Abstract. The combination of a fluoropyrimidine with receptor tyrosine kinase inhibitors in tumor treatment has been proposed to enhance their therapeutic efficiency. The synergism of such a combination in the treatment of colorectal carcinoma is equivocal although the epidermal growth factor receptor (EGFR) is frequently overexpressed in the tumors. We used human colorectal SW 480 cells, to analyze the EGFR phosphorylation levels. We showed that incubation of cells with 5-fluorouracil (5-FU) does not influence EGFR phosphorylation of tyrosine 1173 and the overall phosphorylation after stimulation. Inhibition of EGFR phosphorylation with AG1478 reduced cell proliferation compared to 5-FU. Cells exhibited highest apoptosis rates with 5-FU. AG1479 inhibited cell proliferation more potently than 5-FU alone. Apoptosis rates after incubation with AG1478 alone and AG1478 in combination with 5-FU were significantly lower than apoptosis induction by 5-FU alone. Therefore, no synergism of both substances can be demonstrated. This experimental data argues against the combination of EGFR-inhibitors with 5-FU in a clinical setting.

Colorectal cancer (CRC) remains among the leading causes of cancer-related deaths (1-3). In spite of recent achievements in the treatment of colorectal cancer, further research efforts are essential to achieve better outcomes. The overall 5-year survival rate does not exceed 60% worldwide (4, 5).

One of the most promising recent developments in cancer treatment has been the clinical implementation of targeted therapies, where molecules that block the growth and spread of cancer by interfering with specific signal pathways involved in tumor growth and progression are employed. One example of a targeted therapeutic approach is the combination of endothelial growth factor receptor (EGFR) inhibitors with standard chemotherapeutic agents. The rationale behind such an idea is that tumor cell death may be counteracted by EGFR-induced survival signalling via Phosphatidylinositide 3-kinase (PI3K)-AKT (6, 7). EGFR dimerization and phosphorylation leads to downstream stimulation of the PI3K/AKT pathway activation. This pathway activation then blocks apoptosis and promotes cellular proliferation (8, 9). Targeted inhibition of the EGFR/PI3K/AKT cell survival pathway has been shown to induce tumor cell apoptosis and slow tumor cell migration in various cancer cell lines (10, 11). In parallel, cell lines with high basal EGFR phosphorylation (H630, LoVo, and A431) were more sensitive to gefitinib treatment compared to cell lines with low basal EGFR phosphorylation (7).

However, there are contradictory in vitro and in vivo reports concerning the efficacy and the synergism of such combination in the treatment of CRC, although EGFR is frequently found to be overexpressed in tumor tissues (12). We have previously shown that EGFR phosphorylation inversely correlates with EGFR expression in certain CRC cell lines, while in pancreatic carcinoma cells EGFR expression is a direct indicator of EGFR phosphorylation (13, 14). Since the combination of EGFR inhibitors with cytotoxic agents remains controversial in the clinical treatment of CRC, independently of EGFR expression levels, in the present study we used an in vitro approach, using the model colon carcinoma cell line SW 480 in order to investigate the effect of 5-fluorouracil induced (5-FU) in combination with EGFR tyrosine kinase inhibition included by AG1478.
Materials and Methods

Reagents. Human recombinant EGF and the EGFR kinase inhibitor AG 1478 was purchased from Calbiochem (Gibbstown, NJ, USA). Vanadate was obtained from Sigma (Taufkirchen, Germany). EGFR antibodies F4 and generic phosphotyrosine antibodies PY72 were obtained from the monoclonal cell facility of Cancer Research UK (London, UK). The monoreactive dyes Cy3 and Cy5 were purchased from Amersham Biosciences (Buckinghamshire, UK). All other reagents were purchased from Sigma unless indicated otherwise.

Cell culture. SW 480 human colon cancer cells were provided by the American Type Culture Collection (Manassas, VA, USA) and were grown in monolayer culture in RPMI medium containing 10% fetal bovine serum (FBS), 1% P/S and 1% glutamine (ICN, Irvine, CA, USA). The cell cultures were maintained in plastic flasks and incubated in 5% CO₂, 95% air at 37°C. Stock cultures were stored in liquid nitrogen and used for experiments within five passages. Cell viability (at least 95%) was assessed by trypan blue dye exclusion.

After incubation with 2 mmol vanadate or 2 mmol vanadate and 100 ng/ml EGF and 25 μM 5-FU, 50 μM AG1478 with vanadate and EGF, SW 480 human colon cancer cells were washed twice with phosphate-buffered saline (PBS), pH 7.4 and immediately fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, the cells were washed twice with PBS to remove the remaining fixative PFA and then incubated for 5 min in quenching solution to quench the fluorescence of the remaining aldehyde groups. Immediately after quenching, the cells were incubated in permeabilization solution (0.1% Triton X-100) for 15 min to extract plasma membranes. The cells were then washed twice with PBS to remove the remaining permeabilization solution. Cells were then incubated for 24 h at 4°C with Cy3-tagged F4 and Cy5-tagged Py72 antibodies diluted in 1% bovine serum albumin in PBS. The next day cover slips were harvested from a culture plate by trypsinizing, centrifuged (1200 × g for 5 min) and the cell number was counted. A total of 1×10⁶ cells were added to each well and incubated for 24 h. On the next day 25 μM 5-FU, 50 μM AG1478 or their combinations were added into the wells and incubated for 72 h to induce apoptosis. Flow cytometric detection of apoptosis. SW 480 cells were harvested from a culture plate by trypsinizing, centrifuged (1200 × g for 5 min) and the cell number was counted. A total of 1×10⁶ cells were added to each well and incubated for 24 h. On the next day 25 μM 5-FU, 50 μg AG1478 or their combinations were added into the wells and incubated for 72 h to induce apoptosis. Flow cytometric detection of apoptosis

Acceptor Photobleaching. Acceptor photobleaching imaging of fluorescence resonance energy transfer (FRET). The phosphorylation state of endogenous EGFR was imaged in the cells after exposure of the cells to vanadate, EGF, 5-FU, AG1478 or combinations as described above by detecting fluorescence resonance energy transfer (FRET) between fluorphores conjugated to antibodies against the receptor and phosphotyrosine respectively. The FRET efficiency between two fluorphores allows quantitative evaluation of EGFR phosphorylation. For this a confocal laser scanning microscope Leica SP2 (Leica, Bensheim, Germany) was used. All measurements were obtained using a 63×/1.32 NA objective. Simultaneous images with the pinholes set at 2 Airy units were acquired from the donor (Cy3) and the acceptor (Cy5) using 10% of the maximal 554 nm HeNe laser line power for excitation of Cy3. 15% of the 633 nm laser line power was used for excitation of Cy5. Detection of fluorescence was in the spectral window of 560-595 nm for Cy3 and 650-700 nm for Cy5. Acceptor photobleaching of Cy5 was performed in a region of interest (zoom=25) with the 633 nm laser line set at maximum intensity for eight rounds with a line average of four. Post-bleached donor (Cy3) images were acquired by reverting back to the original acquisition settings.

The FRET efficiency (E) following different kind of stimulations was calculated as described (13): 

\[ E = \frac{\text{Intensity}_{\text{post bleach}} - \text{Intensity}_{\text{pre bleach}}}{\text{Intensity}_{\text{pre bleach}}} \]

Flow cytometric detection of apoptosis. SW 480 cells were harvested from a culture plate by trypsinizing, centrifuged (1200 × g for 5 min) and the cell number was counted. A total of 1×10⁶ cells were added to each well and incubated for 24 h. On the next day 25 μM 5-FU, 50 μg AG1478 or their combinations were added into the wells and incubated for 72 h to induce apoptosis. For control, cells were incubated in normal and DMSO added medium in the absence of 5-FU. After the incubation time, the supernatants, which contained detached cells, were collected and added to labeled FACs tubes. Cells were washed with cold PBS and trypsinized. The harvested cells were added to the supernatants. Then the tubes were
centrifuged (1200 × g for 5 min) and supernatants were carefully aspirated. Staining with Alexa Flour 488-conjugated annexin V and propidium iodide (PI) (Invitrogen Carlsbad, CA, USA) was carried out according to the manufactures’ instructions. Unstained cells were used as the negative control. The tubes were placed on ice and analyzed by flow cytometry with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells were gated on forward scatter and side scatter to eliminate cell debris and clumps. A total of 20,000 cells were counted. Data acquisition and analysis were carried out CellQuest software (BD Bioscience, San Jose, CA, USA). Only annexin V-stained cells that were PI-negative were considered to be apoptotic.

Cell proliferation assay. Cell viability and proliferation were measured using a CellTiter 96 AQUeous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany). This is a colorimetric method for determining the number of viable cells in a proliferation assay. For measurements, SW 480 human colon cancer cells were grown in 25 mm tissue culture flasks, rinsed with PBS, trypsinized and diluted in DMEM to 2000 cells/100 μl. Cells were thereafter plated into each well of a 96-well plate by adding 100 μl of the cell suspension and incubated overnight at normal maintenance conditions to let the cells seed. The next day, the cells were exposed to 25 μM 5-FU, 50 μM AG 2478 or their combinations for 72 hours. For negative control, the cells were incubated in normal medium and DMSO. At the end of the incubation time 20 μl of CellTiter 96 AQUeous One Solution reagent (Promega) was added in each well and the plate was placed in a cell culture incubator for 2-4 hours. Thereafter the absorbance was recorded at 490 nm using a 96-well DPC ELISA plate reader (Los Angeles, CA, USA).

Statistics. All experiments were reproduced at least five times. We compared each treatment group versus controls and other groups by one-way analysis of variance (ANOVA). If ANOVA indicated a significant difference between groups, pairwise multiple comparison of all means and post-hoc testing using Tukey’s method were employed to determine significant differences between groups and controls. The data were analyzed for statistical significance using the software package SPSS™, (Chicago, IL, USA). p-Values of <0.05 were considered significant. In the box plots used for graphical data representation a box is drawn around the interquartile range. A line inside the box indicates the median value. Error bars are drawn at the 5% and the 95% confidence intervals.

Results

AG1478 is a highly potent inhibitor of EGFR phosphorylation. To detect EGFR phosphorylation a phosphorylation ELISA assay was used. Specific antibodies were used which bind to tyrosine residues 1173 after they are phosphorylated. SW 480 cells showed a significant increase in EGFR tyrosine 1173-phosphorylation after starvation when stimulated by EGF. Inhibition of EGFR dephosphorylation by the phosphatase inhibitor vanadate also increased EGFR phosphorylation. As shown in Figure 1. EGFR stimulation can be potently inhibited when cells are incubated with AG1478. When cells were incubated with EGF, vanadate and 5-FU, phosphorylation of EGFR was increased. For control, parallel incubation with AG1478 reduced tyrosine phosphorylation levels to normal.

For validation, we observed the overall EGFR phosphorylation using an antibody based acceptor-photobleaching FRET assay. After the corresponding incubation, cells were stained with an EGFR antibody. This
antibody was coupled to the chromophore Cy3. As second antibody we used PY72, an antibody which binds to all phosphorylated tyrosine residues. This second antibody was coupled to Cy5. When both antibodies bind to phosphorylated EGFR, the chromophors conjugated to the respective antibodies undergo fluorescence resonance energy transfer. The presence of FRET can be detected after the acceptor chromophore is photobleached by an increase in donor fluorescence. Figure 2 shows examples of such an experiment.

The efficiency of FRET can be calculated by comparing donor and acceptor intensities before and after photobleaching. Cells showed an increase in phosphorylation level after stimulation which was abrogated by AG1478. Here again, incubation with 5-FU did not influence phosphorylation of EGFR (Figure 3).

We then investigated cell proliferation and apoptosis induction by 5-FU in the presence of AG1478. The effect of 5-FU and EGFR inhibition by AG1478 on cell proliferation is summarized in Figure 4. After incubation of the cells for three days with AG1478 (50 μM), a pronounced inhibition of cell proliferation was found as compared to controls and incubation with 5-FU (25 μM) alone (p<0.05). Proliferation was not further reduced when cells were incubated with 5-FU and AG1478 as compared to AG1478 alone.

Apoptosis was measured using flow cytometric quantification of cells after staining with annexin V and propidium iodine. Cells were incubated for three days with 5-FU, AG1478, or 5-FU and AG1478 in combination. As compared to controls, the highest apoptosis rate was found in cells after incubation with 5-FU alone. The combined incubation of cells with AG1478 and 5-FU significantly (p<0.05) reduced the rate of apoptosis; more necrotic cells were also found in this group. When cells were incubated with AG1478 alone, cells showed a significantly higher apoptosis rate than the controls; significantly fewer cells were positive for annexin V. Therefore AG1478 alone induced less apoptosis than the combined exposure to 5-FU and AG1478 (Figure 5).

Discussion

While neoadjuvant radiotherapy has been shown to be effective in the treatment of locally advanced rectal carcinoma (15, 16), the efficiency of such a therapy has so far not been apparent for colon cancer. Here, surgical excision remains the mainstay for any curative approach. For advanced CRC, adjuvant therapy is commenced usually soon after the operation. If general fitness and wound healing allow, a therapy with oxaliplatin and a fluoropyrimidin are administered (17). In patients with already metastasized cancer, regression of the primary tumor may be achieved under chemotherapy alone (18). In this context, recently, the combination of standard chemotherapeutic agents with tyrosine kinase inhibitors has been proposed. One of the promising targets is the tyrosine kinase receptor EGFR which relays signals of proliferation, angiogenesis and metastasis from the cell surface within the cell. Combination therapies with EGFR-TKIs and cytotoxic agents have been considered a therapeutic option for patients with NSCLC expressing wild-type EGFR (19). In gastric cancer, the combination of
S-1 and human epidermal growth factor receptor-2 (Her2)-
targeting agents was reported as a promising treatment option
for tumors with HER2 amplification (20).

For colorectal cancer there are presently only limited data.
The efficiency of oxaliplatin and 5-FU (FOLFOX) or irinotecan
and 5-FU (FOLFIRI) with or without gefitinib has been
analyzed. Human tumor primary culture microspheroids
isolated from colorectal cancer surgical specimens or
cytologically-positive fluids were exposed to the single
chemotherapeutics or to combinations of them. Gefitinib
demonstrated significantly less synergy with oxaliplatin than
with irinotecan (21). The clinical results of a single EGFR-
targeting therapeutic approach were not promising either:
Cetuximab and panitumumab are both EGFR antibodies which
have been recently licenced for the treatment of patients with
colorectal cancer. However, so far response rates have been low
(22). Here, mutations of the GTPase V-Ki-ras2 Kirsten rat
sarcoma viral oncogene homolog KRAS have been implicated
for the therapeutic failure. These may activate the
RAS/RAF/MAPK cascade downstream of EGFR. This could
render targeting EGFR irrelevant (23). Since selected patients
appear to profit from EGFR inhibitor-based therapy, the search
for more biomarkers is ongoing, looking for suitable patients
for such a therapy (24). One important factor is the level of
basal and endogenous EGFR phosphorylation within the
colorectal cancer cells (13). EGFR expression does not
necessarily correlate with the level of EGFR phosphorylation.
Interestingly for pancreatic carcinoma cells, a clear correlation
was found (14). EGFR is highly expressed in SW 480 cells and
shows only a moderate level of basal phosphorylation (13).

Here we used two different approaches to quantify
phosphorylation within the SW 480 cells. Upon the binding of
a ligand, EGFR forms homo- and heterodimers which activate
the kinase domain, resulting in receptor autophosphorylation
(25). In human EGFR, a glycoprotein of 1186 amino acids,
three major autophosphorylation sites (Tyr 1068, 1148, and
1173), and two minor sites (Tyr 992 and 1086) have been
identified (26-29). ELISA-based detection of single-site
phosphorylation and FRET imaging assay of overall
phosphorylation were employed. The optical FRET assay used
here is an accurate way to determine receptor phosphorylation
within single cells (13). Neither assay indicated that 5-FU
influences EGFR phosphorylation within the cells. While
AG1478 significantly lowered cell proliferation, this effect was
not further enhanced by 5-FU. We found less apoptosis
induction in cells exposed to combination of 5-FU and AG1478
as compared to 5-FU alone. One potential explanation may be
the cell-cycle arrest induced by tryphostines such as AG1478.
These agents have been shown to arrest cells in the G1 phase,
similarly to other known inhibitors of EGFR (30). This would
consequently hinder apoptosis induction by 5-FU.

In summary, in the present study we show that the
combined exposure of SW 480 colon cancer cells to the
EGFR tyrosine kinase inhibitor AG1478 and the cytotoxic
agent 5-FU leads to less apoptosis than incubation with 5-
FU alone. 5-FU does not influence EGFR phosphorylation. A
combination of AG1478 and 5-FU does not increase
inhibition of cell proliferation as compared to AG1478 alone.
Therefore, no synergism of both substances was found. In
conclusion, experimental data argue against the clinical
combination of 5-FU with EGFR-tyrosine kinase inhibitors.

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