Monepantel considerably enhances the therapeutic potentials of PEGylated liposomal doxorubicin and gemcitabine in ovarian cancer: in vitro and in vivo studies

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Abstract: Ovarian cancer is a lethal disease since treated patients often die from relapse. Resistance to current treatment regime involving doxorubicin and gemcitabine is well known. Hence, we set forth to develop a more effective therapy by combining current treatment drugs with monepantel, an antihelminth drug with proven anticancer effect. In vitro cytotoxicity were first investigated with pegylated liposomal doxorubicin (PLD), gemcitabine, monepantel as single agents and then in combination with monepantel on ovarian tumor cells. Drug effect on oncogenic proteins was determined by western blot analysis and resistance to drugs by colony formation assays. Using in vivo model (nude mice), a similar study, as above, was carried out to determine correlation to in vitro findings. Close correlation existed between in vitro and in vivo studies with the latter indicating that combination of monepantel with either low or high dose PLD was more effective compared to single drug therapy. A similar finding existed for gemcitabine, with gemcitabine showing a more superior efficacy (100% ablation) in combination with MPL. Western blot analysis indicated p-mTOR, p70s6K and 4E-BP1 were severely inhibited by combination of MPL with either PLD or gemcitabine. Colony formation assay indicated a dramatic reduction of colonies with combination treatment suggesting a considerable reduction of resistance. After 28 days, treatment using a combination of MPL with either PLD or gemcitabine showed tumor regression. Hence, the combination of gemcitabine or doxorubicin with monepantel may serve as a more effective therapy for ovarian cancer.

Keywords: Monopentel, doxorubicin, gemcitabine, combination therapy, ovarian cancer

Introduction

Ovarian cancer (OC) is a highly lethal gynecological malignancy and the fifth leading cause of cancer deaths in women [1, 2]. The standard treatment for OC involves rigorous cytoreductive surgery combined with postoperative chemotherapy [3, 4]. Although most patients are initially responsive to this therapy, more than 70% of patients have relapse eventuating in death [5]. Despite recent advancements in OC management including novel and more effective antineoplastic drugs, there remains a high risk for recurrence owing to resistance [6]. Moreover, attempts to improve the efficacy of standard therapy by incorporation of additional cytotoxic agents have yielded limited success [3]. Pegylated liposomal doxorubicin (PLD) and gemcitabine are currently used for the treatment of recurrent and progressive ovarian cancer [6, 7]. However, these agents generally have low response rates with poor survival. Hence, these limitations clearly necessitate the development of novel treatment strategies for ovarian cancer.

Monepantel [(1S)-1-Cyano-2-(5-cyano-2-trifluoromethyl-phenoxy)-1-methyl-ethyl]-4-trifluoromethylsulfanyl-benzamide] (MPL) is a new anthelmintic drug [8]. We have recently reported that MPL has potent anti-tumorigenic properties on human ovarian cancer [9]. In preclinical models of ovarian cancer, MPL modulates specific transcriptional and signal transduction cascades that lead to cell-cycle arrest, autophagy, and down regulation of DNA synthesis [9, 10]. Although, the exact mechanism by which MPL mediates these anticancer effects remain un-
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clear, we have shown its ability to inhibit the mTOR/p70S6K signaling pathway that is consistently involved in tumor genesis [10].

Up regulation of mTOR signaling has been implicated in a large number of malignancies including ovarian cancer [11, 12]. Intrinsically or constitutively active mTOR has been defined critical in the promotion of cell growth, proliferation [13], motility, angiogenesis [14] and development of drug resistance [15] in ovarian cancer. It is well established that activated mTOR exerts these effects through phosphorylation of two master downstream molecules: the translational regulator eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and P70S6 Kinase (P70S6K) which are crucial to the translation of numerous proteins involved in tumor pathogenesis [16]. Therefore, the blockade of mTOR activity may serve as a therapeutic target for this malignancy.

Numerous clinical studies are currently evaluating mTOR inhibitors for the treatment of ovarian cancer. Phase I and II trials have so far shown modest response to mTOR inhibitors in relapsed or refractory ovarian cancer [17, 18]. Due to the multifaceted crosstalk between signaling cascades in ovarian carcinogenesis, it is conceivable that mTOR inhibitors as single agent therapy may be inadequate to yield the desired clinical responses. As such, several preclinical and clinical studies have focused on the combination of mTOR inhibitors with other chemotherapeutic agents [19, 20]. Since MPL has an inhibitory effect on mTOR pathway, we postulated that in combination with PLD or gemcitabine, it might enhance the therapeutic efficacy of these clinically approved chemotherapeutic agents.

Materials and methods

Chemicals and antibodies

Unless otherwise stated, all drugs and chemicals used in this study were obtained from Sigma-Aldrich (Australian subsidiary). The following primary antibodies were used throughout this study: rabbit polyclonal antibodies specific for mTOR, phospho-mTOR (Ser2448), P70S6K, phospho-P70S6K (Thr389), 4E-BP1, phospho-4E-BP1 (Thr37/46), Cyclin A2, Cyclin E2 (Cell Signaling Technology), CDK2, Cyclin D1, Ki67 and c-Myc (Santa Cruz Biotechnology), and mouse monoclonal GAPDH. Secondary antibodies were goat anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP; Santa Cruz Biotechnology).

Cell culture

The human ovarian cancer cell lines, A2780, CAOV-3 and OVCAR-3; and human ovarian surface epithelial cell line, HOSE were obtained from American Type Culture Collection. These cell lines were authenticated by DNA short tandem repeat profiling, and experiments were carried out within 6 months of resuscitation. Ovarian cancer cells were maintained in RPMI-1640 medium with 2 mmol/L L-glutamine, 2 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate (Invitrogen) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin (50 U/mL) at 37°C in a humidified atmosphere containing 5% CO₂ as suggested by the manufacturer. HOSE cells were maintained in OEpiCM (ScienCell) supplemented with 10% OEpiCGS (Ovarian Epithelial Cell Growth Supplement) (ScienCell) and penicillin-streptomycin (50 U/mL) as suggested by the manufacturer. Cell lines were routinely assessed by cell morphology and their average doubling time.

Cell proliferation assay

Effects on cell proliferation were determined using the Sulforhodamine B (SRB) assay at an initial cell density of 3,000 cells/well in a 96-well plate as previously reported [9].

Colony formation assay

Colony formation assay was performed using standard agar coated plates. A2780 and SOVCAR-3 cells were seeded at a low density in the presence of doxorubicin (10 nm) or gemcitabine (10 µM), either alone or together with MPL (5 µM) for 72 h. Equal numbers of viable cells from each treatment group were seeded following drug treatment and incubated in the absence of drug for two weeks. The growth of resistant colonies from single cells was then analyzed by counting the number of developed colonies in each treatment group.

Western blot analysis

50 µg of cellular lysate (collected as previously described; ref. [19]) were subjected to SDS-
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Figure 1. MPL and doxorubicin/gemcitabine show a synergistic effect on reducing viability of ovarian cancer cells. (A) MPL reduces cell viability of ovarian cancer cells A2780, CAOV-3 and OVCAR-3 cells but has minimal effect on immortalised human ovarian surface epithelial cells (HOSE). Cells were treated with MPL at the indicated concentrations for 3 days before SRB assays were performed. Data from three independent experiments were represented as mean ± SD. (B and C) Co-treatment with MPL increases the sensitivity of ovarian cancer cells but not HOSE cells to doxorubicin (B) or gemcitabine (C). Cells were treated with various concentration of doxorubicin/gemcitabine as indicated in the absence or presence of 5 µM MPL for 3 d before SRB assays were performed. Data from three independent experiments were represented as mean ± SD. (D) Combination indexes were calculated for various cells at 5 µM PLX4720 and 0.1 µM doxorubicin/gemcitabine by using the CompuSyn software.

PAGE. Following electrophoresis, polyvinylidene difluoride membrane (Millipore Corporation) was probed with specific antibodies. Immune complexes were detected using HRP conjugated with either anti-mouse or anti-rabbit followed by chemiluminescence detection (Perkin Elmer Cetus). To show equal protein loading, blots were stripped and reprobed with specific antibodies recognizing GAPDH.

Animal studies

Female nude athymic Balb C nu/nu mice (6 weeks old) were purchased from Biological Resources (Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the University of New South Wales Animal Ethics Committee.
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Evidence

South Wales Animal Care and Ethics Committee (ACEC). All procedures carried out on mice were in strict accordance with the protocols approved by ACEC (approval numbers: 14/35A and 14/80A), and all efforts were made to minimize suffering. 2 × 10^6 log-phase growing OVCAR-3 cells suspended in 0.1 ml matrigel matrix were injected subcutaneously into the right lateral flank of each mouse. Treatment began when the tumor volume reached between 80 and 100 mm$^3$. Tumor volumes were calculated from caliper measurements by using the following ellipsoid formula: (D × d$^2$)/2, in which D represents the large diameter of the tumor, and d represents the small diameter. To assess the therapeutic effects of the combination of MPL with either of chemotherapeutics used in this study (PEGylated lysosomal doxorubicin and gemcitabine), animals were randomly assigned to nine groups that were administered vehicle [0.5% (wt/vol) hydroxypropylmethylcellulose (HPMC) in PBS], MPL 25 or 50 mg/kg, chemotherapy agents 2 or 5 mg/kg, or MPL/chemotherapy agents in combination (both dose as used in the single-agent groups) by intra-peritoneal injection. MPL was administered 3 times per week for the duration of the experiment. PLD was used weekly for three doses. Gem was administered 2 times per week for 2 weeks. Mice were weighed before each treatment, and drug doses were adjusted accordingly. At the end of both the treatment periods, animals were euthanized using Lethabarb R (100 mg/kg) intraperitoneal (i.p.) injection (VIRBAC) and tumors were dissected immediately and preserved at -80°C for analysis.

Results

MPL in combination with pegylated doxorubicin (PLD) or gemcitabine show synergistic effect in reducing ovarian cancer cells viability

To examine the effect of MPL on cell viability as single agent, SRB cytotoxicity assays were performed on several ovarian cancer cells with different molecular characteristics and clinical behavior (A2780, CAOV-3 and OVCAR-3) along with normal (non-malignant) human ovarian surface epithelial cells (HOSE). MPL had limited effect on reducing cell viability in HOSE cells, when used at concentrations of up to 100 µM. In contrast, this agent was much more potent on ovarian cancer cells, with an estimated IC$_{50}$ in the range of 7-10.5 µM (Figure 1A). Based on these findings, we next examined the effects of MPL combination with pegylated doxorubicin or gemcitabine on ovarian cancer cells viability. As shown in Figure 1B and 1C, co-treatment of A2780, CA0V-3 and OVCAR-3 cells with 5 µM...
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Monepantel (MPL) enhances the cytotoxicity of doxorubicin and gemcitabine in ovarian cancer cells. A combination of MPL with doxorubicin or gemcitabine led to a more pronounced decrease in cell viability compared with either of the chemotherapeutic agents alone. In addition, a combination index of ~0.3, 0.8 and 0.6, respectively, as calculated by the CalcuSyn software was found for these cells co-treated with MPL and doxorubicin. These figures were calculated at ~0.2, 0.4 and 0.1 for A2780, CAOV-3 and OVCAR-3, respectively, when MPL was combined with gemcitabine (Figure 1D). The combination index is a measurement of the combined drug interaction and is defined as <1 for synergism, 1 for additivity and >1 for antagonism [20]. Importantly, combining MPL with either pegylated doxorubicin or gemcitabine did not increase the cytotoxic effects of these agents on normal HOSE cells.

**Combination of MPL with PLD or gemcitabine delays the emergence of chemotherapy resistance in cultured ovarian cancer cells**

Based on the synergistic effect of combining MPL with pegylated doxorubicin or gemcitabine, we hypothesized that combining MPL with these agents may also influence the emergence of acquired resistance to them in ovarian cancer cells. As shown in Figure 2A and 2B, treatment with MPL plus doxorubicin or gemcitabine significantly reduced the number of colonies formed compared with treatment with either chemotherapy agents alone. Combining MPL resulted in 35 ± 4% and 40 ± 3% decrease in the number of A2780 and OVCAR-3 colonies recovering from doxorubicin (P<0.001). MPL also reduced the number of A2780 and OVCAR-3 colonies recovering from gemcitabine by 31 ± 4% and 34 ± 3% (P<0.001), respectively. These results suggest that co-treatment with MPL and doxorubicin/gemcitabine suppresses the emergence of resistance to these chemotherapy options in ovarian cancer cells.

**Mechanism**

To explore the molecular mechanisms underlying the synergistic effects of MPL and doxorubicin (PLD) or gemcitabine, western blot analyses were performed to evaluate the activities of relevant signaling pathways in ovarian cancer cells treated with MPL and doxorubicin or gemcitabine in combination compared with each drug individually.

These analyses revealed that MPL alone inhibited mTOR activity significantly, and co-treatment of MPL with either PLD or gemcitabine led...
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Figure 4. Combination treatment of MPL and doxorubicin/gemcitabine lead to tumour regression in mouse models. Nude mice bearing OVCAR-3 xenograft tumours were subjected to either (A) vehicle, MPL (25 and 50 mg/kg), doxorubicin (2 and 5 mg/kg), and the combination of MPL and doxorubicin; or (B) vehicle, MPL (25 and 50 mg/kg), gemcitabine (2 and 5 mg/kg), or the combination of MPL and gemcitabine when tumour volume reached between 80 and 100 mm³. (C) and (D) net tumour growth calculated by subtracting tumour volume on the first treatment day from that on the final day. Data are representative of mean volumes ± SD from 5 to 6 mice per group. *P<0.05, **P<0.01.
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Table 1. Summary of effect of MPL and its combinations with gemcitabine & doxorubicin on the growth of OVCAR-3 xenografts in nude mice

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Before</th>
<th>After</th>
<th>Tumour Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of mice</td>
<td>Wt (g)</td>
<td>No of mice</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>17.3±1.22</td>
<td>6</td>
</tr>
<tr>
<td>MPL</td>
<td>6</td>
<td>17.7±0.75</td>
<td>6</td>
</tr>
<tr>
<td>PLD (2 mg/kg)</td>
<td>6</td>
<td>18.1±0.65</td>
<td>6</td>
</tr>
<tr>
<td>PLD (5 mg/kg)</td>
<td>6</td>
<td>17.6±0.95</td>
<td>6</td>
</tr>
<tr>
<td>MPL+PLD (2 mg/kg)</td>
<td>6</td>
<td>17.5±0.96</td>
<td>6</td>
</tr>
<tr>
<td>MPL+PLD (5 mg/kg)</td>
<td>6</td>
<td>17.6±0.61</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>17.2±0.76</td>
<td>6</td>
</tr>
<tr>
<td>MPL</td>
<td>6</td>
<td>17.4±0.93</td>
<td>6</td>
</tr>
<tr>
<td>Gem (2 mg/kg)</td>
<td>6</td>
<td>17.7±0.75</td>
<td>6</td>
</tr>
<tr>
<td>Gem (5 mg/kg)</td>
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<td>16.8±0.39</td>
<td>6</td>
</tr>
<tr>
<td>MPL+Gem (2 mg/kg)</td>
<td>6</td>
<td>17.3±0.65</td>
<td>6</td>
</tr>
<tr>
<td>MPL+Gem (5 mg/kg)</td>
<td>6</td>
<td>16.8±1.0</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2. Fractional tumor volume relative to control

<table>
<thead>
<tr>
<th>MPL (50 mg/kg)</th>
<th>DOX (2 mg/kg)</th>
<th>Expected (E)</th>
<th>Observed (O)</th>
<th>E/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.82</td>
<td>0.297</td>
<td>0.243</td>
<td>0.149</td>
<td>1.631 (SYN)</td>
</tr>
<tr>
<td>MPL (50 mg/kg)</td>
<td>DOX (5 mg/kg)</td>
<td>Expected (E)</td>
<td>Observed (O)</td>
<td>E/O</td>
</tr>
<tr>
<td>0.82</td>
<td>0.337</td>
<td>0.276</td>
<td>0.093</td>
<td>2.97 (SYN)</td>
</tr>
<tr>
<td>MPL (50 mg/kg)</td>
<td>GEM (2 mg/kg)</td>
<td>Expected (E)</td>
<td>Observed (O)</td>
<td>E/O</td>
</tr>
<tr>
<td>0.681</td>
<td>0.448</td>
<td>0.305</td>
<td>0.206</td>
<td>1.481 (SYN)</td>
</tr>
<tr>
<td>MPL (50 mg/kg)</td>
<td>GEM (5 mg/kg)</td>
<td>Expected (E)</td>
<td>Observed (O)</td>
<td>E/O</td>
</tr>
<tr>
<td>0.681</td>
<td>0.476</td>
<td>0.324</td>
<td>0.125</td>
<td>2.592 (SYN)</td>
</tr>
</tbody>
</table>

Table 1 shows the percentage tumour growth inhibition in the various treatment groups as compared to control and the weight of the animals before and after treatment. MPL = Monepantel; PLD = Doxorubicin; Gem = gemcitabine.

Table 2 shows the results of fractional tumour volume analysis using the ratio of expected (E) to observed (O) tumour volume. Results indicate synergy in all combination regimes. MPL = Monepantel; DOX = Doxorubicin (PLD); SYN = Synergy.

to further reduction of p-mTOR levels. To further confirm the inhibitory effect of combination treatment on mTOR pathway, we next examined the effects on the phosphorylation of the downstream targets of mTOR namely P70S6K and 4E-BP1. As shown in Figure 3A, using either drug alone resulted in only modest inhibition of P70S6K and 4E-BP1 phosphorylation levels. However, when applied in combination, they essentially abolished the activation of P70S6K and 4E-BP1. These results support the presence of a strong synergistic effect on mTOR inhibition for the MPL and doxorubicin or gemcitabine combinations.

MPL, PLD, gemcitabine and combination therapy effects on subcutaneous tumor growth

Effects on in vivo tumor growth were evaluated in OVCAR-3 subcutaneous tumor xenografts in athymic Balb/C nude mice. As shown in Figure 4A and 4B, although tumor growth in the vehicle-treated animals progressed steadily over 26-days, those tumors treated with MPL alone only showed slight inhibition of tumor growth. Moreover, in the first experiment, the % inhibition in tumor growth by MPL = 6.6; PLD (LD) = 50; PLD (HD) = 47; MPL + PLD (LD) = 63 and MPL + PLD (HD) = 70. In subsequent experiment, the percentage inhibition in tumor growth by MPL = 46; Gem (LD) = 79; Gem (HD) = 75; MPL + Gem (LD) = 98 and MPL + Gem (HD) = 96.

On a comparative basis, the tumor response to MPL although the same dosage was used in both the experiments, the response in the second experiment was much higher compared to the first (46-6.6 = 39.4%). Further as individual agents, Gem seems to show a slightly better response for both the LD and HD, as compared to PLD (Gem -79 and 75 vs PLD- 50 and 47).

However, treatment with both doses of PLD or Gem led to significant reduction in tumor growth, but without evidence of tumor regression. In contrast, animals treated with the combination of PLD (L.D.- low dose and H.D.-
high dose) or Gem (L.D. and H.D.) with MPL showed a much greater reduction in tumor size. In the case of MPL + Gem, the tumor weight measurement at completion of therapy was similar to the final day tumor volume data indicating the arrest of tumor growth after treatment. Similar to the tumor volume, the ability of MPL to overcome PLD and Gem resistance was also observed in terms of the tumor weight (Figure 4C and 4D). Remarkably, MPL with Gem shows a much better response almost close to 100% as compared to MPL with PLD (63-70%). In addition, no significant differences in animal body weight were observed during the experiment (Table 1).

Further, the anti-tumor efficacy of both doses of PLD/GEM in combination with MPL was also assessed by a fractional product method. At day 26 after treatment, the ratio between the expected final tumor volume (FTV) and the observed FTV of the combination regimens was calculated 1.63, 2.97, 1.48, 2.60 for combination of MPL with PLD (L.D.), PLD (H.D.), Gem (L.D.) and Gem (H.D.), respectively (Table 2), clearly indicating synergy between MPL and PLD/GEM treatment.

After 28 days, some of the treatment groups began to show tumor growth indicating that resistance has developed. Growth resumes after 28 days in MPL treated group however, treatment with either PLD (LD) or PLD (HD) showed no growth and their combinations with MPL showed regression. In the case of Gemcitabine, both LD and HD overcome treatment and tumor resumes growth after 28 days; however their combinations with MPL showed regression (Figure 4A and 4B). This is an important finding indicating that tumors can be completely eradicated with long term treatment.

Combination treatment with MPL and PLD/gemcitabine cooperatively induce apoptosis and suppresses the activation of mTOR pathway

To assess whether the inhibitory effect of combination treatment on mTOR pathway could occur in vivo, we next evaluated the effect of MPL when combined with PLD or gemcitabine on mTOR activity in an experimental model of ovarian cancer in mice. As shown in Figure 5A and 5B, a modest decrease in p-mTOR expression was observed in tumors excised from mice treated with single agents; MPL (P<0.05), PLD (H.D.) (P<0.01) and gemcitabine (L.D. and H.D.) (P<0.01). However, combining MPL with both doses of PLD and gemcitabine dramatically attenuated p-mTOR levels in the tumors (P<0.001). In line with this, phosphorylation of the downstream targets of mTOR, P70S6K and 4E-BP1 were strongly inhibited in all combination treatment groups.

These in vivo observations fully support our in vitro results described above, suggesting effective inhibition of mTOR signaling by combination treatment leading to the observed suppressive effects on tumor growth in ovarian cancer.

Discussion

In vitro toxicity studies on tumor cell lines (A2780, CAOV-3, OVCAR-3) have indicated that the combination of MPL with either PLD or GEM have dramatic effect on tumor cell viability as compared to the use of single agents. The combination index (CI) values of 0.3, 0.8, 0.6 for MPL-PLD combination on cell lines A2780, CAOV and OVCAR-3, respectively was indicative of synergy with further implication that A2780 was highly sensitive compared to the other two
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tumor cells. Further, MPL combination with GEM gave a CI value of 0.2, 0.4 and 0.1, respectively on similar cell lines indicating that MPL-GEM combination has greater synergy on these cell lines compared to MPL-PLD mixture. Hence these results indicate that the combination therapy involving MPL with PLD or GEM has therapeutic advantage over the use of any of the single agents in these ovarian tumor cell lines.

The variability of toxicity (as indicated by the CI values) that is shown by the three cell lines may be attributed to their differences in individual molecular features. Although several mechanisms have been proposed for Doxorubicin's mode of action, none of these seems to be applicable at the clinical drug concentration achievable [21]. More, recently it has been suggested to act through proteolytic activation of a transcription factor cAMP response element-binding protein 3-like 1 (CREB3L 1) [22, 23] and cells with higher expression of this protein are most probably more responsive. On the other hand, gemcitabine has been shown to inhibit phosphatidylinositol 3-kinase (PI3K)-Akt survival cascade in A2780 cells as well as in other cell lines [24]. Hence, the differential expression of these molecular features along with the expression of m-TOR that is responsive to MPL may contribute to the differences observed in response. This needs further investigation in future studies.

In these cell lines, it is quite clear that mTOR has a significant impact on cell survival and hence targeting this oncogene has profound effects and this is further enhanced in combination with these two chemotherapeutic agents. This is consistent with other studies using a combination of mTOR inhibitors with other agents in liver and breast cancers [25, 26].

Chemo-resistance is a common feature of most cancers and this has been attributed to a sub-population of resistant stem cells that are able to overcome chemotherapy through a variety of mechanisms [27-29]. Hence, we examined the growth of remnant cells by colony formation assay after treatment with individual and combination of agents in order to identify resistant colonies. A combination of MPL with either PLD or GEM significantly reduced the number of colonies as compared to individual agents. MPL with PLD resulted in 35% and 40% colony reduction in A2780 and OVCAR-3 cells, respectively. Similarly, treatment with MPL and GEM showed a reduction of 31% and 34% colony formation in the above cell lines. Hence, the present results suggest that the combination of MPL with PLD or GEM may have substantial effect in the reduction of resistance colonies and on the treatment of ovarian cancers with perhaps minimal recurrence.

The activation of the PI3K/Akt pathway and downstream signaling of mTOR has been shown to be related to drug resistance with poor prognosis in a variety of cancers [30, 31]. High expression of PI3K and Akt is a common feature in 12-68% of ovarian cancer and is associated with mTOR signaling [32-34]. Rapamycin a commonly used mTOR inhibitor has been shown to enhance the effects of a number of chemotherapeutic agents in a variety of cancers [35, 36]. Hence, in the present study, the use of MPL, an mTOR inhibitor, in combination with chemotherapeutic agents such as doxorubicin or gemcitabine seems to have a similar effect in the reduction of resistance and enhance the anti-cancer effect.

Subsequent western blot analysis revealed that MPL alone dramatically inhibited the expression of mTOR in agreement with our past work [10]. In combination with either PLD or GEM, there was a further reduction of mTOR expression. Additional examination of the phosphorylation of downstream targets of mTOR such as P70S6K and 4E-BP1 indicated that single drugs only showed a modest inhibition of phosphorylation, in comparison to combination with MPL that had a dramatic effect.

In vivo studies using OVCAR-3 subcutaneous tumor xenografts in nude mice showed that MPL in the first experiment had only a slight effect (7.3%) on tumor growth whilst Doxorubicin (PLD) or GEM treatment showed significant effect but without evidence of tumor regression. However, combination treatment of MPL with either Doxorubicin (PLD) or GEM treatment showed significant effect but without evidence of tumor regression. The anti-tumor efficacy of the combination regime as calculated using fractional product method showed that at day 26, the ratio of expected final tumor volume (FTV) to the observed FTV was 1.63, 2.97 for MPL/PLD at low dose and high dose respectively. Similar
analysis for MPL with GEM was 1.48 and 2.60. These results indicate synergy for the combination drug regime.

The difference in response of OVCAR-3 tumor to MPL (comparing the first experiment, 6.6% to the second 46%), a clear difference of 39.4% was seen. This may indicate the heterogeneity of the tumor composition. This needs further investigation to determine the reason for the large differences in the response. Clearly, the combination of MPL with Gem seems to produce a much better response (tumor regression) that was almost close to a 100%, as compared to MPL with PLD. A difference of almost 30% was seen and this may be contributed by the tumor heterogeneity between the two experiments. This heterogeneity of the tumors was clearly indicated by the differences in tumor response between the two experiments when treated with similar dose of MPL. On the other hand the differences in molecular mechanistic action of the two chemotherapeutic agents PLD (Doxorubicin) and Gemcitabine may have also contributed to the final differences in tumor response.

Treatment after 28 days showed that in all treatment groups, except the combination with MPL, growth resumed. This indicates that resistance developed in all groups except in the combination groups. (MPL + PLD and MPL + Gem). Notably, after 28 days, treatment groups with PLD (LD & HD) or Gemcitabine (LD & HD) in combination with MPL showed tumor size regression. This is a very important finding that indicates that tumors can be completely abolished with long term treatment with these combinatorial regimes.

Further, there were no significant variations in body weight between the treatment groups over the 26 days and this may indicate that animals were least affected by these treatments, suggesting that adverse reactions may also be minimal.

Finally, the analysis of the tumor tissues from in vivo studies showed that the combination treatment severely inhibited the p-mTOR, P70-s6K and 4E-BP1 as compared to single agents and this is in agreement to our earlier in vitro investigation. In conclusion, MPL an m-TOR inhibitor, in combination with either Doxorubicin (PLD) or GEM, may serve as a useful treatment for ovarian cancer since it not only acted synergistically enhancing the cytotoxicity but was also capable of reducing chemo-resistance. Reduction of chemo-resistance could have considerable effect on patient recovery, relapse and survival [37-39].

Since MPL is a potent mTOR inhibitor, it may serve as a useful agent in combination with a number of other current chemotherapeutic agents that target tumor cells in a variety of cancers. Further, in combination, it seems to attenuate resistance and hence, it may serve as a useful agent for the treatment of a number of resistant and recurring cancers such as pancreatic, breast, prostate and lung cancer [40, 41]. This needs further investigation.

**Disclosure of conflict of interest**

None.

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**References**


[5] Edwards SJ, Barton S, Thurgar E and Trevor N. Topotecan, pegylated liposomal doxorubicin hydrochloride, paclitaxel, trabectedin and gemcitabine for advanced recurrent or refractory ovarian cancer: a systematic review and
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Monepantel enhances cytotoxicity of doxorubicin and gemcitabine in ovarian cancer