Co-targeting the EGFR and IGF-IR with anti-EGFR monoclonal antibody ICR62 and the IGF-IR tyrosine kinase inhibitor NVP-AEW541 in colorectal cancer cells

MATTHEW P. CUNNINGHAM¹, HILARY THOMAS¹, CHRISTOPHER MARKS², MARGARET GREEN², ZHEN FAN³ and HELMOUT MODJTAHEDI¹,⁴

¹Division of Oncology, Postgraduate Medical School, Guildford, ²Colorectal unit, Royal Surrey County Hospital, Guildford, UK; ³Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; ⁴School of Life Sciences, Kingston University London, Kingston-upon-Thames, UK

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Abstract. The aberrant expression of the epidermal growth factor receptor (EGFR) has been reported in a wide range of epithelial tumours. In some studies, co-expression of insulin-like growth factor receptor-I (IGF-IR) have been associated with resistance to the EGFR inhibitors. Here, we investigated the sensitivity of a panel of human colorectal tumour cell lines, including two newly established lines Colo2 and Colo13, to treatment with anti-EGFR mAb ICR62 and IGF-IR tyrosine kinase inhibitor NVP-AEW541 alone and in combination. We also determined the association between the expression levels of EGFR and IGF-IR with their responses to ICR62 and/or NVP-AEW541. In contrast to DiFi cells, which contained high levels of EGFR but lower level of IGF-IR, the remaining 11 colorectal tumour cells expressed low levels of both EGFR and IGF-IR and such cells were relatively resistant to ICR62 or NVP-AEW541 when used alone. Interestingly, compared to the results with the single agent, the effect of combination of NVP-AEW541 and ICR62 was found to be additive on inhibiting the growth of Colo13, CCL235, CCL244 cells but antagonistic in other (CCL218) cells. While overexpression of the EGFR seems to be associated with response to ICR62, no clear correlation was found between the expression levels of EGFR and IGF-IR, or the levels of phosphorylated EGFR and response to treatment with NVP-AEW541, in single or combination setting with ICR62. Our results suggest that combining EGFR and IGF-IR inhibitors may enhance anti-tumour response in a fraction of colorectal cancer cells and warrants further study in colorectal cancer.

Introduction

Despite continued advances in early diagnosis and improvements of new treatments, colorectal cancer remains one of the most common types of human cancer in terms of incidence and mortality. In 2000, there were 945,000 cases of colorectal cancer and colorectal cancer was responsible for 492,000 deaths worldwide (1).

Over the past three decades, aberrant expression of cell surface receptors with intrinsic protein tyrosine kinase activity has been found in a wide range of epithelial tumours including colorectal cancers, and, in some cases, it has been shown to correlate with poor prognosis. The insulin-like growth factor (IGF) receptor (IGF-IR) is a receptor tyrosine kinase that transmits the mitogenic signals of IGF-I and IGF-II (2-4). Binding of IGF-I or IGF-II to the cysteine-rich extracellular IGF-IR α subunits causes a conformational change in the intracellular domain of the IGF-IR ß-subunits, leading to activation of intrinsic receptor tyrosine kinase and subsequent receptor tyrosine autophosphorylation and phosphorylation of the insulin receptor substrate molecules (e.g. IRS-1, IRS-2) that triggers a cascade of mitogenic cell signal activation via the PI-3K/Akt pathway and via the Ras-Raf-MAPK pathway (2-6).

Deregulation of signalling via the IGF-IR and/or its ligands has been associated with initiation and maintenance of the transformed phenotype, including cell invasion and motility, tumour angiogenesis and metastases, protection against tumour micro-environmental stress, and resistances to chemotherapy and radiotherapy (7-12). Expression of the IGF-IR is found in many types of malignancy including colorectal cancers (13-17). Consequently, therapeutic strategies targeting the IGF-IR have been explored, including the use of mAbs, TKIs, and anti-sense oligonucleotides, which can inhibit the growth of IGF-IR expressing tumour cells (e.g. breast, lung, multiple myeloma) in vitro and in vivo, and enhance responses of cultured or xenografted human cancer cells to treatments with cytotoxic drugs or radiotherapy (9,18-24).

Ample evidence has shown that the IGF-IR cross-talks with other growth factor tyrosine kinase receptors, such as the EGFR, VEGFR, and HER-2, to coordinate the malignant...
behaviour of cancer cells (25-30). IGF-IR expression has also been associated with resistance to anti-EGFR and anti-HER-2 based therapies in several experimental models, and co-targeting the IGF-IR with the EGFR or HER-2 may achieve better therapeutic effects in several types of cancer model (31-33). We have recently reported that the IGF-IR, EGFR, and HER-2 are co-expressed in 75% of patients with Dukes' C (stage III) colorectal cancer and that targeting of the EGFR in human colorectal tumour cells by a combination of ICR62 and gefitinib was not superior to the result of either agent alone (17,34). The aim of the present study was therefore to examine the cell surface expression of the IGF-IR and EGFR in a panel of human colorectal cancer cell lines, including 2 new cell lines recently established in our laboratory, and to evaluate the sensitivity of these cancer cells to treatment with an IGF-IR tyrosine kinase inhibitor (TKI), NVP-AEW541 (35) and our anti-EGFR mAb ICR62 (36). We explored the therapeutic advantage of a combination of ICR62 and NVP-AW541 over the single inhibitor together with the relationship between receptor expression and growth inhibition by the EGFR and/or IGF-IR inhibitors.

Materials and methods

Cancer cell lines. The human colorectal cancer cell line CCL247/HCT-116 was purchased from the European Collection of Cell Culture (ECCAC; Porton Down, UK), and other colorectal cancer cell lines CCL218/HIT-29, CCL221/DLD-1, CCL225/HCT-15, CCL227/SW620, CCL228/SW480, CCL231/SW48, CCL235/SW837, CCL244/HCT-8/HRT-18 were purchased from The American Type Culture Collection (Manassas, VA, USA). Two new human colorectal tumour cell lines Colo2 and Colo13 were recently established in our laboratory: the Colo2 cell line was established from a patient with a Dukes' C invasive and moderatively differentiated tumour. The EGFR-overexpressing DiFi colorectal cell line was previously reported (34). Other reference human cancer cell lines included 2 new cell lines recently established in our laboratory, and to evaluate the sensitivity of these cancer cells to treatment with an IGF-IR tyrosine kinase inhibitor (TKI), NVP-AEW541 (35) and our anti-EGFR mAb ICR62 (36). We explored the therapeutic advantage of a combination of ICR62 and NVP-AW541 over the single inhibitor together with the relationship between receptor expression and growth inhibition by the EGFR and/or IGF-IR inhibitors.

Reagents. The rat mAb ICR62 (IgG2b) and the mouse mAb HM43.16B were raised against the external domain of the EGFR on the breast carcinoma cell line MDA-MB-468 and the HC2 20d2/c cell line, respectively (36, unpublished data). The mouse anti-IGF-IR antibody (α subunit) was purchased from Calbiochem (Merck Biosciences Ltd., Nottingham, UK). The mouse anti-EGFR mAb clone F4 and rabbit anti β-actin polyclonal antibody were purchased from Sigma (Sigma-Aldrich). Antibodies to phospho-tyrosine (P-Tyr-100), Tyr1068-phosphorylated EGFR, and Thr202/Tyr204-phosphorylated MAPK p44/p42 were purchased from New England Biolabs Ltd. (Hitchin, UK). Antibodies to phosphorylated EGFR in other sites (Tyr1173, 1148, 1086, and 845), and Ser473-phosphorylated Akt were purchased from Biosource (Biosource Europe S.A., Belgium). The FITC-conjugated goat anti-mouse IgG secondary antibody was purchased from Southern Biotechnology Associates Inc. (AL, USA). The IGF-IR TKI NVP-AEW541 was kindly provided by Novartis (35) and the inhibitors to PI3K (LY294002) and MEK (U0126) were purchased from Sigma. EGFR and HB-EGF were from R&D Systems, MN, USA, whereas IGF-I and IGF-II were from Astral Biologicals, CA, USA.

Flow cytometric analysis. The cell surface levels of EGFR or IGF-IR were determined using FACS analysis as described previously (34). A minimum of 10,000 events were recorded by excitation with an argon laser at 488 nm, and analyzed using the FL-1 detector (FITC detector; 525 nm) of a Beckman Coulter Epics XL flow cytometer (Becton-Dickinson, UK) and the CellQuest™ software.

Cell proliferation and inhibition assays. The effect of ICR62, NVP-AEW541, LY294002 and/or U0126 on the growth of human tumour cell lines was investigated using a colorimetric assay, as described previously (36). Briefly, tumour cells were seeded at a density of 5x10^4/well in 100 μl DMEM containing 2-10% FCS in a 96-well plate. Following 3-h incubation at 37°C, 100 μl aliquots of the inhibitors were added to triplicate wells and the cells were incubated at 37°C until the cells in the wells containing control medium were confluent. Tumor cells were then fixed with glutaraldehyde, washed with tap water, air dried and stained with 0.05% methylene blue. The absorbance of each well was measured at 620 nm using a Labsystems MultiSkan RC plate reader (Thermo Electron Corporation, UK). To determine the initial number of cells, an extra plate of cells was set up and processed after 3-h incubation at 37°C without the inhibitors.

Western blot analysis. Cells grown to near confluence in 6-well tissue culture plates (Greiner Bio-One, UK) were washed once and then incubated in 5 ml DMEM/0.1% FCS containing medium, ICR62 (400 nM) and/or NVP-AEW541 (400 nM) for 24 h at 37°C, prior to no treatment or the addition of 10 nM EGFR, HB-EGF (R&D Systems), IGF-I or IGF-II (Austral Biologicals) for 15 min at 37°C. Cells were washed with PBS and then solubilized with 400 μl of LDS sample buffer (Invitrogen, UK) supplemented with 4 μl of protein inhibitor cocktail containing 104 mM AEBSF, 80 μM aprotonin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich). The cell lysates were heated at 72°C for 10 min and their viscosity was reduced by several passages through a 25x5/8 gauge needle. Equal amounts of cell lysate were separated on 4-12% Bis-Tris-gels (Invitrogen) using the XCell II™ Surelock™ Mini-Cell system (Invitrogen) and transferred to PVDF membranes using the XCell II™ Mini-Cell Blot Module kit (Invitrogen). The PVDF membranes were blocked to prevent non-specific binding, incubated with primary antibody for
1 h at room temperature. Specific signals were detected using the WesternBreeze® chemiluminescent anti-rabbit and anti-mouse kits (Invitrogen).

**Results**

**Expression of EGFR and IGF-IR in human colorectal cancer cells.** The expression levels of EGFR and IGF-IR in the panel of human colorectal cell lines used in this study were determined using FACS analysis. As shown in Fig. 1, among the human colorectal cancer cell lines, DiFi cells express the highest levels of EGFR, with a mean fluorescence intensity (MFI) of 169.6, but the value of EGFR expression ranged from only 8.4 (CCL 218) to 23.0 (Colo13) in the other 11 colorectal tumour cell lines. While all cell lines were positive for IGF-IR expression, the expression level was generally low in the panel of colorectal cancer cell lines, ranging from an MFI of 9.0 (Colo13) to 20.3 (CCL221) (Fig. 1).

**Response of human colorectal cancer cells to treatment with NVP-AEW541.** The IGF-IR-expressing control cell lines MCF-7 and HN5 were found to be highly sensitive to NVP-AEW541. At 500 nM, NVP-AEW541 inhibited the growth of MCF-7 by 80% (IC₅₀=123 nM), and HN5 cells by 54% (IC₅₀=470 nM), respectively (Fig. 2A). However, of the 12 human colorectal cancer cells examined, only colo13, CCL218 and CCL235 were sensitive to treatment with NVP-AEW541 (Fig. 2B). For example, at 500 nM, NVP-AEW541 inhibited the growth of CCL218 cells by 67% (IC₅₀=426 nM) (Fig. 2A). No clear correlation was found between the levels of IGF-IR expression determined by FACS analysis (Fig. 1) and responses to NVP-AEW541 (Fig. 2B).

**Effect of combination treatment with NVP-AEW541 and ICR62.** In contrast to NVP-AEW541 treatment, by which CCL218 and CCL235 were found to be the most sensitive colorectal tumour cell lines (Fig. 2B), the EGFR-overexpressing cell line DiFi was the only colorectal tumour cell line that was highly sensitive to anti-EGFR mAb ICR62 (Fig. 2C). Complete growth inhibition of DiFi cells by mAb ICR62 was achieved at a concentration of 3.2 nM.
We next investigated the effect of combination treatment with the EGFR and IGF-IR inhibitors on the growth of these tumour cells, and found that treatment with a combination of NVP-AEW541 and mAb ICR62 achieved better growth inhibition of Colo13, CCL227, CCL235 and CCL244 cells, compared with the result by either inhibitor alone. Interestingly, the combination treatment was found to be antagonistic in the case of CCL218 cells (Fig. 2C), and similar antagonistic results were found when NVP-AEW541 was used in combination with small molecules EGFR inhibitors in CCL218 cells (data not shown).

Phosphorylation of EGFR by stimulation with EGF, HB-EGF, IGF-I and IGF-II. We found no clear correlation between cell surface expression levels of EGFR and IGF-IR in colorectal cancer cells and their response to the combination of EGFR and IGF-IR inhibitors. We next determined the levels of phosphorylated EGFR in these cell lines to determine whether the IGF-IR ligands are capable of transactivating the EGFR. Compared to DiFi cells, other human colorectal cell lines contained low or undetectable levels of EGFR (Fig. 3). In DiFi cells, EGFR phosphorylation was further increased following stimulation with EGF or HB-EGF, but not with the IGF-IR ligands IGF-I and IGF-II. Interestingly, in the EGFR and IGF-IR dual positive CCL235 cell line, exposure of the cells to IGF-I or IGF-II induced trans-phosphorylation of the EGFR (Fig. 3). Similar finding was observed in the EGFR-overexpressing control cell line HN5 but not in the EGFR-low expressing cell line MCF-7 or in the other human colorectal cell lines. The IGF-IR ligands were found to be less effective than EGF and HB-EGF at inducing tyrosine phosphorylation of the EGFR at the time-points studied (Fig. 3).

Effects of ICR62 and NVP-AEW541 on the phosphorylation levels of EGFR, MAPK and Akt in DiFi cells. Fig. 4 shows that DiFi cells contain a high basal level of phosphorylation at all 5 known distinct tyrosine residues on the EGFR, as well as phosphorylated MAPK p44/p42, but not that of Akt. ICR62 treatment of DiFi cells reduced the levels of total EGFR, phosphorylated EGFR levels at the 5 distinct tyrosine residues, and phosphorylated MAPK p44/p42 in the cells. In contrast, such results were not found upon NVP-AEW541 treatment (Fig. 4). However, NVP-AEW541 was effective in preventing IGF-I-stimulated phosphorylation of MAPK p44/p42 and Akt in DiFi cells, and such effects were more evident when NVP-AEW541 was used in combination with ICR62 (Fig. 4).
LY294002 and, a MEK inhibitor, U0126, on the proliferation of cell lines that were found to be sensitive (DiFi, HN5) or resistant (CCL225, CCL228) to ICR62 and/or NVP-AEW541. Fig. 5 shows that DiFi, HN5, CCL225, and CCL228 were all sensitive to LY294002 or U0126 treatment. At 50 μM, LY294002 inhibited completely the growth of DiFi, CCL225, and HN5 cells and growth of CCL228 cells by 72% (Fig. 5). At 20 μM, the MEK inhibitor U0126 inhibited the growth of DiFi, CCL225, CCL228, and HN5 cells by 81, 39, 33 and 79%, respectively (Fig. 5). Using a combination of the 2 inhibitors, there was increased growth inhibition in each of the cell lines when compared to treatment with either single agent (Fig. 5). The findings suggest that the lack of response to ICR62 or NVP-AEW541 in resistant cell lines, such as CCL225 and CCL228, was due to EGFR or IGF-IR independent activation of these downstream signal pathways.

**Discussion**

In recent years, several EGFR inhibitors have been approved by the FDA for the treatment of cancer patients (40,41). Of these, anti-EGFR mAbs cetuximab and panitumumab were approved for the treatment of patients with metastatic colorectal cancer (42). Despite positive responses observed in approximately 10-20% of patients treated, a major obstacle is the lack of overall response of colorectal cancer to therapy with the anti-EGFR mAbs. In addition, there are currently no reliable markers for response to therapy with the EGFR inhibitors. In several experimental studies, co-expression of the IGF-IR has been associated with resistance to treatment with anti-EGFR and anti-HER-2 therapies and co-targeting the IGF-IR and EGFR or HER-2 with a combination of the 2 agents inhibiting respective targets has shown advantages of therapeutic effect over the single inhibitor alone (27,31-33). Consistent with these findings, in the present study, we found co-expression of the EGFR and IGF-IR in a fraction of human colorectal cancer cell lines and potential therapeutic advantage of co-targeting the EGFR and IGF-IR with an anti-EGFR mAb (ICR62) and an IGF-IR TKI (NVP-AEW541).

Of the 12 human colorectal cancer cell lines examined, only DiFi expressed high levels of the EGFR (Fig. 1) and was the only human colorectal tumour cell line that was highly sensitive to the anti-EGFR mAb ICR62 (Fig. 2C). In addition, there was no clear association between the levels of phosphorylated EGFR in the panel of colorectal cancer cells and response to the anti-EGFR mAb ICR62. For example, unlike DiFi or HN5 cells which contained high levels of pEGFR and were highly sensitive to ICR62 treatment, this antibody did not inhibit the growth of SKBR3 cells which like DiFi and HN5 cells contained high level of pEGFR (Figs. 2C and 3). In addition, while all of the colorectal cancer cell lines were IGF-IR positive, the levels of IGF-IR expression were not predictive of sensitivity/resistance to the EGFR inhibitors. Interestingly, NVP-AEW541 in combination with ICR62 was found to be more effective at inhibiting the growth of some human colorectal cancer cell lines (e.g. Colo13, CCL235, CCL244) than either agent alone. Others have reported enhanced antitumour activity in human breast and prostate cancer cell models when an IGF-IR TKI was used in combination with anti-EGFR or anti-HER-2-based therapies (2,31). More recently, Hopfner and colleagues have also reported that NVP-AEW541 in combination with anti-EGFR mAb cetuximab was more effective than treatment with either agent alone at inhibiting the growth of 2 human colorectal tumour cell lines, HCT116 and HT29 (43). Similar to responses achieved with either single treatment, no clear association was found between EGFR or IGF-IR expression and response to the combination of NVP-AEW541 and ICR62 in our study. Interestingly, of the 12 human colorectal tumour cell lines used in the present study, both IGF-I and IGF-II induced tyrosine phosphorylation of the EGFR in several cell lines. This was more evident in CCL235 cells, which were also growth inhibited by the greatest extent by the combination of EGFR and IGF-IR inhibitors (Fig. 3).

While the combination of the EGFR and IGF-IR inhibitors did not completely inhibit the growth of any colorectal
tumour cell line in this study (Fig. 2C), the growth of tumour cells resistant to the EGFR and/or IGF-IR inhibitors could be inhibited using inhibitors of the MEK/MAPK (U0126) and/or PI3-K/Akt (LY294002) pathways (Fig. 4). These data are consistent with a previous study in which the EGFR TKI gefitinib was shown to be more effective than inhibitors of the PI3-K and MAPK pathways at inhibiting the growth of EGFR overexpressing A431 cells (44). Therefore, taken together, these results suggest that in EGFR overexpressing tumour cells (e.g. DiFi, HN5, A431), such tumour cells are likely to be dependent upon the EGFR for their proliferation and survival and that EGFR inhibition is more effective than PI3-K and MEK inhibition (34,45). In contrast, in other cell lines which expressed low levels of EGFR and IGF-IR (Fig. 1), direct inhibition of the MAPK and PI3-K pathways may be superior to targeting with the EGFR and/or IGF-IR inhibitors as such cells may rely on alternative/multiple receptors for activation of the MAPK or PI3-K pathways (44,46,47).

In a previous study, IGF-I was shown to delay the occurrence of apoptosis induced by anti-EGFR mAb cetuximab in DiFi cells but the protection was not sustained with the expansion of treatment due to the high sensitivity of the cells to cetuximab (48). Similarly, we found in our current study that IGF-I or IGF-II failed to protect DiFi cells from ICR62-mediated growth inhibition in extended cell culture. This was in contrast to our as-yet-unpublished data using 6 different EGFR ligands that protected DiFi cells from ICR62-induced growth inhibition (data not shown). In addition, either stimulation of IGF-IR with ligand or inhibition of the receptor with NVP-AEW541 had no major effect on phosphorylation levels of EGFR or MAPK p44/p42 in DiFi cells. While the anti-EGFR mAb ICR62 was able to attenuate basal and IGF-stimulated phosphorylation of Akt in DiFi cells. Because IGF-I induced Akt phosphorylation was decreased by NVP-AEW541 and more effectively when used in combination with ICR62, the results suggest that the combination of EGFR and IGF-IR inhibitors may have potential for enhanced inhibition on selective signal transducing molecules (i.e. Akt) in colorectal cancer cells.

The lack of apparent additive or synergistic growth inhibition of DiFi cells with the combination of NVP-AEW541 and ICR62 is likely due largely to high sensitivity of the cells to EGFR inhibition alone, which may under-estimate the potential utility of combinatorial therapy against the 2 receptors. We have shown recently that co-expression of IGF-IR and EGFR is common in patients with colorectal cancer (17). In addition to its role in up-regulating the EGFR autocrine loop and in mediating resistance to the EGFR inhibitors, IGF-I has also been shown to up-regulate the production of VEGF in several types of human cancers, including colorectal cancer (25,32,33). Moreover, we have shown previously that mAb ICR62, although not being able to inhibit the growth of cells expressing the type-III deletion mutant EGFR (EGFRvIII) in cell culture, has strong activity in inhibiting the metastasis of EGFRvIII expressing cells to the lung in athymic mice through mediating antibody-dependent cellular cytotoxicity (ADCC) (49). Thus, there might be additional antitumor effects of the combination of ICR62 and NVP-AEW541 mediated via immunological mechanisms such as ADCC, or via disturbing tumour blood supply that certainly warrants further investigation (47,49,50). It will also be interesting to determine whether the combination of NVP-AEW541 and ICR62 produces an antagonistic effect against CCL218 cells in vivo.

In summary, our results show that combinatorial treatment with the anti-EGFR mAb ICR62 and IGF-IR TKI NVP-AEW541 resulted in an enhanced growth inhibition in a fraction of colorectal cancer cell lines. Since co-expression of the EGFR, HER-2 and IGF-IR is common in patients with Dukes’ C colorectal cancer (17), further in-depth studies are warranted to elucidate the full therapeutic potential of combining the EGFR and IGF-IR inhibitors in experimental colorectal cancer models and the human setting.

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References


