ECRONICON OPEN ACCESS

EC GYNAECOLOGY
Research Article

A Combination of EGFR/HER-2 and c-MET Inhibitors Reduced Cell Adhesion of Ovarian Cancer Clusters to the Extracellular Matrix (ECM)

Wafaa Hassan[1], Kenny Chitcholtan[2]*, Peter Sykes[2] and Ashley Garrill[3]

[1]College of Medicine, lbn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq

[2]Gynaecological Cancer Research Group, Department of Obstetrics and Gynaecology, University of Otago, Christchurch, New Zealand

[3]School of Biological Sciences, University of Canterbury, Christchurch, New Zealand

***Corresponding Author:** Kenny Chitcholtan, Gynaecological Cancer Research Group, Department of Obstetrics and Gynaecology, University of Otago, Christchurch, New Zealand.

Received: April 24, 2026; **Published:** May 08, 2026

Abstract

Background: Advanced ovarian cancer cell clusters, aggregates, and spheroids are the primary source of metastatic dissemination in advanced ovarian cancer. A critical early step in this process is the adhesion of ovarian cancer cells to the peritoneal surface, which consists of a mesothelial cell layer overlying the basement membrane. Elucidating the molecular mechanisms underlying adhesion may provide novel strategies to prevent the widespread dissemination of disease. This study investigated the role of the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2), and the hepatocyte growth factor receptor (c-MET) axis in regulating the adhesion of ovarian cancer cell clusters and aggregates.

Methods: Human ovarian cancer cell lines OVCAR-5 and SKOV-3 were cultured on poly-HEMA-coated surfaces to promote the formation of cell clusters and aggregates. These clusters were subjected to adhesion assays on collagen matrices in the presence of epidermal growth factor (EGF) or hepatocyte growth factor (HGF), and receptor-specific inhibitors. Adherent cells were quantified, and signalling pathways were investigated using immunoblotting and immunofluorescence analyses.

Results: Adhesion of OVCAR-5 and SKOV-3 cell clusters and aggregates to the extracellular matrix (ECM) was growth-factor-dependent and was significantly inhibited by the dual EGFR/HER-2 inhibitor canertinib and the c-MET inhibitor PHA-665752. Combined inhibition of EGFR/HER-2 and c-MET further impaired cell adhesion, likely through disruption of interactions between EGFR/HER-2/c-MET signaling and β4 integrins.

Conclusions: These findings identify β4 integrins as key mediators of ovarian cancer cell cluster adhesion and suggest that targeting the EGFR/HER2/c-MET-β4 integrin axis may be a therapeutic strategy to limit metastatic spread in advanced ovarian cancer.

Keywords: *Ovarian Cancer; EGFR; HER-2; Canertinib; PHA665752; β4 Integrin; Cell Adhesion*

Introduction

Metastatic ovarian cancer primarily progresses within the peritoneal cavity, where malignant cells exfoliate from the primary tumour surface and spread with the movement of peritoneal fluid [1]. Detached ovarian cancer cells frequently form small clusters or aggregates that facilitate metastasis and contribute to treatment resistance [2]. These clusters can generate drug-resistant clones that adhere to the

peritoneal wall and establish secondary tumours. The initial adhesion of ovarian cancer clusters to the peritoneal membrane, which is formed by a single layer of mesothelial cells covering the internal organs, is a critical step in metastasis [3,4].

Multiple mechanisms have been proposed to explain this adhesion, including CD44-hyaluronan binding, mesothelial cell apoptosis via TNF-α signalling, and mesothelial retraction induced by ascitic fluid components [5-7]. These events expose the underlying extracellular matrix (ECM), enabling cancer cells to bind to collagen [8,9]. Ovarian cancer cells express various integrins that can recognise laminins and collagens I and IV [10-13]. Integrins are αβ heterodimers that transduce ECM contacts into intracellular signalling through activation of their β-subunits [14,15]. Upon ECM binding, the β-subunits can convert mechanical contacts into cellular signalling information via the activation of numerous signalling proteins responsible for tumour progression [16]. Among them, the β4 subunit is particularly associated with tumorigenesis, yet its role in the early adhesion of ovarian cancer clusters remains unclear [17,18]. The β4 subunit can associate with receptor tyrosine kinases, such as EGFR, HER2, and c-MET, suggesting that disrupting these interactions could prevent initial adhesion to the peritoneal wall and limit tumour progression [19,20].

Ovarian cancer cells express variable levels of EGFR, HER-2, and c-MET, and receptor overexpression correlates with a poor disease prognosis [21-25]. Although inhibitors targeting these receptors have been tested clinically, their results have been inconsistent [21,26,27]. Canertinib, an irreversible dual EGFR/HER-2 inhibitor, has shown strong *in vitro* antitumour effects in ovarian cancer [2,27-29], but a phase II trial (CI-1033) showed limited benefits in patients [21]. PHA665752 is a selective c-MET inhibitor that binds reversibly to the ATP-binding pocket of the c-MET kinase domain [30]. Preclinical studies have shown that PHA665752 has anti-tumour activity in both *in vitro* and *in vivo* ovarian cancer models, yet it has never reached clinical study [26,27]. Targeting these pathways in late-stage, disseminated disease has been shown to be ineffective [31]. However, blocking the adhesion of metastatic cells during early dissemination could offer a more rational therapeutic approach.

In the present work, in view of the above, we hypothesise that ovarian cancer cell adhesion to the ECM depends on co-activation of EGFR, HER2, and c-MET with the β4 subunit and that inhibition of these receptor tyrosine kinases will disrupt this process.

Materials and Methods

Cell lines and cell culture, and generating 3D cell clusters

Human ovarian cancer cell lines, media for cell culture, cell maintenance and the culturing of cells in 3D cell clusters were as previously described [31]. Briefly, OVCAR-5 and SKOV-3 cells were maintained in DMEM media (GIBCO", Life Technologies, New Zealand) supplemented with 10% foetal bovine serum (FBS) (GIBCO", Life Technologies, New Zealand), Pen/Strep (GIBCO", Life Technologies, New Zealand) at a working concentration of 100 units/mL penicillin, 100 units/mL streptomycin, 2 mM glutaMAX™ (GIBCO", Life Technologies, New Zealand) and 1 µg/mL Fungizone (Life Technologies, New Zealand). The final concentration of glucose in the media was 5.5 mM. The respective supplemented media is hereafter referred to as working media. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cell lines were authenticated by short tandem repeat (STR) testing (CellBank, Children's Medical Research Institute, New South Wales, Australia). Cell lines were tested for *Mycoplasma* contamination using a PCR-based assay with generic primers, as described by Timenetsky., *et al*. [32].

Adhesion assays of ovarian cancer cell clusters

Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) were purchased from Thermo Fisher (Auckland, New Zealand). 3D cell clusters/aggregates were cultured for six days in working medium prior to incubation in a serum-free medium (SFM) for 24h. Cells were then incubated in SFM that contained 0.2 or 20 ng/mL growth factor and canertinib (LC Laboratories, MA, USA), PHA665752 (LC Laboratories, MA, USA), RGDS (Sigma- Aldrich, Auckland, New Zealand), RGES (Sigma-Aldrich, Auckland, New Zealand) alone or combined

with these inhibitors for four hours. After this incubation, cell adhesion assays were carried out, in which cells with conditioned media were then transferred onto a freshly pre-coated collagen-gel matrix (a mixture of 2 mg/mL collagen I and 25% (v/v) GelTrex™, Thermo Fisher, New Zealand) in cell culture wells, and the cells were incubated for an additional four hours in a CO_2 incubator at 37°C. This meant they were incubated for a total of 8h prior to cellular metabolism assays and cell counting. For Western blotting and immunofluorescence, cells were treated for 4h before protein lysate collection or fixation with a cold solution of 50% acetone:50% methanol. The Alamar Blue assay was used to determine cellular metabolism. The numbers of non-adherent and adherent cells were counted by a haemocytometer.

Determination of protein expression using Western blotting

Protein expression was assessed by Western blotting as described previously [33]. Primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The antibodies used were anti-EGFR (SC-03), pEGFR (SC-101668), HER-2 (SC-284), pHER-2 (SC-12352-R), c-MET (SC-10), p-MET (SC-101736), GAPDH (SC-25778), and β4 integrin (SC-9090). For Western Blotting, the primary antibody dilution was 1/1000. The secondary antibodies, donkey anti-mouse IgG-HRP (SC2314) and mouse anti-rabbit IgG-HRP (SC2357), were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) at a 1/5000 dilution.

Detection of cellular proteins using immunofluorescence

The protocols for preparation of OVCAR-5 clusters and SKOV-3 cell aggregates before the sectioning and detection of protein using immunofluorescence were as previously described [2]. For immunofluorescence, the primary antibody dilution was 1/500. The secondary antibodies, goat anti-mouse IgG-FITC (F0257) and goat anti-rabbit IgG-Atto594 (77671), were from Merck (Auckland, New Zealand) at a 1/500 dilution.

Statistical analysis

Statistical analyses were performed using GraphPad Prism® (La Jolla, CA, USA). One-way ANOVA and a student's t-test were carried out when appropriate, where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) indicate levels of statistical significance. All data are presented as Mean ± SEM. Each experiment was independently repeated at least 3 times.

Results

Effects of EGF and HGF on cell adhesion to ECM

Adhesion of OVCAR-5 clusters and SKOV-3 aggregates depended on growth factor concentration. In OVCAR-5 clusters, only the super-physiological concentration of EGF (20 ng/mL) significantly increased adhesion ($p < 0.05$). In contrast, the physiological level (0.2 ng/mL) had no effect (Figure 1A). In contrast, SKOV-3 aggregates showed increased adhesion at both concentrations, with a stronger response at 20 ng/mL ($p < 0.0001$). A similar trend was observed with HGF, the c-MET ligand: super-physiological HGF significantly enhanced adhesion in both lines ($p < 0.01$ for OVCAR-5, $p < 0.0001$ for SKOV-3; Figure 1C and 1D). Only SKOV-3 cells were sensitive to physiological HGF ($p < 0.01$).

Effects of canertinib and PHA665752 on cell numbers and metabolism before adhesion

Before the adhesion assay, we evaluated the effects of single and combined inhibitor treatments on cell number and metabolism in OVCAR-5 clusters and SKOV-3 aggregates under mixed growth-factor conditions. After 4h of exposure to various concentrations of canertinib, with or without 1 μM PHA665752, neither cell numbers nor metabolic activity changed significantly in OVCAR-5 clusters (Supplement 1A-1D). Similar results were obtained for SKOV-3 aggregates (Supplement 1E-1H), except for a small but significant decrease in cell number following combination treatment ($p < 0.05$). When PHA665752 was tested at different concentrations, alone or with 3 μM canertinib, no reductions in cell number or metabolism were observed in either line, except for a small decrease in SKOV-3 cell number

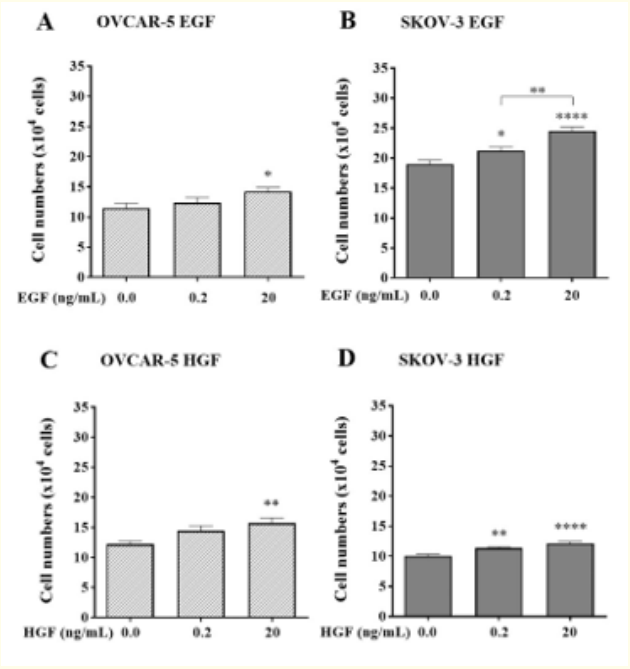
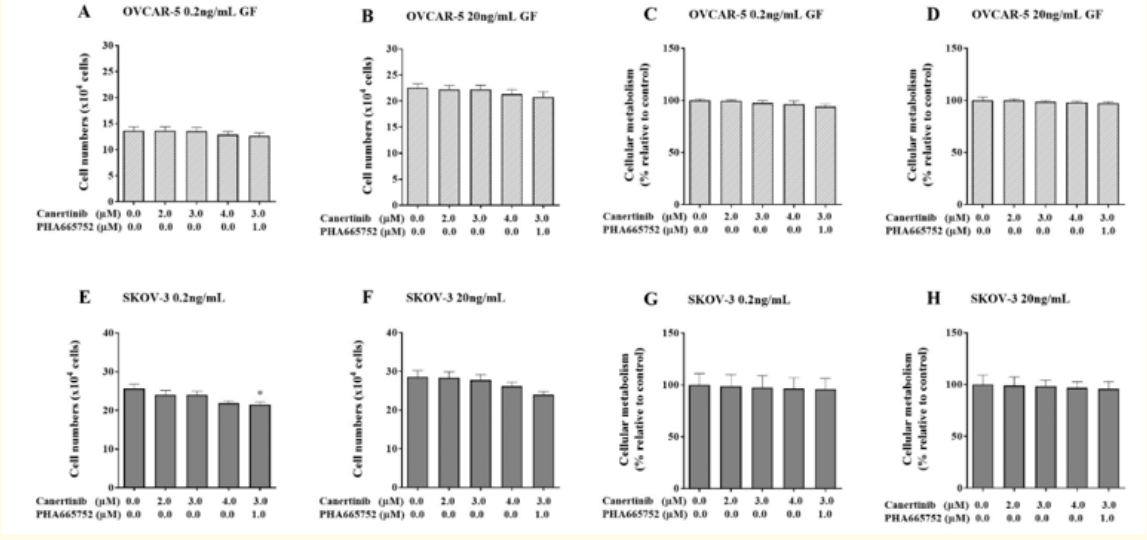
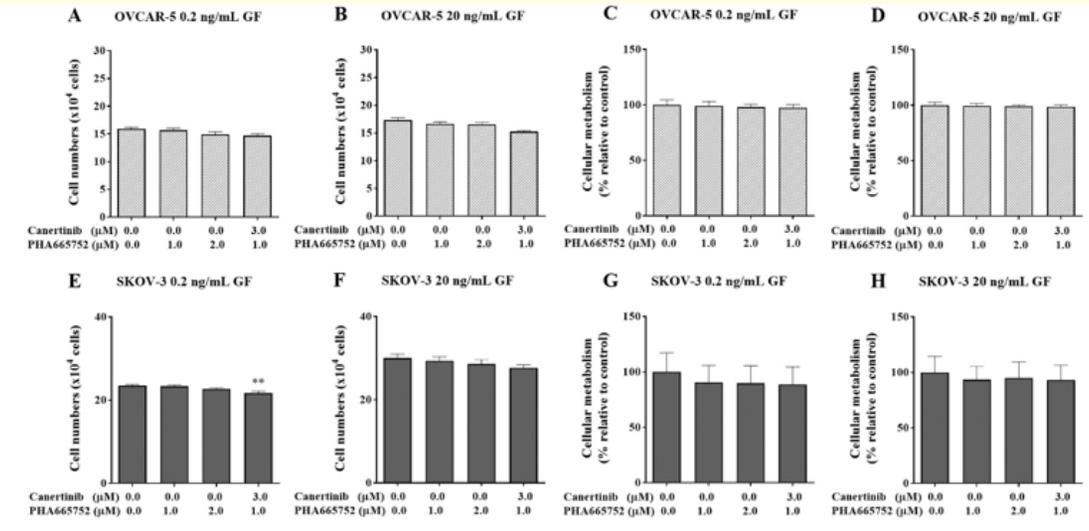


Figure 1: *Effects of EGF and HGF on OVCAR-5 (A, C) and SKOV-3 (B, D) ovarian cancer cell line adherence. Results show the number of adherent OVCAR-5 clusters and SKOV-3 aggregates treated with 0.2 and 20 ng/mL of EGF and HGF. Data are expressed as means ± S.E.M (n = 3). Statistically significant differences in cell numbers are indicated as p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****) compared to the control or other concentrations.*

with the combination (p < 0.01; Supplement 2E). These findings confirm that drug treatments did not affect total cell number before the adhesion assay, indicating that subsequent inhibitor effects reflected altered adhesion mechanisms rather than reduced cell viability.



Supplementary Figure 1: *Cell numbers and cellular metabolism of OVCAR-5 and SKOV-3 after 4 hr treatment with various canertinib, 1μM PHA665752 and combination before cell adhesion assay. Cell numbers for OVCAR-5 (A, B) are unaffected. SKOV3 cells are affected by the combination treatment at 0.2 ng/mL growth-factor-activated condition (E); however, at 20 ng/mL, it does not statistically reduce cell number (F). Cellular metabolism of both cell lines does not change (C, D, G, H).*



Supplementary Figure 2: *Cell numbers and cellular metabolism of OVCAR-5 and SKOV-3 after 4 hr treatment with 3μM canertinib, various concentrations of PHA665752 and combination before cell adhesion assay. Cell numbers for OVCAR-5 (A, B) are unaffected. SKOV3 cells are affected by the combination treatment at 0.2 ng/mL growth-factor-activated condition (E); however, at 20 ng/mL, it does not statistically reduce cell number (F). Cellular metabolism of both cell lines does not change (C, D, G, H).*

Effects of canertinib alone with the mixed GF on cell adhesion to ECM

We next assessed the effect of canertinib on the adhesion of OVCAR-5 clusters and SKOV-3 aggregates in the presence of a growth factor (GF) mixture. Canertinib reduced adhesion in both cell lines in a concentration-dependent manner (Figure 2A-2D). At the physiological GF concentration (0.2 ng/mL), OVCAR-5 adhesion decreased significantly at 2 μM ($p < 0.01$) and more strongly at 3 - 4 μM ($p < 0.0001$; Figure 2A). In SKOV-3 aggregates, adhesion was reduced at 3 μM ($p < 0.001$) and 4 μM ($p < 0.0001$; Figure 2B). Under super-physiological GF (20 ng/mL), all canertinib concentrations (1-4 μM) decreased OVCAR-5 adhesion ($p < 0.001$-0.0001; Figure 2C), whereas in SKOV-3 aggregates concentrations >1 μM significant decreased adhesion (Figure 2D). These data suggest that EGFR and HER-2 signalling contributes to the adhesion of ovarian cancer cell clusters and aggregates to the ECM.

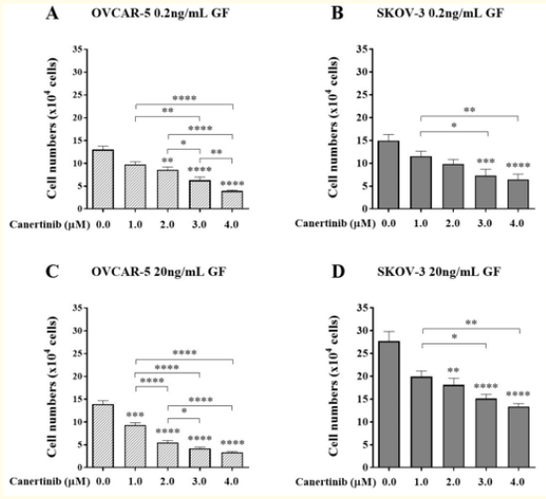


Figure 2: *The effect of canertinib on cell adhesion of OVCAR-5 clusters (A, C) and SKOV-3 aggregates (B, D) in the presence of growth factors (GF). Cells were treated with canertinib in the presence of 0.2 or 20 ng/mL EGF+HGF (GF) for 4h before a further 4h exposure during the adhesion assay. The number of adherent cells was counted. Data are expressed as means ± S.E.M. (n = 3). Statistically significant differences in cell number are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) compared to the control or other concentrations.*

Effects of PHA665752 alone on cell adhesion to ECM

At physiological GF concentration (Figure 3A), PHA665752 significantly reduced OVCAR-5 cluster adhesion at 1 μM ($p < 0.001$) and 2 μM ($p < 0.0001$). In SKOV-3 aggregates, adhesion decreased only at 2 μM (Figure 3B, $p < 0.01$). Under super-physiological GF levels, all PHA665752 concentrations markedly inhibited adhesion in both OVCAR-5 (Figure 3C) and SKOV-3 cells (Figure 3D), consistent with c-MET-mediated adhesion mechanisms.

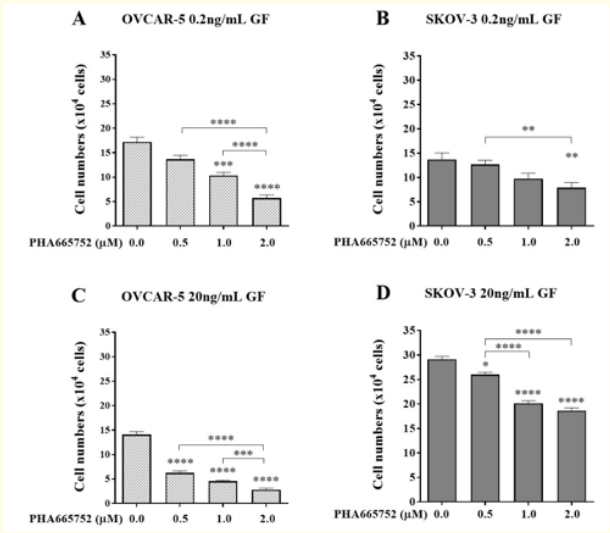


Figure 3: *The effect of PHA665752 on cell adhesion of OVCAR-5 clusters (A, C) and SKOV-3 aggregates (B, D) in the presence of growth factors (GF). Cells were treated with PHA665752 in the presence of 0.2 or 20 ng/mL EGF+HGF (GF) for 4 h before a further 4 h exposure during the adhesion assay. The number of adherent cells was counted. Data are expressed as means ± S.E.M. (n=3). Statistically significant differences in cell number are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) compared to the control or other concentrations.*

Effects of combined canertinib and PHA665752 on cell adhesion to ECM

The combination of canertinib and PHA665752 appeared to inhibit cell adhesion more effectively than either drug alone, suggesting potential for combinatorial therapy in advanced ovarian cancer. At physiological GF levels, 3 μM canertinib with 1 μM PHA665752 reduced OVCAR-5 cluster adhesion more than 4 μM canertinib alone (Figure 4A). In SKOV-3 aggregates, the combination significantly ($p < 0.001$) decreased adhesion compared to 2 μM PHA665752 (Figure 4B). Similar effects were observed under super-physiological GF conditions in both lines (Figure 4C and 4D), supporting dual EGFR/HER-2 and c-MET targeting to disrupt early metastatic adhesion.

Effects of integrin antagonist peptide RGDS with a combination of canertinib and PHA665752 on cell adhesion

Cell adhesion begins with integrin binding to the ECM, a key step for cell survival and progression. To test whether disrupting this interaction reduces adhesion, we added exogenous RGDS peptides-sequences recognised by all β-integrin subunits except the β4 subunit- and examined their effects alone or in combination with inhibitors. As shown in figure 5, RGDS (20 μM, 40 μM) significantly decreased adhesion in OVCAR-5 clusters (Figure 5A, B, $p < 0.0001$) and SKOV-3 aggregates (Figure 5C, D, $p < 0.0001$), whereas controls (DMSO, acetate buffer, RGES peptide) had no effect. The drug combination of canertinib + PHA665752 further reduced adhesion compared with

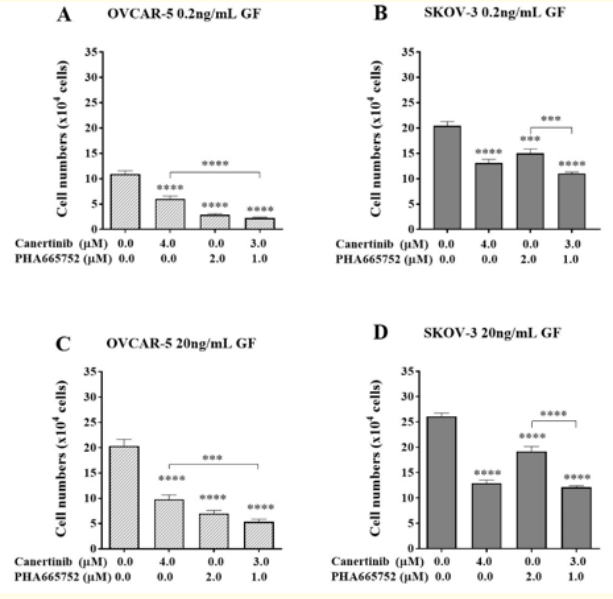


Figure 4: *The effect of a combination of canertinib and PHA665752 on the adherence of OVCAR-5 clusters and SKOV-3 aggregates. Cells were treated with canertinib alone or a combination of canertinib and PHA665752 in the presence of 0.2 or 20 ng/mL GF for 4 h before a further 4 h exposure during the adhesion assay. The number of adherent cells was counted. Data are expressed as means ± S.E.M. (n = 3). Statistically significant differences in cell number are indicated as p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****) compared to the control or other concentrations.*

RGDS alone, but adding RGDS to the drugs produced no additional reduction. These findings suggest β-integrin-RGDS binding is not essential for OVCAR-5 or SKOV-3 adhesion.

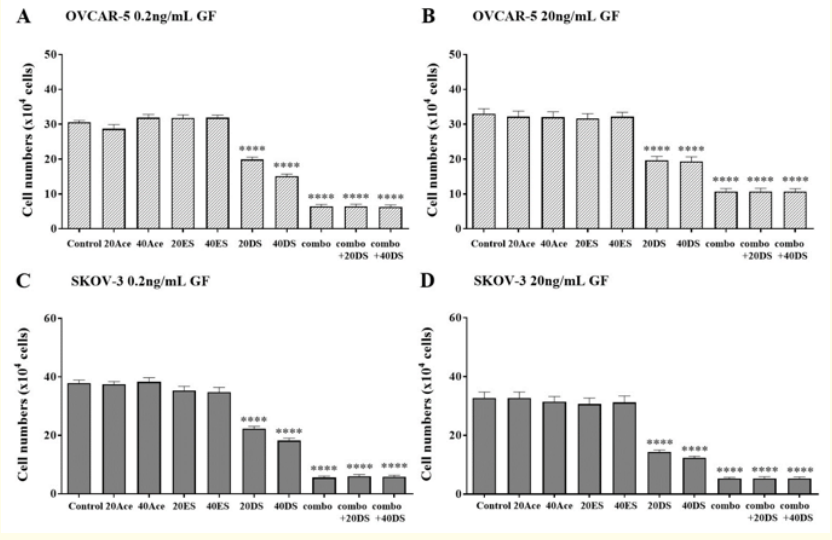


Figure 5: *The effect of exogenous RGDS peptides on cell adhesion in the presence of canertinib and PHA665752. OVCAR-5 (A, B) and SKOV-3 (C, D) clusters were treated with 0.2 ng/mL (A, C) and 20 ng/mL (B, D) of GF in the presence of inhibitors including 20 or 40 μM of acetate (Ace), RGES (ES), RGDS (DS), and a mixture of 3 μM canertinib+1 μM PHA665752 (combo). Data are expressed as means ± S.E.M. (n = 3). Statistically significant differences in cell number are indicated as p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****) compared to the control or other concentrations.*

Effects of a combination of canertinib and PHA665752 on the β4 integrin subunit

These findings suggest that β-integrin subunits that do not bind to the RGDS sequence may mediate adhesion and be influenced by the inhibition of EGFR/HER2 and c-MET. We, therefore, examined the β4 integrin subunit, which binds laminin-5 and contributes to tumour progression, survival, and metastasis [34]. As shown in figure 6, β4 integrin localised to the basal domains of OVCAR-5 clusters and to the outer regions of SKOV-3 aggregates. In canertinib- and PHA665752-treated cells, β4 integrin aggregation was also evident within inner regions of both lines.

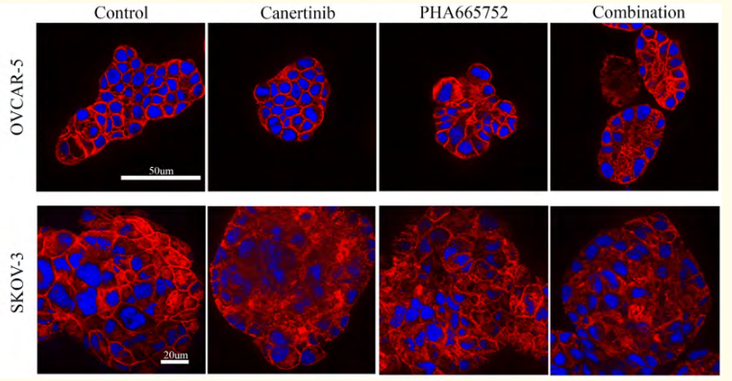


Figure 6: *Immunofluorescent images of the β4 integrin (red) and DNA (blue) of OVCAR-5 clusters and SKOV-3 aggregates after cells were treated with 3 μM canertinib, 1 μM PHA665752 and their combination. Cells were treated with inhibitors for 4h before conducting immunofluorescence.*

Effects of the drug combination on EGFR, HER-2, c-MET and β4 integrin

We previously showed that EGFR, HER-2, and c-MET inhibitors markedly suppressed cell growth [27,29]. We assessed protein levels using Western blot (Figure 7K and 7L). Here, pre-treatment with these inhibitors for 4h before adhesion assays altered protein expression. In OVCAR-5 cells stimulated with 0.2 ng/mL GF, all treatments significantly decreased pEGFR (Figure 7A), while only canertinib reduced total EGFR (Figure 7B). Both pMET and total c-MET were significantly lowered by PHA665752 and the combination (Figure 7C and 7D). At 20 ng/mL GF, all inhibitors reduced pEGFR (Figure 7F), but total EGFR declined only with the combination (Figure 7G). Although pMET showed a decreasing trend, it was not significant (Figure 7H), whereas total c-MET was reduced by PHA665752 and the combination (Figure 7I). β4 integrin levels remained unchanged under all treatments (Figure 7E and 7J).

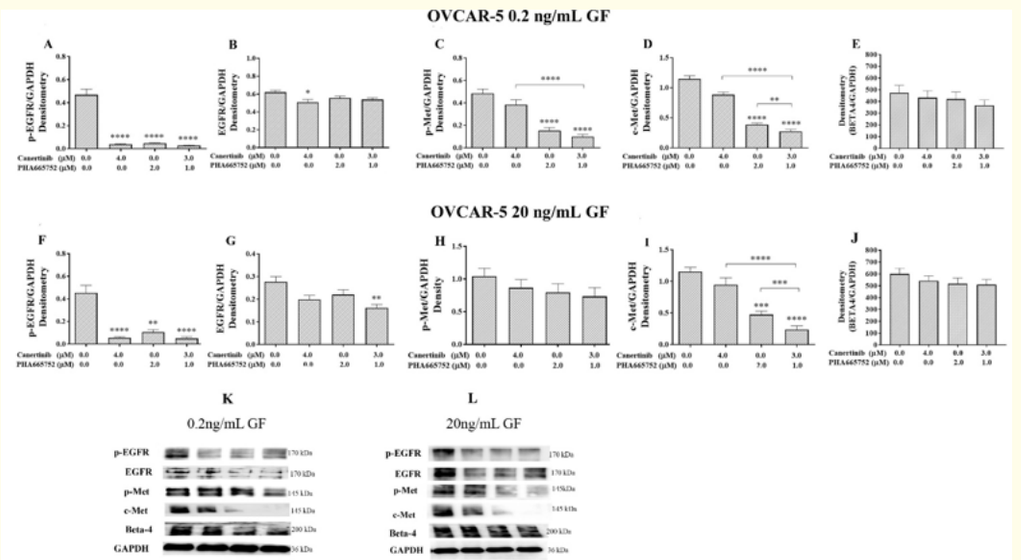


Figure 7: *Western blot and densitometry indices of selective proteins from OVCAR-5 cell clusters after treatment with canertinib and PHA665752 alone and in combination in the presence of 0.2 (A, B, C, D, E, K) and 20 (F, G, H, I, J, L) ng/mL GF. The levels of p-EGFR (A, F), EGFR (B, G), p-MET (C, H), MET (D, I) and β4 integrin subunit (E, J) are representative of the densitometry indices, expressed as mean ± SEM (n=9). Statistically significant differences are indicated as p < 0.05 (*), p < 0.01(**), p < 0.001(***), and p < 0.0001(****) compared to the control or the other concentrations. GAPDH was used as a housekeeping protein.*

In the SKOV-3 cell line stimulated with 0.2 and 20 ng/mL GF, there was a significant reduction of pEGFR (Figure 8A and 8H), EGFR (Figure 8B and 8I), pHER-2 (Figure 8C and 8J), HER-2 (Figure 8D and 8K), pMET (Figure 8L) and c-Met (Figure 8F and 8M). The pMET level at 0.2 ng/mL GF tended to decrease during treatment, but the difference was not statistically significant (Figure 8E). However, the β4 integrin level was not significantly reduced as detected by immunoblotting (Figure 8G and 8N).

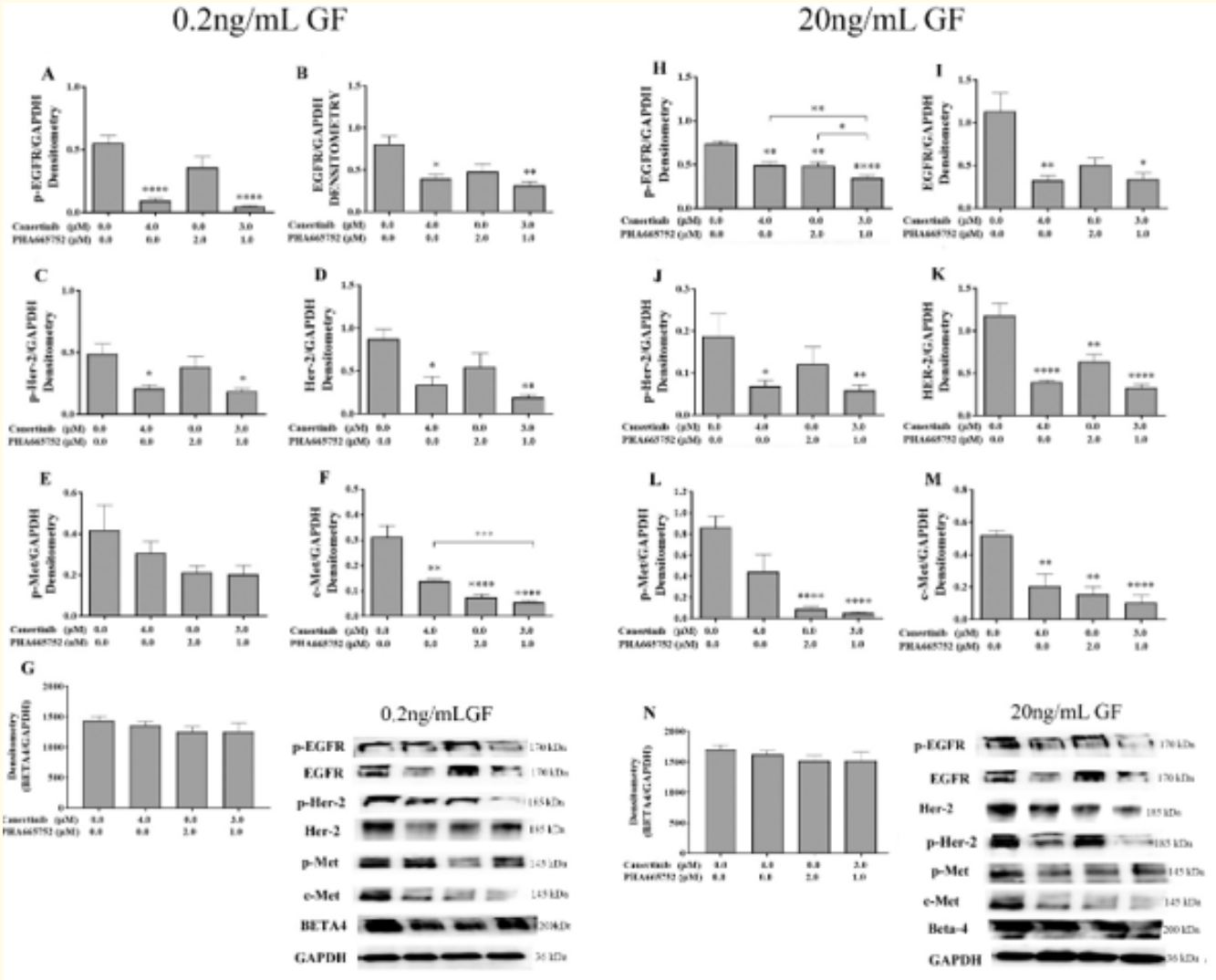


Figure 8: *Densitometry indices of selective proteins of SKOV-3 cell clusters after treatment with canertinib and PHA665752 alone and in combination in the presence of 0.2 (A, B, C, D, E, F, G) and 20 (H, I, J, K, L, M, N) ng/mL GF. The levels of p-EGFR (A, H), EGFR (B, I), p-HER-2 (C, J), HER-2 (D, K), p-MET (E, L), MET (F, M) and β4 integrin subunit (G, N) are representative of densitometry indices as mean ± SEM (n = 9). Statistically significant differences are indicated as p < 0.05 (*), p < 0.01(**), p < 0.001(***), and p < 0.0001(****) compared to the control or the other concentrations. GAPDH is used as a housekeeping protein.*

Effects of the drug combination on co-localisation of EGFR, HER-2 and β4 integrin subunit

These results show that total and phosphorylated EGFR and HER2 levels were significantly reduced by single- or combined-inhibitor treatments, resulting in decreased cell adhesion. In contrast, β4 integrin levels were unchanged. This suggests that inhibition of EGFR/HER-2 may disrupt their physical interaction with β4 integrin rather than its expression, implying this interaction is critical for adhesion. To test this, possibly, the co-localisation of the β4 integrin subunit with EGFR and HER-2 was examined. Figure 9 shows strong co-localisation of EGFR with β4 integrin in OVCAR-5 clusters and SKOV-3 aggregates, while figure 10 shows HER-2 and β4 integrin co-localisation in SKOV-3 cells. As OVCAR-5 is HER-2-negative, no staining was performed.

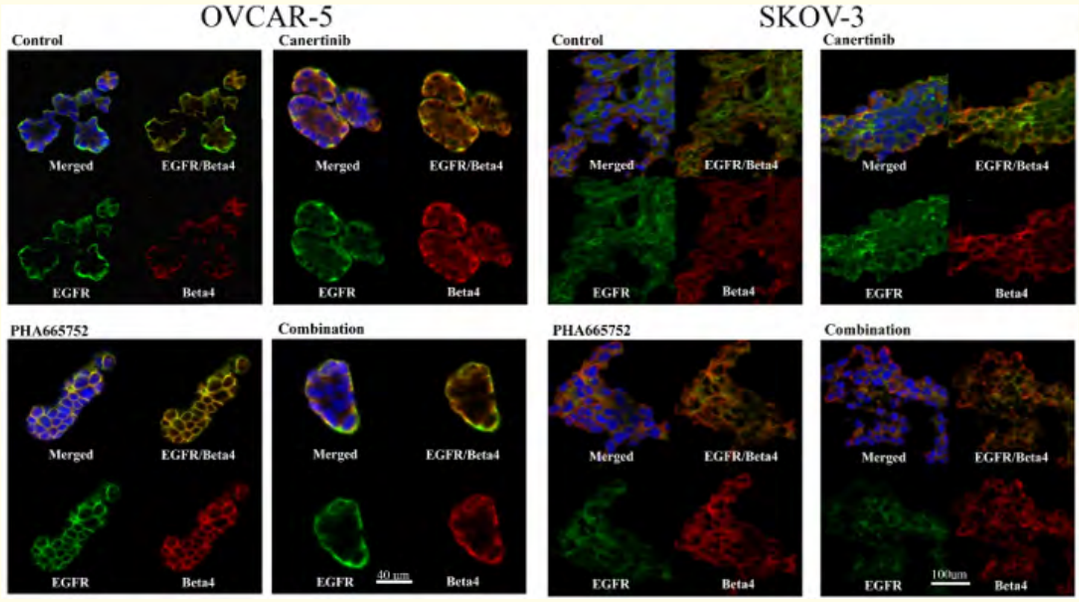


Figure 9: *Co-localisation of EGFR and β4 integrin subunit in cell clusters of OVCAR-5 and cell aggregates of SKOV-3 cell lines. Cells were treated with 4 µM canertinib, 2 µM PHA665752, and a combination of the inhibitors (3 µM Canertinib + 1 µM PHA665752) for 4 h before processing for immunofluorescence. Green fluorescence is EGFR, red fluorescence is β4 integrin, and blue fluorescence is the nucleus.*

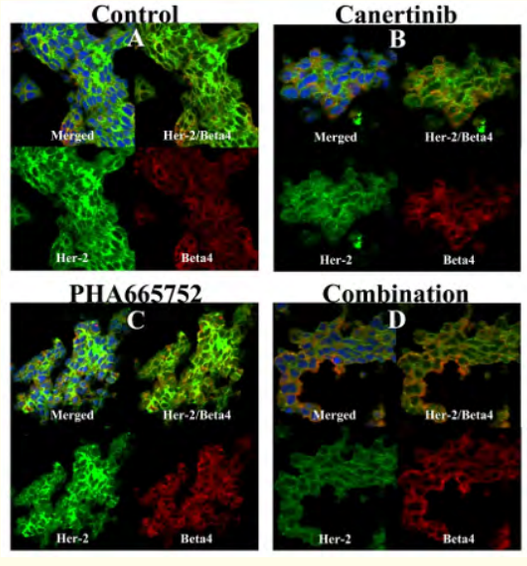


Figure 10: *Co-localisation of HER-2 and β4 integrin subunit in cell aggregates of SKOV-3 cell lines. Cells were treated with 4 µM canertinib, 2 µM PHA665752, and a combination of the inhibitors (3 µM Canertinib + 1 µM PHA665752) for 4h before processing for immunofluorescence. Green fluorescence is HER-2, red fluorescence is β4 integrin, and blue fluorescence is the nucleus.*

We analysed immunofluorescent co-staining of EGFR and HER-2 with β4 integrin, quantifying fluorescence intensity and co-localisation. Both single and combination inhibitor treatments significantly reduced EGFR-β4 integrin co-localisation in OVCAR-5 clusters (Figure 11A, $p < 0.0001$) and SKOV-3 aggregates (Figure 11B, $p < 0.0001$), as well as HER-2-β4 integrin co-localisation in SKOV-3 (Figure 11C, $p < 0.0001$).

Fluorescence intensity of EGFR decreased in canertinib and combination treatments in OVCAR-5 (Figure 11D) and SKOV-3 (Figure 11E), while HER-2 intensity declined in SKOV-3 (Figure 11F). β4 integrin intensity remained unchanged across treatments, consistent with immunoblotting results (Figure 8).

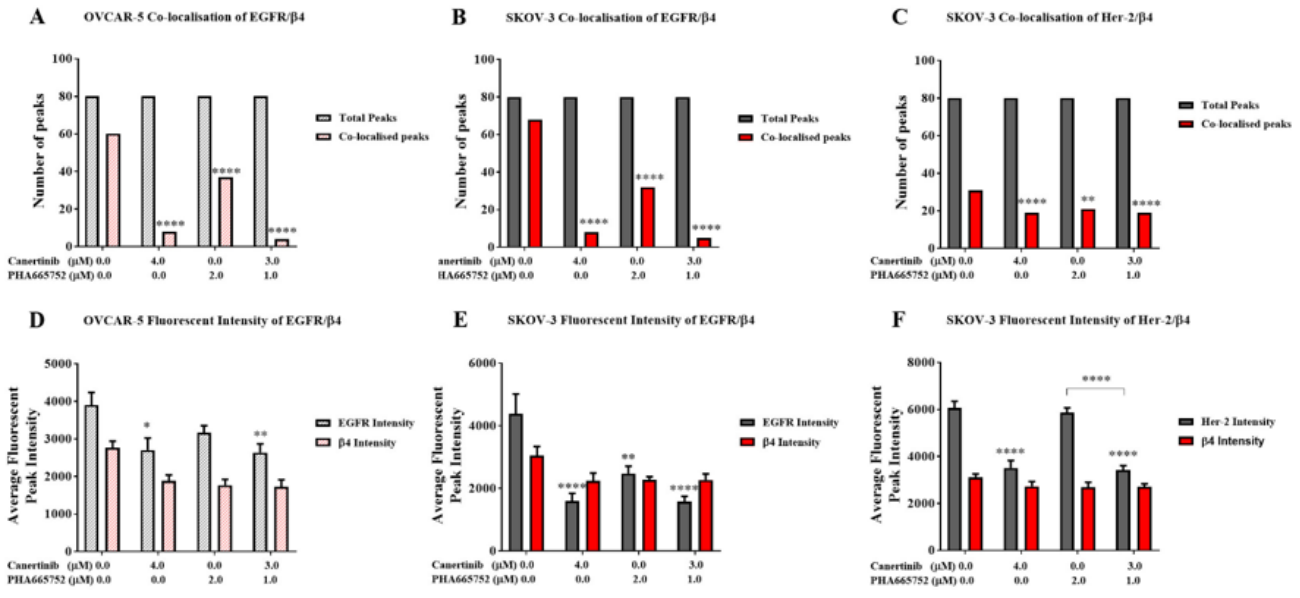


Figure 11: *Co-localisation and total fluorescent intensity of EGFR, HER-2 and β4 integrin subunit in OVCAR-5 and SKOV-3 cells after treatment with canertinib and PHA665752 alone and in combination in 20 ng/mL GF. The co-localisation of EGFR/β4 in OVCAR-5 (A) and its total fluorescent intensity (D), co-localisation of EGFR/β4 in SKOV3 (B) and its total fluorescent intensity (E), co-localisation of HER-2/β4 in SKOV-3 (C), and its total fluorescent intensity (F). The total fluorescent intensity (peaks) of 80 (n = 3) were randomly counted and pooled to generate graphs (A, B, C). Statistically significant differences are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) compared to the control or the other concentrations.*

Discussion

Advanced ovarian cancer often disseminates through the peritoneum, forming multiple secondary nodules. Despite surgery and chemotherapy, recurrence is frequent due to therapy resistance. Understanding early adhesion of cancer cells to the peritoneal membrane is vital for preventing tumour implantation and regrowth [33,35]. Adhesion begins when single cells or spheroids attach to exposed basement membrane proteins following mesothelial disruption, a process further facilitated by ascitic fluid that weakens the mesothelial barrier [10,36-38]. Integrin-ECM interactions then trigger focal adhesion signalling that promotes survival and spread, with the β4 integrin subunit playing a central role [39]. Its co-activation with EGFR, HER-2, and c-MET is cell line-dependent [16,17,40].

We investigated how EGF and HGF influence the adhesion of ovarian cancer clusters to ECM components (collagen I, IV, and laminin). These growth factors, which are abundant in patients' ascites, were tested alongside the EGFR/HER-2 inhibitor canertinib and the c-MET

inhibitor PHA665752 [41-46]. Adhesion increased with higher growth-factor concentrations in OVCAR-5 and SKOV-3 cells, but was suppressed by both inhibitors in a dose-dependent manner. Combined inhibition produced the most potent effect, implicating cooperative EGFR/HER-2/c-MET signalling in ECM-mediated adhesion, but the efficacy of combined drug treatment at specific concentrations may be additive in this study. Treatments were applied 4 hours before assays to exclude effects on proliferation. Previous reports have linked EGFR and HER-2 activation to invasion and spheroid survival [46-49], yet few have addressed the adhesion step in cell clusters. We hypothesised that integrins mediate this process via cross-talk with EGFR, HER-2, and c-MET [47-50].

Consistent with earlier findings on integrin expression in ovarian cancer [14,36,50-52], our data show that RGDS peptides, mimicking β1-integrin binding, reduced adhesion in both lines, but dual drug treatment had more potent effects [14,37,51-53]. This suggests both RGDS-dependent and non-RGDS interactions may mediate adhesion. Since β4 integrin does not bind RGDS, it likely mediates non-RGDS adhesion [54]. Inhibitors of EGFR, HER-2, and c-MET may disrupt β4 integrin interactions without altering its expression, as β4 protein levels remained stable (Figure 7, 8 and 11). Reduced phosphorylation of EGFR, HER-2, and c-MET following treatment supports a functional relationship between receptor kinase activity and β4 integrin-associated adhesion. Disruption of β4 integrin-receptor co-localization (Figure 11A-11C) further suggests that spatial proximity of these molecules contributes to adhesion-related signalling. β4 integrin is therefore implicated as a potential mediator, rather than the sole determinant, of RGDS-independent adhesion under EGFR/HER-2/c-MET inhibition.

As these inhibitors target ATP-binding sites, they may interfere with cytoplasmic β4 integrin-receptor associations, thereby impairing extracellular matrix attachment [55,56]. While our findings support a role for β4 integrin-associated adhesion, integrin-mediated ECM interactions are inherently complex and likely involve multiple integrin subtypes. Future studies employing integrin subtype-specific functional perturbations will be important to delineate the relative contributions of β1, β4, and other integrins to ovarian cancer cell cluster adhesion. Although β4 integrin associations with EGFR, HER-2, and c-MET have been reported, supporting data in ovarian cancer remain limited. Downstream signalling pathways-including PI3K/AKT, FAK, and MAPK/ERK-activated by these receptor-integrin complexes are well established in other cancer types [16,57-59]. Further investigation is required to define the specific downstream signalling events activated during the early adhesion of ovarian cancer cell clusters, which may represent critical targets for therapeutic intervention to limit early metastatic dissemination.

It is important to be mindful that this study has several limitations. First, direct mechanistic validation of β4 integrin involvement was not performed, as molecular deletion of the β4 integrin extracellular domain and Förster Resonance Energy Transfer (FRET)- based interaction analyses were not conducted. Functional perturbation approaches-including β4 integrin knockdown, knockout, or function-blocking antibody strategies-would provide stronger evidence to define causal relationships between β4 integrin and receptor tyrosine kinase signalling. Second, the experimental models used do not fully recapitulate the cellular complexity of the tumour microenvironment. More advanced co-culture systems incorporating extracellular matrix (ECM)-producing stromal cells, such as fibroblasts and endothelial cells, would provide a more physiologically relevant context for studying integrin-mediated adhesion [60-62]. Future studies will further investigate how ovarian cancer cell clusters respond to EGFR and c-MET inhibition in regulating adhesion and invasion using an *ex vivo* chorioallantoic membrane (CAM) model. This platform more closely mimics the early ovarian tumour microenvironment and may help clarify the mechanisms that contribute to the establishment of advanced disease [33].

Conclusion

In summary, adhesion of OVCAR-5 and SKOV-3 multicellular clusters is regulated by EGFR, HER-2, and c-MET signalling pathways. Combined inhibition of these receptor axes significantly reduces adhesive capacity, likely through disruption of β4 integrin-associated receptor interactions. These findings underscore the importance of early tumour cell-extracellular matrix (ECM) adhesion in the

metastatic cascade and identify receptor-integrin crosstalk as a potential therapeutic vulnerability. Targeting this adhesion machinery may represent a promising strategy to limit peritoneal dissemination and metastatic progression in advanced ovarian cancer.

Author Contributions

WF designed, conducted and analysed the research study. KC designed, conducted and analysed the research study. PS designed the experiment and prepared the manuscript; AG designed and prepared the manuscript.

Acknowledgment

Version 1 of the manuscript has previously been published in a Preprint [63]. The manuscript is based on a PhD thesis by Dr Wafaa Hassan [64]. We would like to thank the government of Iraq for supporting Wafaa Hassan's PhD scholarship.

Funding Support

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Bibliography

1. Rickard BP., *et al*. "Malignant ascites in ovarian cancer: cellular, acellular, and biophysical determinants of molecular characteristics and therapy response". *Cancers* 13.17 (2021): 4318.

2. Chitcholtan K., *et al*. "Sensitivity of EGFR/HER-2 positive cells isolated from ascitic fluid of advanced ovarian cancer patients to EGFR/HER-2 inhibitors". *Applied Sciences* 10.7 (2020): 2343.

3. Ayantunde AA and Parsons SL. "Pattern and prognostic factors in patients with malignant ascites: a retrospective study". *Annals of Oncology* 18.5 (2007): 945-949.

4. Latifi A., *et al*. "Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors". *PLoS One* 7.10 (2012): e46858.

5. Sun X., *et al*. "Detection of type IV collagenase activity in malignant ascites". *World Journal of Gastroenterology* 9.11 (2003): 2592-2595.

6. Ritch SJ and Telleria CM. "The transcoelomic ecosystem and epithelial ovarian cancer dissemination". *Frontiers in Endocrinology* 13 (2022): 886533.

7. Mikuła-Pietrasika J., *et al*. "Malignant ascites determine the transmesothelial invasion of ovarian cancer cells". *International Journal of Biochemistry and Cell Biology* 92 (2017): 6-13.

8. Worzfeld T., *et al*. "The unique molecular and cellular microenvironment of ovarian cancer". *Frontiers in Oncology* 7 (2017): 24.

9. Kenny HA., *et al*. "Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extra-cellular matrices on adhesion and invasion of ovarian cancer cells to the omentum". *International Journal of Cancer* 121.7 (2007): 1463-1472.

10. Ween MP., *et al*. "Role of versican, hyaluronan and CD44 in ovarian cancer metastasis". *International Journal of Molecular Sciences* 12.2 (2011): 1009-1029.

11. Bottinis C., *et al*. "Polarization of the alpha6 beta 4 Integrin in ovarian carcinomas". *International Journal of Cancer* 54.2 (1993): 261-267.

12. Skubitz APN., *et al*. "Expression of a6 and f34 integrins in serous ovarian carcinoma correlates with expression of the basement membrane protein laminin". *American Journal of Pathology* 148.5 (1996): 1445-1461.

13. Ahmed N., *et al*. "Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment". *Clinical and Experimental Metastasis* 22.5 (2005): 391-402.

14. Sawada K., *et al*. "Integrin inhibitors as a therapeutic agent for ovarian cancer". *Journal of Oncology* (2012): 915140.

15. Barczyk M., *et al*. "Integrins". *Cell and Tissue Research* 339.1 (2010): 269-280.

16. Merdek KD., *et al*. "Intrinsic signaling functions of the beta 4 integrin intracellular domain". *Journal of Biological Chemistry* 282.41 (2007): 30322-30330.

17. Bertotti A., *et al*. "B4 integrin is a transforming molecule that unleashes Met tyrosine kinase tumorigenesis". *Cancer Research* 65.23 (2005): 10674-10679.

18. Bon G., *et al*. "Loss of B4 integrin subunit reduces the tumorigenicity of MCF7 mammary cells and causes apoptosis upon hormone deprivation". *Clinical Cancer Research* 12 (2006): 3280-3287.

19. Giancotti FG. "Targeting integrin b4 for cancer and anti-angiogenic therapy". *Trends in Pharmacological Sciences* 28.10 (2007): 506-511.

20. Bertotti A., *et al*. "β4 integrin activates a Shp2–Src signaling pathway that sustains HGF-induced anchorage-independent growth". *Journal of Cell Biology* 175.6 (2006): 993-1003.

21. Campos S., *et al*. "Multicenter, randomized phase II trial of oral CI-1033 for previously treated advanced ovarian cancer". *Journal of Clinical Oncology* 23.24 (2005): 5597-5604.

22. Vergote IB., *et al*. "Randomized phase III study of erlotinib versus observation in patients with no evidence of disease progression after first-line platin-based chemotherapy for ovarian carcinoma: a European organisation for research and treatment of cancer-gynaecological cancer group, and gynecologic cancer intergroup study". *Journal of Clinical Oncology* 32.4 (2013): 320-326.

23. Siwak DR., *et al*. "Targeting the epidermal growth factor receptor in epithelial ovarian cancer: current knowledge and future challenges". *Journal of Oncology* (2010): 568938.

24. Amler LC., *et al*. "HER2 as a therapeutic target in ovarian cancer". Ovarian Cancer - Clinical and Therapeutic Perspectives (2012).

25. Davies S., *et al*. "High incidence of ErbB3, ErbB4 and MET expression in ovarian cancer". *International Journal of Gynecological Pathology* 33.4 (2014): 402-410.

26. Bu R., *et al*. "HGF/c-Met pathway has a prominent role in mediating antiapoptotic signals through AKT in epithelial ovarian carcinoma". *Laboratory Investigation* 91.1 (2011): 124-137.

27. Hassan W., *et al*. "A combination of two receptor tyrosine kinase inhibitors, canertinib and PHA665752 compromises ovarian cancer cell growth in 3D cell models". *Oncology and Therapy* 4.2 (2016): 257-274.

28. Prasasya RD., *et al*. "A multivariate model of ErbB network composition predicts ovarian cancer cell response to canertinib". *Biotechnology and Bioengineering* 109.1 (2012): 213-224.

29. Hassan W., *et al*. "Ascitic fluid from advanced ovarian cancer patients compromises the activity of receptor tyrosine kinase inhibitors in 3D cell clusters of ovarian cancer cells". *Cancer Letters* 420 (2018): 168-181.

30. Kim HJ. "Therapeutic strategies for ovarian cancer in point of HGF/c-met targeting". *Medicina* 58.5 (2022): 649.

31. Dunn E., *et al*. "The anti-proliferative effect of PI3K/mTOR and ERK inhibition in monolayer and three-dimensional ovarian cancer cell models". *Cancers* 14.2 (2022): 395.

32. Timenetsky J., *et al*. "Detection of multiple mycoplasma infection in cell cultures by PCR". *Brazilian Journal of Medical and Biological Research* 39.7 (2006): 907-914.

33. Chitcholtan K., *et al*. "Effects of resveratrol on *in vivo* ovarian cancer cells implanted on the chorioallantoic membrane (CAM) of a chicken embryo model". *International Journal of Molecular Sciences* 25.8 (2024): 4374.

34. Weaver VM., *et al*. "b4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium". *Cancer Cell* 2.3 (2002): 205-216.

35. Moghaddam SM., *et al*. "Significance of vascular endothelial growth factor in growth and peritoneal dissemination of ovarian cancer". *Cancer and Metastasis Reviews* 31.1-2 (2012): 143-162.

36. Kwon Y., *et al*. "Differential expressions of adhesive molecules and proteases define mechanisms of ovarian tumor cell matrix penetration/invasion". *PLoS ONE* 6.4 (2011): e18872.

37. Barbolina MV., *et al*. "Downregulation of connective tissue growth factor by three-dimensional matrix enhances ovarian carcinoma cell invasion". *International Journal of Cancer* 125.4 (2009): 816-825.

38. Kipps E., *et al*. "Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research". *Nature Reviews* 13.4 (2013): 273-282.

39. Stewart RL and O'Connor KL. "Clinical significance of the integrin α6β4 in human malignancies". *Laboratory Investigation* 95.9 (2015): 976-986.

40. Ross RS. "Molecular and mechanical synergy: cross-talk between integrins and growth factor receptors". *Cardiovascular Research* 63.3 (2004): 381-390.

41. Matte I., *et al*. "Profiling of cytokines in human epithelial ovarian cancer ascites". *American Journal of Cancer Research* 2.5 (2012): 566-580.

42. Kim S., *et al*. "Ascites modulates cancer cell behavior, contributing to tumor heterogeneity in ovarian cancer". *Cancer Science* 107.9 (2016): 1173-1178.

43. Chudecka-Głaz AM., *et al*. "Assessment of selected cytokines, proteins, and growth factors in the peritoneal fluid of patients with ovarian cancer and benign gynecological conditions". *OncoTargets and Therapy* 8 (2015): 471-485.

44. Santala M., *et al*. "Synthesis and breakdown of fibrillar collagens: concomitant phenomena in ovarian cancer". *British Journal of Cancer* 77 (1998): 1825-1831.

45. Li M., *et al*. "Microenvironment remodeled by tumor and stromal cells elevates fibroblast-derived COL1A1 and facilitates ovarian cancer metastasis". *Experimental Cell Research* 394.1 (2020): 112153.

46. Trachana S., *et al*. "The development of an angiogenic protein "signature" in ovarian cancer ascites as a tool for biologic and prognostic profiling". *PLoS ONE* 11 (2016): e0156403.

47. Bourgeois DL., *et al*. "High-grade serous ovarian cancer cell lines exhibit heterogeneous responses to growth factor stimulation". *Cancer Cell International* 15 (2015): 112.

48. Zhou HY., *et al*. "Synergistic effects of epidermal growth factor and hepatocyte growth factor on human ovarian cancer cell invasion and migration: role of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase". *Endocrinology* 148.11 (2007): 5195-5208.

49. Alper Ö., *et al*. "Epidermal growth factor receptor signaling and the invasive phenotype of ovarian carcinoma cells". *Journal of the National Cancer Institute* 93.18 (2001): 1375-1384.

50. Parashar D., *et al*. "Peritoneal spread of ovarian cancer harbors therapeutic vulnerabilities regulated by FOXM1 and EGFR/ERBB2 signaling". *Cancer Research* 80.24 (2020): 5554-5568.

51. Villegas-Pinedaa JC., *et al*. "Integrins and haptoglobin: Molecules overexpressed in ovarian cancer". *Pathology- Research and Practice* 211.12 (2015): 973-981.

52. Casey RC., *et al*. "b1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids". *American Journal of Pathology* 159.6 (2001): 2071-2080.

53. Shield K., *et al*. "α2β1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis". *Journal of Carcinogenesis* 6 (2007): 11.

54. Litjens SHM., *et al*. "Current insights into the formation and breakdown of hemidesmosomes". *Trends in Cell Biology* 16.7 (2006): 376-383.

55. Spector N., *et al*. "HER2 therapy small molecule HER-2 tyrosine kinase inhibitors". *Breast Cancer Research* 9.2 (2007): 205.

56. Seshacharyulu P., *et al*. "Targeting the EGFR signaling pathway in cancer therapy". *Expert Opinion on Therapeutic Targets* 16.1 (2012): 15-31.

57. Song QH., *et al*. "Role of epidermal growth factor and epidermal growth factor receptor on hemidesmosome complex formation and integrin subunit b4". *Cell and Tissue Research* 312.2 (2003): 203-220.

58. Mercurio AM and Rabinovitz I. "Toward a mechanistic understanding of tumor invasion-lessons from the a6b4 integrin". *Cancer Cell* 11.2 (2001): 129-141.

59. Gambaletta D., *et al*. "Cooperative signaling between a6b4 integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion". *Journal of Biological Chemistry* 275.14 (2000): 10604-10610.

60. Plesselova S., *et al*. "Multicompartmentalized microvascularized tumor-on-a-chip to study tumor-stroma interactions and drug resistance in ovarian cancer". *Cellular and Molecular Bioengineering* 17 (2024): 345-367.

61. Gauduchon P., *et al*. "Transmigration of human ovarian adenocarcinoma cells through endothelial extracellular matrix involves v integrins and the participation of MMP2". *International Journal of Cancer* 114.4 (2005): 531-543.

62. Medeiros M., *et al*. "Mimicking the tumor microenvironment: Fibroblasts reduce miR-29b expression and increase the motility of ovarian cancer cells in a coculture model". *Biochemical and Biophysical Research Communications* 516.1 (2019): 96-101.

63. Hassan W., *et al*. "A combination of EGFR/HER-2 and CMET inhibitors reduced cell adhesion of ovarian cancer clusters to the extracellular matrix (ECM)". Preprints (2025).

64. Hassan W. "The effects of a combination of two receptor tyrosine kinase inhibitors on two ovarian cancer cell lines". University of Canterbury: Canterbury (2017).

Volume 15 Issue 5 May 2026