

The effect of a dimeric Affibody molecule ($Z_{\text{EGFR:1907}}$)₂ targeting EGFR in combination with radiation in colon cancer cell lines

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Abstract. The epidermal growth factor receptor (EGFR) is frequently overexpressed in colorectal cancer and is therefore an attractive target for treatment. ($Z_{\text{EGFR:1907}}$)₂ is a newly developed dimeric affibody molecule with high affinity to the extracellular part of EGFR. In this study, we evaluated the cytotoxic effects of ($Z_{\text{EGFR:1907}}$)₂ in combination with external radiation and the possible inhibitory effects in the EGFR signalling pathways in the colon cancer cell lines HT-29 and HCT116. The effects were compared with an EGFR antibody (cetuximab) and the tyrosine kinase inhibitors (erlotinib and sunitinib). These cell lines are genotypically different with respect to e.g. *KRAS* and *BRAF* mutational status, recently shown to be of clinical significance for therapeutic effects. Both cell lines express approximately 100,000-150,000 EGFRs per cell but differ in the radiation response (HCT116, SF2=0.28 and HT-29, SF2=0.70). Exposure to ($Z_{\text{EGFR:1907}}$)₂ produced a small, but significant, reduction in survival in HCT116 but did not affect HT-29 cells. Similar results were obtained after exposure to EGF and the EGFR antibody cetuximab. The EGFR tyrosine kinase targeting inhibitor erlotinib and the multi-tyrosine kinase inhibitor sunitinib reduced survival in both cell lines. However, none of the drugs had any significant radiosensitizing effects in combination with radiation. Akt and Erk are central proteins in the EGFR downstream signalling and in the cellular response to ionizing

radiation. The activation of Akt (Ser 473) and Erk (Thr202/Tyr204) by radiation was both dose- and time-dependent. However the activation of EGFR was not clearly affected by radiation. Neither ($Z_{\text{EGFR:1907}}$)₂ nor any of the other drugs were able to completely inactivate Akt or Erk. On the contrary, erlotinib stimulated Akt phosphorylation in both cell lines and in HCT116 cells Erk was activated. Overall the results illustrate the complexity in response to radiation and drugs in cells with differential phenotypic status.

Introduction

Colorectal cancer is the third most frequent cancer form in the world with 1.2 million people diagnosed every year (1). The primary treatment is surgery but chemotherapy and/or radiotherapy are used preoperatively to reduce tumour burden or diminish recurrence risk (2,3) or postoperatively to likewise diminish recurrence risk (4-6).

The side effects from radiotherapy can be considerable (7) and agents that specifically radiosensitize tumours would allow dose reduction and thus less damage to surrounding tissues. Chemotherapeutic agents such as 5-FU, cisplatin and oxaliplatin are used in combination with radiation for sensitizing tumor cells to radiotherapy (8,9). However, the additional effects are limited, and resistance against cisplatin and oxaliplatin have been observed which can be associated with mutations in the DNA mismatch repair mechanisms (10). Other potential drugs with radiosensitizing properties are therefore of great interest.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor with an extracellular ligand binding site and an internal tyrosine kinase domain which is often overexpressed in colorectal cancer (11). EGFR expression in tumours has been associated with resistance against conventional drug treatment and radiation and may indicate poor prognosis (12,13). Activation of EGFR, by ligand or radiation, triggers a cascade of signalling events through three major pathways, the PI3 kinase (phosphatidylinositol 3-kinase) cascade, the Ras/Raf/Erk pathway and STAT (Signal transducers and activators of transcription), causing cell proliferation, survival, migration, and

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differentiation (14,15). EGFR is therefore a potential target for cancer treatment and extensive research has been devoted to find targeted therapies such as monoclonal antibodies (mAbs) and tyrosine kinase inhibitors.

Cetuximab is an EGFR antibody approved for clinical use for metastatic colorectal cancer both as monotherapy and in combination with chemotherapy or radiation (16,17). Erlotinib and sunitinib are both tyrosine kinase inhibitors but erlotinib inhibits EGFR (and possibly HER-2) (18) whilst sunitinib is a multiple receptor tyrosine kinase inhibitor. Erlotinib is used as treatment of locally advanced or metastatic non-small cell lung cancer (19) and sunitinib is used as treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumour (GIST) (20). However, not all patients respond to these treatments and generally, tyrosine kinase inhibitors have not shown the same promising results in clinical trials as with antibodies against EGFR (21). The EGFR expression and mutation status of several genes in the EGFR signalling pathway are some of the factors that have been considered to be responsible for the different response in patients.

A newly developed EGFR-targeting molecule is the Affibody protein, $(Z_{\text{EGFR:1907}})_2$, (Affibody AB, Stockholm, Sweden). Affibody molecules are small (~6.5 kDa) proteins consisting of a Z-domain originally derived from the IgG-binding domain of staphylococcal protein A, which has been engineered to be chemically stable and to bind target proteins with high affinity (22,23). Affibody molecules have potential for both therapeutic and diagnostic applications and $Z_{\text{EGFR:1907}}$ labelled with ^{111}In is a good EGFR tracer *in vivo* (24). $(Z_{\text{EGFR:1907}})_2$ is a dimeric affibody molecule with high affinity (K_D of 1.6 nM) to the extracellular domain of EGFR. The exact binding epitope is unknown; however it is not competing with the binding site for cetuximab. Since $(Z_{\text{EGFR:1907}})_2$ is a small molecule it may inhibit the EGFR activation in a different way than EGFR antibodies or tyrosine kinase inhibitors. Previous studies have evaluated the affinity, retention and internalization as well as the effect on cellular signalling in A431 cells (25,26). In this study, the possible cytotoxic effects of $(Z_{\text{EGFR:1907}})_2$ in combination with external radiation and the inhibitory effects in the EGFR signalling pathways were evaluated in the colon cancer cell lines HT-29 and HCT116. Akt and Erk in the PI3 kinase (phosphatidylinositol 3-kinase) cascade and Ras/Raf/Erk pathway, respectively, are central proteins in the EGFR downstream signalling and in the cellular response to ionizing radiation and EGFR-targeting agents. The serine/threonine kinase Akt is an important factor in protecting cancer cells from apoptosis, thereby contributing to resistance to drugs and radiation (27). Therefore, downregulation of Akt is expected to give an increased therapeutic effect of the drugs, leading to increased apoptosis and radiosensitization. Activated Erk is found both in the cytoplasm and nucleus, where it has a direct effect on the transcription by phosphorylation of transcription factors associated with increased radiation tolerance. HCT116 and HT-29, similar to most cancer cells, have several mutations in the EGFR signalling pathway, such as *KRAS* and *BRAF*, which may affect drug sensitivity and radiation response. Clinical studies have shown that colorectal cancer patients with a *KRAS* mutation, in general, respond poorly or not at all to EGFR inhibitor treatments (28). Moreover, the presence of mutated *BRAF* is associated with poor prognosis in metastatic colorectal cancer (29).

Also, this study investigated the possible radiosensitizing or even synergistic effects between radiation and $(Z_{\text{EGFR:1907}})_2$ exposure. According to the 'additive model' the survival fraction of cells exposed to a combination of radiation and drug was compared with the 'expected additive survival fraction' calculated by multiplying the survival fraction after treatment with radiation and drug alone (30). The effect of $(Z_{\text{EGFR:1907}})_2$ on cell survival, radiation response and cell signalling was compared with the EGFR binding antibody cetuximab and the EGFR tyrosine kinase inhibitor erlotinib as well as the multi-receptor tyrosine kinase inhibitor sunitinib.

Materials and methods

Cell culture. The colon cancer cell lines HT-29 and HCT116, purchased from the American Type Culture Collection (Rockville, MD, USA), were cultured in 75 cm² culture flasks (Nunclon surface, Roskilde, Denmark) in McCoy's 5A medium (Flow Irvine, UK) with 10% fetal bovine serum (Sigma Aldrich), L-glutamine and PEST (penicillin 100 IU/ml and streptomycin 10 µg/ml) all from Biochrom Kg, Berlin, Germany. All cells were cultured in a humidified incubator with 5% CO₂ at 37°C and trypsinized with trypsin-EDTA (0.25% trypsin, 0.02% EDTA, Biochrom Kg).

Drugs and reagents. Human recombinant EGF (Chemicon), was kept in stock in PBS at 1 mg/ml and diluted in cell culture medium to 10 nM before use. $(Z_{\text{EGFR:1907}})_2$ which was kindly provided by Affibody AB, was kept in stock in PBS at 2 mg/ml and diluted in cell culture medium to 1 nM–1 µM before use. Cetuximab, which was purified from Erbitux (Roche) on a NAP-10 column, was kept in stock in PBS at 1 mg/ml and diluted in cell culture medium to 20 nM before use. Erlotinib, hydrochloride salt (LC laboratories) was dissolved in DMSO/water 96/4 to 1 mg/ml and diluted in cell culture medium to 4 µM and sterile filtered before use. Sunitinib, malate salt (LC laboratories) dissolved in DMSO to 1 mg/ml, was diluted in cell culture medium to 0.8 µM and sterile filtered before use.

Saturation assay for EGFR. To analyse the number of EGFR per cell a saturation assay was performed using a fixed number of cells (100,000 cells) and different concentrations (0.125–250 ng/ml) of ^{125}I -labelled human EGF (Sigma Aldrich). EGF dissolved in PBS (25 µl, 0.1 mg/ml) was mixed with 1 MBq of ^{125}I (Amersham Biosciences, Sweden). Reaction was initiated by adding chloramine T (10 µl, 1 mg/ml, Sigma, St. Louis, MO, USA) and was quenched after rigorous vortexing during 1 min by adding sodium metabisulfite (20 µl, 1 mg/ml, Aldrich, USA). Labelled EGF was separated from non-reacted ^{125}I and low-molecular-weight reaction components by using NAP-5 column (Sephadex G-25, Amersham Biosciences) pre-equilibrated with PBS. Cells were seeded in 24-well plates using four wells for each concentration. An 100-fold excess of unlabeled EGF was added to one of the four wells per concentration for unspecific binding correction. The cells were washed with serum-free medium before adding medium supplemented with the varying amounts of labelled and unlabeled EGF. The cells were then incubated on ice for 4 h followed by washing twice with cell culture medium. The cells were then detached using trypsin-EDTA solution for 10 min at 37°C, resuspended in complete

medium and counted (see below). Radioactivity was measured in a γ -well counter (1480 Wallack wizard, Perkin Elmer, MA, USA). The data were analyzed by non-linear regression curve fit using GraphPad Prism 5. Bmax correspond to the total number of EGFR per cell.

Mutation analyses of KRAS and BRAF. Genomic DNA were extracted from the cell lines ($0.5\text{--}9 \times 10^6$ cells) using the QIAamp DNA mini kit (Qiagen AB, Solna, Sweden). DNA concentration and purity were determined with a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). Pyrosequencing mutational analysis was performed according to the manufacturer's protocols for the PyroMark™ Q24 KRAS and PyroMark Q24 BRAF assays (Qiagen). Briefly, 10 ng DNA from each cell line was used for analyses of KRAS (codons 12/13 and 61) and BRAF (codon 600). 20 μ l of each PCR product was subjected to Pyrosequencing analysis using Streptavidin Sepharose High Performance (GE Healthcare, Uppsala, Sweden), PyroMark Gold Q96 reagents, PyroMark Q24 1.0.9 software, and a Q24 instrument (Qiagen).

Immunocytochemistry for MRE11. HT-29 and HCT116 cells were harvested by trypsin and 1 drop of the cell suspension was transferred on a microscope glass slide. The cells were allowed to attach to the surface (2 h incubation at 37°C). Then, the cells were grown in complete media for 1 to 2 days and treated with a radiation dose of 1 Gy, inducing approximately 25 DNA DSB. After 30 min repair time at 37°C the cells were fixed in ice-cold methanol for 20 min and washed in PBS 3 times for 5 min. Then, the slides were rinsed in acetone for 10 sec and let dry at room temperature. The areas where the cells grew were surrounded with a fat pen to keep the used antibody solution in place. After a washing step with PBS the microscope slides were put in blocking solution containing 10% FBS for 1 h. The primary antibody (MRE11, Calbiochem, San Diego, CA) was diluted 1:100 in ddH₂O with 1% FBS, applied to the slides and incubated overnight in a moisture chamber at 4°C. On the next day the slides were washed 3 times for 5 min in PBS and the secondary antibody (Alexa Fluor anti-rabbit) was applied for 1 h at 37°C in a dark, moisturized atmosphere. After washing, the slides were stained with the nuclei dye DAPI (0.1 μ g/ml). Finally the cells were sealed with a cover slip and soft mounting media (Vecta Shield), for protection of the cover slip was fixed by using polish (L'Oreal). The Zeiss 510 META confocal and the 2-photon Zeiss 710 NLO microscope were used for evaluation and taking images.

Cell survival. To study the effect on cell survival of radiation, drugs or their combination, clonogenic survival assays were performed using standard techniques (21). Cells were harvested by use of trypsin for cell detachment followed by counting in a Z2 Coulter Counter Analyzer (Beckman Coulter, FL, USA) and a certain number of cells (300 up to 20,000 depending on treatment) were pre-plated in 25 cm² tissue culture flasks with 10 ml complete medium. The cells were allowed to attach during culture conditions in humidified air with 5% CO₂ overnight. The following day, the cells were exposed to drugs for 2 h followed by γ -radiation using a 137Cs source (Gammacell® 40 Exactor, Best Theratronics, Ottawa, Canada) at a dose rate of 1 Gy/min. Control cultures were left unexposed and some cultures were

exposed to drugs or radiation only. After 8-14 days incubation (depending on the doubling time of the cell lines) in the continuous presence of drug, cells were washed in 1X PBS and fixed with 99.5% ethanol and stained with Mayer's Haematoxylin. Colonies containing more than 50 cells were counted manually. The plating efficiency, PE (number of colonies formed/number of cells seeded) in the untreated control and the survival fraction, SF (number of colonies formed after treatment/number of seeded cells \times PE) were calculated. All experiments were repeated at least three times with triplicates each time. The survival curve was analysed using the linear-quadratic formula ($S_{\text{Dose}}/S_0 = \exp(\alpha D + \beta D^2)$) (31).

Detection of DNA double strand breaks by pulsed-field gel electrophoresis. Cells for pulsed-field gel electrophoresis (PFGE) were plated in 3-cm dishes and labelled with 2 kBq/ml [methyl-¹⁴C] thymidine (Perkin Elmer) for approximately two doubling times. The dishes were put on ice 20-30 min before irradiation and were kept on ice during the entire irradiation. Cells were prepared for PFGE as described previously (32). After irradiation and repair in incubation at 37°C, cells were trypsinized and mixed with low gelling-point agarose (InCert, Cambrex) to a final concentration of $1.5\text{--}2.5 \times 10^6$ cells/ml in 0.6% agarose. The mixture was transferred into plug-moulds. The plugs with cells were then transferred to 10 plug volumes of ESP lysis buffer at 4°C [2% N-lauroylsarcosine (Sigma), 1 mg/ml proteinase K (Roche), all diluted in 0.5 M EDTA (Na₃) at pH 8.0]. After >20 h the ESP buffer was removed and replaced with 20 plug volumes HS-buffer and incubated overnight at 4°C (HS, High Salt; 1.85 M NaCl, 0.15 M KCl, 5 mM MgCl₂, 2 mM EDTA, 4 mM Tris, 0.5% Triton X-100, pH 7.5, Triton X-100 is added just before use). Plugs were washed in 0.1 M EDTA and once in 0.5XTBE at 4°C prior to electrophoresis. The plugs were then loaded into wells in a chilled (4°C) agarose gel (0.8% SeaKem Gold, Lonza). The gel was placed into a PFGE unit (Gene Navigator, Amersham Pharmacia Biotech, Uppsala, Sweden) with 120° between the fields. Following electrophoresis, the gels were sliced at the position of the 5.7 Mbp chromosome from *S. pombe* (BMA), and ¹⁴C in the gel segments was measured by liquid scintillation. The fraction of radioactivity corresponding to DNA of size less than 5.7 Mbp was divided by the total radioactivity in the lane, giving the fraction of DNA <5.7 Mbp which is a relative measure of DNA double-strand breaks.

ELISA for phosphorylated EGFR. ELISA against phosphorylated EGFR was performed according to DuoSet IC Human Phospho-ERbB1 ELISA (DYC 1095, R&D Systems) protocol. In short, a 96-well plate was prepared with EGFR capture antibody in room temperature overnight. The plate was washed with wash buffer (0.05% Tween-20 in PBS) five times before blocking the wells with 0.5% BSA in PBS. The lysates (see below for lysate preparation) were added in duplicates to the wells and incubated for 2 h. After washing the plate as previously, the wells were incubated with detection antibody for two hours. The plate was washed again and incubated with substrate (R&D Systems) and the reaction stopped with 2 M H₂SO₄. The optical density was determined at 450 nm using wavelength correction at 570 nm. The experiment was repeated three times with duplicates of every lysate at every reading.

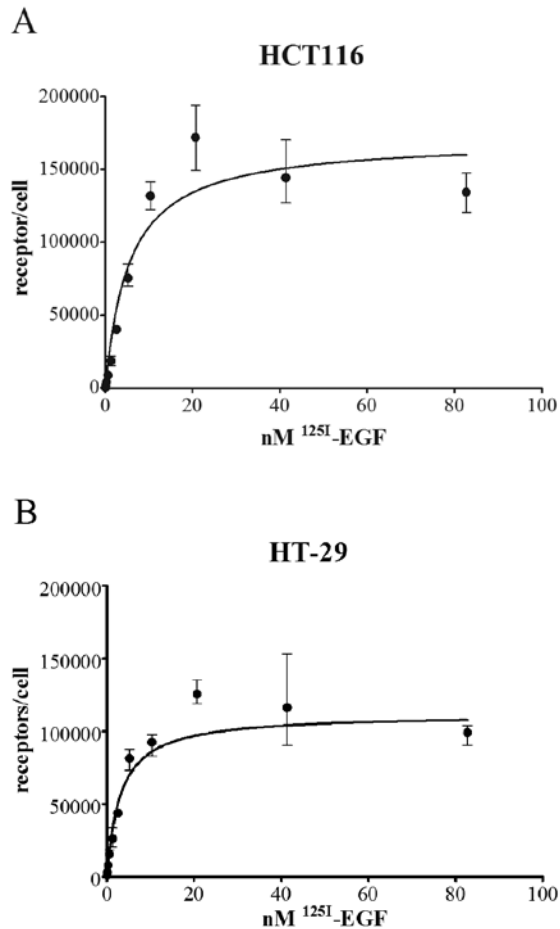


Figure 1. Expression of EGFR in (A) HCT116 and (B) HT-29 cells using saturation assay analysis. Cells were exposed to 0.125-250 ng/ml of ^{125}I -labeled human EGF (in triplicates) and a 100-fold excess of unlabeled EGF was added at each concentration for unspecific binding correction. The cells were incubated on ice for 4 h. The number of cells was counted and radioactivity measurements were performed in a γ -well counter.

Western blotting for Akt and Erk. Cells were cultivated in 3 cm petri-dishes for at least three doubling times before exposure to drug, radiation or combined treatment. Lysates were prepared after treatment by washing the cells with ice-cold PBS followed by addition of 10^7 cells/ml lysis buffer [1% Tween-20, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate (Sigma) and protease inhibitor cocktail (P8340, Sigma) and incubation on ice for 30 min. The lysates were centrifuged at 14,000 rpm for 10 min in 4°C . The supernatant was transferred to new tubes and the pellet discarded. The protein concentration of the lysate was determined by BCA protein assay (Pierce). Equal amounts of protein was loaded on an SDS PAGE and afterwards transferred to a nitrocellulose membrane by wet blotting. The nitrocellulose membrane was blocked for 1 h in 5% BSA, PBS and then incubated with the primary antibody overnight at 4°C . Antibody specific for Akt1/2/3 (sc8312) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies recognising the phosphorylated forms of Akt (Ser473) (9271) and Erk1/2 (Thr202/Tyr204) (9101) were from Cell Signaling Technology (Beverly, MS, USA). Erk antibody was kindly provided by the

Table I. List of mutations detected in HCT116 and HT-29 colorectal carcinoma cell lines, according to the Sanger Institute (34).

Mutations	HCT116	HT-29
<i>APC</i>	wt	mut
<i>BRAF</i> ^a	wt	mut
<i>CDKN2A</i>	mut	wt
<i>CTNNB1</i>	mut	wt
<i>KRAS</i> ^a	mut	wt
<i>MLH1</i>	mut	wt
<i>PIK3CA</i>	mut	mut
<i>SMAD4</i>	wt	mut
<i>TP53</i>	wt	mut
<i>MRE11</i> ^b	mut	wt
<i>MSI</i>	mut	wt

^aMutation verified by our lab. ^bPresence of *MRE11* was verified by our lab using immunohistochemistry.

Ludwing Institute. Antibody against β -actin (A5441) was from Sigma. After washing in PBS with 1% Tween-20, the membrane was incubated with horseradish peroxidase-labelled secondary antibody (626520 and 656120) (Invitrogen) for 1 h at room temperature. Immunoreactive bands were visualized in a CCD camera (SuperCCD HR, Fujifilm, Japan) after treatment with electrochemiluminescent solution (Immobilon) for 5 min. The intensity of the bands was compared to β -actin using Image J software.

Statistical analysis. The data were processed with Microsoft office Excel 2007 (Microsoft, Redmond) and all graphs were plotted in GraphPad Prism 5 (GraphPad Software, San Diego). For statistical analysis of cell survival after treatment with drug and radiation alone or in combination, GraphPad Prism 5 was used to perform a 2-way ANOVA coupled with a Bonferroni post-test. This analysis evaluated whether the effects of drugs were significantly different from the untreated controls and if the effects of drugs in combination with radiation significantly differed from radiation alone. If the observed SF after combined treatment (SF_{obs}) was lower than the SF after treatment with only radiation (SF_{rad}), $\text{SF}_{\text{obs}} < \text{SF}_{\text{rad}}$, the response was defined as radio-sensitizing. Synergistic/sub-additive/antagonistic interactions were analysed, with Student's t-test, using a version of the 'additive model' where the SF after treatments of drug and radiation alone were multiplied to each other to form an 'expected value' of additive SF ($\text{SF}_{\text{exp.add}}$) (30,33). When the observed SF after combined treatment was significantly lower than the expected additive SF, $\text{SF}_{\text{obs}} < \text{SF}_{\text{exp.add}}$, the response was defined as synergistic. However, when the observed SF after combined treatment was higher than the expected additive SF but lower than the SF after treatment with radiation alone, $\text{SF}_{\text{rad}} > \text{SF}_{\text{obs}} > \text{SF}_{\text{exp.add}}$, the response was defined as sub-additive. If the observed SF after combined treatment was higher than radiation alone, $\text{SF}_{\text{obs}} > \text{SF}_{\text{rad}}$, the response was defined as radio-desensitising or interference. In this case, since the drugs had the lowest response in

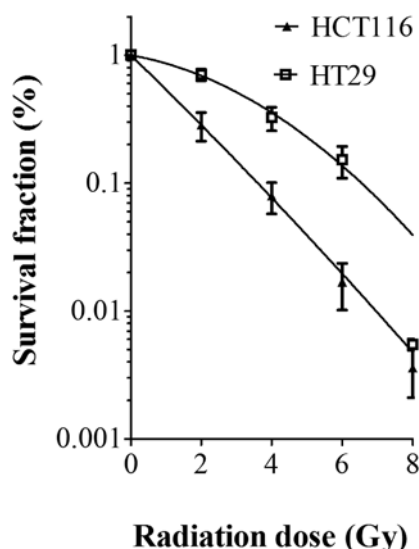


Figure 2. Clonogenic survival curves for HCT116 and HT-29 cells. The cells were preplated in triplicates and irradiated with different doses (2, 4, 6 and 8 Gy). The error bars represent standard deviation. Both curves are normalized to the plating efficiency of the controls without radiation treatment using Linear Quadratic equation model [$S(D) = e^{-\alpha D + \beta D^2}$]. Survival fraction at 2 Gy (SF2) is 0.70 for HT-29 and 0.28 for HCT116.

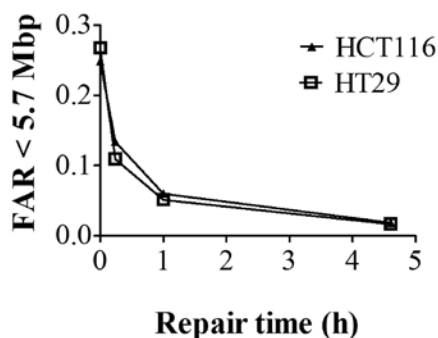


Figure 3. Rejoining of DNA double-strand breaks. Cells were irradiated with 40 Gy and allowed to repair for the stated times and the fraction of DNA fragments <5.7 Mbp was analyzed by pulsed-field gel electrophoresis. Error bars represent standard deviation.

the cells, antagonism was considered if the observed SF after combined treatment was higher than the SF after treatment of only drug (SF_{drug}), SF_{obs} > SF_{drug}.

Results

Cell line expression of EGFR and mutational status. To quantify the number of EGFR in the two cell lines we applied a saturation assay using ¹²⁵I-labelled EGF. The level of EGF receptors was estimated to approximately 150,000 and 100,000 receptors per cell in HCT116 and HT-29 cells, respectively (Fig. 1).

Both cell lines carry mutations that may affect the response to drugs targeting the EGFR signalling pathways. In this study, *KRAS* and *BRAF* mutations and presence of MRE11 were confirmed from previous analyses of these cell lines. Table I lists

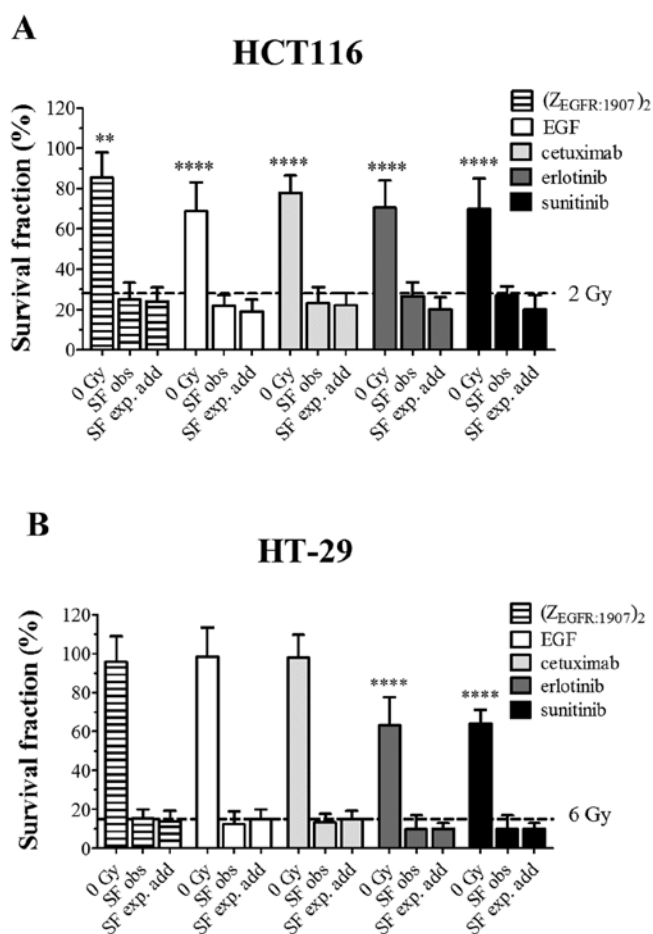


Figure 4. Clonogenic survival assays on (A) HCT116 and (B) HT-29 cells with drugs in combination with radiation. Preplated cells were exposed to drugs (in triplicates) 2 h before irradiation. After 10 to 14 days, the number of cell colonies was counted and the survival fraction (SF) calculated. The experiment was repeated at least three times. The drug concentrations used were 20 nM (Z-EGFR:1907)₂, 10 nM EGF, 20 nM cetuximab, 4 μ M erlotinib and 0.8 μ M sunitinib. The SF of the untreated cells were set to 100%. The SF of radiation alone is shown as a dotted line (SF=15% at 6 Gy in HT-29 and SF=28% at 2 Gy in HCT116). SF obs, survival fraction after treatment with both drug and radiation. SF exp. add, expected survival fraction using the 'additive model'. The difference in SF between drug treatment and untreated cells and between the combined treatment and radiation treatment alone was analyzed with 2-way ANOVA. Error bars represent standard deviation. *P<0.05, **P<0.01 and ***P<0.001 and ****P<0.0001.

these and additional mutations which have been identified in the two cell lines (34).

Sensitivity to radiation and drugs. To evaluate sensitivity to radiation and drugs the clonogenic assay was applied. The HCT116 cells were more sensitive to radiation than the HT-29 cells (Fig. 2). At 2 Gy the survival fraction (SF2) was 0.70 for HT-29 and 0.28 for HCT116. The DSB repair rate for both cell lines was evaluated using pulsed-field gel electrophoresis. However no difference was detected (Fig. 3), as also seen in previous studies (35). To study the effects of radiation in combination with drugs, using only one concentration and radiation dose, the irradiation dose and drug concentrations were chosen to yield a survival fraction of around 20% and 60-80%, respectively.

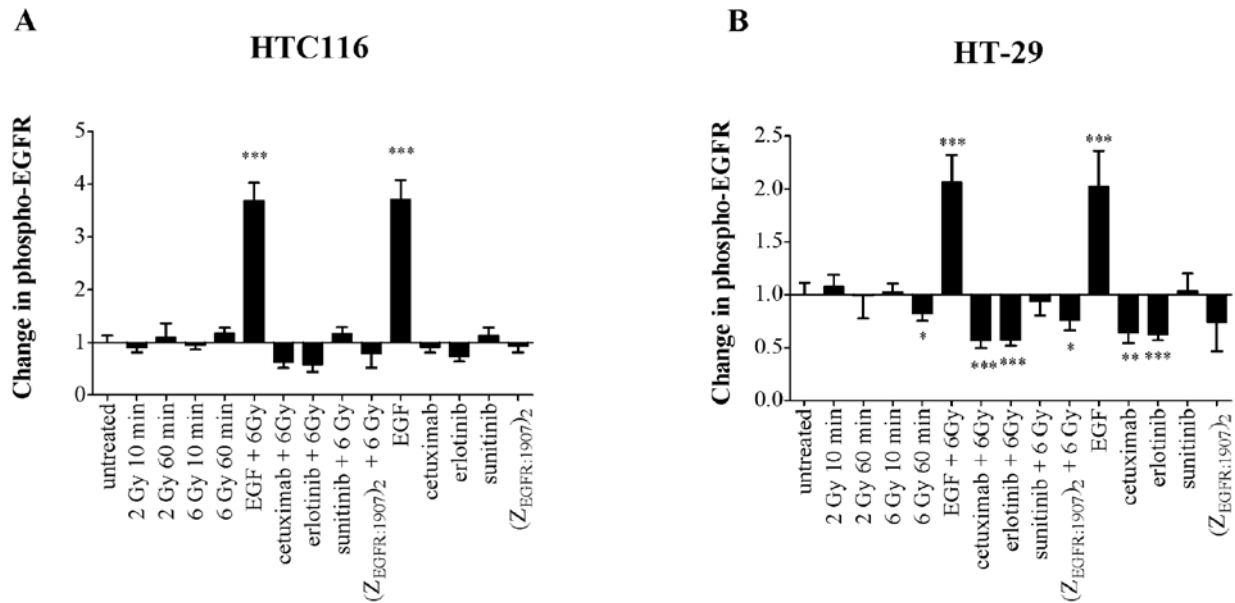


Figure 5. ELISA of phosphorylated-EGFR of (A) HCT116 and (B) HT-29 cells irradiated with 2 Gy or 6 Gy and lysed after 10 or 60 min, cells treated with different substances (10 nM EGF, 20 nM cetuximab, 4 μ M erlotinib, 0.8 μ M sunitinib and 20 nM (Z_{EGFR:1907})₂ for 2 h before lysis or irradiated and lysed after an additional 60 min. The signal was normalized to the untreated cells and the significant difference from untreated was analyzed with Student's t-test. *P<0.05, **P<0.01 and ***P<0.001 and ****P<0.0001. Error bars represent standard deviation.

Therefore HCT116 and HT-29 cells were exposed to 2 and 6 Gy, respectively in these experiments. The affinity of (Z_{EGFR:1907})₂ is approximately 1.6 nM however in the concentration range of 0.3 nM to 1 μ M, it had only a weak effect on the cell survival (data not shown). In following studies, 20 nM of (Z_{EGFR:1907})₂ was used which gave a survival fraction of about 85% in HCT116 cells (Fig. 4A).

Exposure to cetuximab at 20 nM resulted in a survival fraction of 78% in HCT116 cells, whereas no effect was observed in HT-29 cells (Fig. 4). At exposure to 0.8-4 μ M of erlotinib the survival fraction was steadily around 60-70% in both cell lines, therefore 4 μ M was used for further studies. Sunitinib exposure had a dose-dependent effect on cell survival. At a concentration of 0.8 μ M the cell survival fraction was 70% and 64% in HCT116 and HT-29 cells, respectively. EGF was used as a control at a concentration of 10 nM. Interestingly, EGF reduced the survival fraction in HCT116 to 70%, but had no effect in HT-29 cells (Fig. 4).

Effects of drugs in combination with radiation. In HCT116 cells, there was no significant radiosensitizing effect with (Z_{EGFR:1907})₂. Similar results were obtained with EGF, cetuximab, erlotinib and sunitinib. Exposure to erlotinib and sunitinib resulted in large reductions in cell survival as single treatments. However, combined with radiation they were not radiosensitizing. The survival fractions of combined treatment with erlotinib or sunitinib in combination with radiation were significantly higher than the expected additive survival fraction (Fig. 4A).

In HT-29 cells, there were no significant radiosensitizing effects after treatment with neither (Z_{EGFR:1907})₂ nor any of the other substances. Exposure to erlotinib and sunitinib as single treatments resulted in similar reductions in cell survival as observed for HCT116 cells. However, the survival fraction after

the combined treatment of erlotinib or sunitinib with radiation showed tendencies to be radiosensitizing/sub-additive but they were not significantly different from neither the expected additive survival fraction nor the response to radiation alone (Fig. 4B).

Unfortunately, due to the low drug response or the high variance of the data, it was not always possible to statistically determine whether the combined treatment was additive, sub-additive or equal to radiation alone.

Radiation and drug induced changes in the EGFR signaling pathway. Both cell lines have a relatively high basal phosphorylation of Akt and Erk. The activation of Akt and Erk increased with radiation dose (2-6 Gy) and time after radiation (10-60 min), see Fig. 6A. (Z_{EGFR:1907})₂, cetuximab and erlotinib were expected to block EGFR signalling and thus down-regulate the activation of Erk and Akt. In the HT-29 cells cetuximab (P<0.001), erlotinib (P<0.001) and (Z_{EGFR:1907})₂ (P<0.05) all reduced the phosphorylation of EGFR. The same trend was seen for the HCT116 cells but the decrease in phosphorylation was not statistically significant (Fig. 5). However, none of these drugs were able to completely inhibit the basal phosphorylation of Akt and Erk. On the contrary, exposure to 4 μ M erlotinib increased the phosphorylation of Akt, in both cell-lines, as well as Erk in HCT116 cells. As expected, EGF exposure resulted in a significant increase in phosphorylation. Drug treatment for two hours before irradiation with 6 Gy and lysis after additionally 60 min did not change the activation pattern in terms of pEGFR, pAkt or pErk (Figs. 5, 6B and C).

Discussion

The aim of this study was to investigate the possible cytotoxic effect of the EGFR targeting affibody molecule (Z_{EGFR:1907})₂ in

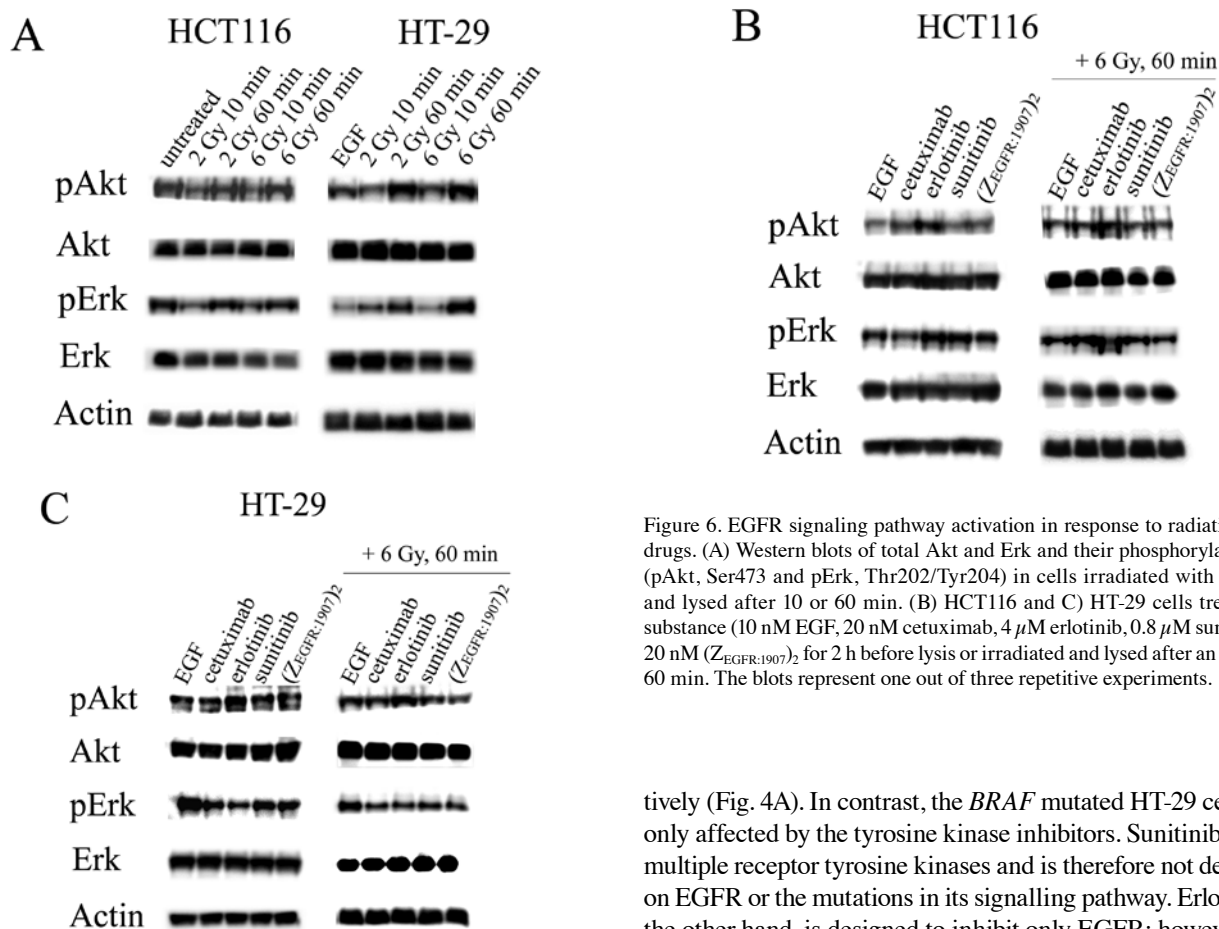


Figure 6. EGFR signaling pathway activation in response to radiation and/or drugs. (A) Western blots of total Akt and Erk and their phosphorylated forms (pAkt, Ser473 and pErk, Thr202/Tyr204) in cells irradiated with 2 or 6 Gy and lysed after 10 or 60 min. (B) HCT116 and (C) HT-29 cells treated with substance (10 nM EGF, 20 nM cetuximab, 4 μ M erlotinib, 0.8 μ M sunitinib and 20 nM (Z-EGFR:1907)₂ for 2 h before lysis or irradiated and lysed after an additional 60 min. The blots represent one out of three repetitive experiments.

combination with external radiation and the inhibitory effects in the EGFR signalling pathways in two colon cancer cell lines. For comparison, the EGFR antibody, cetuximab, and the tyrosine kinase inhibitors, erlotinib and sunitinib, were included in the study.

The two colon cancer cell lines, HCT116 and HT-29, express approximately 100,000-150,000 EGFRs per cell (Fig. 1) and have several genetic mutations in the EGFR signalling pathways which may affect the drug and radiation sensitivity by promoting aberrant survival signals (e.g. *KRAS*, *BRAF*, *PIK3KCA*, *TP53*) and may make the targeting of EGFR or receptor tyrosine kinases obsolete. The two cell lines had different sensitivity to radiation but showed the same DSB repair rate (Figs. 2 and 3) and the result for the HCT116 cells are in line with previous studies (35). However, HCT116 have an MRE11 deficiency which may affect the repair fidelity. MRE11 is believed to be a major sensor of DSBs and subsequently recruits ATM (36). HT-29 cells have a *TP53* mutation (R273H) which presumably can cause an aberrant function of the protein leading to increased resistance to radiation.

KRAS and *BRAF* mutations are associated with low response to EGFR inhibition and with poor prognosis in metastatic colorectal cancer (28,29,37). (Z-EGFR:1907)₂, as well as the other EGFR targeting substances and the multi receptor tyrosine kinase inhibitor reduced the clonogenic survival of the *KRAS* mutated HCT116 cells. However, the reductions in cell survival of (Z-EGFR:1907)₂ and cetuximab were only 15% and 22%, respec-

tively (Fig. 4A). In contrast, the *BRAF* mutated HT-29 cells were only affected by the tyrosine kinase inhibitors. Sunitinib inhibits multiple receptor tyrosine kinases and is therefore not dependent on EGFR or the mutations in its signalling pathway. Erlotinib, on the other hand, is designed to inhibit only EGFR; however it has low activity against other tyrosine kinases as well (HER2/neu, KDR, c-flt, or serine/threonine kinases including protein kinase C, MEK-1, and Erk) which could explain its effect in these cell lines.

Interestingly, treatment with 10 nM EGF resulted in significant cell death in HCT116 cells, which has also been observed in an epidermoid carcinoma (A431) as well as in other cell lines at high amounts of EGF (38-40). One possible explanation behind this counter interactive mechanism may be the activation of STAT1 which is involved in apoptosis and anti-proliferation (41).

The effects of EGFR targeting drugs in combination with radiation are believed to be dependent on the way EGFR is inhibited, such as by small molecules or monoclonal antibodies, as well as by the specific phenotype of the tumor cell. It was therefore of interest to compare the possible effects of the antibody molecule with other drugs with radiosensitizing effects. In this study, neither (Z-EGFR:1907)₂ nor any of the other substances had any significant radiosensitizing effects on the cells (Fig. 4). However, erlotinib and sunitinib showed tendencies to be radiosensitizing/additive in HT-29 cells. Both sunitinib and erlotinib have been observed to have radiosensitizing effects or synergistic interactions in several other tumour cell lines (42-44). The extent of erlotinib-induced radiosensitization may be proportional to EGFR expression, as well as autophosphorylation of EGFR (45). This could explain the absence of radiosensitizing effect of erlotinib on these cell lines which do not have a high EGFR expression. Another possible explanation is that both the drug and irradiation may partly affect the same cellular pathways that could lead to saturation in the response. An additional aspect is the timing of drug treatment before radiation that has been shown

to be crucial with e.g. cisplatin and radiation (46). In this study, the cells were exposed to radiation two hours after drug treatment and the drug was kept in the cell-medium during the whole experiment. A different treatment drug and radiation regimen might give a different result. From a clinical point of view, the present and previous data illustrate the complexity of radiation and drug interactions. This calls for extensive preclinical investigations in relevant models prior to clinical trials, in e.g. rectal cancer, with the aim to improve the anti-tumor effect of radiation by simultaneous exposure to drugs targeting important cellular signal pathways.

Both cell lines have a constitutive activity of Erk (pErk) and Akt (pAkt) consistent with mutations in *PIK3CA*, *KRAS* and *BRAF* (47). ($Z_{\text{EGFR:1907}}$)₂ as well as cetuximab and erlotinib reduced the EGFR phosphorylation in both cell lines (Fig. 5), but the activation of Akt or Erk was not inhibited by any of the drugs (Fig. 6). On the contrary, the phosphorylation of Akt could be further induced by erlotinib. Erlotinib also increased Erk phosphorylation in HCT116 cells. Overall there was no apparent link between cell survival and inhibition of EGFR, Akt or Erk. Since these drugs had a clear effect on cell survival they probably inhibit other signals activated by EGFR or other tyrosine kinases in the cells.

The activation by radiation of Akt and Erk was dose- and time-dependent (Fig. 6A) as seen in previous studies on HUVEC cells (48). Since there was no clear effect on radiation-induced activation of EGFR, the Akt and Erk phosphorylation induced by radiation is probably through other signalling pathways, such as DNA damage response.

In conclusion, improved effect of radiation in tumor cells with mutations in the EGFR or its signalling pathways seems to require a combination with drugs targeting other pathways such as DNA repair. Future studies should also evaluate the drug and radiation schedules and find biomarkers which can predict interaction effects.

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