [13, 16, 17]. Additionally, a recent chronic study in mice, rats, rabbits and dogs reported negative results in all genotoxicity and carcinogenicity studies carried out [18]. Furthermore, pharmacokinetic studies conducted in various mammals have revealed that MPL has a well-accepted pharmacokinetic profile enabling it to be used as an oral medication [19].

We have previously shown that, under cell culture conditions, MPL is highly effective at suppressing proliferation of a range of molecularly diverse ovarian cancer cells [20]. In vitro treatment of human ovarian cancer cells with MPL led to concentration and time-dependent inhibition of growth, proliferation and colony formation [20]. Further studies revealed that, by reducing the expression of cyclin dependent kinases (CDKs), MPL arrests cells in the G1 phase of the cell cycle thus disrupting progression. For the cell cycle to proceed through the G1/S transition, activation of cyclin/CDK would

be essential as expression of cyclin D and CDK4 in the G1 phase of the cell cycle acts as the primary sensor of positive and negative environmental signals responsible for triggering G1 cell cycle progression [21-23]. Intrigued by our microscopic observations on the structural changes and the potent concentration-dependant inhibition of cellular thymidine incorporation by MPL in various ovarian cancer cells, we thus interrogated the phenomenon further. Through molecular experiments it was found that, MPL suppresses cell growth and eventually causes cell death by activating the cellular autophagic pathway a process mediated through the inhibition of the mTOR/p70S6K signaling pathway [24]. We next looked for changes in the gross appearance of cancer cells during MPL treatment. Here, extensive intracellular vacuole formation highly suggestive of autophagy were observed. The in vitro information on MPL anticancer activity collectively suggest that the drug may have the potential to suppress vital signaling pathways within the tumor. Consequently, the current study was designed with the aim to investigate the effects of MPL on the tumor growth mechanisms and signaling pathways. To do this, the well-established ovarian OVCAR-3 xenograft model in female nude mice was employed [25]. Already knowing that the drug is quite safe in rodents and mammals [19, 26], our primary goal therefore was to evaluate the efficacy of MPL in an experimental model of ovarian cancer. Additionally, we wanted to gain insight into the molecular effects of MPL on tumor signaling pathways. Here we report novel evidence on the activity of MPL in suppressing the growth of OVCAR-3 xenografts in nude mice through disruption of major signaling pathways within the tumor. The data presented in the present study coupled with the very recent safety reports indicate that clinical trials on MPL would be highly credible and potentially forthcoming.

### Material and methods

#### *Ethics statement*

All animal procedures conducted meet the standards outlined by the University of New South Wales Animal Care and Ethics Committee.

#### *Chemicals and antibodies*

Monepantel was purchased from a contract manufacturer. All other drugs and chemicals

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were acquired from Sigma-Aldrich (Australia). All primary and secondary antibodies used were purchased from either Cell Signaling Technology or Santa Cruz Biotechnology, Sydney, Australia. Mouse monoclonal anti-GAPDH was purchased from Sigma-Aldrich, Australia.

### *Preparation of drug*

In the suspension formulation, MPL was dispersed in sterile PBS containing 0.5% (w/v) hydroxypropyl methylcellulose (HPMC) and tween 80 to the required final concentrations of 25 and 50 mg/ml. The suspension was then stirred for 24 hours at room temperature followed by 20 min sonication for 5 times. The MPL suspension was vigorously shaken before administration to the mice. MPL suspension was freshly prepared every time before administration.

### *Mice*

Ten-week-old female BALB/c Nu/Nu nude mice were purchased from the Biological Resources and Imaging Laboratory (University of New South Wales, Sydney, Australia). All animals were maintained under standard conditions at the purpose-built university facilities. All experiments were closely supervised in accordance with the approved current protocols of the Ethics Committee.

### *Cell line*

OVCAR-3 cell line was originally acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI 1640 according to standard procedures. Supplemented with 10% fetal bovine serum, cells were incubated in an incubator with 5% CO<sub>2</sub> at 37°C with saturated humidity.

### *Sub-cutaneous tumour induction and drug treatment*

Mice were initially left under standard conditions to acclimatize for a week. This was followed by, injection of two million OVCAR-3 cells suspended in 0.1 ml of matrigel subcutaneously (SC) into the left flank of the animals [27]. Tumor growth was monitored 3 times per week. Tumors were measured externally with Vernier callipers. When the tumors reached 100 mm<sup>3</sup>, animals were randomly assigned into 3 treatment groups of 5 mice each and treated intra-

peritoneally for 21 days. Animals in the treatment arms received MPL suspended in HPMC (1 ml/20 g body weight), whereas those in the control group received equivalent volumes of the vehicle (HPMC/PBS).

### *Evaluation of MPL effect on tumour growth*

During the 3 weeks of treatment, body weight and tumour indices were measured every three days and tumour volumes were calculated using the following formula:

$$\text{Tumour volume} = (\text{shortest diameter})^2 \times \text{longest diameter} \times 0.5$$

Animals were euthanized if the tumour size reached 1000 mm<sup>3</sup> any time during the investigation.

### *Sample collection and analysis*

At the end of treatment period, animals were sacrificed and their tumors were excised, weighed and snap frozen in liquid nitrogen or paraffin embedded for subsequent immunohistochemistry and molecular analysis [27].

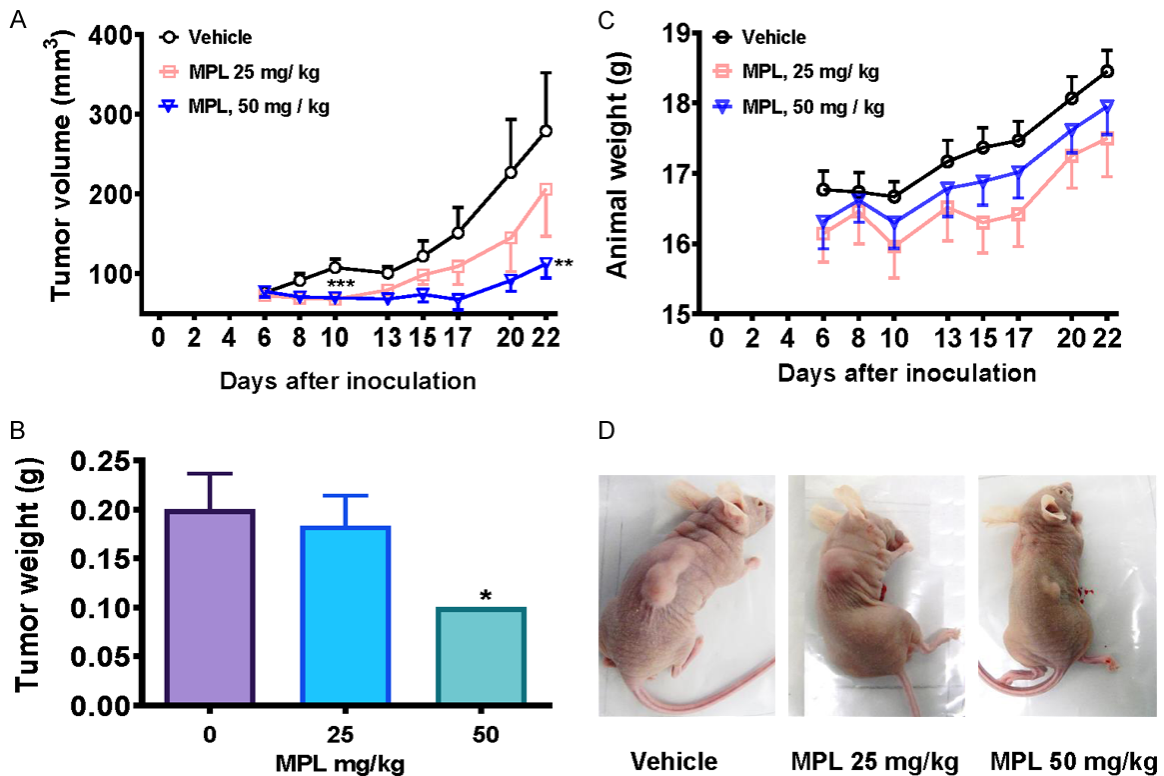
### *Immunohistochemistry*

Tumors were fixed in neutral buffered 10% formalin (24 h) for hematoxylin and eosin (H&E) staining. Antigen retrieval was performed by heating in citrate buffer (pH 6) and sections were then washed in TBS (50 mM Tris, 150 mM NaCl pH 7.5), incubated with biotinylated anti-mouse immunoglobulins for 30 min followed by treatment with streptavidin peroxidase (Dako Corporation) and counter-stained with haematoxylin.

### *Western blot analysis*

To determine the in vivo effect of MPL on various tumor proteins involved in growth and proliferation, western blot analysis was performed using standard methods previously described [28]. Tumor tissues extracted from the various animal drug or vehicle treated groups were washed for 30 minutes using ice-cold PBS. Samples were then extracted with a RIPA buffer cocktail containing 10% phosphatase inhibitor and 10% protease inhibitor (Sigma, St. Louis, MO). Following centrifugation (13,000 g for 30 minutes) and protein concentration determination equivalent amounts of the extracts were resolved by SDS-polyacrylamide gel electro-

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**Figure 1.** Monepantel suppresses growth of OVCAR-3 xenografts in nude mice. Antitumor effects of MPL was tested in nude mice bearing rapidly growing subcutaneous OVCAR-3 xenografts. Mice were treated (IP injections; 0.1 mL) for 2 weeks with the vehicle (control group) or MPL (25 or 50 mg/kg). Vehicle or drug administration took place 3 times weekly starting on day 7 post cell inoculation and termination on day 20 of the experiment. Sterile 0.5% HPMC was used as the vehicle for MPL and also as the treatment for the control group. Calliper measurement of tumour volumes were recorded 3 times weekly. Values are mean  $\pm$  SD from the measurements over the course of the experiment. (A) Tumor volumes during the 22 day course of MPL or vehicle administration. (B) MPL treatment did not affect animal weights. (C) Tumor weights in nude mice weight in nude mice at the time of sacrifice (day 22 of study) and (D) representative photos of mice bearing OVCAR-3 tumors and being treated with either the vehicle or monepantel (25 or 50 mg/kg).

phoresis and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, MA, USA). Membranes washed and incubated with horseradish peroxidase-conjugated secondary antibodies were then probed. To detect immune-complexes formed, we used horseradish peroxidase conjugated with either anti-mouse or anti-rabbit antibody and blot detection was performed using chemiluminescence detector (Perkin Elmer Cetus, Foster City, CA, USA). In order to ascertain equal protein loading, all blots were stripped and re-probed with a specific antibody recognizing GAPDH.

### Statistical analysis

Statistical analysis on the data obtained was conducted using GraphPad Prism Software version 6.0. Values presented are mean  $\pm$  SD. To

compare 2 independent group means, student t test was used. To determine statistical difference between various groups, one-way ANOVA followed by Bonferroni's post-test was employed. Statistical significance was established at the  $P < 0.05$  level.

### Results

#### MPL treatment suppresses tumor growth

Here we sought molecular evidence for MPL in vivo efficacy in OVCAR-3 xenografts grown subcutaneously in female nude mice. For this, mice were treated with the vehicle or MPL at 25 or 50 mg/kg (IP) administered thrice weekly for 2 weeks (days 7 to 20). Final tumor volumes were measured 2 days after the last drug injection. Presented in **Figure 1A** are the tumor volumes

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over the course of the experiment, demonstrating dose-dependent suppression of tumor growth in these animals. Even at the relatively low doses employed, MPL treatment led to substantial inhibition of tumor growth. The average tumour volumes were  $206.28 \pm 59.26 \text{ mm}^3$  and  $112 \pm 18.14 \text{ mm}^3$  in MPL 25 and 50 mg/kg treated cohorts compared to  $279.5 \pm 73.16 \text{ mm}^3$  in vehicle treated controls. Consistent with this, were the tumor weights excised from mice. These were in the range  $0.183 \pm 0.037$  and  $0.100 \pm 0.02 \text{ g}$  in MPL 25 and 50 mg/kg-treated mice respectively (**Figure 1B**) compared to  $0.200 \pm 0.037$  in vehicle treated control group. Animals appeared healthy throughout the study and did not lose weight either (**Figure 1C**). These data indicate that MPL does significantly reduce growth of OVCAR-3 xenograft in nude mice without causing any observable side effects (**Figure 1D**).

### *MPL inhibits mTOR/p70S6K signaling pathway*

The ability of MPL to inhibit mTOR and its downstream mediators (p70S6K and 4EBP1) in ovarian cancer cells under in vitro cell culture conditions was highly suggestive that, at the right doses the drug may potentially be highly effective in suppressing the mTOR pathway in vivo. So, the phenomenon was interrogated further resulting in the observation that, MPL does indeed suppress tumor growth under the stated conditions. To further characterize this observation, we next looked at the MPL induced signaling effects within the tumor. Starting with mTOR signaling pathway and its downstream proteins p70S6K and 4EBP1 (**Figure 2**). Western blot analysis of tumor tissue demonstrated substantially reduced expression of tumoral phosphorylated (Ser2448) mTOR. The percentage of inhibition relative to vehicle treated tumors were  $75 \pm 12.09$  and  $18.84 \pm 3.7$  in mice treated with 25 and 50 mg/kg MPL, respectively ( $P = 0.0036$  for MPL 50 mg/kg). Subsequently, MPL highly suppressed phosphorylation of both p70S6K (at Thr389) and 4EBP1 (Thr37/46). Animals treated with 25 mg/kg MPL showed about 2.5-fold decrease in p-p70S6K expression while treatment with the 50 mg/kg suppressed the molecular active phosphorylated form by 7-fold. Similarly, MPL induced inhibition of 4EBP1 compared to vehicle treated-group was  $44.83 \pm 6.85$  ( $P = 0.0015$ ) and  $48 \pm 5.5$  ( $P = 0.0009$ ) in MPL 25

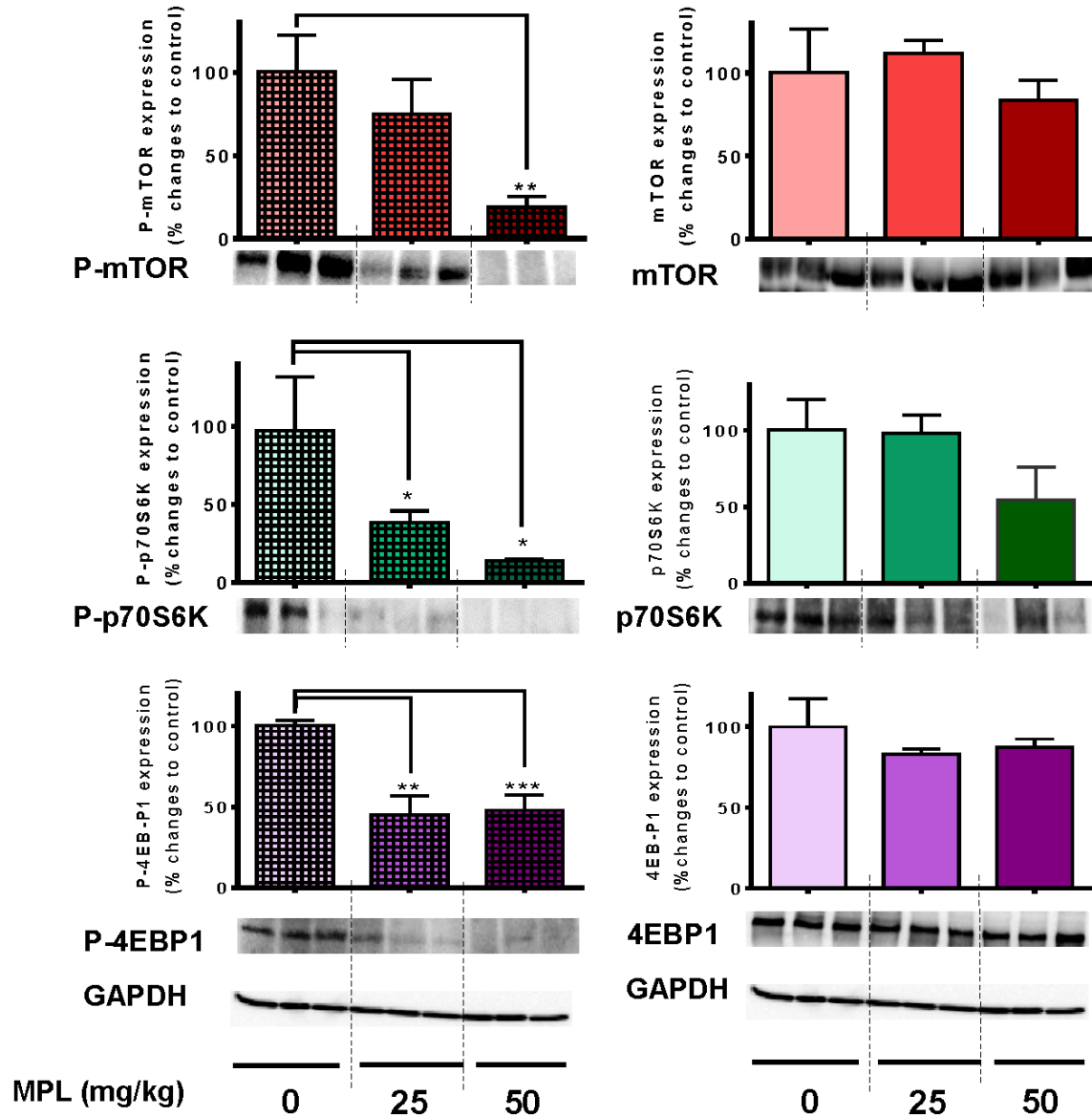
and 50 mg/kg groups, respectively. These results are firmly indicative of the potent suppressive activity of MPL on mTOR and its downstream signaling pathway under in vivo conditions.

### *MPL affects cell cycle regulators*

Progression and transcriptional processes within the cell cycle are highly regulated by cyclin-dependent kinases (CDKs) thus rendering them as potential therapeutic targets for anticancer drug development. The balance between cyclins, CDKs and CDK inhibitors are frequently de-regulated in cancer cells [29]. In our in vitro investigations we found that, MPL profoundly affects the expression of not only 4EBP1 and p70S6K but also those of cyclin D1, cyclin E2 and CDK2. Another protein tightly controlling the interaction amongst the flow of messages and the interaction between these cell cycle regulatory proteins is the retinoblastoma protein (Rb). Often regarded as the cell cycle clock, it has been proposed that Rb controls the process through critical regulatory check points in the G1 phase by its sequential phosphorylation [30]. We therefore examined the protein levels of these crucial cell cycle mediators within the tumor specimens after MPL treatment.

Results from tumor western blot analysis are depicted in **Figure 3**. MPL induced suppression of cyclin D1, E2 and CDK2. It was found that treatment with MPL leads to reduced levels of cyclin D1 protein to  $75.46 \pm 13.68$  and  $65.44 \pm 3.57$  (relative to control) in the 25 and 50 mg/kg MPL treated groups respectively. Similarly, the protein levels of cyclin E2, one of the main transcriptional targets of 4EBP1 and its related cyclin dependent kinase, CDK2 were also decreased by 30%-40%. These results confirm the induction of G1 cell cycle arrest resulting from the inhibitory effect of MPL on mTOR/p70S6K signaling. Additional novel information resulting from tumor analysis are the elicited in the increased levels of phosphorylated retinoblastoma protein. A tumor suppressor protein, which operates in the midst of the cell cycle and plays a pivotal role in the negative control of the cell cycle and tumor progression. It has been well established that, by blocking S-phase entry and cell growth, active Rb protein is regarded as a major G1 checkpoint [31]. Considering the time lapse (48 h) between the

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**Figure 2.** MPL inhibits mTOR/p70S6K signaling pathway. Immunoblot analysis of ovcAR-3 tumor lysates to determine levels of mTOR, P70S6K and 4EBP1 together with their related phosphorylated proteins in tumor lysates. Blots were cropped to ensure clearer presentation of the data. Densitometry was performed to quantify changes arising from MPL treatment (25, 50 mg/kg three times weekly for two weeks). Vehicle treated values (control) are expressed as 100% and the drug treated values are presented relative to control. GAPDH was used as the housekeeping gene confirm similar protein loading and blot transfer. \* < 0.05 compared to control (vehicle treated).

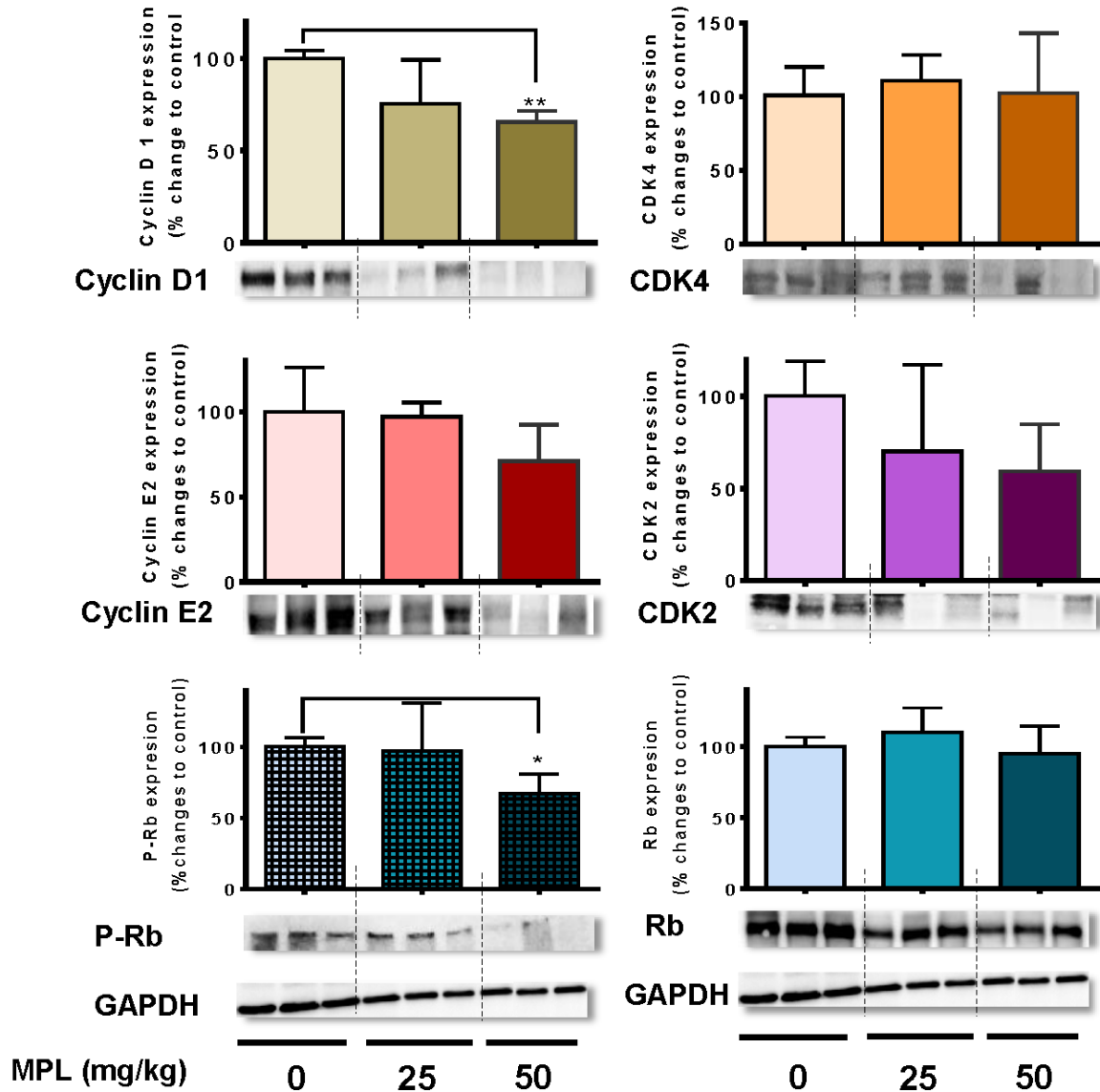
last MPL administered dose and the excision of tumors, this observation seems quite rational, revealing that the drug effect has almost completely weaned off. We therefore investigated the implications of such an effect on IGF-1R receptor, TGF- $\beta$  and c-MYC expressions at later stages of tumor lysate analysis. Another aspect that was considered and investigated was

the structural changes within the tumors extracted.

### *MPL treatment induces necrosis in OVCAR-3 subcutaneous tumors*

To characterize the mechanism behind MPL-induced suppression of tumour growth, immu-

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**Figure 3.** MPL affects cell cycle regulators. Treatment of mice bearing OVCAR-3 tumors with monepantel (25 or 50 mg/kg × 3 weekly) for two weeks led to down-regulation of cell cycle regulatory proteins. Western blot analysis of lysates prepared from the tumours demonstrate down-regulation of cyclins D1 and E2, CDK2 and the phosphorylated form of the retinoblastoma protein. This aligns well with the cell cycle data showing substantial arrest of the cells at the G1 phase of the cell cycle. The cell cycle is tightly controlled at specific points by CDKs, and in particular CDK2 and CDK4 which seem essential for entry in G1 and G1-S transition.

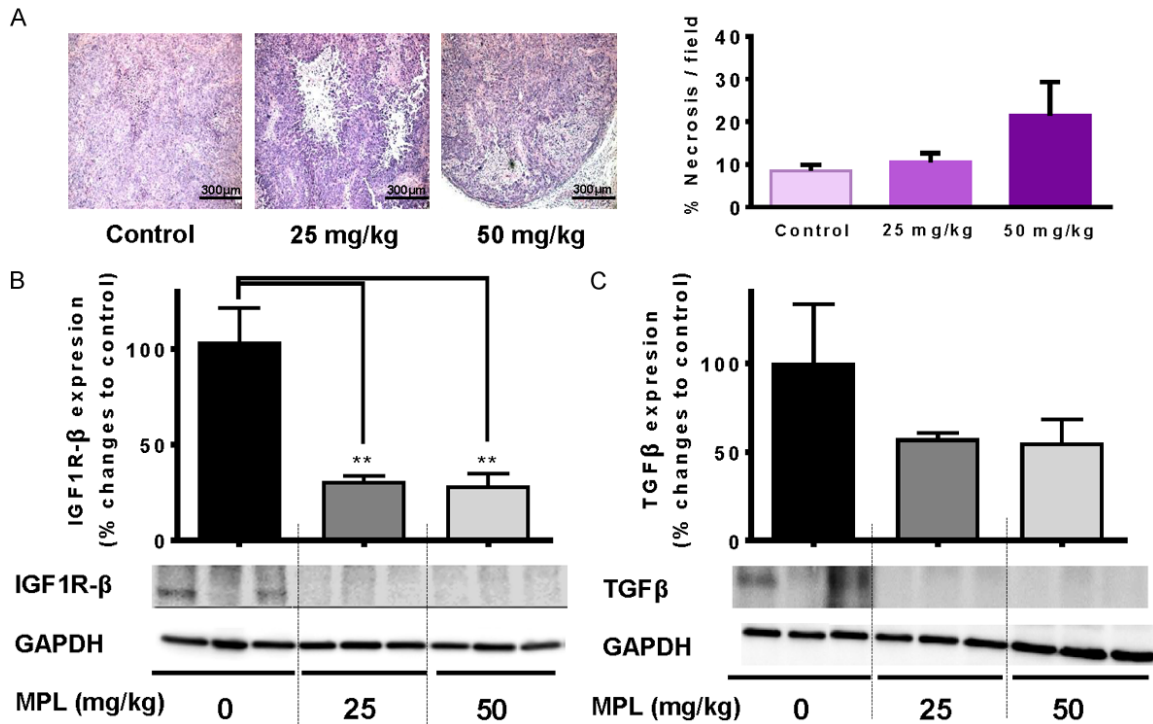
nohistochemical analyses was performed on tumour tissues through Hematoxylin and eosin (H&E) staining. Results are presented in **Figure 4A**, with the blue and purple portions representing the cell nucleus and cytoplasm, respectively. Histological images of tumors indicate profound drug-induced necrosis, where a significant change can only be seen at the 50 mg/kg dose level. The cellular nuclei shrank in the tumor cells of the MPL-treated mice (percentage of shrinkage were from  $8.5 \pm 1.3$  in

control animals to  $10.5 \pm 2.08$  and  $21.5 \pm 7.75$  in 25 and 50 mg/kg MPL-treated mice, respectively).

### *MPL down-regulates IGF1R-β and TGF-β signaling*

It has been suggested that, association, interplay and the frequent mutation of mTOR and IGF-1 signaling pathways in tumors play a critical role in tumorigenesis [32].

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**Figure 4.** MPL induces necrosis and down-regulates IGF1R and TGF- $\beta$ . (A) Histological images of H&E stained tumors shown on top, indicating profound drug-induced necrosis (black arrow; magnification, 10  $\times$ ). Analysis of necrosis was performed based on number of the field/section, section = 5, % of necrosis = necrosis area/Total area  $\times$  100. Western blot analysis of monepantel treated tumors show down-regulation of IGF1R- $\beta$  (B) and TGF $\beta$  (C) proteins. Blots were cropped to ensure clearer presentation of the data.

Furthermore, to ensure, the balance of both a reasonable level of nutrients and a positive signal for cell growth and division from mitogen stimulation where disruption in the later leads to non-functionality of the mTOR. Consistent with these effects is that, the activation of the of IGF-1R by its tyrosine kinase ligand (IGF-1) results in the activation of mTOR through the IGF-1/AKT pathway [33]. Similarly, acting through Akt, TGF- $\beta$  as one of the most important regulators of PI3K/mTOR pathway leads to the activation of the mTOR signaling thus culminating in cell survival, growth and proliferation.

We therefore investigated the effects of MPL treatment in the context of IGF-1 receptor (IGF1R- $\beta$ ) and TGF- $\beta$  protein expression. Results obtained are presented in **Figure 4B** and **4C** respectively. Western blot analysis IGF1R- $\beta$  demonstrate profound down-regulation of this protein at both 25 and 50 mg/kg dose levels. Quantitatively, 25 and 50 mg/kg MPL reduced IGF1R- $\beta$  expression to  $30.84 \pm 2.1$  and  $28.53 \pm 1.45$  of control values (both values significant

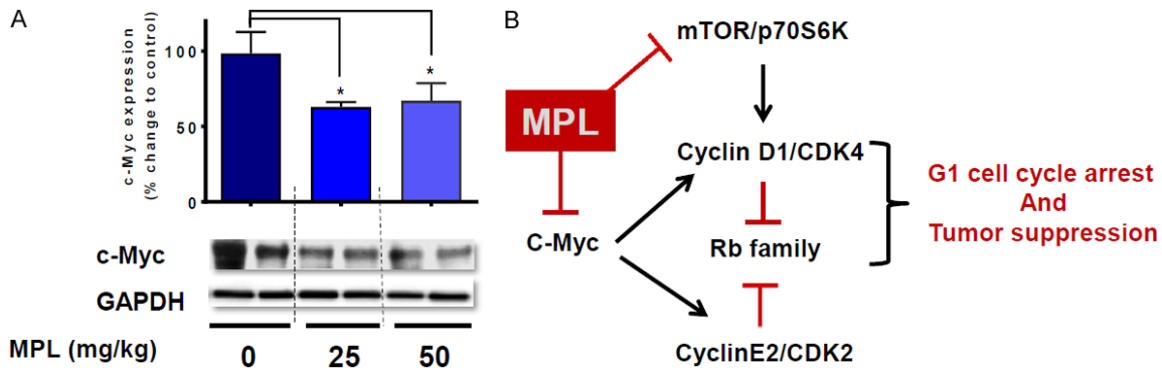
at  $P < 0.01$ ). The effect of MPL treatment on TGF- $\beta$  expression, while values were reduced to  $57.51 \pm 1.81$  and  $55.20 \pm 7.51$  of the control values, but the changes were not statistically significant. These novel findings indicate that MPL may also be interfering with expression of c-MYC, the proto-oncogene cap-dependent translation initiation machinery [34].

### MPL suppresses c-MYC levels in tumors

A protein involved in the activation of cell cycle protein biosynthesis, c-MYC triggers the entrance of quiescent cells into the cell cycle. Consequently, its blockade or under expression affects cell cycle dynamics and ultimately leads to the arrest of the cell cycle [35, 36]. In order to examine the effects of MPL on this pivotal cell cycle molecule, here we looked at the levels of the c-MYC expression in tumors (**Figure 5**). Western blot analysis of tumor tissue revealed that MPL modestly represses expression of c-MYC levels within the tumors. The values for MPL 25 and 50 mg/kg treated tumors were



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**Figure 5.** MPL suppresses c-MYC. Overexpression of the MYC oncogene is a key feature of many human malignancies. Treatment of mice ( $n = 5$  per treatment group) bearing SC OVCAR-3 tumours with monepantel for a period of two weeks led to significant ( $P < 0.05$ ) inhibition of c-MYC. Monepantel was administered 3 times weekly as a bolus IP injection at the doses of 25 or 50 mg/kg. Dosing was initiated on day 7 post cell inoculation. The control group received 0.5% HPMC as the vehicle. Western blot analysis for detection of c-MYC protein was performed on lysates prepared from tumors excised from mice treated with the vehicle or the drug. Blots were cropped to ensure clearer presentation of data. Based on the results obtained in this investigation, a tentative mechanism has been proposed to describe the antitumor effects of monepantel in this experimental model of ovarian cancer.

decreased by  $35.96 \pm 8.39$  ( $P < 0.05$ ) and  $31.40 \pm 10.6$  ( $P < 0.05$ ) respectively. No significant difference was observed between the two drug doses used.

### Discussion

We have previously reported that the novel amino-acetonitrile derivative MPL exerts various important concentration - dependant anticancer properties in a number of ovarian cancer cells under in vitro conditions. In particular, it was found that, MPL inhibits growth, proliferation and colony formation of ovarian cancer cells and more specifically, interfered with the cyclin kinases leading to G1 phase cell cycle arrest. Inhibition of thymidine incorporation coupled with the increased PARP-1 activity collectively imply potent and possibly multifaceted anticancer activity of this novel small molecule. Furthermore, it was discovered that the cytotoxic effects of MPL were not mediated through the apoptotic process but were a consequence of induction of autophagy. It was found that in cancer cells, MPL induces persistent autophagy through the inhibition of the mTOR signaling pathway [20, 24]. Furthermore, we observed significant enhancement of doxorubicin and gemcitabine antitumor activity when MPL was added to these agents [37]. To follow these developments, we sought further insight and evidence on the in vivo antitumor activity of MPL. Here we report preclinical data demonstrating that MPL exerts antitumor effects th-

rough suppressing the expression of several important mediators of tumor growth.

In the current study, we essentially found that MPL at 25 and 50 mg/kg doses retards tumor growth in these mice. More importantly, results obtained through analysis of excised tumors not only confirmed our in vitro observations but also revealed new aspects on the molecular effects of MPL on signaling pathways in rapidly growing tumors. Initially it was found that, in MPL treated tumors, the mTOR/p70S6K/4EBP1 pathway is highly suppressed. It is well established that, mTOR a highly conserved 289-kDa serine/threonine protein kinase integrates both intracellular and extracellular signals to serve as a central regulator of cellular growth. Modulation of cell metabolism leading to anabolic effects, growth and proliferation are initiated by mTOR activation [38]. Essentially, mTORC1 phosphorylates substrates that increase the production of proteins and lipids and ensures the supply of energy required for cell growth and proliferation. Activated mTOR phosphorylates key regulators of protein translation namely 4EBP1 and p70S6K. 4EBP1 enhances the translation of several growth factors including cyclin D1, c-MYC and VEGF whereas phosphorylated p70S6K leads to the translation of pyrimidine-enriched mRNA translation, with a net result of protein synthesis [39]. This is consistent with the literature showing the antitumor activity of other mTOR inhibitors.

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To identify the potential effects of MPL on other tumor signaling pathways, further tests were conducted. It was thought conceivable that MPL may exert other effects on tumors which may or may not be directly connected to mTOR inactivation. The presence of multiple growth signaling pathways in tumors generally accounts for the development resistance. Here we found that, levels of active Rb (phosphorylated form) were significantly ( $P < 0.05$ ) reduced in the 50 mg/kg treated mice. Retinoblastoma is a signal transducer which connects the cell cycle clock to the transcriptional machinery within the cell thus controlling the expression of genes that mediate progress of the cell cycle through a critical phase essential for growth [40]. A highly complex and regulated process of cyclin D, cyclin E, their kinases and Rb protein interact to control cell cycle regulation and regulate the appropriate timing of G1 length [41, 42]. We also found that in MPL treated animals the tumor levels of TGF- $\beta$  are relatively depressed (no statistical significance).

Down-regulation of tumor IGF-1R protein by MPL was another novel and highly significant observation of this study. A downstream target of p70S6K and 4EB-P1, IGF1R regulates serine threonine kinase receptors and is highly susceptible to mTOR activation status. Perhaps this explains the magnitude of inhibition seen as it is in line with the P70S6K and 4EBP1 results. This observation corroborates well with the MPL effect in causing tumor cell cycle arrest in the G1 phase. IGF-1 activation of IGF-1R leads to enhanced cyclin D1 expression and thus cell cycle advancement from G0/G1 to the S phase. Up-regulation of cyclin D1 that is triggered by activation of the IGF-1 receptor which in turn propels signaling cascades leading to increased proliferation. The event is thought to take place through its binding partner CDK4 [43, 44]. The mTOR/p70S6K pathway plays a critical role as one of the regulators of downstream IGF-1R and TGF- $\beta$  in regulating protein synthesis in response to signals from mitogenic factors, nutrients, cellular energy levels, and stress. Furthermore, activation of both IGF-1R and the PI3K/Akt/mTOR pathways is thought to be indicative of rapid tumor progression resulting in poor prognosis [45, 46]. To determine whether other prominent tumor modulating factors are also affected by MPL, we next at the expression levels of c-MYC. Another mTOR con-

nected molecule recognized as a mediator of cell cycle progression, oncogenesis and tumor growth is c-MYC. Indeed, a potent proto-oncogene whose deregulation is one of the most frequently encountered events present in epithelial ovarian carcinoma and believed to be a major contributing factor in sustained tumour growth [47]. Promotion of cell proliferation and hindering of cell differentiation are thought to be of the primary functions of c-MYC in tumor growth [48-51]. In accordance, it has been shown that, c-MYC is involved in modulating several of the cell cycle cyclins including cyclins D1 and D2, cyclin E and CDK4 [52-56]. In the G1 phase of the cell cycle, c-MYC accelerates cell proliferation enhancing the cyclin D-CDK2 activity [57, 58]. It is thought that, CDK4 also contributes to the oncogenic and the cell-cycle regulatory effects of c-MYC [54]. The finding that the levels of c-MYC are significantly reduced in MPL treated mice is therefore well in line with the cell cycle effects of MPL. It is generally accepted that, the c-MYC - retinoblastoma network coordinate two independent control modes of cell cycle progression [59]. From a molecular perspective, it is imminent that these two pathways play a crucial role in regulating cell cycle exit [60-62]. Therefore, the finding that MPL suppresses both c-MYC and the active form of retinoblastoma protein may suggest MPL-induced events leading to a quick exit from the cell cycle thus resulting in suppressed cell proliferation and inhibition of tumor growth (**Figure 5**). Collectively, these data reveal MPL as a potential drug well worthy of further pre-clinical anticancer assays particularly in the variety of cancers where mTOR, IGF-1 and c-MYC pathways are known to be hyperactive. From a drug development perspective, inhibition of multiple tumor growth signaling pathways provide solid framework for future clinical trials utilizing MPL in combination therapy to reduce drug resistance and ensure enhanced therapeutic success.

### Disclosure of conflict of interest

None.

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