MEK Pathway Inhibition Increases the Efficacy of a PI3K and HDAC Inhibitor in Endometrial Cancer Cells

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Abstract

Background: Endometrial cancer (EC) is the commonest gynaecologic malignancy in many countries and its incidence is rising. Although excellent prognosis is associated with early stage disease, response to systemic treatment for metastatic or recurrent EC is often low and treatment options are limited.

Aims-Methods: The aim of the study was to propose improved targeted drug treatments suitable for subsequent testing in pre-clinical models of EC. Cell proliferation assay (MTS) was used to assess viability of EC cell lines following treatment with drug inhibitors and Western blotting to explore the effect of inhibitors in molecular pathways.

Results: We identified that CUDC-907, a PI3K and HDAC inhibitor, was the most effective monotherapy treatment of a panel of drugs screened in EC cells. Moreover, several combination treatments showed synergism in EC cell lines, with the most efficacious being CUDC-907 combined with the MEK inhibitor PD0325901. This indicates that simultaneous inhibition of two main oncogenic pathways, PI3K and MEK, could improve drug sensitivity in EC.

Conclusions: In summary, we propose a range of targeted inhibitory drugs, alone or in combination, showing in vitro efficacy in endometrial cancer cells, which could provide novel therapeutic strategies for advanced EC.

Background

The incidence of endometrial cancer (EC) is rising in many countries, with an estimated of 417,367 new cases and 97,370 deaths worldwide in 2020 (1). Unlike many other cancers, where the prognosis has improved over time, mortality from EC is rising in many countries, including the UK (2). In the USA, endometrial and cervical cancer are the only malignancies for which the five-year survival has decreased when comparing the 1975-77 period with 2006-12 (from 86.9–83.4% for EC) (3). At present the first-line systemic therapy for advanced EC is platinum-based chemotherapy, however response rates are modest. A Cochrane review (4) identified three main combinations: Cisplatin with doxorubicin, cisplatin with doxorubicin and paclitaxel and cisplatin with doxorubicin and cyclophosphamide and although adjuvant chemotherapy following surgery did improve overall survival there is still uncertainty as to the optimum regimen. New agents, in particular immune checkpoint inhibitors, appear to hold great potential for EC cases with microsatellite instability (MSH-H)/mis-match repair gene deficiencies (dMMR), either as monotherapy or in combination with other agents, for example a multireceptor tyrosine kinase inhibitor, however outcome in microsatellite stable (MSS) tumours is lower (5).

EC is reported to contain the greatest proportion of PI3K mutations of all cancers (6). Of these, PTEN, PIK3CA and PIK3R1 are the most commonly mutated, with rates of 63.5%, 52.2% and 30.9% respectively, much higher to any other cancer studied in The Cancer Genome Atlas dataset (7). It is therefore unsurprising that many novel targeted therapies for recurrent/advanced EC focus on this pathway.

PI3K is a family of kinases that are categorised into three classes. Class I is the best described and most involved in tumourigenesis. The Class I catalytic subunit (p110a) is encoded by the PIK3CA gene, while its regulatory subunit (p85) is encoded by the PIK3R1-3 genes (8). Signals from tyrosine kinase receptors (RTK) and G-protein-coupled receptors in the cell membrane lead to PI3K activation, which in turn phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) and converting it to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 can be dephosphorylated by the PTEN phosphatase and reverted back to PIP2, which halts the downstream effects of PIP3. In the absence of this action, PIP3 recruits phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (AKT) in the inner cell membrane. PDK1 subsequently phosphorylates AKT and this activity increases even further following AKT's phosphorylation by the mammalian target of rapamycin complex 2 (mTORC2) (9).

The activated AKT has several downstream effectors (10). One of the most important is the activation of the mammalian target of rapamycin complex 1 (mTORC1), which results in increased synthesis of proteins and survival, common feature of most cancers (11).

Another well-studied intracellular signalling pathway is the MAPK, which is responsible for several key cellular functions, including proliferation, differentiation and apoptosis (12), and is commonly involved in many cancers (13). Studies have also proposed a direct association between oestrogenic stimulation (common feature in the majority of endometrial cancers) and MAPK activation (14). Each MAPK pathway is a phosphorylating cascade that involves a minimum of three kinases (MAP3K, MAP2K and MAPK) (15). Five MAPK cascades have been characterized in mammals (16) with the most extensively investigated being the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). The MAPK pathway is triggered by cell surface RTK stimulation, followed by Ras activation (16). Ras will then recruit Raf and MEK1/2 phosphorylation subsequently ensues, followed by ERK1/2 phosphorylation.

Several PI3K and/or mTOR inhibitors have been tested *in vitro* with encouraging results (17–19), however, to date have failed to demonstrate efficacy in clinical trials when tested as monotherapies (20–23). Similar outcomes have been reported with MEK inhibitors, with *in vitro* efficacy demonstrated with several agents (Cobimetinib, UO126, Sorafenib and Selumetinib) (24–27) but failure in early phase clinical trials (28, 29). This therefore raises the question as to whether combination therapy, rather than monotherapy, would be a better strategy for any of these targeted agents in clinical practice, as well as highlighting the need for personalized therapies matching targeted inhibitors to driver mutations within the tumour.

To explore novel potential therapies for EC, we tested a PI3K and histone deacetylase (HDAC) inhibitor (CUDC-907) together with a panel of MEK inhibitors, aiming to identify synergistic combinations for killing endometrial cancer cells carrying different driver mutations.

Methods

Cell culture. The Ishikawa cancer cell line was obtained from Merck KGaA (Darmstadt, Germany) and the HEC-1-A cell line from American Type Culture Collection (ATCC® HTB-112™, Manassas, Virginia, USA). Ishikawa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% Fetal

Bovine Serum (FBS) and 1% Non Essential Amino Acids (GibcoTM). HEC-1-A cells were cultured in McCoy's 5A (Modified) medium (GibcoTM), supplemented with 10% FBS. All cells were incubated at 37oC in 5% CO2.

Cell proliferation assay. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay, PromegaTM) was used to assess cell viability following treatment with various inhibitory drugs. 5,000-10,000 cells were transferred to 96-well, flat bottom plates, in triplicates. When reached 70-80% confluence, they were treated with serial concentrations of various inhibitors. Three control wells were used by adding DMSO to it, as all the drugs of our study were diluted in DMSO. Finally, three wells were filled with culture medium only in order to measure the background absorbance. 48-hours after the drug treatment, the reagent was added to the cells at a concentration of 5:1. Following a 2-3 hours incubation in a humidified, 5% CO2 atmosphere, the absorbance of the plate was read at 490nm using Infinite® F50 / Robotic ELISA plate reader (TECANTM) and the data recorded with the MagellanTM data analysis software. The percentage of cell viability was subsequently calculated with Microsoft Excel 2013 by dividing the absorbance (optical density) of the sample with the one of the control and multiplying this by 100. Graphs were created using GraphPad Prism 7.0 software.

Immunoblotting. After treating cells with drug inhibitors, their pellets were collected and lysed with RIPA lysis buffer containing 150mM NaCl, 50mM Tris HCl pH 8.0, 1% Tergitol-type NP-40, 0.1% Sodium Dodecyl Sulfate, 0.5% sodium deoxycholate diluted in distilled water, along with 1% protease inhibitor and 1% phosphatase inhibitor cocktails (Sigma-AldrichTM). Following centrifugation, the supernatants were collected and the protein concentration was calculated using the Bradford assay. Polyacrylamide gel electrophoresis was subsequently performed and the proteins from the gel were transferred to nitrocellulose filter membrane (Amersham; GE). The membranes were incubated with primary antibodies overnight. They were subsequently washed and incubated with secondary antibodies [Goat anti-Rabbit for rabbit primary antibodies (IRDye® 680RD Goat anti-Rabbit IgG, Li-CORTM) and Goat anti-Mouse for mouse primary antibodies (IRDye® 800CW Goat anti-Mouse IgG, Li-CORTM), concentration 1:10,000]. The protein bands analyzed and quantified using Image Studio Lite software version 5.2 (Li-CORTM).

Statistical analysis. IC50 values of inhibitory drugs were calculated after converting the cell viability values derived from MTS assay to percentage of inhibition. This was then converted to a logarithmic value and from the linear regression graph created with GraphPad Prism version 7.0, the IC50 value was calculated. Calculation Index (CI) for combination drug treatments was calculated using the Chou-Talalay method (30). If CI<1, there is synergism, if CI>1 there is antagonism and when CI=1, there is additive effect. CI was calculated using CalcuSyn Version 2.0 software (Biosoft).

Results

EC cancer cells are sensitive to dual PI3K/HDAC inhibition but not to MEK inhibitors.

In order to screen for potential targeted therapies in EC, two endometrial cancer cell lines (Ishikawa and HEC-1-A) were chosen, due to their different properties and genetic background: Ishikawa is known to

carry PTEN and PIK3R1 mutations (18), while HEC-1-A carries KRAS and PIK3CA mutations. Moreover, Ishikawa was derived from a well differentiated uterine adenocarcinoma of an Asian patient, while HEC-1-A was derived from a moderately differentiated papillary adenocarcinoma of a Caucasian background. We first tested the sensitivity of the cell lines to different targeted monotherapies. Starting with CUDC-907, a novel PI3K and HDAC inhibitor shown to be effective in stopping the proliferation of malignant B cells (31). Both cell lines showed sensitivity to CUDC-907 at low concentrations (Figure 1A). As expected, the efficacy was greater in cells carrying PI3K pathway mutations (Ishikawa, IC50=23nM) compared to cells with wild-type PI3K signalling (HEC-1-A, IC50=129nM). We also treated these cells with a panel of MEK inhibitors (Selumetinib, Trametinib and PD0325901, Figure 1A). These inhibitors did not induce cell death in Ishikawa when used as monotherapy. However, in HEC-1-A cells, reduced viability was observed, consistent with a dependence on an activated RAS-MEK-ERK pathway, but only at the higher concentrations tested (over 1μM). Of note, HEC-1-A cells had different sensitivity to each MEK inhibitor used, with PD0325901 being the most effective and Selumetinib having no significant effect.

To better understand the effect of these drugs in EC cells, we analysed how they changed the activation of the PI3K and MAPK pathways. As expected, CUDC-907 reduced the basal levels of AKT phosphorylation in Ishikawa (Figure 1B). On the other hand, none of the MEK inhibitors blocked activation of the MAPK pathway in Ishikawa and instead, MAPK and MEK phosphorylation was increased (Figure 1C). In contrast, a high concentration of PD0325901 reduced MAPK phosphorylation in HEC-1-A cells (Figure 1C). This shows that the toxicity of the MEK inhibitors is not proportional to their inhibition of the pathway. Our results together confirm the relevance of choosing targeted inhibitors based on the pathways mutated in each tumour. They also confirm that inhibiting the PI3K pathway may be in general more useful than inhibiting RAS-MEK-ERK in EC and highlight the varied responses to different MEK inhibitors.

Trametinib has a synergistic effect with CUDC-907 in cells with oncogenic MAPK activation. We next explored potential synergistic combinations between PI3K and MEK inhibitors. We selected Trametinib and PD0325901, but discarded Selumetinib due to its lack of efficacy in our models. As shown in Figure 2A, adding Trametinib did not change the sensitivity of Ishikawa to CUDC-907, however, the combination had a synergistic effect in HEC-1-A cells, combination index (CI) of 0.878, suggesting that PI3K and MEK inhibition together may be a good therapeutic option for EC cells that carry mutations in both pathways. Although Trametinib alone did not suppress the basal levels of phosphorylated MAPK (see Figure 1C), when combined with CUDC-907, it reduced MAPK activity in a dose-dependent manner (Figure 2B). Interestingly, this effect was seen in both cell lines, and therefore independent of the oncogenic activation of the pathway. This indicates that the degree of MAPK inhibition is not proportional to the increase in cell death observed with these combination therapies, suggesting that other off-target effects of these drugs may be contributing to the results.

PD0325901 synergizes with CUDC-907 regardless of the mutation status of the MAPK pathway.

We also tested the combination of CUDC-907 and PD0325901 in EC cells. As shown in Figure 3A, the treatment had synergistic effects in both cell lines. Of note, this was the only combination of CUDC-907 with a MEK inhibitor that showed strong synergism in the Ishikawa cells, CI of 0.381, as it reduced viability from 66% (when Ishikawa cells are treated with CUDC 0.01μM alone) to 36% or 25% when adding 0.1μM or 1μM of PD0325901, respectively. This suggests that certain MEK inhibitors may be effective even in cells with no mutations in the RAS-MEK-ERK pathway. The synergism was still associated with a strong suppression of the basal MAPK pathway activity in these cells (Figure 3B). These data together show that some MEK inhibitors can enhance the effects of PI3K inhibitors in EC cells, even those with no mutations in the MAPK pathway.

Discussion

There is a pressing clinical need for more efficacious systematic therapies for endometrial cancer. In this study we have identified the PI3K and HDAC inhibitor CUDC-907 as being active in EC cell lines.

We have identified that CUDC-907, a PI3K and HDAC inhibitor, was very effective against EC cells, with IC50 values in the nanomolar level. CUDC-907 is currently under investigation in early phase trials, primarily in haematological malignancies, but also in solid tumours (32). To our knowledge, it has not been investigated previously in endometrial cancer and since it has a favourable safety profile (33, 34), it is an attractive candidate for further testing in pre-clinical models.

Our rationale for investigating combinations with MEK inhibitors was born out of the disappointing results of PI3K and mTOR inhibitors tested as monotherapies in clinical trials, for example everolimus, pilaralisib and others (20–23) but also evidence suggesting that MAPK is activated in EC following oestrogen receptor stimulation (14, 35).

Our results suggest that, while monotherapies with MAPK inhibitors may have a limited use in EC, they can successfully enhance other targeted drugs. We have shown that the combination of PI3K and MAPK inhibition can be synergistic and, in some circumstances even independently of the activation status of the pathway. This indicates that phosphorylation of different members of the MAPK pathway would not be sufficient to predict sensitivity to these drugs. In our study we identified the paradoxical activation of the MEK-ERK pathway when cells were treated with MEK inhibitors, as shown by the increased basal levels of MEK and MAPK phosphorylation (Figure 1C). This was also observed previously in a study of our lab made on chronic lymphocytic leukemia, which proposed an upstream reactivating loop as a possible cause (36). This paradoxical activation was more prominent in Ishikawa cells, possibly as a result of wild type genes controlling the MAPK pathway. On the contrary, HEC-1-A cells, which carry KRAS mutations, have a constitutively more active MAPK pathway, and hence this paradoxical activation is more subtle and can be reversed with higher concentrations of MEK inhibitors.

The association between KRAS mutations and MEK inhibitor resistance has been studied previously. In colorectal cancer, a direct correlation between the strength of ERK1/2 signalling and the sensitivity of colorectal cells to MEK1/2 inhibitors (37). This is in keeping with our results, which confirmed that the

KRAS-mutant HEC-1-A cells are more sensitive to MEK inhibitors than the Ishikawa cells. Similarly, a study on 13 EC cell lines concluded that KRAS-mutant cell lines (including HEC-1-A), were more sensitive to the MEK inhibitor Cobimetinib compared to the PTEN-mutant cell lines (24), again in keeping with our results.

The most efficacious combination treatment was CUDC-907 and PD0325901, a drug shown to be safe in a phase 1 clinical trial (29), with efficacy independent of the mutational status of the MAPK pathway. In contrast, the combination of Trametinib and PI3K inhibitor (CUDC-907) was only synergistic in cells carrying a mutated MEK pathway. Interestingly, the synergism observed with these two drug combinations was irrespective of the degree of MAPK inhibition, as both these MEK inhibitors, when used as monotherapy, do not suppress the MAPK pathway. This might be related to complex and variable off-target and signaling effects associated with MEK inhibitors (38).

Study limitations.

The cells lines (HEC-1A and Ishikawa) were chosen because of their different mutational profiles and suited the screening nature of this study. It would arguably be better to perform the experiments in a larger panel of cell lines, which could be more representative of EC, and confirm the prediction of any sensitivity to a certain drug by selectively silencing the appropriate pathway using CRISPR-Cas9 or other genetic tools. Also, there is a longstanding and ongoing debate as to the value of cell lines as a cancer model, although they do have a proven track record of successes (39). Proponents of cell line work emphasize the fact that it is a cheap and easy to grow screening tool available to almost every laboratory, enabling extensive testing, which would be very challenging and costly to conduct in human or animal models.

A negative of cell lines is that they are unable to capture the tumour microenvironment, which is known to impact on tumour behaviour. In vitro cells are also a very poor model in assessing drug-induced toxicities, as these can only be reliably assessed in in vivo models (40).

Conclusions

In summary, the novel PI3K and HDAC inhibitor (CUDC-907), was very effective in reducing viability of endometrial cancer cell lines both as monotherapy and in combination with the MEK inhibitor PD0325901. We propose this drug and combination be taken forward for further testing in pre-clinical models with a view to a potential new combination in the systemic treatment of EC.

Abbreviations

AKT Protein kinase B

CI Calculation Index

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl Sulfoxide

EC Endometrial cancer

ERK Extracellular signal-regulated kinase

FBS Fetal Bovine Serum

HDAC Histone deacetylase

IC50 The concentration of a drug that is required for 50% inhibition in vitro

MAPK Mitogen-activated protein kinase

MMR Mismatch repair

mTOR mammalian target of rapamycin

PDK Phosphoinositide-dependent kinase

PI3K Phosphoinositide 3-kinase

PIP2 Phosphatidylinositol 4,5-bisphosphate

PIP3 Phosphatidylinositol 3,4,5-triphosphate

RIPA Radio immunoprecipitation assay

RTK Receptor tyrosine kinase

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests: EM serves on advisory boards for Inivata and GlaxoSmithKline; received speaker fees from GlaxoSmithKline; has received research grants from Intuitive Surgical and Hope Against Cancer for unrelated work. All other authors have no competing interests.

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Authors' contributions: Experiments were designed and analysed by SM and KP, with DG and EM. All experiments were performed by KP. The manuscript was written by KP, SM and EM. All authors revised and edited the manuscript.

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Figures

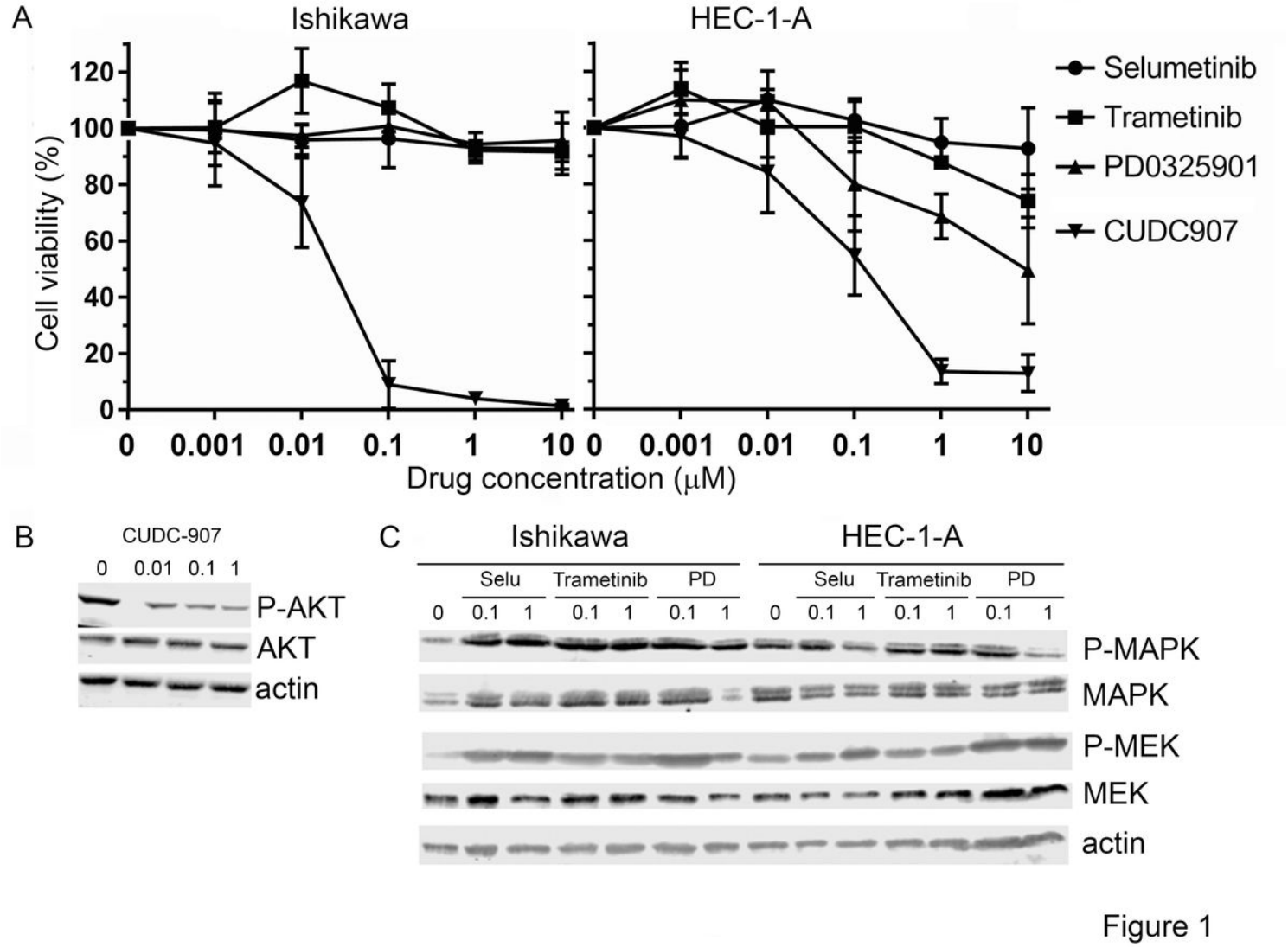

Figure 1

Figure 1

(A) Effect of treatment with serial concentrations of MEK inhibitors (Selumetinib, Trametinib and PD0325901) and the PI3K and HDAC inhibitor CUDC-907 on two endometrial cancer cell lines (Ishikawa and HEC-1-A). Viability was measured 48 hours after treatment using MTS assay. Experiments were performed in triplicates and repeated a minimum of three times. Error bars represent standard deviation of the mean. (B) Western blotting showing the expression of phospho-AKT and AKT in Ishikawa endometrial cancer cells treated with serial concentrations of CUDC-907 (PI3K and HDAC inhibitor) for 24 hours. (C) Western blotting showing the effect on MAPK and MEK phosphorylation of endometrial cancer cells (Ishikawa and HEC-1-A) after treating with serial concentrations of the MEK inhibitors Selumetinib, Trametinib and PD0325901 for 24 hours.

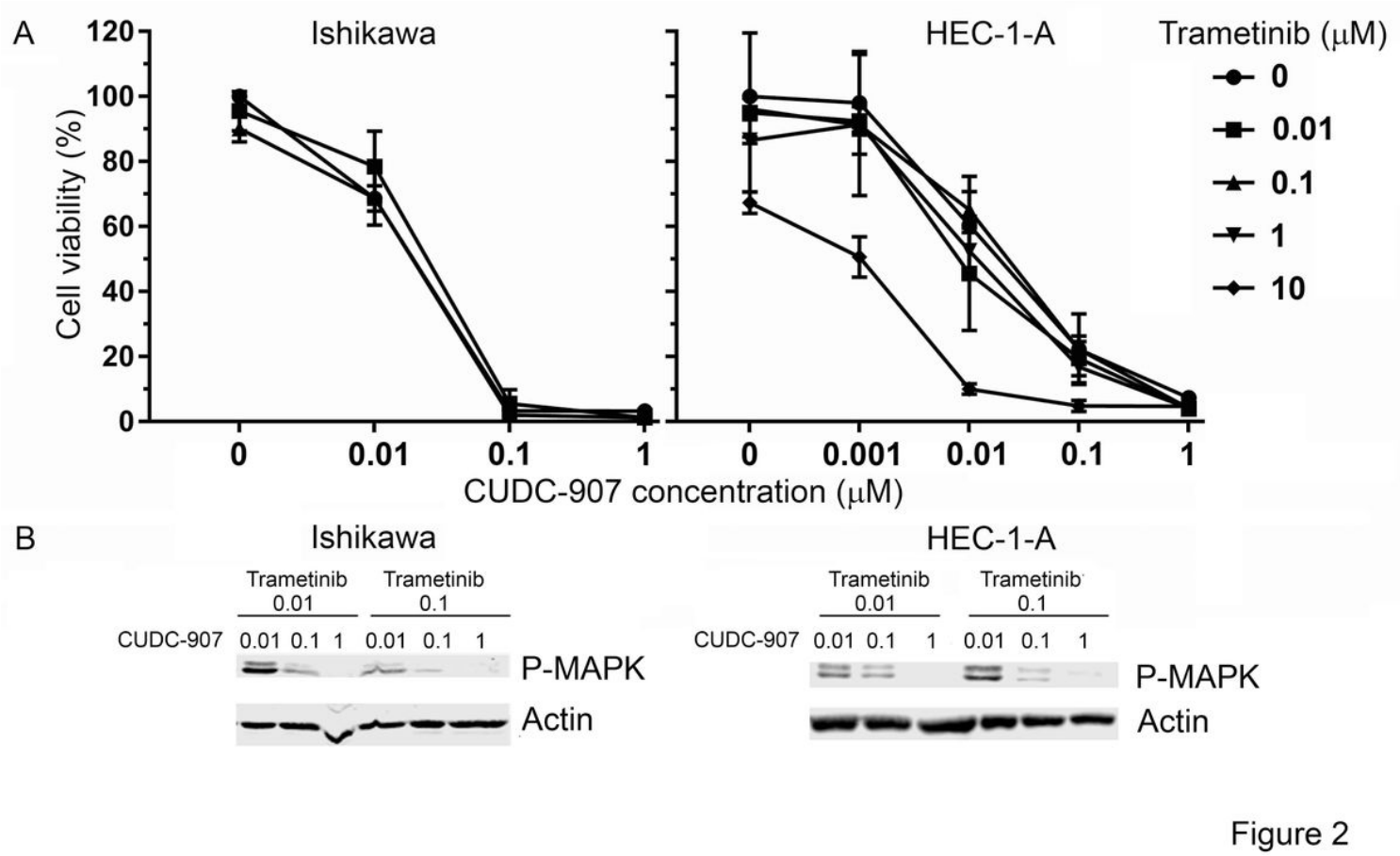

Figure 2

Figure 2

(A) Effect of Trametinib (MEK inhibitor) on viability of endometrial cancer cells. Viability was measured 48 hours after treatment using MTS assay. Experiments were performed in triplicates and repeated a minimum of three times. Error bars represent standard deviation of the mean. (B) Effect of dual MEK and PI3K inhibition (Trametinib and CUDC-907) on MAPK phosphorylation of endometrial cancer cells. Western blotting 24 hours after treatment with serial concentrations of the previously mentioned inhibitors.

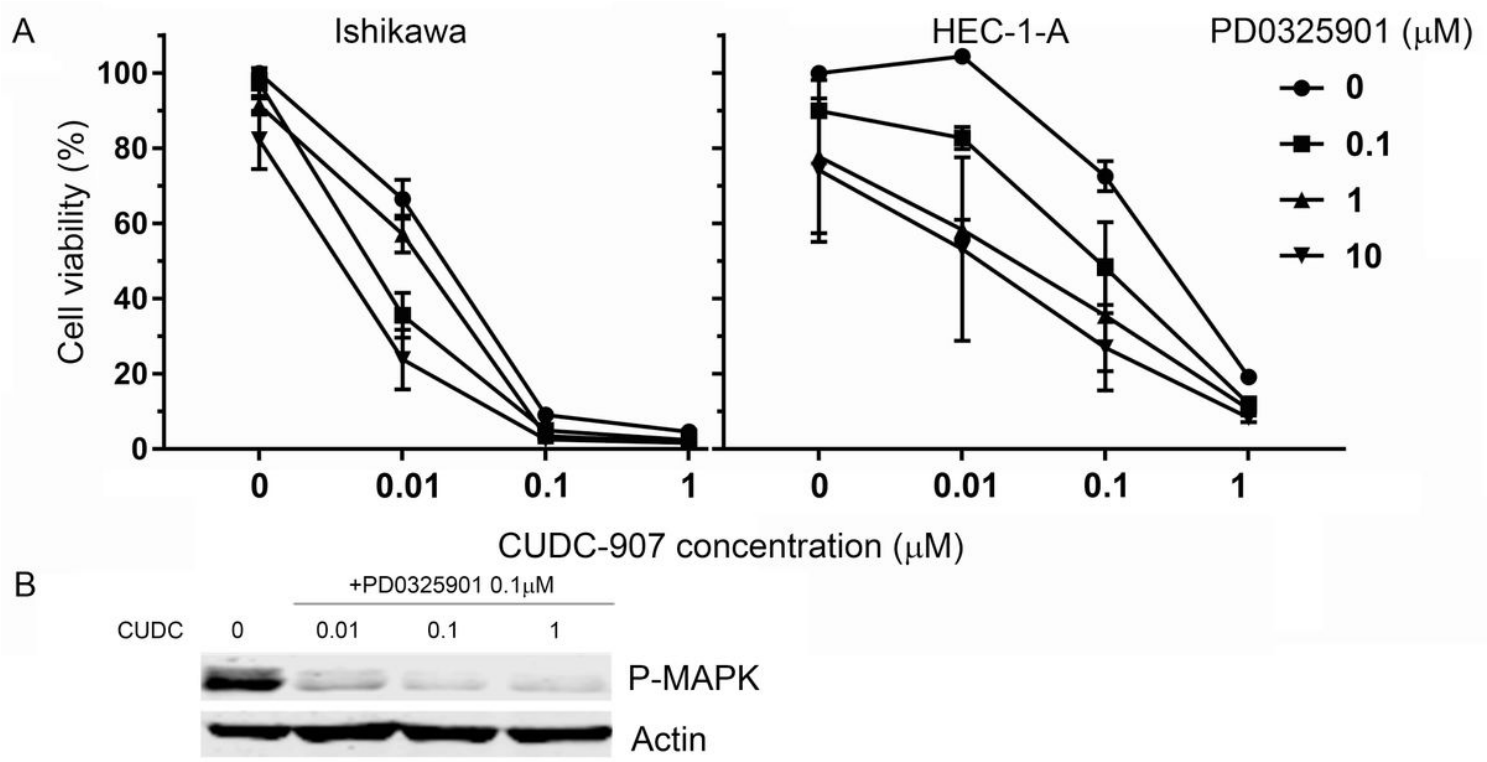

Figure 3

Figure 3

(A) Effect of PI3K and HDAC inhibitor (CUDC-907) combined with MEK inhibitor (PD0325901) in viability of endometrial cancer cells after 48 hours of treatment, using MTS assay. Experiments were performed in triplicates and repeated a minimum of three times. Error bars represent standard deviation of the mean. (B) Effect of dual MEK and PI3K inhibition (PD0325901 and CUDC-907) on MAPK phosphorylation of Ishikawa endometrial cancer cells. Western blotting 24 hours after treatment with serial concentrations of the previously mentioned inhibitors.

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