ABT-737 and pictilisib synergistically enhance pitavastatin-induced apoptosis in ovarian cancer cells

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Abstract. There is considerable interest in redeploying drugs for use in combination with other oncology therapeutics. The single-agent activity of statins in ovarian cancer has been widely reported, however the drug concentration required to cause cell death is considerably higher than that achieved in patients receiving statin treatment for hypercholesterolemia. Unfortunately, statins can cause myopathy when administered in high doses. One solution to this is to identify drugs that could be used in combination with statins to reduce the dose required and those that may potentially reduce the incidence of adverse side effects. When the BH3 mimetic ABT-737, or the phosphatidylinositol 3-kinase inhibitor pictilisib, were combined with pitavastatin in cell growth assays using Ovcar-3 and Igrov-1 cells, the drug combinations were more effective than pitavastatin alone. In support of this, ABT-737 or pictilisib markedly increased cell death induced by pitavastatin in several ovarian cancer cell lines. The drugs were also synergistic in apoptosis assays. These observations suggested that either BH3 mimetics or pictilisib in combination with pitavastatin could be used in a subset of ovarian tumours, particularly those sensitive to BH3 mimetics, and phosphatase and tensin homolog inhibition, in the treatment of ovarian cancer.

Introduction

Statins are a class of drug used for the treatment of hypercholesterolaemia. They reduce plasma cholesterol by competitively inhibiting 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), an enzyme involved in the mevalonate pathway that is responsible for cholesterol synthesis. Inhibition of HMGCR also leads to a reduction in other products of the mevalonate pathway including farnesyl diphosphate (FPP)

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and geranylgeranyl diphosphate (GGPP). These isoprenoids are used in the post-translational modification and membrane localization of several important GTPases, such as Ras, Rho, Rac, Rab and Cdc42, many of which have been identified as oncogenes. Consequently, statins reduce the recruitment of small GTPases to the cell membrane. We have previously reported the anti-cancer activity of statins in ovarian cancer cells, showing that both high concentrations and continuous exposure to statins were required to induce cell death (1). More recently, we showed that pitavastatin, a hydrophobic statin with a relatively long half-life, demonstrated potent anti-cancer activity, inhibiting the growth and promoting apoptosis in ovarian cancer cell lines and causing regression of tumour xenografts (2). Pitavastatin has been evaluated in phase II clinical trials for the treatment of hypercholesterolaemia at doses up to 64 mg daily, with dose-limiting toxicities observed after 2-4 weeks reversible within 2 weeks of discontinuing therapy (3). The concentrations of pitavastatin required to cause cell death [0.2-7.6 μM depending on cell line (2)] are similar to the expected plasma concentration *in vivo* following administration of 64 mg pitavastatin [C_{max}, ~3 μM, assuming linear pharmacokinetics and using data from (4-6)]. Despite this, the anti-cancer activity of pitavastatin can be suppressed by exposure to geranylgeraniol, an isoprenoid found in many common foodstuffs, thereby raising the possibility that dietary isoprenoids may impede the effectiveness of statins in clinical trials (2). One possible solution is to control patients' diet in oncology clinical trials. However, the potential for pitavastatin to cause myopathy, particularly at high doses, makes it desirable to identify drugs which could be used in combination with pitavastatin to reduce the dose required and potentially reduce the incidence of adverse drug effects.

The BH3 mimetics ABT-737 and obatoclax have been employed to overcome the pro-survival effects of anti-apoptotic proteins by competitively binding to and inhibiting the Bcl-2 family of proteins (7). We have previously shown that ABT-737 and the orally bioavailable analogue, ABT-263, can enhance the cell death induced by carboplatin or paclitaxel in ovarian cancer cells (8,9). A closely related selective Bcl-2 inhibitor, venetoclax, has been approved for the treatment of chronic lymphocytic leukemia. However, we have shown that inhibitors of Bcl-x$_L$, a member of the Bcl-2 family, are likely to be needed for the treatment of ovarian cancer (10). These observations suggest that BH3 mimetics which inhibit Bcl-x$_L$

may be useful in combination with statins, which have also be shown to induce apoptotic cell death (1,2,11,12).

The phosphatidylinositol 3-kinase (PI3K) pathway plays an important role in cell survival, proliferation, migration and metabolism, and has been recently reported to be frequently activated in advanced epithelial ovarian cancers (13,14). Pictilisib is an orally active PI3K inhibitor which is more than 100 times more potent against class I PI3K compared to class II, III and IV family members (15). Statins have also been shown to interfere with PI3K signalling by inhibiting NFκB, and consequently increasing transcription of PTEN and reducing Akt phosphorylation (11). This suggests that pitavastatin in combination with PI3K inhibitors could synergistically inhibit PI3K signalling, leading to an increase in cell death.

To evaluate whether ABT-737, obatoclax or pictilisib could potentiate the activity of pitavastatin, we evaluated the anti-cancer activity of pitavastatin alone and in combination with these drugs. We found that ABT-737 and pictilisib combined additively with pitavastatin in cell growth assays, and potentiated the cell death induced by pitavastatin, in several ovarian cancer cell lines.

Materials and methods

Cell culture. Human ovarian cancer cells (A2780, Ovcar-3, Ovcar-8 and Igrov-1; American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI 1640; Lonza Group, Ltd., Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin/streptomycin and 2 mM glutamine. In addition, Ovcar-3 cells were supplemented with 0.11 g/l sodium pyruvate and 0.01 mg/ml insulin. Cells were incubated at 37°C and in a humidified 5% CO_2 atmosphere.

Cell growth/survival assays. ABT-737 (Abbott Laboratories, Chicago, IL, USA) and obatoclax (Active Biochem, Maplewood, NJ, USA) were prepared as 10 and 5 mM solutions respectively in dimethyl sulfoxide (DMSO). Pitavastatin (Sequoia Research Products, Pangbourne, UK) and pictilisib (LC Laboratories, Woburn, MA, USA) were prepared as 20 mM solutions in DMSO. Single-agent and combination studies were completed as previously reported (8). Fixed concentrations of ABT-737, which had been determined to inhibit cell growth by 5% (A2780, 3 μM; Ovcar-3, 1 μM; Ovcar-8, 1 μM; Igrov-1, 0.6 μM), were added to 18 different concentrations of pitavastatin in cell growth assays (8). Obatoclax or pictilisib and pitavastatin were combined at a fixed ratio of their IC_{50} values as determined from single-agent studies. Combination indicies (16) were calculated to measure the combined effect of pitavastatin with ABT-737, pictilisib or obatoclax, and quoted at a fraction affected of 0.5 or 0.75, which is the concentration of the drug combination that inhibited 50 or 75% of cell growth respectively.

Cell death assays. Ovcar-3 and Igrov-1 cells were incubated with DMSO, pitavastatin (12 and 6 μM respectively), ABT-737 (1 and 0.6 μM), obatoclax (2 and 3 μM), pictilisib (2 and 0.7 μM) alone or in combination with pitavastatin for 48 h (caspase-3/7 assay) or 72 h (trypan blue assay). Cells were collected by centrifugation (150 g, 3 min), and resuspended

in phosphate-buffered saline (PBS) containing 0.2% trypan blue (Sigma-Aldrich, St. Louis, MO, USA). Cells which did not exclude trypan blue were considered dead. Alternatively, an equal volume of caspase-Glo-3/7 substrate (Promega Corporation, Madison, WI, USA) was added directly to the cells, and after 30 min incubation, a microplate reader was used to measure luminescence, as per the manufacturers protocol. For caspase-3/7 assays, a paired *t*-test was used to compare the effect of the drug combination to the effect of single agent pitavastatin. For trypan blue assays, a paired t-test was used to compare the observed effect of the drug combination to the expected additive effect calculated using the Bliss independence criterion (17).

Western blotting. For western blotting, Igrov-1 cells were exposed to pitavastatin or ABT-737 or solvent (DMSO) alone or in combination. After 48 h, cell lysates were prepared as described (18) and total protein was quantified using the Bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Membranes were incubated with rabbit anti-human polyclonal PARP antibody (1:1,000) (#9542), rabbit anti-human polyclonal Bim antibody (1:1,000) (#2819), rabbit anti-human polyclonal Bcl-x$_L$ antibody (1:1,000) (#2762), or rabbit anti-human monoclonal Mcl-1 (D35A5) antibody (1:1,000) (#5453; all from Cell Signaling Technology, Inc., Danvers, MA, USA), and mouse anti-human monoclonal GAPDH antibody (1:5,000) (MAB374; EMD Millipore, Billerica, MA, USA) as a loading control. Bands were quantified using AlphaView SA (ProteinSimple, San Jose, CA, USA) and normalized to GAPDH. The amount of cleaved PARP was expressed as a fraction of uncleaved-PARP, and all other proteins were expressed as a fraction of the solvent control.

Statistical analysis. Data are presented as the mean ± standard deviation. Differences were analysed using a t-test (Microsoft Excel, 2010; Microsoft Corporation, Redmond, WA, USA). At least three experimental replicates were performed. P<0.05 was considered to indicate a statistically significant difference.

Results

ABT-737, obatoclax or pictilisib additively combine with pitavastatin in ovarian cancer cells. To investigate the activity of drug combinations with pitavastatin, the potencies of pitavastatin, ABT-737, obatoclax and pictilisib as single agents in cell growth assays were first determined in a panel of four ovarian cancer cell lines. Pitavastatin (IC_{50}=0.26-5.8 μM), ABT-737 (IC_{50}=2.1-4.0 μM), obatoclax (IC_{50}=0.15-0.36 μM) and pictilisib (IC_{50}=0.072-0.88 μM) inhibited the growth of ovarian cancer cell lines (Fig. 1A).

Concentrations of ABT-737 above 20 μM have previously been shown to inhibit cell growth independently of Bcl-x$_L$ (8). Thus, drug combination experiments using ABT-737 were performed using a fixed concentration of ABT-737 which inhibits cell growth by <10%. When combined with pitavastatin, ABT-737 either caused additive effects or in A2780 cells, an antagonistic interaction was observed. Although synergy was not observed between these drugs in any of the cell lines evaluated, an additive interaction was obtained in Igrov-1 cells (Fig. 1B).
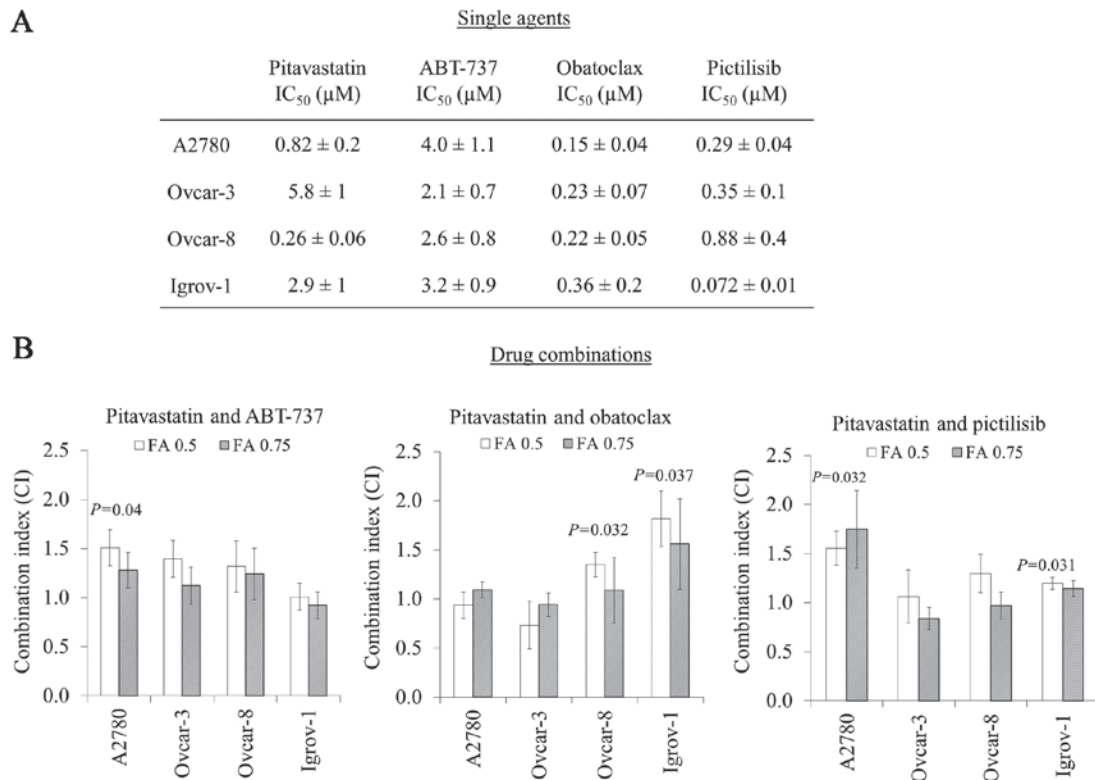
Figure 1. Potency of pitavastatin, ABT-737, obatoclax and pictilisib as single agents or in combination in cell growth assays. (A) The potency of pitavastatin, ABT-737, obatoclax and pictilisib as single agents were measured in cell proliferation/growth assays (columns 1-4, mean ± standard deviation; n=3-9). (B) To measure the activity of pitavastatin in combination with ABT-737, obatoclax or pictilisib, cells were exposed to a range of concentrations of pitavastatin and either, a fixed concentration of ABT-737, or a range of concentrations of either obatoclax or pictilisib, combined at a ratio of their single agent IC$_{50}$s. CIs (mean ± standard deviation; n=3-9) are quoted at the indicated FA and differed significantly from unity where indicated (paired t-test). IC$_{50}$, half maximal inhibitory concentration; CIs, combination indices; FA, fraction affected.

Obatoclax in combination with pitavastatin did not show a significant synergistic interaction in any of the cell lines tested (measured at fraction affected 0.5) (Fig. 1B). However, in Ovcar-8 and Igrov-1 cells, there was significant antagonism (P=0.032 and P=0.037 respectively, fraction affected, 0.5), which was reduced at higher drug concentrations (fraction affected, 0.75; Fig. 1B). Pictilisib and pitavastatin were additive in Ovcar-3 and Ovcar-8 cells, even at higher drug concentrations (fraction affected, 0.75). However, significant antagonism was observed in A2780 and Igrov-1 cells (P=0.032 and P=0.031, fraction affected, 0.5, Fig. 1B).

Pitavastatin in combination with ABT-737 or pictilisib increases cell death in Igrov-1 or Ovcar-3. The activity of the most promising of these drug combinations was evaluated in cell death assays. Ovcar-3 or Igrov-1 cells were exposed to pitavastatin in combination with ABT-737, and the Bliss independence criterion was used to estimate the effect of the drugs. In Igrov-1 cells, but not Ovcar-3 cells, significantly more cell death was observed by trypan blue staining (P=0.0004) than would have been expected if the drugs had demonstrated an additive effect. (Fig. 2A). However, in Ovcar-3 cells, the combination of pitavastatin and pictilisib resulted in significantly more cell death than would be expected from an additive combination (P=0.018, Fig. 2A).

To confirm that the drug combinations resulted in apoptosis, activation of caspase-3/7 was measured. Directly reflecting the results obtained in the trypan blue assays, there was also a significant increase in caspase-3/7 activity in Ovcar-3 and Igrov-1 cells exposed to pitavastatin and pictilisib or ABT-737 respectively compared to pitavastatin alone (P=0.046 and P=0.029), suggesting that apoptosis contributes to the cell death caused by these drug combinations (Fig. 2B). Unsurprisingly, pitavastatin in combination with obatoclax resulted in a significant increase in caspase-3/7 activity in ovarian cancer cells compared to single agent pitavastatin (P=0.022 and P=0.0051, Fig. 2B); obatoclax as a single agent also significantly activated caspase-3/7 activity, suggesting that this was not a synergistic interaction.

The synergy observed in the trypan blue studies was most striking in the Igrov-1 cells exposed to ABT-737 and pitavastatin, so further studies focused on this drug combination. The synergistic activation of caspase-3/7 was confirmed by measuring the cleavage of its substrate, PARP. ABT-737 on its own caused no PARP cleavage, but when combined with pitavastatin, ABT-737 potentiated the PARP cleavage caused by pitavastatin (Fig. 3).

The mechanism by which ABT-737 potentiated the apoptosis induced by pitavastatin was explored by measuring the levels of several Bcl-2 family members. Pitavastatin, when used as a single agent, increased the level of the pro-apoptotic protein, Bim, and decreased the level of Bcl-x$_L$ (Fig. 3) In contrast, ABT-737, used as a single agent, resulted in the increase in Bcl-x$_L$ and Mcl-1 (Fig. 3). Overall, Bcl-x$_L$ was reduced in cells exposed to the combination of pitavastatin and ABT-737 (Fig. 3).
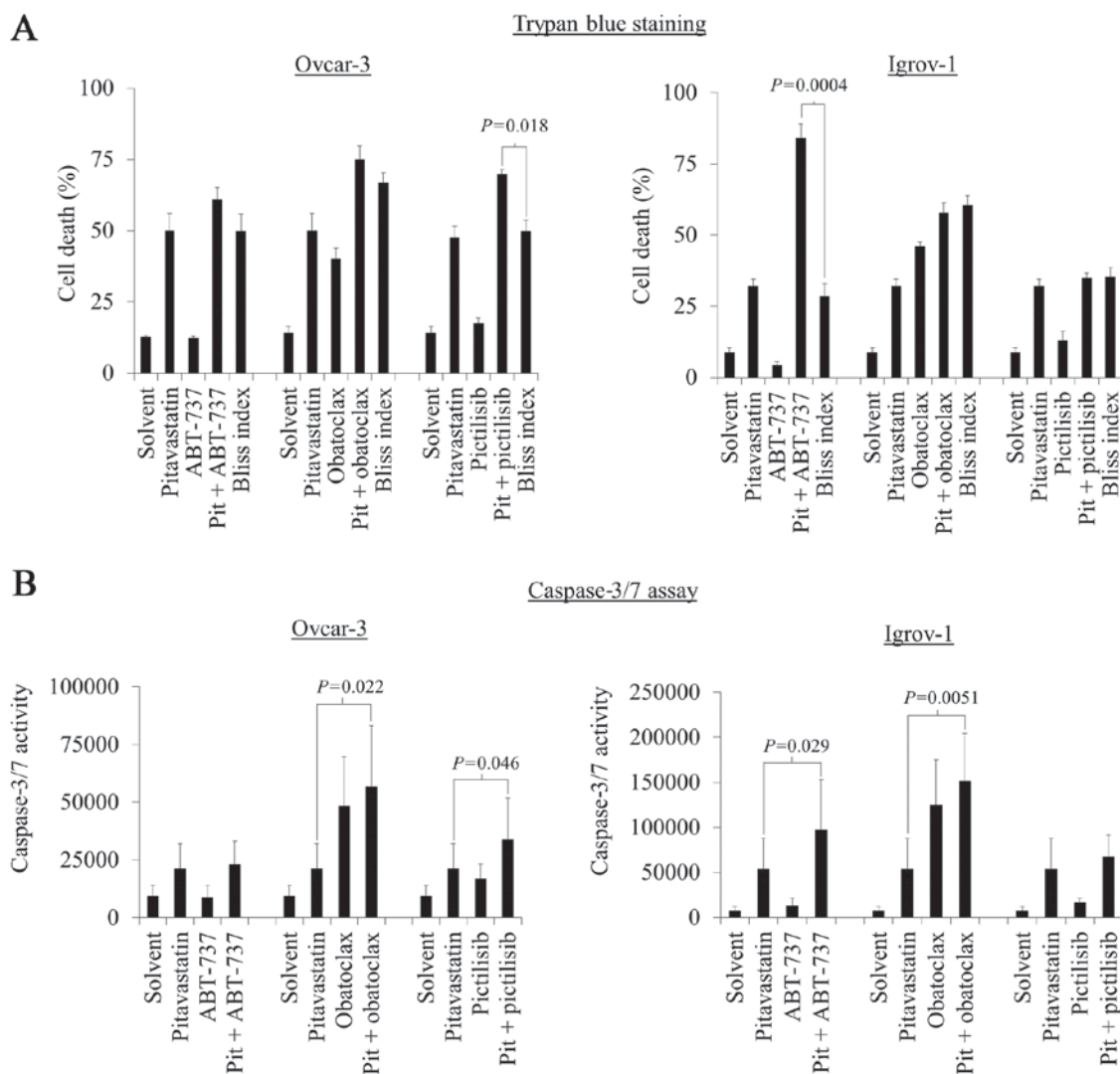
Figure 2. Cell death following exposure to different combinations of pitavastatin and ABT-737, obatoclax or pictilisib. (A) The number of dead Ovcar-3 and Igrov-1 cells was determined by trypan blue staining following exposure for 72 h to either solvent (DMSO), or 12 and 6 μM pitavastatin, and 1 and 0.6 μM ABT-737, 2 and 3 μM obatoclax, or 2 and 0.7 μM pictilisib. The number of dead cells (mean ± standard deviation; n=3) was significantly different from the Bliss independence calculated for each drug combination where indicated (paired t-test). (B) Caspase-3/7 activity in Ovcar-3 and Igrov-1 cells was measured following exposure to either solvent (DMSO), or 12 and 6 μM pitavastatin, and 1 and 0.6 μM ABT-737, 2 and 3 μM obatoclax, or 2 and 0.7 μM pictilisib for 48 h. Caspase-3/7 activity (mean ± standard deviation; n=3) in each drug combination was significantly different from cells exposed to the single agent pitavastatin where indicated (paired t-test).

Discussion

We have investigated the potential for ABT-737, obatoclax and pictisilib to potentiate the activity of pitavastatin. This is of particular concern because we have previously shown relatively high doses of statins are likely to be necessary to treat patients with ovarian cancer (1). Statins have been previously evaluated in combination with various chemotherapeutic agents including cisplatin and doxorubicin, resulting in an additive or synergistic reduction in ovarian cancer cell proliferation (19,20). ABT-737 or pictilisib combined additively with pitavastatin in cell growth assays. In shorter term cell death and apoptosis assays, both drugs potentiated the activity of pitavastatin. This suggests that these drugs increase the rate of apoptosis caused by pitavastatin. We have previously observed this phenomenon with other drug targets. Inhibition of autotaxin speeds up apoptosis induced by carboplatin, whilst having less pronounced effects in longer duration cell growth assays (21).

The PI3K pathway contributes to proliferative and anti-apoptotic effects on tumour cells, and is deregulated in 45% of high-grade serous ovarian cancers (14). Statins have also been shown to inhibit PI3K signalling by inhibiting NF-κB, which results in an increase in the expression of PTEN and a reduction in Akt phosphorylation (11,22). Dual inhibition of PI3K/Akt/mTOR signalling with a combination of pictilisib and pitavastatin increased apoptosis in Ovcar-3 cells, whilst demonstrating antagonism in A2780 and Igrov-1 cells. Ovcar-3, Ovcar-8, Igrov-1 and A2780 cells have PI3K/Akt pathway alterations consistent with activation of PI3K/Akt signalling, suggesting that these cell lines may be particularly sensitive to PI3K pathway inhibition (23,24). This was supported by the submicromolar IC_{50} values obtained for pictilisib in all cell lines tested. The lack of synergy between pitavastatin and pictilisib in A2780 and Igrov-1 cells may be due to *PTEN* deletions in these cell lines, resulting in low or undetectable levels of PTEN protein (23). This may hyperactivate the Akt
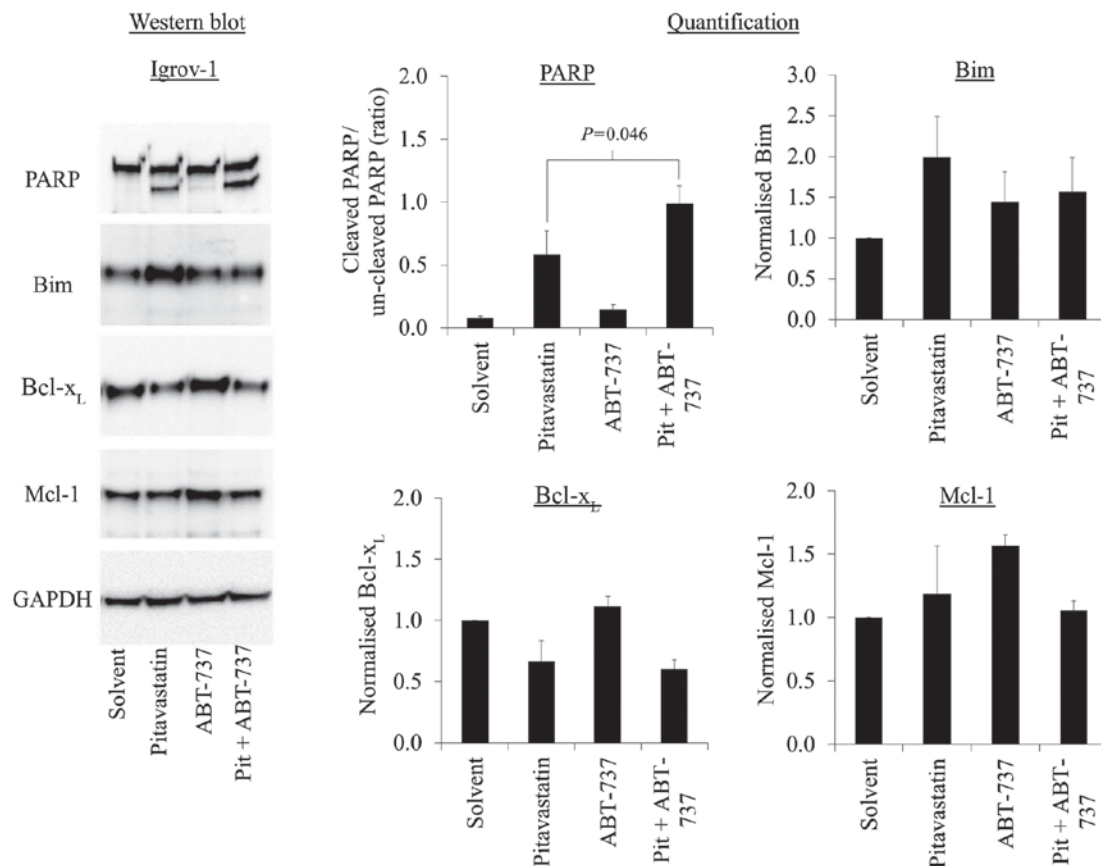
Figure 3. Effects of pitavastatin in combination with ABT-737 on proteins involved in the apoptosis signalling pathway. Igrov-1 cells were exposed to solvent (DMSO) or 6 μM pitavastatin (Pit) or 0.6 μM ABT-737, as single agents or in combination, for 48 h. The levels of PARP, Bim, Bcl-x$_L$ and Mcl-1 were measured by western blotting (mean ± standard deviation; n=3). PARP cleavage was significantly increased in cells exposed to pitavastatin and ABT-737 compared to cells exposed to pitavastatin alone (paired t-test). GAPDH was used as a loading control. PARP, poly (ADP-ribose) polymerase 1; Bcl-x$_L$, B-cell lymphoma X$_L$ protein; Bim, Bcl-2-like protein 11; Mcl-1, myeloid leukemia cell differentiation protein 1.

pathway and prevent further inhibition of PI3K signalling through PTEN modulation by statins.

The activity of ABT-737 has previously been attributed to inhibition of the pro-apoptotic mediators, Bcl-2, Bcl-x$_L$ or Bcl-w, of which Bcl-x$_L$ is overexpressed in ovarian cancer (8,25). Statins have been shown to induce apoptosis through a number of pathways, including suppression of Akt/Erk activation (11,12), increased phosphorylation of the p38 MAPK pathway (12), and attenuation of Mcl-1, probably through the inhibition of NF-κB (26). The most synergy between ABT-737 and pitavastatin was observed in Igrov-1 cells. It is noticeable that, of a panel of ovarian cancer cell lines, these cells were the ones in which the most pronounced synergy between ABT-737 and carboplatin was observed (8). It is possible that these cells are 'primed' for cell death (27). In 'primed' cells, ABT-737 prevents Bcl-x$_L$ from sequestering pre-existing pro-apoptotic mediators, thereby enabling apoptosis to occur more readily. In contrast to previous reports, pitavastatin did not decrease Mcl-1 levels, but instead reduced the levels of Bcl-x$_L$ and increased Bim. This suggests a mechanism by which the drug combination is synergistic. Pitavastatin increases the ratio of Bim to Bcl-x$_L$, facilitating the release of Bim from Bcl-x$_L$ by ABT-737, and consequently activating the intrinsic apoptosis pathway. Additivity or mild antagonism was observed in the other cell lines exposed to this drug combination, and these differences could be related

to the expression of apoptosis inhibitiors. Previous research demonstrated that expression of Bcl-x$_L$ was markedly lower in A2780 cells, and this, together with increased Mcl-1 levels (8), previously linked to cellular resistance to ABT-737 (28), could account for the antagonism observed in this cell line. Mcl-1 can cause resistance to ABT-737 because it is able to suppress apoptosis by sequestering pro-apoptotic BH3-only proteins such as Bim, but it is not inhibited by ABT-737. We have also previously observed antagonism between ABT-737 and carboplatin in A2780 cells, despite observing synergy between these drugs in other cell lines (8).

Obatoclax is a pan-Bcl-2 inhibitor which also inhibits Mcl-1, and therefore, may overcome the resistance to ABT-737. However, obatoclax in combination with pitavastatin was additive at best in A2780 and Ovcar-3 cells, with antagonism observed in Ovcar-8 and Igrov-1 cells. We have previously shown that obatoclax has off-target effects. It accumulates in lysosomes causing their alkalinisation (29). This may have contributed to the antagonism observed in these and previous combination studies (30). Taken together, these results suggest that the combination of obatoclax and pitavastatin may be of limited value in a clinical setting.

Taken together, pictilisib or ABT-737 in combination with pitavastatin could be used in a subset of ovarian tumours in a clinical setting. This has several implications. Although the synergy between these drugs may reduce the dose of statin that

is necessary to treat patients, consideration should be given to identifying which patient groups may benefit most from these combination treatments. 'BH3 profiling' is one method that can be used to determine the sensitivity of cancer cells to Bcl-2 inhibitors by determining the effects of the drug or related peptide on mitochondria isolated from the cancer cells (31). For the PI3K inhibitor and pitavastatin combination, measurement of PTEN expression and other pathway markers may help to select which patients may respond to this combination.

In conclusion, these promising results demonstrate that combinations of pitavastatin and BH3 mimetics or inhibitors of the PI3K pathway warrant further studies as potential therapeutics for ovarian cancer. There is a legitimate concern, however, that the inclusion of pictisilib or a BH3 mimetic in combination with pitavastatin will result in additional toxicity. Whether the therapeutic window for the use of the proposed combinations is an improvement over that with pitavastatin alone is something that can best be addressed in clinical trials.

Acknowledgements

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