Preclinical Drug Testing of the CDK 4/6 Inhibitor Palbociclib in Combination With a PI3K or MEK Inhibitor in Colorectal Cancer Cell Lines

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PRECLINICAL DRUG TESTING OF THE CDK 4/6 INHIBITOR PALBOCICLIB IN COMBINATION WITH A PI3K OR MEK INHIBITOR IN COLORECTAL CANCER CELL LINES

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ABSTRACT

Background: Studies have demonstrated the efficacy of Palbociclib (CDK 4/6 inhibitor), Gedatolisib (PI3K/mTOR dual inhibitor) and PD0325901 (MEK1/2 inhibitor) in colorectal cancer (CRC), however single agent therapeutics are often limited by resistance. The main purpose of this in vitro study is to comprehensively test two drug combinations [Palbociclib with Gedatolisib (P+G) and Palbociclib with PD0325901 (P+PD)] to determine the most synergistic combination for clinical development.
Methods: We compared the anti-proliferative effects of both drug combinations in five CRC cell lines with various mutations (Caco-2, DLD-1, LS1034, SNUC4 and LS411N). Reverse Phase Protein Arrays was used to investigate the effects of P+G on the total and phosphoproteins of the signalling pathways. Results: Our results from toxicology experiments indicated that the P+G is a superior combination. The combination of P+G had synergistic anti-proliferative effects in all cell lines [CI range: 0.11-0.69]. The combination of P+PD is also synergistic in all cell lines [CI range: 0.06-0.44], except LS411N with BRAF V600E mutation [CI=14.7]. The combination of P+G caused significant suppression of S6rp(S240/244) in all cell lines, without AKT reactivation. This indicated efficient blockage of PI3K/AKT/mTOR pathway, even in PIK3CA mutated cell lines. The combination of P+G induced BAX and Bcl-2 levels in PIK3CA mutated cell lines. The combination of P+G caused MAPK/ERK reactivation, as seen in total EGFR increase and this was not mutationally exclusive. Conclusion: This in vitro study demonstrated that the combination of P+G has synergistic anti-proliferative effects in both wild-type and mutated CRC cell lines. This data provides good rationale for further in vivo studies for P+G novel combinative therapy in CRC. Separately, the S6rp(S240/244) may serve a promising biomarker of responsiveness.

Keywords: combinative therapy, Palbociclib, Gedatolisib, S6rp(S240/244), colorectal cancer
BACKGROUND

Colorectal cancer (CRC) is the second most common cause of cancer death in Europe. It is estimated that the global burden of CRC will increase by 60%, with 2.2 million new cases and one million deaths worldwide by 2030.\(^1\) In the metastatic CRC setting, compound agent chemotherapies given in combination with the anti-EGFR (epidermal growth factor receptor) or anti-VEGF (vascular endothelial growth factor) monoclonal antibodies are standard clinical practice, providing improvement in patients outcomes reaching median OS between 29 and 36 months (in wild-type).\(^2,3\) However, more than 50% of cases would eventually relapse and subsequent treatment options rarely offer high clinical impact, especially in patients with RAS mutations.\(^4\)

Treatment for CRC is complexed and often limited by resistance, which can be intrinsic or acquired. The crosstalk between the PI3K/AKT/mTOR (phosphatidylinositol-3-kinase/acutely transforming retrovirus/mammalian target of rapamycin) and MAPK/ERK (mitogen activated protein kinase) pathways has been recognised as a key mechanism of resistance in Oncology therapy.\(^5\) Other mechanisms that lead to resistance evolution include: 1) Formation of new secondary site resistance mutations within the target kinase; 2) Activation of escape bypass routes involving pathways such as the MAPK/ERK and PI3K/AKT/mTOR; 3) Dysregulation of downstream effectors; 4) Transformation into pro-metastatic phenotypes which enable the cancer cells to survive the effects of treatment; 5) Immune adaption within the tumour microenvironment to enable cancer cell survival, via either immune-dependent or immune-independent processes.\(^6\) Theoretically,
when compared to the single agents, combined therapies can produce synergistic inhibition in a relatively safe manner to reduce multiple growth signal transmission responsible for development of drug resistance. There is now a growing appreciation for using the combinative therapeutic approaches which can be exploited through multiple modalities such as radiotherapy, chemotherapy, immunotherapy or targeted agents.

The PI3K/AKT/mTOR and MAPK/ERK signalling pathways are highly implicated in CRC pathogenesis with key mutations like RAS, BRAF and PIK3CA arising from both pathways. There are data from Phase I/II trials to support the use of Palbociclib (CDK 4/6 inhibitor)\(^7\)-\(^9\), Gedatolisib (PI3K/mTOR dual inhibitors)\(^7\),\(^12\),\(^13\) and PD0325901 (selective MEK1/2 inhibitor)\(^14\)-\(^16\) in various types of cancers. These inhibitors failed to cause significant tumour regression when used as single agents because of resistance. As shown in breast cancer models, the combination of CDK 4/6 inhibitor with a PI3K/mTOR inhibitor can produce synergistic effects. This specific drug combination can overcome treatment-related resistance by preventing RSK activation and subsequent MAPK/ERK pathway activation.\(^17\)-\(^20\) Currently, there are three active Phase I trials evaluating the combination of Palbociclib with Gedatolisib in patients with refractory malignancies including CRC.\(^7\)-\(^9\) Similarly, another Phase Ib trial is evaluating the effectiveness of combining a different PI3K/mTOR dual inhibitors (LY3023414) with a CDK4/6 inhibitor (Abemaciclib) in multiple common cancers.\(^21\) The combined approach of Palbociclib with Gedatolisib to prevent the emergence of resistance in breast cancer, is hypothetically applicable to
other cancers including CRC. In parallel, there is strong preclinical evidence for evaluation of co-inhibition using Palbociclib with PD0325901 in CRC.\textsuperscript{14-16} The combination of Gedatolisib with PD0325901 is toxic in humans and not advisable as previously shown.\textsuperscript{22}

In summary, combined drug inhibition have multiple advantages over single agents since it can maximise anti-proliferative efficacy within an acceptable overlapping drug toxicity limit. In comparison to single agents, the combined drug therapies exhibit multiple-target inhibitions and cellular regulatory actions, thus more likely to be effective in attenuating drug resistance pathways. This method has been exploited in various cancers, particularly in breast cancer. As we have noted there is a research-gap in CRC, therefore we want to explore novel therapeutics using combinative drug approaches. We hypothesise that the combination of Palbociclib with either Gedatolisib or PD0325901 can produce synergistic benefits with potential clinical application in refractory CRC.

**METHODS**

**Aims**

**Specific Aim 1:** To demonstrate \textit{in vitro} that Palbociclib can act synergistically with either Gedatolisib or PD0325901 as reflected by improved anti-proliferative effects in five CRC cell lines with different mutational backgrounds. This preclinical information will be utilised for future \textit{in vivo} evaluation and clinical trial efforts.
**Specific Aim 2:** To examine the effects of the drug combination on the PI3K/AKT/mTOR, MAPK/ERK and apoptotic pathways using Reverse Phase Protein Arrays (RPPA) technology. The RPPA analysis will likely identify biomarker(s) which may be used as monitoring tool(s) of the combinative therapy. This information will also offer insight into the specific mechanisms of two rather than single agent effects in mediating and reducing the effects of drug resistance development.

**Cell Lines**

Five human CRC epithelial cell lines with varying mutational backgrounds were used [refer Table 1]. The cell lines were obtained from the American Tissue Type Collection (ATCC, USA) lines (DLD-1, LS411N, LS1034, Caco-2) and Korean Cell Line Bank (KCLB, South Korea) (SNUC4). The cell line mutational status was determined using Cancer Cell Line Encyclopaedia (CCLE) and mutations were verified by us, by using the MassARRAY (Agena Bioscience™) system platform.

Caco-2 cells were *wild-type* for mutations in PI3K/AKT/mTOR and MAPK/ERK pathways. DLD-1 cells have *KRAS* G13D and *PIK3CA* E545K mutations. LS411N cells have a *BRAF* V600E mutation. LS1034 cells have a *KRAS* A146T mutation. SNUC4 cells have a *PIK3CA* E545G mutation. Cell line characteristics and culture details are detailed in Additional File 1.

**Drug Inhibitors**

Palbociclib (CDK 4/6 inhibitor), Gedatolisib (PI3K/mTOR dual inhibitors) and PD0325901 (selective MEK 1/2 inhibitor) were purchased directly
from Selleckham. The drugs were prepared in 100% dimethylsulfoxide (DMSO) at the stock concentrations of 10mM, 5mM and 10mM, respectively. The two drug combinations tested were Palbociclib with Gedatolisib (P+G) and Palbociclib with PD0325901 (P+PD).

**Proliferation Assays and Drug Combination Analysis**

The acid phosphatase assay was used to test the anti-proliferative effects of Palbociclib, Gedatolisib and PD0325901, alone and in combination in each cell line. This was performed over a 6-day period. Cell lines were plated at 1x10^4 cells/mL into 96-well plates (100µL per well) and incubated at 37°C with 5% CO₂ for 24 hours. 200µL of sterile H₂O was added around the edges of the plate to prevent it from drying out. Following 24 hours incubation, drugs were added at the indicated concentrations and incubated at 37°C for 5 days. On Day 6, all drug was removed, washed and processed for absorbance measurement at 405nm using a 96-well plate reader. Inhibition of proliferation was calculated relative to untreated controls to obtain the dose of half maximal inhibitory concentration (IC₅₀). The individual inhibitors IC₅₀ values were used for dosing guidance in the drug combinations analysis. The doses used for the two drugs combinations in each cell lines are listed in Table 1.

**Protein extraction from cell lines**

For RPPA investigations, we selected the Palbociclib and Gedatolisib because both drugs demonstrated synergism as combinations in all the cell lines tested in our study. The Caco-2, DLD-1, LS1034 and SNUC4 cell lines were selected. The cell lysates were prepared according to the
MD Anderson protocol as previously described by us. The complete methods of protein extraction from cell lines are detailed in Additional File 2.

**Reverse Phase Protein Array (RPPA)**

Cell lysates with protein concentration of 1.5μg/μL for each replicate were prepared. Before RPPA processing, each sample was solubilised in sodium dodecyl sulfate (SDS) sample buffer (40% Glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8, 50nM Bond-breaker TCEP solution) and heated to 80°C for 5 minutes. RPPA analysis was carried out using triplicate biological replicates following 30 minutes and 4 hours treatment with Palbociclib, Gedatolisib and the combination in Caco-2, DLD-1, LS1034 and SNUC4 cell lines. The cell lines were treated at the same fixed ratio doses used in the drug combination proliferative analysis. The full lists of antibodies used are listed in Additional File 3.

**Statistical Analysis**

CalcuSyn software version 3.1 (Biosoft) was used to calculate IC\textsubscript{50} and combination index at effective dose 50 (CI). The CI values were determined using the Chou-Talalay equation on CalcuSyn. A CI<0.9 is considered synergistic, between 0.9 and 1.0 additive and >1.1 is antagonistic. Each experiment was repeated 3 to 4 times. For the RPPA experiments, the mean and standard error of mean (SEM) were calculated from three biologically independent protein samples analysed on the same RPPA slide. The mean and SEM were normalised to the vehicle-treated control samples. Based on our internal precision studies, we can detect changes in protein expression with a
coefficient of variance (CV) of less than 20%.$^{25}$ To evaluate the effect of the combination treatment, a one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test was used (GraphPad PRISM version 8). To compare the effects of Palbociclib, Gedatolisib and the combination treatment on protein expression and phosphorylation, the Kruskal-Wallis non-parametric test was used. The p<0.05 value was considered statistically significant.

RESULTS

Effects of Palbociclib, Gedatolisib and PD0325901 in CRC cell lines

The corresponding IC$_{50}$ values of single-agent Palbociclib, Gedatolisib and PD0325901 in CRC cell lines are summarised in Table 1. Arbitrarily defining the peak of plasma concentration 15µM as a cut off sensitivity to Palbociclib, the Caco-2, DLD-1 and LS1034 cells were resistant to Palbociclib (IC$_{50}$ >15µM). The LS411N and SNUC4 cells were sensitive to Palbociclib (IC$_{50}$: LS411N=0.8µM; SNUC4=1.7µM). The LS1034 cells was relatively resistant to Gedatolisib (IC$_{50}$=7.2µM). All other cell lines were more sensitive to Gedatolisib with IC$_{50}$ values generally within nanomolar ranges (IC$_{50s}$: LS411N=76nM; DLD-1=183nM; SNUC4=400nM; Caco-2=1200nM). The cell lines also have high sensitivity to PD0325901 (IC$_{50s}$: LS411N=0.001µM; LS1034=0.013µM; SNUC4=0.014µM; Caco-2=4.0µM; DLD-1=13µM). The Caco-2 wild-type cell line was relatively resistant to all drugs, in particular Palbociclib (IC$_{50}$ >15µM).
Effect of Palbociclib in combination with Gedatolisib (P+G) or PD0325901 (P+PD) in CRC cells lines

Drug combination analysis showed that combination P+G has a synergistic anti-proliferative effect in all CRC cell lines tested [refer Table 2; Figure 1]. The combination of P+G is very synergistic in LS1034 cells (KRAS mutation; CI=0.11). The combination of P+G is also synergistic in DLD-1 (KRAS and PIK3CA mutated; CI=0.58) and Caco-2 (wild-type; CI=0.33) cells. The combination of P+G is minimally synergistic in LS411N (BRAF V600E mutated; CI=0.64) and SNUC4 (PIK3CA mutated; CI=0.69) cells.

The combination of P+PD produces a synergistic growth inhibitory response in all cell lines, apart from LS411N cells (CI values: DLD-1=0.06; Caco-2=0.17; LS1034=0.29; SNUC4=0.44; LS411N=14.7) [refer Table 2; Figure 2].

RPPA Analysis

We conducted RPPA analysis using 40 primary antibodies representing multiple nodes of the PI3K/AKT/mTOR, MAPK/ERK and intracellular apoptotic signalling pathways, following 30 minutes and 4 hours treatment with Palbociclib, Gedatolisib and the combination in the Caco-2, DLD-1, LS1034 and SNUC4 cell lines. We selected Palbociclib and Gedatolisib because this drug combination was synergistic in all cell lines tested in our cell proliferative study.

Overall, there was little difference between the protein levels measured at the 30-minute and 4-hour timepoints following exposure to
Palbociclib, Gedatolisib or the combination, therefore we focused on the 4-hour timepoint.

**Effect of the combination of Palbociclib with Gedatolisib (P+G) on inhibition of the PI3K/AKT/mTOR pathway**

Following 4 hours of treatment, the combination of P+G showed synergistic inhibition of some components of the PI3K/AKT/mTOR pathway compared to other treatment arms [refer Figures 3A to 3D]. The combination of P+G caused significant suppression of phosphorylated S6 Ribosomal protein S6rp(S240/244) across all cell lines [Caco-2 fold change= -4.55±0.01, *p*<0.001; DLD-1 fold change= -3.85±0.05, *p*=0.012; LS1034 fold change= -2.70±0.02, *p*<0.001 and SNUC4 fold change= -2.86±0.07, *p*<0.001]. The combination of P+G also caused significant suppression of S6rp(S235/236) in Caco-2 [fold change= -4.70±0.08, *p*=0.001], DLD-1 [fold change= -2.85±0.18, *p*=0.021] and a close to significant suppression in LS1034 [fold change= -1.74±0.02, *p*=0.050] compared to control. The suppression of S6rp(S240/244) and S6rp(S235/236) was much more marked with the combination of P+G than either Palbociclib or Gedatolisib alone.

We did not observe increases in phosphorylated PDK1(S241) or AKT(S473 and T308) with the combination of P+G, suggesting that there was no feedback activation of AKT signalling [refer Figures 3A to 3D]. In contrast, AKT(S473) and PDK1(S241) did increase in some cells (Caco-2 and DLD-1) following treatment with single agent Gedatolisib, possibly reflective of feedback loop activity. In SNUC4 cells, PDK1(S241) was significantly reduced by P+G in combination [fold
change= -1.52±0.02, \( p=0.006 \) compared to control. In comparison to control, the combination of P+G appeared to exert greater suppression of phosphorylated mTOR(S448) than Palbociclib or Gedatolisib alone, especially in DLD-1 cells [fold change= -1.18±0.13, \( p=0.236 \)] and LS1034 cells [fold change= -1.41±0.36, \( p=0.316 \)]. However, these changes were not statistically significant. The combination of P+G caused no levels changes of phosphorylated mTOR(S2481) in all cell lines after 4 hours of treatment compared to control.

The combination of P+G caused significant increased \textit{PTEN} levels compared to control in LS1034 cells [fold change=1.29±0.05, \( p=0.011 \)] and Caco-2 cells [fold change=1.17±0.02, \( p=0.022 \)].

\textbf{Effect of the combination of Palbociclib with Gedatolisib (P+G) on apoptosis and cell cycle}

The expression and phosphorylation of the intracellular apoptotic signalling proteins were assessed to determine if the addition of Gedatolisib can enhance the actions of Palbociclib during cell cycle progression to increase apoptosis [refer Figures 4A to 4D]. In comparison to control and in contrast with single agent Palbociclib or Gedatolisib, the combination treatment for 4 hours resulted in increased BAX and Bcl-2 markers. The combination of P+G significantly increased BAX levels in DLD-1 [fold change=1.26±0.06, \( p=0.039 \)], SNUC4 [fold change=1.17±0.09, \( p=0.019 \)] and Caco-2 cells [fold change=1.21±0.07, \( p<0.001 \)]. We also observed a significantly increased Bcl-2 levels in DLD-1 [fold change=1.09±0.08, \( p=0.017 \)] and SNUC4 [fold
change=1.07±0.05, \ p=\lt 0.001 \) cells treated with drug combination again unlike with single agent Palbociclib or Gedatolisib alone.

The increase in BAX and Bcl-2 markers in DLD-1 and SNUC4 cells by the combination therapy were not associated with an increase in the levels of caspase 3, caspase 8, cleaved caspase 7, cleaved caspase 9 and cleaved PARP, possibly indicating they were not sufficient to induce total apoptosis. However, it is possible that the 4-hour timepoint was too early to measure the late proteomic alterations associated with apoptosis. Of interest, the addition of Gedatolisib to Palbociclib did not induce any changes on the phosphorylated Ribosomal protein Rb(S807/811).

**Effects of the combination of Palbociclib with Gedatolisib (P+G) on EGFR and the MAPK/ERK pathway**

One of the mechanisms of the development of resistance to PI3K-targeted inhibitors is reactivation of membrane RTKs and/or the MAPK/ERK signalling cascade. Our RPPA data showed an increase in total EGFR in all cell lines after 4 hours of drugs treatment [refer Figures 5A to 5D]. For example, after the combination treatment, the total EGFR upregulation levels compared to control arms were as follows: Caco-2 fold change=2.67±0.12; \ p\lt 0.001, \ DLD-1 fold change=2.64±0.49; \ p\lt 0.001, \ SNUC4 fold change=2.39±0.10; \ p\lt 0.001 and LS1034 fold change=1.71±0.14; \ p=0.072. Although MAPK(T202/Y204) phosphorylation did not increase following any treatment, MEK1/2(S217/221) phosphorylation did increase after single agent and combination treatments in DLD-1 cells, as did levels of E2F1,
suggesting global activation of MAPK/ERK signalling, including with the combination treatment. In details, the fold changes for MEK1/2(S217/221) with the combination treatment=2.09±0.30; \( p<0.001 \), Gedatolisib=1.81±0.20; \( p=0.003 \) and Palbociclib=1.64±0.19; \( p=0.013 \) whilst the fold changes for E2F1 with the combination treatment=1.50± 0.03; \( p<0.001 \), Gedatolisib=1.40±0.12; \( p=0.005 \) and Palbociclib=0.94±0.07;\( p=0.845 \).

Of note, there was also an increase in Ribosomal S6 Kinase RSK, in particular with the combination treatment compared to control arm e.g. in DLD-1 [fold change=1.65±0.20; \( p=0.025 \)] and SNUC4 [fold change=1.54±0.12, \( p<0.001 \)] cells. This may also reflect the MAPK/ERK reactivation which occurs in some cell lines, particularly after treatment with P+G in combination.

All the p-values for the 4-hour timepoint RPPA analysis are provided in Tables 3A and 3B. The full list of p-values for the comparison of all antibodies measured between 30-minute and 4-hour treatment timepoints are in Additional File 4.
Table 1: The inhibitory concentration 50 (IC$_{50}$) values and subsequent combinative drug doses used for Palbociclib (CDK 4/6 inhibitor), Gedatolisib (PI3K/mTOR dual inhibitors) and PD0325901 (selective MEK 1/2 inhibitor) against the tested colorectal cancer cell lines with various mutational backgrounds. The IC$_{50}$ values highlighted in bold are indicative of resistant cell lines to the drug. Each experiment was repeated 3 to 4 times. ATCC=American Tissue Type Collection; KCLB=Korean Cell Line Bank

| Cell Line       | Mutational Status | IC$_{50}$ Palbociclib | IC$_{50}$ PD0325901 | IC$_{50}$ Gedatolisib | Palbociclib + PD0325901 Doses | Palbociclib + Gedatolisib Doses |
|-----------------|-------------------|------------------------|---------------------|-----------------------|-------------------------------|---------------------------------|
| Caco-2 (ATCC HTB-37) | Wild-Type          | >15µM                  | 4.0µM               | 1200nM                | 5µM + 5µM                     | 5µM + 1µM                      |
| DLD-1 (ATCC CCL-221) | KRAS G13D, PIK3CA E545K | >15µM                  | 13µM                | 183nM                 | 2µM + 5µM                     | 2µM + 200nM                    |
| LS411N (ATCC CRL-2159) | BRAF V600E         | 0.8µM                  | 0.001µM             | 76nM                  | 2µM + 5µM                     | 2µM + 200nM                    |
| LS1034 (ATCC CRL-2158) | KRAS A146T         | >15µM                  | 0.013µM             | 7200nM                | 3µM + 500nM                   | 3µM + 400nM                    |
| SNUC4 (KCLB 0000C4) | PIK3CA E545G       | 1.7µM                  | 0.014µM             | 400nM                 | 3µM + 25nM                    | 3µM + 400nM                    |
Table 2: Combination indexes at effective dose 50 (CI) for two drug combinations i.e. Palbociclib with Gedatolisib (P+G) and Palbociclib with PD0325901 (P+PD) tested in this study. The CI effects are in vitro drug response in five colorectal cancer cell lines. A CI < 0.9 is indicative of synergistic, between 0.9 and 1.0 is additive, and >1.1 is antagonistic

| Cell Line  | Drug Combinations | CI         |
|------------|-------------------|------------|
| Caco-2     | P+PD              | 0.17       |
|            | P+G               | 0.33       |
| DLD-1      | P+PD              | 0.06       |
|            | P+G               | 0.58       |
| LS411N     | P+PD              | 14.7 (antagonistic) |
|            | P+G               | 0.64       |
| LS1034     | P+PD              | 0.29       |
|            | P+G               | 0.11       |
| SNUC4      | P+PD              | 0.44       |
|            | P+G               | 0.69       |
Figure 1: Cell growth inhibitory effects of the combination of Palbociclib with Gedatolisib in DLD-1, LS1034, SNUC4, LS411N and Caco-2 cell lines. Each cell line was treated with increasing concentrations of Palbociclib, Gedatolisib and the combination at various ratio doses which were pre-determined by the single agent. IC50 values. The x-axis represents the combined drugs doses in the ratio of Palbociclib's dose. Cell viability was assessed using a 6-day acid phosphatase assay. The graphs showed the mean cell growth +/- standard error of mean (SEM) from minimum 3 repeats in each cell lines. CI=Combination Index at effective dose 50
**Figure 2:** Cell growth inhibitory effects of the combination of Palbociclib with PD0325901 in DLD-1, LS1034, SNUC4, LS411N and Caco-2 cell lines. Each cell line was treated with increasing concentrations of Palbociclib, PD0325901 and the combination at various ratio doses which were pre-determined by the single-agent IC50 values. The x-axis represents the combined drugs doses in the ratio of Palbociclib's dose. Cell viability was assessed using a 6-day acid phosphatase assay. The graphs showed the mean cell growth +/- standard error of mean (SEM) values from minimum 3 repeats in each cell lines. CI=Combination Index at effective dose 50
Figures 3A to 3D: RPPA analysis in Caco-2, DLD-1, LS1034 and SNUC4 cell lines following 4 hours treatment with Palbociclib, Gedatolisib and the combination. Significant suppression of S6rp(S240/S244) and S6rp(S235/S236) without an increase in phosphorylated AKT(S473 and T308) was observed in cell lines with the combination P+G. Error bars are representative of independent triplicate experiments. All p-values were generated by the Kruskal Wallis test. *p< 0.05, **p< 0.002, ***p< 0.001
Figures 4A to 4D: RPPA analysis in Caco-2, DLD-1, LS1034 and SNUC4 cell lines following 4 hours treatment with Palbociclib, Gedatolisib and the combination. Elevation of BAX and Bcl-2 markers was observed in Caco-2, DLD-1 and SNUC4 cells with the combination P+G, in contrast to therapy with Palbociclib or Gedatolisib alone. No significant change in pRb(S807/S811) was observed following treatment in the cell lines. Error bars are representative of independent triplicate experiments. All p-values were generated by the Kruskal Wallis test. *p< 0.05, **p< 0.002, ***p< 0.001
Figures 5A to 5D: RPPA analysis in Caco-2, DLD-1, LS1034 and SNUC4 cell lines following 4 hours treatment with Palbociclib, Gedatolisib and the combination. Significant total EGFR upregulation in all cell lines was observed. Error bars are representative of independent triplicate experiments. All p-values were generated by the Kruskal Wallis test. *p< 0.05, **p< 0.002, ***p< 0.001
**Table 3A:** Adjusted p-values for the antibodies in Caco-2 and DLD-1 cell lines in Reverse Phase Protein Arrays (RPPA) analysis following 4 hours of treatment with Palbociclib (P), Gedatolisib (G) and the combination in comparison to DMSO-treated control cells. DMSO= dimethylsulfoxide.

| Cell Lines | Caco-2 | DLD-1 |
|------------|--------|-------|
| Treatment  | Combination | P | G | Combination | P | G |
| Antibodies | | | | | | |
| Total AKT  | 0.865 | 0.941 | 0.852 | 0.101 | **0.013** | 0.392 |
| AKT(S473)  | 0.998 | 0.065 | 0.002 | 0.766 | 0.972 | 0.196 |
| AKT(T308)  | 0.066 | 0.323 | 0.361 | 0.689 | 0.942 | 0.986 |
| Total mTOR | 0.110 | 0.931 | 0.992 | 0.278 | 0.968 | 0.994 |
| mTOR(S2448) | 0.255 | 0.757 | 0.365 | 0.236 | 0.097 | 0.779 |
| mTOR(S2481) | 0.542 | 0.999 | 0.886 | 0.368 | 0.279 | 0.402 |
| P70S6K(T389) | 0.849 | 0.956 | 0.950 | 0.753 | 0.775 | >0.999 |
| GSK3β(S9)  | 0.006 | 0.005 | 0.021 | 0.844 | 0.429 | 0.786 |
| PDK1       | 0.007 | 0.048 | 0.346 | 0.284 | 0.284 | 0.084 |
| PDK1(S241) | 0.937 | 0.017 | 0.010 | 0.224 | 0.365 | **0.001** |
| PI3-Kinase110α | 0.947 | **0.046** | 0.875 | 0.997 | **0.036** | 0.076 |
| S6rp(S235/236) | 0.011 | 0.931 | 0.539 | 0.021 | 0.998 | 0.927 |
| S6rp(S240/244) | <0.001 | <0.001 | **0.012** | 0.012 | 0.245 | 0.878 |
| PTEN       | 0.022 | 0.881 | 0.695 | 0.259 | 0.939 | 0.985 |
| EGFR       | <0.001 | <0.001 | <0.001 | <0.001 | **0.002** | **0.010** |
| c_RAF      | 0.057 | 0.510 | 0.692 | 0.993 | 0.379 | 0.253 |
| MAPK-ERK1/2 | 0.462 | 0.438 | 0.829 | 0.343 | 0.637 | 0.902 |
| MAPK(T202/Y204) | 0.055 | 0.084 | 0.845 | 0.265 | 0.610 | 0.465 |
| Total MEK1/2 | 0.452 | 0.061 | 0.497 | 0.558 | >0.999 | 0.583 |
| MEK1/2(S217/221) | 0.355 | 0.244 | 0.592 | <0.001 | **0.019** | **0.004** |
Table 3A (continued): Adjusted p-values for the antibodies in Caco-2 and DLD-1 cell lines in Reverse Phase Protein Arrays (RPPA) analysis following 4 hours of treatment with Palbociclib (P), Gedatolisib (G) and the combination in comparison to DMSO-treated control cells. DMSO =dimethylsulfoxide.

| Cell Lines | Caco-2 | DLD-1 |
|------------|--------|-------|
| Treatment  | Combination | P   | G   | Combination | P   | G   |
| Antibodies |         |      |     |            |     |     |
| Total Rb   | 0.034  | <0.001| <0.001| 0.497       | 0.254| 0.087|
| pRb(S807/811) | 0.487 | >0.999| 0.877 | 0.902 | 0.322 | 0.529 |
| E2F1       | <0.001| 0.872 | 0.236| <0.001 | 0.845| 0.005|
| RSK        | 0.819  | 0.836| 0.963| 0.025 | 0.281 | 0.951|
| cMET       | 0.700  | 0.221| 0.344| 0.407 | 0.314 | 0.727|
| p53        | 0.999  | 0.876| 0.955| 0.605 | 0.744 | 0.095|
| BAX        | <0.001| >0.999| 0.916| 0.039 | 0.862 | 0.048|
| Bcl-2      | 0.580  | 0.027| 0.933| 0.017 | 0.742 | 0.798|
| Bcl-xl     | 0.884  | 0.540| 0.160| 0.757 | 0.679 | 0.555|
| Caspase 3  | >0.999 | 0.988| 0.996| 0.681 | 0.162 | 0.539|
| Caspase 9  | >0.999 | 0.986| 0.686| 0.782 | 0.871 | 0.980|
| Caspase 8  | >0.999 | 0.986| 0.686| 0.799 | 0.819 | 0.982|
| c_Caspase 7(D198) | 0.966 | 0.962| 0.359| 0.064 | 0.570 | 0.123|
| c_Caspase 9(D315) | 0.811 | 0.070| 0.998| 0.983 | 0.054 | 0.983|
| Total PARP | 0.107  | 0.731| 0.999| 0.709 | 0.881 | 0.969|
| c_PARP     | 0.993  | 0.954| 0.778| 0.692 | 0.187 | 0.997|
Table 3B: Adjusted p-values for the antibodies in LS1034 and SNUC4 cell lines in Reverse Phase Protein Arrays (RPPA) analysis following 4 hours of treatment with Palbociclib (P), Gedatolisib (G) and the combination in comparison to DMSO-treated control cells. DMSO= dimethylsulfoxide.

| Cell Lines | LS1034 |   | SNUC4 |   |
|------------|--------|---|-------|---|
| Treatment  | Combination | P | G | Combination | P | G |
| Antibodies |        |   |   |        |   |   |
| Total AKT | 0.976  | >0.999 | 0.982 | 0.280 | 0.670 |
| AKT(T308) | 0.959  | 0.135 | 0.979 | 0.775 | 0.233 | 0.895 |
| AKT(T308) | 0.264  | 0.966 | 0.873 | 0.604 | 0.438 | 0.665 |
| Total mTOR| 0.105  | 0.100 | 0.019 | 0.052 | 0.971 | 0.867 |
| mTOR(S2448)| 0.316 | 0.325 | >0.999 | 0.832 | 0.002 | 0.010 |
| mTOR(S2481)| 0.999 | 0.987 | 0.948 | 0.999 | 0.984 | >0.999 |
| P70S6K(T389)| 0.255 | 0.995 | 0.277 | >0.999 | 0.010 | 0.340 |
| GSK3β(S9) | >0.999 | 0.984 | 0.921 | 0.647 | >0.999 | 0.987 |
| PDK1      | 0.961  | 0.970 | 0.037 | 0.996 | 0.930 | 0.381 |
| PDK1(S241)| >0.999 | 0.868 | 0.610 | 0.006 | 0.775 | 0.322 |
| PI3-Kinasep110α | 0.800 | 0.032 | 0.986 | 0.998 | 0.997 | 0.035 |
| S6rp(S235/236)| 0.050 | 0.992 | 0.090 | 0.593 | 0.930 | 0.993 |
| S6rp(S240/244)| <0.001 | 0.090 | 0.995 | <0.001 | 0.025 | 0.124 |
| PTEN      | 0.011  | 0.583 | 0.497 | 0.950 | 0.894 | 0.986 |
| EGFR      | 0.072  | 0.241 | 0.001 | 0.001 | 0.006 | 0.002 |
| c_RAF     | 0.929  | 0.220 | 0.998 | 0.323 | 0.746 | 0.972 |
| MAPK-ERK1/2| >0.999 | 0.998 | 0.404 | 0.459 | 0.627 | 0.976 |
| MAPK(T202/Y204)| 0.427 | 0.167 | 0.382 | 0.968 | >0.999 | 0.119 |
Table 3B (continued): Adjusted p-values for the antibodies in LS1034 and SNUC4 cell lines in Reverse Phase Protein Arrays (RPPA) analysis following 4 hours of treatment with Palbociclib (P), Gedatolisib (G) and the combination in comparison to DMSO-treated control cells. DMSO= dimethylsulfoxide.

| Cell Lines | LS1034 | SNUC4 |
|------------|--------|-------|
| Treatment  | Combination | P | G | Combination | P | G |
| Antibodies |               |    |   |           |    |   |
| Total MEK1/2 | 0.990 | 0.797 | 0.362 | 0.080 | 0.296 | 0.070 |
| MEK1/2(S217/221) | <0.001 | 0.924 | 0.675 | 0.363 | 0.155 | 0.841 |
| Total Rb | 0.001 | <0.001 | <0.001 | 0.984 | 0.641 | 0.02 |
| pRb(S807/811) | 0.822 | 0.100 | 0.241 | 0.415 | 0.938 | 0.112 |
| E2F1 | 0.316 | 0.192 | 0.388 | 0.996 | 0.027 | 0.656 |
| RSK | 0.097 | 0.591 | 0.355 | <0.001 | 0.088 | 0.382 |
| cMET | 0.540 | 0.861 | 0.838 | 0.918 | 0.995 | 0.011 |
| p53 | >0.999 | 0.543 | 0.010 | 0.919 | 0.925 | >0.999 |
| BAX | 0.339 | 0.524 | 0.996 | 0.019 | 0.884 | 0.611 |
| Bcl-2 | 0.951 | >0.999 | 0.938 | <0.001 | 0.852 | 0.346 |
| Bcl-xl | 0.905 | 0.459 | 0.997 | 0.971 | 0.901 | >0.999 |
| Caspase 3 | 0.041 | 0.702 | 0.380 | 0.904 | 0.069 | 0.964 |
| Caspase 9 | 0.935 | 0.924 | 0.953 | 0.807 | 0.536 | 0.922 |
| Caspase 8 | 0.935 | 0.924 | 0.953 | 0.997 | 0.376 | 0.865 |
| c_Caspase 7(D198) | 0.817 | 0.206 | 0.997 | >0.999 | >0.999 | 0.092 |
| c_Caspase 9(D315) | 0.973 | 0.002 | 0.675 | 0.939 | <0.001 | 0.028 |
| Total PARP | 0.869 | 0.452 | 0.028 | 0.988 | 0.932 | 0.514 |
| c_PARP | 0.938 | 0.212 | 0.077 | 0.996 | 0.007 | 0.808 |
DISCUSSION

Resistance evolves dynamically within 6 to 12 months\textsuperscript{5} into treatments owing to various mechanisms and combined drug inhibition are shown to be a promising strategy for tackling resistance.\textsuperscript{7-10,26-29} In this \textit{in vitro} study, we tested three inhibitors, Palbociclib (CDK 4/6 inhibitor), Gedatolisib (PI3K/mTOR dual inhibitors) and PD0325901 (selective MEK 1/2 inhibitor) to assess the therapeutic strategy of PI3K/AKT/mTOR and MAPK/ERK pathways combined inhibition in CRC cell lines. We also aimed to investigate the proteomic effects of the combination of Palbociclib with Gedatolisib (P+G) in the CRC cell lines with various mutational backgrounds to identify potential biomarker(s) for this novel therapy.

In our experiments, the IC\textsubscript{50} values for single agent Gedatolisib were between 76nM to 7200nM. The values were comparatively higher than other studies which reported IC\textsubscript{50} values of less than 100nM.\textsuperscript{30,31} We also obtained a higher range of IC\textsubscript{50} values for the single agent Palbociclib in our tested cell lines, in comparison to other studies.\textsuperscript{32} PD0325901 demonstrated a relatively low IC\textsubscript{50} range in our cell lines tested, suggesting more innate sensitivity to the MEK inhibitor.

Our results demonstrated that the combination of Palbociclib with either Gedatolisib (P+G) or PD0325901 (P+PD) exhibits synergistic anti-proliferative effects, relative to the single agents, in all cell lines including LS1034 (\textit{KRAS} mutated) and DLD-1 (co-occurring \textit{KRAS} with \textit{PIK3CA} mutated) cells. These are important findings since \textit{KRAS} and \textit{PIK3CA} mutations are seen in approximately 60\% and 20\% of CRC, respectively.\textsuperscript{33} In LS411N (\textit{BRAF} V600E mutated) cells, the combination of P+G may have mild synergistic effect whilst the combination of P+PD failed to show any
synergistic effects (CI=0.64 versus 14.7). In LS1034 cells, both drug combinations demonstrated synergistic effects, but the combination of P+G appears to be superior (CI=0.11 versus 0.29). In the Caco-2 wild-type cells, both drug combinations demonstrated comparable synergistic effects. Taken together, we considered the combination of P+G of a higher superiority for clinical assessment, compared to the combination of P+PD, thus decided to focus our studies on the P+G. The subnanomolar IC\textsubscript{50} range of Gedatolisib makes it a more favourable companion drug than PD0325901 to avoid excessive overlapping toxicities. Furthermore, we have strong efficacy and safety data from two Phase I clinical trials involving combination of P+G.\textsuperscript{7,9} In these trials, Palbociclib (125 mg) was administered orally, daily for 3 weeks with Gedatolisib (110mg) administered intravenously once during the 4-week cycle.

In our RPPA study following 4 hours of exposure to the combination of P+G, we observed suppression of S6rp(S240/S244) across all cell lines. There was also significant suppression of S6rp(S235/S236) in Caco-2, DLD-1 and LS1034 cells. This suppression was much more marked with P+G in combination than with either single agent alone. This was not associated with any increased expression or phosphorylation of AKT, which are indicative of upstream PI3K reactivation. We thus believe, the combination of P+G acts primarily at the level of mTOR, which is a downstream effector of the PI3K/AKT signalling cascade. It also demonstrated that the combination of P+G likely exhibits stronger inhibition of the PI3K/AKT/mTOR pathway in comparison to control or single agent therapy. For example, we observed increased levels of AKT(S473) and PDK1(S241) in some cell lines with single agent treatment, possibly reflective of feedback loop activity.
In view of the global suppression of S6rp(S240/S244) in all tested cell lines, this may be a promising predictive marker of clinical responsiveness for this combinative therapy. As reported by Iwenofu et al.\textsuperscript{34}, S6rp is considered a better surrogate biomarker of mTOR activity in comparison to p70S6K, which is also known as Ribosomal protein S6 kinase beta-1 (S6K1). This is because p70S6K has structural similarity to p90S6K, which is not phosphorylated by mTOR.\textsuperscript{34,35} As a key component of the PI3K/AKT signalling cascade, mTOR plays a crucial role in the regulation of energy metabolism and protein synthesis. It acts by directly activating p70S6K.\textsuperscript{36-49} p70S6K is a serine-threonine kinase which controls S6rp phosphorylation at the five serine residues (S235/S236, S240/S244 and S247), leading to initiation of protein synthesis.\textsuperscript{40} In contrast to the S240/244 residues which are solely regulated by p70S6K, the phosphorylation at S235/S236 residues is controlled by multiple kinases including p70S6K, p90RSK and PKA.\textsuperscript{41} This may explain the suppression of S6rp(S235/S236) which was significant in Caco-2, DLD-1 and LS1034 but not in SNUC4 cells, in contrast to S6rp(S240/S244).

Emerging experimental data has suggested utilising PI3K/mTOR inhibitors to induce non-cell autonomous actions by modulating signal transduction during G\textsubscript{1} to S phase, leading to increased cell death.\textsuperscript{42-45} Interestingly, we did not observe any increase in pRb(S807/S811) following treatment with P+G in combination. This is consistent with what was previously described by Vora et al.\textsuperscript{46} It appeared that PI3K inhibition suppress AKT phosphorylation, but sometime failed to suppress CDK 4/6 activity, as measured by Rb phosphorylation\textsuperscript{47}. Nonetheless, the regulation of Rb function by phosphorylation during cell cycle is not fully understood. Rb in mammalian cells has 15 known phosphorylation sites and it seems that Rb phosphorylation at specific
sites is required for the ability of Rb to regulate apoptosis.\textsuperscript{48,49} As shown in \textit{in vitro}, Rb may be phosphorylated at few additional sites (S608, S795) in addition to S807/S811 in the role of apoptosis.\textsuperscript{50} Furthermore, there is evidence to suggest that dephosphorylation of Rb has been widely observed during apoptosis.\textsuperscript{51} This may explain the equivocal level of pRb(S807/S811) we observed in our RPPA analysis.

Unlike the single agents, the combination of P+G induced (early) pro-apoptotic effects, as demonstrated by increased BAX and Bcl-2 levels in most cell lines. The increase in both markers was significant in DLD-1 and SNUC4 cells, but was not significant in LS1034 cells, suggesting that the magnitude of effect may be dependent on the cells’ mutational status. We did not observe any increase in caspase 3, caspase 8, cleaved caspase 7, cleaved caspase 9 or cleaved PARP levels, which are indicative of total apoptosis. However, it is possible that the 4-hour timepoint used for our analysis was too early to evaluate the proteomic alterations related to the late stage of apoptosis.

Finally, we observed EGFR and RSK upregulation in all cell lines after 4 hours of drugs treatment, which may be associated with upstream MAPK/ERK reactivation. Total EGFR and RSK upregulation were observed with both single agents and combination therapy in some of the cell lines. This suggests that the mechanism promoting resistance to PI3K-targeted inhibitors (which was Gedatolisib in this study) include feedback loops which lead to reactivation of membrane RTKs and the contralateral MAPK/ERK pathway. This further supports our hypothesis that multiple target inhibition strategy, rather than single agent, is better for tackling resistance occurrence.
It is important to note the limitations of our study. Firstly, it was an *in vitro* study and limited to five cell lines. Secondly, not all specific exon mutations were tested in this study, specifically *PIK3CA* mutations in exon 20 which may be biologically more relevant than exon 9 mutations from an epidemiology standpoint.\(^{52-54}\) Thirdly, the RPPA analysis with 40 preselected antibodies was performed at only two timepoints (i.e. 30 minutes and 4 hours) post drug exposure. The specific mechanism of synergism for the combination of P+G could not be completely defined; however, several possible mechanisms were observed by our RPPA analysis, including more complete inhibition of protein synthesis-related signalling e.g. S6rp(S240/S244) and increased activation of early apoptotic signalling. Despite these limitations, our study has produced evidence to support further *in vivo* evaluation, which is in progress.

**CONCLUSION**

In summary, the novel combination of Palbociclib with Gedatolisib (P+G) displays clear synergistic anti-proliferative effects in both wild-type and mutated CRC cell lines, relative to the single agents. Our results offer good rationale for further *in vivo* study and clinical development of P+G as emerging therapeutics in metastatic CRC patients. S6rp(S240/S244) may be a marker of responsiveness for this novel combination therapy.
ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIAL

All data generated or analysed during this study are included in this published article and its supplementary files.

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COMPETING INTERESTS

The authors declare that there is no competing interest.

AUTHORS CONTRIBUTIONS

BH and ST conceived the study and co-ordinated the experiments and revised the manuscript. CLL coordinated the experiments, performed the experiments, analysed the data and wrote the manuscript. RA performed parts of the drug combination cell assays. MC carried out the RPPA experiments. SM performed statistical analyses.
SK, AC, AF and JW performed the cell protein lysate experiments. All authors read and approved final manuscript.

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**Additional File 1:** The characteristic and culture of the colorectal cancer epithelial cell lines.

The cell lines mutational information was determined using Cancer Cell Line Encyclopaedia (CCLE) and mutations were verified by using the Agena MassARRAY platform. All cell lines are maintained at 37°C with 5% CO₂. Cell lines were Mycoplasma tested before and after the *in vitro* experiments. RPMI 1640=Rowell Park Memorial Institute 1640 medium (Gibco, USA); FBS= fetal bovine serum (Gibco, USA); EMEM=Eagle’s Minimum Essential Medium (ATCC, USA); P/S=Penicillin/Streptomycin.

| Cell Line          | Mutational Status | Media            | Tissue Type                     | Doubling time |
|--------------------|-------------------|------------------|---------------------------------|---------------|
| Caco-2 (ATCC HTB-37) | Wild-Type         | EMEM +20% FBS +1% P/S | Colon, Colorectal adenocarcinoma | 62 hours      |
| DLD-1 (ATCC CCL-221) | KRAS G13D, PIK3CA E545K | RPMI 1640 +10% FBS +1% P/S | Colon, Dukes type C, colorectal adenocarcinoma | 24 hours |
| LS411N (ATCC CRL-2159) | BRAF V600E        | RPMI 1640 +10% FBS +1% P/S | Caecum, Dukes type B, colorectal carcinoma | 24 hours |
| LS1034 (ATCC CRL-2158) | KRAS A146T | RPMI 1640 +10% FBS +1% P/S | Caecum, Dukes type C, colorectal carcinoma | 24 to 33 hours |
| SNUC4 (KCLB 0000C4) | PIK3CA E545G     | RPMI 1640 +10% FBS +1% P/S | Colon, Colorectal adenocarcinoma | 24 hours |
**Additional File 2:** Preparation of cell lysate for RPPA (reverse phase protein array) in 6-well format.

Reagents:

i) **Lysis Buffer:** 1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl$_2$, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na$_3$VO$_4$, 10% glycerol, containing freshly added protease and phosphatase inhibitors from Roche Applied Science Cat.#04693116001 and 04906845001, respectively

ii) **4 xSDS Sample Buffer:** 40% Glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8. Before use, add Bond-breaker TCEP solution at 1/10 of the volume from Thermo-scientific Cat. # 77720

Methods:

1. Seed cells in 6-well plate for 24-hour incubation. Cell number per well is dependent on the cell size, cell growth rate and experimental design.
   
   In this study, the cell counts used to obtain the required protein concentration are as following:
   
   i) Caco-2: $4.0 \times 10^5$ cells /2mls/well
   
   ii) DLD-1: $4.5 \times 10^5$ cells /2mls/well
   
   iii) LS1034: $5.5 \times 10^5$ cells /2mls/well
   
   iv) SNUC4: $5.0 \times 10^5$ cells /2mls/well

2. Treat cells with drugs according to experimental design. Drugs are prepared in 2mls of media per well.

3. Wash the cells twice with phosphate buffered saline (PBS). Add lysis buffer to plate (100-150μl for each wells).

4. Incubate the cells on leveled ice for 20 minutes with occasional shaking every 5 minutes.

5. Centrifuge the cell lysate in microcentrifuge at 14,000 rpm for 10 minutes at 4°C.
6. Carefully collect supernatant. Discard the pellet.

7. Quantify cellular protein concentration by biocinchoninic acid (BCA) protein assay. Adjust protein concentration to 1.5µg/µL. (Use lysis buffer to dilute) and store at –80 °C.

8. Before RPPA processing, mix each cell lysate with 4 × SDS sample buffer without bromophenol blue (3 parts of cell lysate plus one part of 4 × SDS sample buffer) and boil the samples for 5 minutes at 80°C. The samples are ready for RPPA processing.
Additional File 3: List of primary antibodies used in the RPPA experiments.

| Antibody                                | Manufacturer  | Catalogue number | Species | Dilution |
|------------------------------------------|---------------|------------------|---------|----------|
| Total AKT                                | Cell Signalling | 9272             | Rabbit  | 1:3000   |
| AKT (S473)                               | Cell Signalling | 9271             | Rabbit  | 1:250    |
| AKT (T308)                               | Cell Signalling | 9275             | Rabbit  | 1:500    |
| Total mTOR                               | Cell Signalling | 2972             | Rabbit  | 1:400    |
| mTOR (S2448)                             | Cell Signalling | 2971             | Rabbit  | 1:100    |
| mTOR (S2481)                             | Cell Signalling | 2974             | Rabbit  | 1:100    |
| p70 S6 Kinase (T389)                     | Cell Signalling | 9205             | Rabbit  | 1:250    |
| GSK3β (S9)                               | Cell Signalling | 9336             | Rabbit  | 1:500    |
| PDK1                                     | Cell Signalling | 3062             | Rabbit  | 1:100    |
| PDK1 (S241)                              | Cell Signalling | 3061             | Rabbit  | 1:500    |
| PI3-Kinase p110alpha                     | Cell Signalling | 4255             | Rabbit  | 1:100    |
| S6 Ribosomal Protein (S235/236) (2F9)    | Cell Signalling | 2211             | Rabbit  | 1:200    |
| S6 Ribosomal Protein (S240/244)          | Cell Signalling | 2215             | Rabbit  | 1:3000   |
| PTEN                                     | Cell Signalling | 9552             | Rabbit  | 1:1000   |
| EGFR                                     | Cell Signalling | 2232             | Rabbit  | 1:100    |
| EGFR Y992                                | Cell Signalling | 2235             | Rabbit  | 1:100    |
| EGFR Y1068                               | Cell Signalling | 2234             | Rabbit  | 1:100    |
| c_Raf                                    | Millipore      | 04-739           | Rabbit  | 1:250    |
| MAPK_ERK 1/2                             | Cell Signalling | 9102             | Rabbit  | 1:200    |
| MAPK (T202/Y204)-ERK1/2                  | Cell Signalling | 4377             | Rabbit  | 1:1200   |
| MEK1/2                                   | Epitomics      | 1235-1           | Rabbit  | 1:1200   |
| MEK1/2 (S217/221)                        | Cell Signalling | 9121             | Rabbit  | 1:1000   |
| Total Rb                                 | Cell Signalling | 9309             | Mouse   | 1:150    |
| pRb (S807/S811)                          | Cell Signalling | 9308             | Rabbit  | 1:500    |
| E2F1                                     | Cell Signalling | 3742             | Rabbit  | 1:250    |
| RSK                                      | Cell Signalling | 9347             | Rabbit  | 1:500    |
| cMET                                     | Cell Signalling | 3127             | Mouse   | 1:500    |
| p27                                      | Epitomics      | 1591-1           | Rabbit  | 1:250    |
| p53                                      | Cell Signalling | 9282             | Rabbit  | 1:3000   |
| BAX                                      | Cell Signalling | 2772             | Rabbit  | 1:250    |
| Bcl-2                                    | Dako           | MO887            | Rabbit  | 1:250    |
| Bcl-xl                                   | Cell Signalling | 2762             | Rabbit  | 1:250    |
| Caspase 3                                | Cell Signalling | 9662             | Rabbit  | 1:5000   |
| Caspase 9                                | Cell Signalling | 9502             | Rabbit  | 1:1000   |
| Caspase 8                                | Cell Signalling | 9746             | Mouse   | 1:1000   |
| cleaved Caspase-7 (D198)                 | Cell Signalling | 9491             | Rabbit  | 1:100    |
| cleaved Caspase-9 (D315)                 | Cell Signalling | 9505             | Rabbit  | 1:100    |
| Total PARP                               | Santa Cruz     | sc-7150          | Rabbit  | 1:1000   |
| cleaved PARP                             | Cell Signalling | 9546             | Mouse   | 1:250    |
**Additional File 4:** The comparison of the mean fold-change of the antibodies measured at 30-minute and 4-hour treatment timepoints in RPPA analysis. Each treatment arm was compared with the same treatment arms of the different timepoint. The p-values were calculated with the two-way ANOVA test. The p<0.05 is considered statistically significant.

| Antibodies          | Caco-2 | DLD-1 | LS1034 | SNUC4 |
|---------------------|--------|-------|--------|-------|
| Total AKT           | 0.5641 | 0.4618| 0.5641 | 0.6348|
| AKT (S473)          | 0.2836 | 0.3633| 0.0985 | 0.2991|
| AKT (T308)          | 0.5034 | 0.2023| 0.3413 | 0.3559|
| Total mTOR          | 0.4688 | 0.7971| 0.1646 | 0.6938|
| mTOR (S2448)        | >0.9999| 0.1034| 0.3196 | 0.1930|
| mTOR (S2481)        | 0.5508 | 0.3257| 0.8083 | **0.0255**|
| S6rb (S235/S236)    | 0.7397 | 0.6127| 0.5766 | 0.8625|
| S6rb(S240/S244)     | 0.5414 | 0.1378| 0.6865 | 0.4545|
| P70S6K(T389)        | 0.5912 | 0.3611| 0.4545 | 0.4599|
| GSK3β(S9)           | 0.8295 | 0.1886| 0.5999 | 0.9027|
| PDK1                | >0.9999| 0.5440| **0.0427**| 0.7257|
| PDK1(S241)          | 0.1778 | 0.7074| 0.6332 | 0.6673|
| PI3Kinase p110α     | 0.7565 | 0.2811| 0.9029 | 0.6027|
| PTEN                | 0.7949 | 0.3738| 0.5540 | 0.0558|
| MAPK_ERK1           | 0.8643 | 0.3228| 0.5079 | 0.3438|
| MAPK(T202/Y204)     | 0.0866 | 0.1907| 0.7863 | 0.1690|
| EGF-R               | 0.0667 | **0.0229**| 0.0777 | 0.0652|
| Total MEK1/2        | 0.3201 | 0.2987| 0.4831 | 0.5935|
| MEK1/2 (S217/221)   | **0.0349**| 0.6703| 0.4612 | 0.6754|
| c_RAF               | 0.2747 | 0.3936| 0.4931 | 0.8197|
| E2F1                | 0.3824 | 0.3359| 0.1927 | 0.2636|
| Total Rb            | 0.5144 | 0.4733| 0.4105 | 0.8681|
| pRb (S807/811)      | 0.2761 | 0.2256| 0.5467 | 0.7172|
| RSK                 | 0.8214 | 0.4837| 0.2896 | 0.9910|
| BAX                 | 0.2386 | 0.2327| 0.4154 | 0.4739|
| Bcl-2               | 0.4173 | 0.1829| 0.1603 | 0.9067|
| Bcl-xl              | 0.3267 | 0.6329| 0.3875 | 0.4352|
| Caspase 3           | 0.8912 | 0.3528| 0.5139 | 0.7568|
| Caspase 9           | 0.1286 | **0.0306**| 0.3261 | 0.8721|
| Caspase 8           | 0.3691 | 0.1039| 0.3873 | 0.9776|
| c_Caspase 7 (D198)  | 0.1362 | 0.1749| 0.4481 | 0.8623|
| c_Caspase 9 (D315)  | 0.1979 | 0.6391| 0.1113 | 0.3182|
| Total PARP          | 0.6321 | 0.4334| 0.9962 | 0.0870|
| c_PARP              | 0.4813 | 0.9620| 0.9674 | 0.1393|
| p53                 | 0.4359 | 0.1411| 0.6944 | 0.2414|
| cMET                | 0.2233 | 0.0846| 0.2556 | 0.8252|