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Targeting the epidermal growth factor receptor by erlotinib (Tarceva™) for the treatment of esophageal cancer

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Esophageal cancer is the sixth most common cause of cancer-related death worldwide. Because of very poor 5-year survival new therapeutic approaches are mandatory. Erlotinib (Tarceva™), an inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK), potently suppresses the growth of various tumors but its effect on esophageal carcinoma, known to express EGFR, remains unexplored. We therefore studied the antineoplastic potency of erlotinib in human esophageal cancer cells. Erlotinib induced growth inhibition of the human esophageal squamous cell carcinoma (ESCC) cell lines Kyse-30, Kyse-70 and Kyse-140, and the esophageal adenocarcinoma cell line OE-33, as well as of primary cell cultures of human esophageal cancers. Combining erlotinib with the EGFR-receptor antibody cetuximab, the insulin-like growth factor receptor tyrosine kinase inhibitor tyrphostin AG1024, or the 3-hydroxy-3-methylglutaryl coenzyme. A reductase (HMG-CoAR) inhibitor fluvastatin resulted in additive or even synergistic antiproliferative effects. Erlotinib induced cell cycle arrest at the G1/S checkpoint. The erlotinib-mediated signaling involved the inactivation of EGFR-TK and ERK1/2, the upregulation of the cyclin-dependent kinase inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$, and the down-regulation of the cell cycle promoter cyclin D1. However, erlotinib did not induce immediate cytotoxicity or apoptosis in esophageal cancer cells. The inhibition of EGFR-TK by erlotinib appears to be a promising novel approach for innovative treatment strategies of esophageal cancer, as it powerfully induced growth inhibition and cell cycle arrest in human esophageal cancer cells and enhanced the antineoplastic effects of other targeted agents.

Key words: cetuximab; chemoprevention; cell cycle; proliferation; primary cell culture; statins; HMG-CoA reductase; tyrosine kinase; insulin-like growth factor receptor

Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide. Esophageal squamous cell carcinoma (ESCC) is worldwide the most frequent histological subtype of esophageal cancer.[1] However, in Western countries esophageal adenocarcinoma has recently become the most frequent histological subtype.[2] Because of generally advanced tumor stages at diagnosis and the lack of curative treatment modalities in advanced esophageal cancer disease, overall survival is poor. Therefore, innovative approaches are much needed.

The EGFR is a member of a family of 4 closely related receptors: EGFR (ErbB-1), HER-2/*neu* (ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). Upon ligand binding the EGFR dimerizes, leading to subsequent activation of EGFR tyrosine kinase (TK), initiating receptor-mediated signal transduction, cell mitogenesis and cell transformation.[3] Recently, evidence has been accumulated that the EGFR is a promising target for cancer therapy. A great variety of tumors shows abnormal, enhanced and/or constitutive expression of EGFR. Several reports indicate that the EGFR is overexpressed frequently in esophageal cancer, most likely contributing to the aggressive growth characteristics of these tumors and to the poor prognosis.[4] Hence, inhibiting the EGFR and its specific TK activity is a promising principle of targeted cancer therapy.[5]

Erlotinib (*N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine) is a novel orally available compound that acts as a potent inhibitor of EGFR-TK activity. It reversibly competes with ATP at the TK domain of EGFR, resulting in inhibition of EGFR autophosphorylation.[6] Erlotinib is currently being investigated in phase II and phase III clinical studies. Antitumor activity was observed in pretreated patients with nonsmall cell lung cancer and head and neck carcinoma.[7,8] Recently, the results of the BR21 Phase III study showed a significant 42.5% improvement in median survival compared to placebo in patients with advanced nonsmall cell lung cancer.[9]

Insulin-like growth factors (IGFs) are known to display mitogenic, transforming and antiapoptotic properties in various human tumors, including esophageal cancer, by (autocrine) stimulation of distinct intracellular signaling pathways.[10] The expression and activity of IGF-1 receptor (IGF-1R) was shown to be upregulated in esophageal cancer cells, contributing to the process of malignant transformation and growth of esophageal tumors.[11] Increased expression of insulin-like growth factor receptors (IGFRs) may further potentiate the mitogenic effects of IGFs in the development of esophageal cancer. Recently, we and others showed that the coexpression of EGFR and IGF-1R might be critical for EGFR-TK dependent mitogenic effects,[12,13] as IGFR is capable of transactivating EGFR-TK.[14] Accordingly, EGFR-TK inhibition attenuated IGFR-induced extracellular signal-regulated kinase (ERK)1/2 MAPK activation.[15] Thus, IGF-stimulated esophageal cancer growth may also be sensitive to EGFR-TK inhibition.

EGFR-TK inhibition has not been evaluated for the treatment of esophageal cancer. Hence, in the present study on human esophageal cancer cells, we examined the antineoplastic potency of the selective EGFR-TK inhibitor erlotinib, alone or in combination with other agents targeting growth factor receptor pathways.

Material and methods

Cell lines and drugs

The human esophageal squamous carcinoma cell lines Kyse-30, Kyse-70 and Kyse-140[16] were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The human esophageal adenocarcinoma cell line OE-33[17] was grown in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. Cell lines were cultured in a humidified atmosphere (5% CO_2) at 37°C.

Cells were incubated with culture medium containing erlotinib (Roche, Penzberg, Germany). For combination treatment, cells were incubated simultaneously with erlotinib and the tyrphostin AG1024 (Calbiochem-Novabiochem, Bad Soden, Germany), or erlotinib and cetuximab (Merck KgaA, Darmstadt, Germany), or erlotinib and fluvastatin (Calbiochem-Novabiochem). Stock

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; erlotinib, *N*-(3-ethynylphenyl)-6,7-bis (2-methoxyethoxy)-4-quinazolinamine; ESCC, esophageal squamous cell carcinoma; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IGFR, insulin-like growth factor receptor; TK, tyrosine kinase; $\Delta\Psi_M$, mitochondrial membrane potential.

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TABLE I – PRIMER SEQUENCES AND PCR CONDITIONS FOR THE DETECTION OF mRNA EXPRESSION OF EGFRI, EGFRvIII AND IGF-1R IN ESOPHAGEAL CANCER CELLS

Genes	Primers (5′-3′)	Position in the mRNA	Product size (bp)	Denaturating temperature (°C) and time	Annealing temperature (°C) and time	Extension temperature (°C) and time	Number of cycles
EGFR-l	F: TCCTCCCAGTGCCTGAATAC	3442-3462	240	94 (40)[1]	63 (60)[1]	72 (60)[1]	30
	R: TAATTI′GGTGGCTGCCTTTC	3682-3663					
EGFRvIII[2]	F: GGCTCTGGAGGAAAAGAAAG	255-274	226	94 (40)	63 (60)	72 (60)	30
	R: TGATGGAGGTGCAGTTTTTG	1282-1263					
IGF-1R	F: GAAGTGGAACCCTCCCTCTC	1932-1951	241	94 (40)	63 (60)	72 (60)	30
	R: CTTCTCGGCTTCAGTTTTGG	2172-2153					
β-actin	F: ATCATGTTTGAGACCTTCAACAC	437-459	822	94 (40)	63 (60)	72 (60)	30
	R: TCTGCGCAAGTTAGGTTTTGTC	1258-1237					

[1]Values in parentheses in these columns indicate time in seconds.–[2]EGFRvIII does not possess a mRNA-sequence distinct from the EGFR-1 sequence, but Exon 2 to Exon 7 are missing. Using the indicated primers, EGFRvIII is characterized by a product of 226 bp size, whereas the wild-type receptor is recognized with a PCR product of 1028 bp.

solutions (in DMSO, stored at −20°C) were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration was <0.25%.

Isolation and establishment of primary cell cultures from human esophageal cancers

Primary cell cultures of esophageal carcinomas (2 adenocarcinoma, 3 squamous cell carcinomas) from 5 patients (4 male, 1 female; age: 61 ± 12 years) were isolated from endoscopic tumor biopsies as previously described.[18] The human tumor material was used according to the standards set by the Ethical Committee of the Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany. The isolated human esophageal carcinoma cells were maintained in Earle's 199 medium supplemented with 20% FBS, 2 mM L-glutamine, 2% Biotect protective medium, penicillin(100 U/ml), streptomycin(100 μg/ml) and 1% amphotericin B, and were incubated at 37°C in a humidified atmosphere (5% CO_2).

Analysis of growth factor receptor expression

Semiquantitative analysis of EGFR, IGFR and EGFRvIII mRNA expression was carried out by RT-PCR. Total RNA was extracted with RNAClean (Hybaid, London, United Kingdom) and subsequently digested with DNAse I (Gibco, Karlsruhe, Germany). Oligo-dT-primers and the SuperScript Preamplification-Kit (Gibco) were used for cDNA synthesis. PCR reactions were performed as previously described with established primers and conditions as indicated in Table I.[13] Growth factor receptor gene expression was standardized to the expression of the housekeeping gene β-actin.

For analysis of growth factor receptor protein expression, cells were immunostained with a polyclonal anti-EGFR or IGF-1R antibody (5 μg/ml, Santa Cruz Biotechnology, Palo Alto, CA), or isotypic control rabbit IgG1 (DAKO, Hamburg, Germany), as described.[12] Fluorescence was detected by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analyzed using CellQuest software.

Determination of cell number

Cell number was evaluated by crystal violet staining, as described.[19] In brief, cells in 96-well plates were fixed with 1% glutaraldehyde. Then cells were stained with 0.1% crystal violet. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton X-100. Light extinction that increases linearly with the cell number was analyzed at 570 nm using an ELISA-Reader.

Determination of cytotoxicity

Cells were incubated with 1–25 μM erlotinib for 24 and 48 hr. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH),

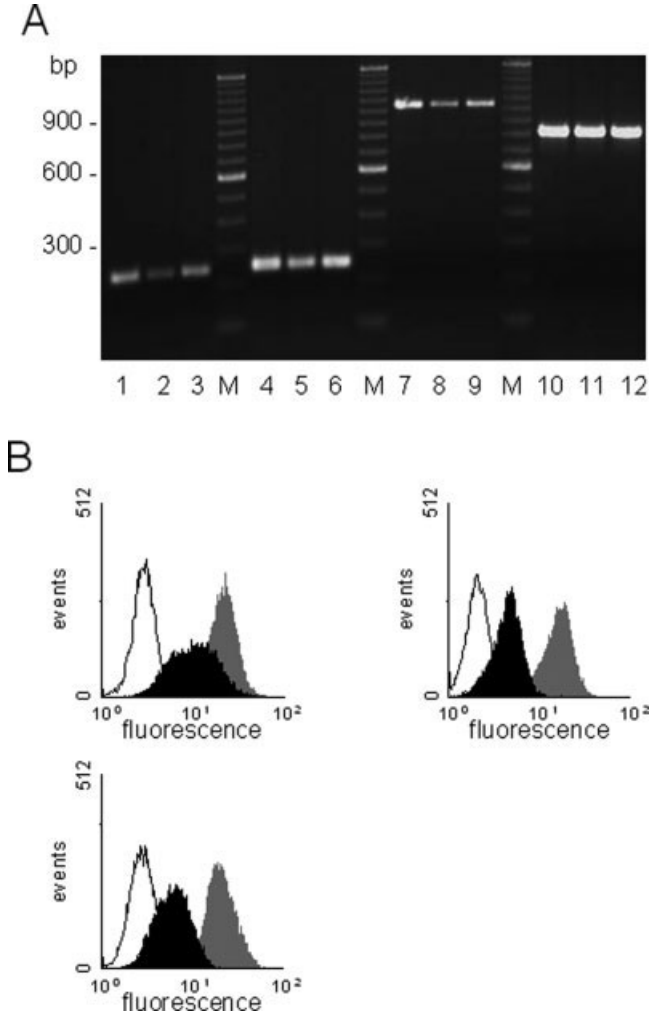

FIGURE 1 – EGFR and IGFR expression in esophageal cancer cells. (*a*) mRNA expression of IGF-1R (lanes 1–3), EGFR (lanes 4–6) and EGFRvIII (lanes 7–9) were evaluated in Kyse-30 (lanes 1,4 and 7), Kyse-70 (lanes 2,5 and 8) and Kyse-140 cells (lanes 3,6 and 9). The expression of the housekeeping gene β-actin (lanes 10–12) was analyzed for standardization. M: 100 bp DNA ladder. (*b*) Flow cytometric analysis of EGFR and IGF-1R protein expression in Kyse-30 (upper left panel), Kyse-70 (upper right panel) and Kyse-140 cells (lower panel). Cells were stained with antibodies against either EGFR (black areas) or IGF-1R (grey areas). Black lines: isotypic controls.

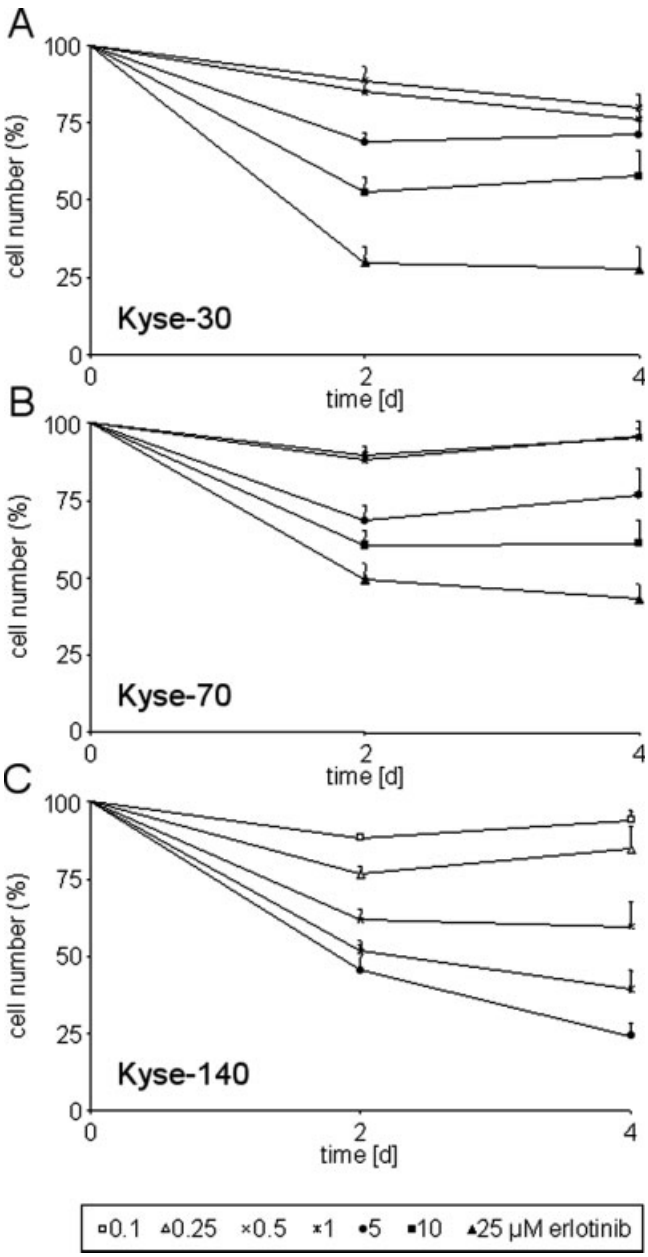

FIGURE 2 – Effects of erlotinib on esophageal cancer cell growth. Erlotinib caused a time- and dose-dependent growth inhibition in esophageal cancer cells. After 4 days of incubation with rising concentrations of erlotinib, cell numbers decreased by 72% (Kyse-30, 25 μM), 57% (Kyse-70, 25 μM) or 76% (Kyse-140, 5 μM) as determined by crystal-violet staining. Data are given as percentage of untreated controls (means ± SEM of 4 independent experiments). Statistical significance ($p < 0.05$) of growth inhibition was shown for 0.5–5 μM (Kyse-140) or 5–25 μM (Kyse-30; Kyse-70) erlotinib.

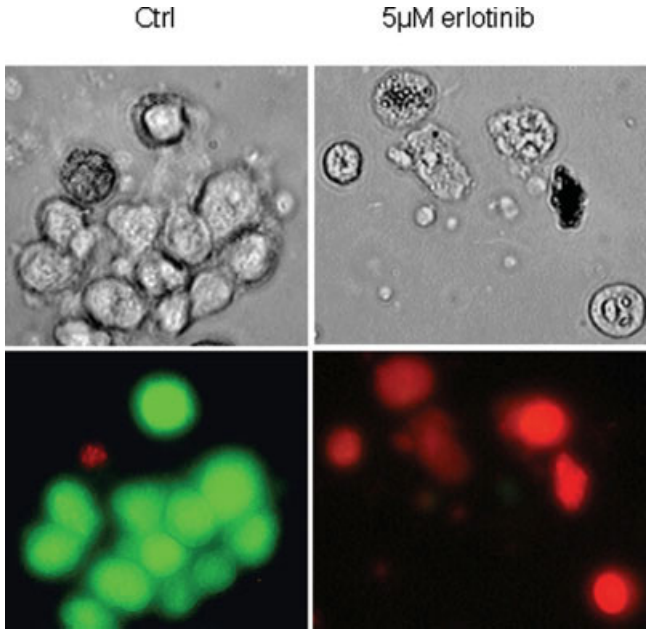

FIGURE 3 – Erlotinib-induced cell death of human primary esophageal cancer cells. Incubation with 5 μM erlotinib for 72 hr decreased both total and viable cell count and led to morphological changes of isolated primary esophageal cancer cells as assessed by Live/Dead-fluorescence microscopy. Viable cells stained green, while cells with impaired cell membrane appeared red. Representative phase-contrast images (upper panel) and corresponding fluorescence micrographs (lower panel) of 1 preparation out of $n = 4$ erlotinib-sensitive primary cell cultures are depicted.

indicating cytotoxicity, was measured in sample supernatants by using a colorimetric kit from Roche (Roche Diagnostics, Mannheim, Germany), as described.[20] Maximum release was measured after adding 2% Triton X-100 to untreated cells. Absorbance was measured at 490 nm (reference wavelengh 690 nm).

Determination of cell viability

Viability of primary esophageal cancer cells was determined by using a cell viability/cytotoxicity assay kit (Live/Dead assay) from Molecular Probes (Eugene, OR) as described elsewhere in detail.[18] In brief, cells were incubated with calcein-AM (160 nM) and EthD-1 (2 μM) for 1 hr at 37°C and examined by fluorescence microscopy. Live cells were identified by the presence of ubiquitous intracellular esterase activity, leading to the conversion of nonfluorescent cell-permeable calcein-AM to the green-fluorescent polyanionic dye calcein (ex/em ~495 nm/~510 nm), which is well retained within live cells. Dead cells were determined by EthD-1 (ex/em ~495 nm/~635 nm), which becomes red-fluorescent upon binding to nucleic acids of cells with damaged membranes.

Detection of apoptosis

Changes in mitochondrial membrane potential ($\Delta\Psi_M$) were assessed as described.[13] In brief, cells were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1, 1 μg/ml, Molecular Probes) for 15 min at 37°C in the dark, prior to analysis by flow cytometry.

Preparation of cell lysates and determination of caspase activity was assessed as described.[13] The activity of caspases was calculated from the cleavage of fluorogenic substrates (caspase-3: DEVD-AMC; caspase-8: Z-IETD-AFC, Calbiochem-Novabiochem).

The proportion of apoptotic cells was determined by quantifying the percentage of sub-G1 (hypodiploid) cells after flow cytometric analysis of propidium iodide-stained isolated nuclei.[21]

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen.[22] Cell nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson). DNA was stained with propidium iodide according to the manufacturers' instructions.

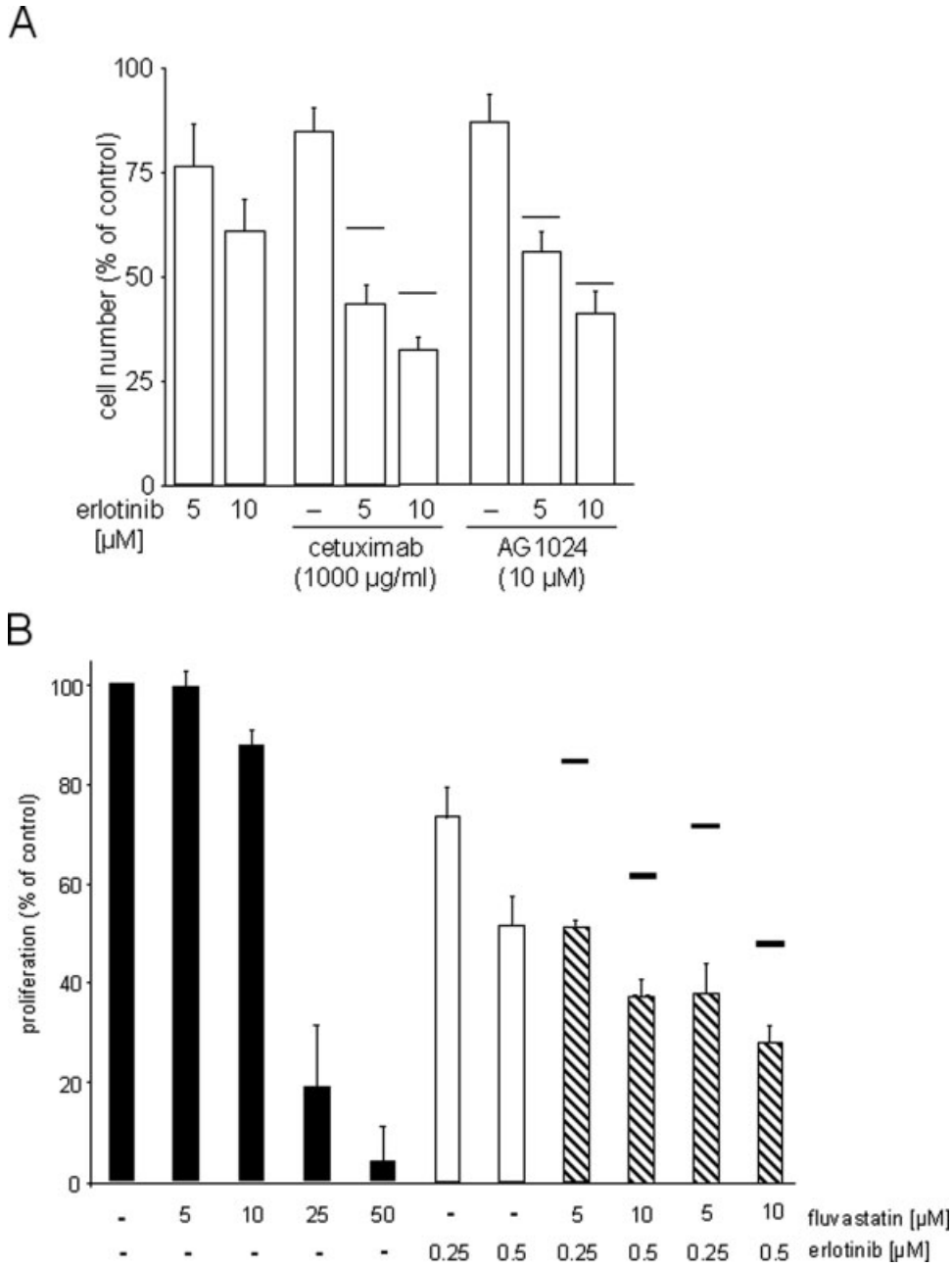

FIGURE 4 – Synergistic growth inhibition of esophageal cancer cells by erlotinib plus cetuximab, or by erlotinib plus AG1024, or by erlotinib plus fluvastatin. (*a*) Combination treatment of Kyse-70 cells with sub-IC_{50} concentrations of erlotinib plus cetuximab or with erlotinib plus AG1024 for 96 hr led to synergistic growth inhibitory effects. Black bars indicate the values of the calculated additive growth inhibition. (*b*) 72 hr of incubation with fluvastatin (0–50 μM) led to a dose-dependent growth inhibition of Kyse-140 cells by >90%. Combination treatment with sub-IC_{50} concentrations of fluvastatin and erlotinib resulted in overadditive growth inhibition. Data are given as percentage of untreated controls (means ± SEM of 3–5 independent experiments each).

The DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson).

Western blotting

Western blotting was performed as described.[13] Blots were blocked in 5% nonfat dry milk for 1 hr, and then incubated at 4°C overnight with anti-cyclin D1 (1:100), $p21^{Waf1/CIP1}$, phospho-EGFR, EGFR (1:1,000), phospho-ERK1/2, ERK1/2 (1:1,000, Santa Cruz Biotechnology, CA), $p27^{Kip1}$ (1:2,500, Becton Dickinson) or β-actin (1:2,500, Sigma, Taufkirchen, Germany).

Statistical analysis

If not stated otherwise, means of 4 independent experiments ± SEM. are shown. Individual drug treatment was compared by the unpaired, two-tailed Mann-Whitney *U* test. *p* values were considered to be significant at <0.05.

Results

Expression of EGFR and IGFR in esophageal cancer cells

EGFR and IGF-1R mRNAs (Fig. 1*a*), as well as protein expression of EGFR and IGF-1R (Fig. 1*b*), was detected in all esophageal cell lines investigated. Moreover, no expression of EGFRvIII, a mutant EGFR variant that has previously been observed in other cancer types,[23] was detected in either model (Fig. 1*a*).

Erlotinib induces growth inhibition of esophageal cancer cells

EGFR-TK inhibition by erlotinib inhibited the growth of Kyse-30, Kyse-70 and Kyse-140 cells in a time- and dose-dependent way (Fig. 2). The IC_{50} values of erlotinib, as determined after 96 hr, were 13.9 ± 3.8 μM (Kyse-30), 19.2 ± 5.3 μM (Kyse-70) and 0.7 ± 0.2 μM (Kyse-140). In addition, erlotinib potently inhibited the growth of OE-33 esophageal adenocarcinoma cells (IC_{50} = 0.43 μM after 96 hr treatment). Similarly, erlotinib treatment

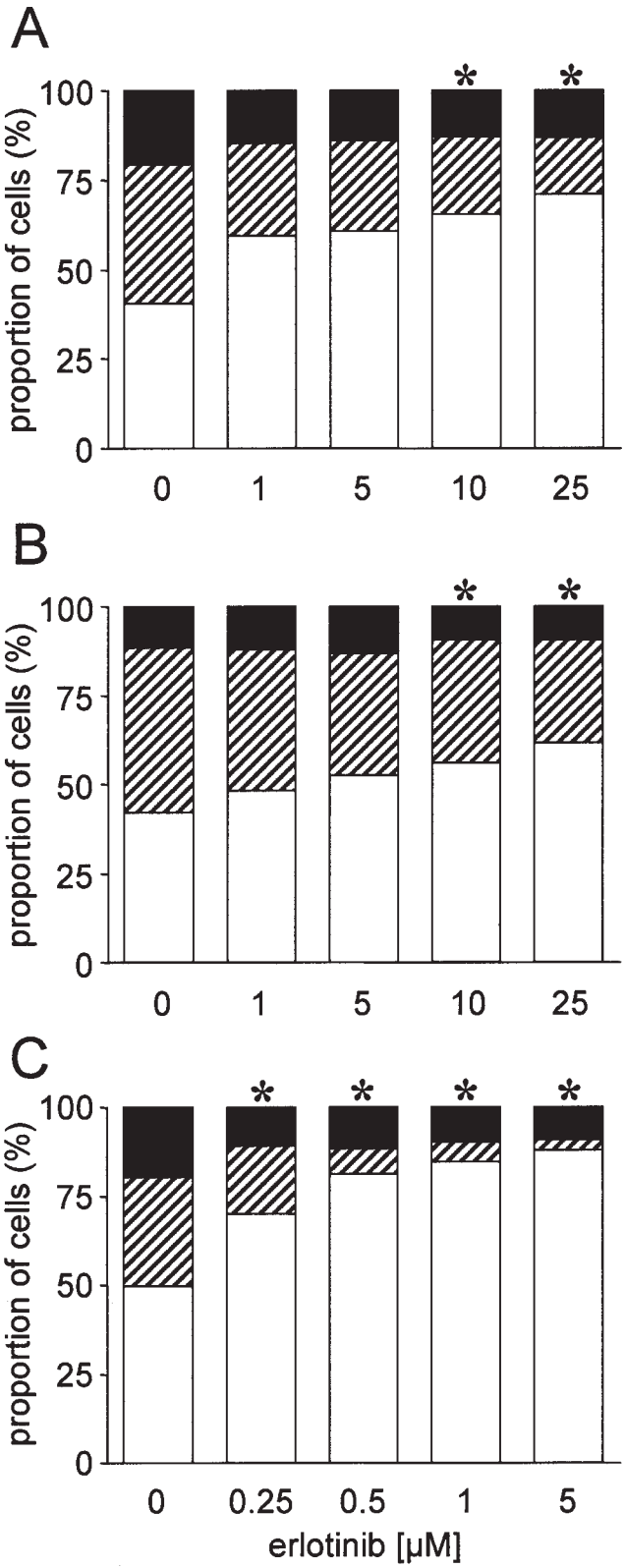

FIGURE 5 – Effects of erlotinib on cell cycle in esophageal cancer cells. After 24 hr of incubation with erlotinib Kyse-30 (*a*), Kyse-70 (*b*) and Kyse-140 cells (*c*) dose-dependently accumulated in the G0/G1-phase of the cell cycle (white bars). Accordingly, the proportions of cells in the S- and G2/M-phases (hatched and black bars) decreased. Means of 4 independent experiments are shown. *Statistically significant ($p < 0.05$) difference of the proportion of cells in the G0/G1 phase compared to untreated controls.

(0–5 μM for 72 hr) reduced the number of human primary esophageal carcinoma cells by up to 63.7 ± 12.7% as assessed by direct cell counting. Using Live/Dead-fluorescence microscopy, we visualized the antineoplastic effects of erlotinib in isolated primary esophageal carcinoma cells (Fig. 3). Interestingly, 1 out of the 5 tested primary cultures was insensitive to erlotinib treatment (<5% growth inhibition).

Antineoplastic potency of erlotinib in combination with other targeted agents

Next we investigated whether the combination of erlotinib with either cetuximab, a humanized monoclonal antibody against EGFR, or with the tyrphostin AG1024, an IGFR-TK inhibitor, was superior to the antiproliferative treatment with each agent on its own. Cells were treated with 2 different concentrations of erlotinib below its respective IC_{50} value and cetuximab (1,000 μg/ml) or AG1024 (10 μM) for 96 hr.

Both the combinations cetuximab plus erlotinib and AG1024 plus erlotinib resulted in an additive or even synergistic growth inhibition. Erlotinib at concentrations inducing 15–40% growth inhibition on its own caused growth inhibition by 30–70% when it was combined with either cetuximab or AG1024. Overall, the most pronounced synergism (57% observed *versus* 38% calculated growth inhibition) was seen for erlotinib (5 μM) plus cetuximab (1,000 μg/ml) in Kyse-70 cells (Fig. 4*a*). We additionally investigated the antiproliferative potency of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) inhibitor fluvastatin, alone and in combination with erlotinib, in Kyse-140 cells. Fluvastatin (0–50 μM) caused a dose-dependent growth inhibition of more than 90%. Combinations of sub-IC_{50} concentrations of fluvastatin and erlotinib resulted in synergistic growth inhibitory effects (Fig. 4*b*).

Erlotinib induces cell cycle arrest but neither immediate cytotoxicity nor apoptosis in esophageal cancer cells

Erlotinib dose-dependently arrested Kyse-30, Kyse-70 and Kyse-140 cells in the G1/G0 phase of the cell cycle after 24 hr of treatment, thereby decreasing the proportion of cells in the S and G2/M-phases (Fig. 5).

We next evaluated potential cytotoxic effects of erlotinib. Incubating esophageal cancer cells with erlotinib (Kyse-30, Kyse-70: 1–25 μM; Kyse-140: 0.1–5 μM) for 24 hr did not result in an increase of LDH release, indicating that erlotinib does not directly affect cell membrane integrity and does not have immediate cytotoxic effects, even at high concentrations (data not shown).

Finally, a decrease of cell number could also be due to apoptotic cell death. However, incubating esophageal cancer cells with erlotinib (Kyse-30, Kyse-70: 1–25 μM; Kyse-140: 0.1–5 μM) for 24–96 hr did not result in an induction of apoptosis. We neither observed any mitochondrial alterations, nor caspase-8 activation, nor caspase-3 activation (data not shown), nor DNA fragmentation in either cell line (Fig. 6), indicating that erlotinib-induced growth inhibition of esophageal cancer cells was not due to apoptosis induction.

Erlotinib treatment leads to diphosphorylation of EGFR and ERK1/2

We next investigated changes in the phosphorylation of EGFR, the target of erlotinib, and ERK1/2, which is a member of the MAPK-family known to be involved in EGFR-mediated mitogenic and antiapoptotic signaling,[24] to explore erlotinib-modulated signaling pathways in esophageal cancer cells. Erlotinib (0.1–5 μM) dose-dependently decreased EGFR- and ERK1/2 phosphorylation in Kyse-140 cells after 24 hr as shown by Western blot analysis (Fig. 7*a*).

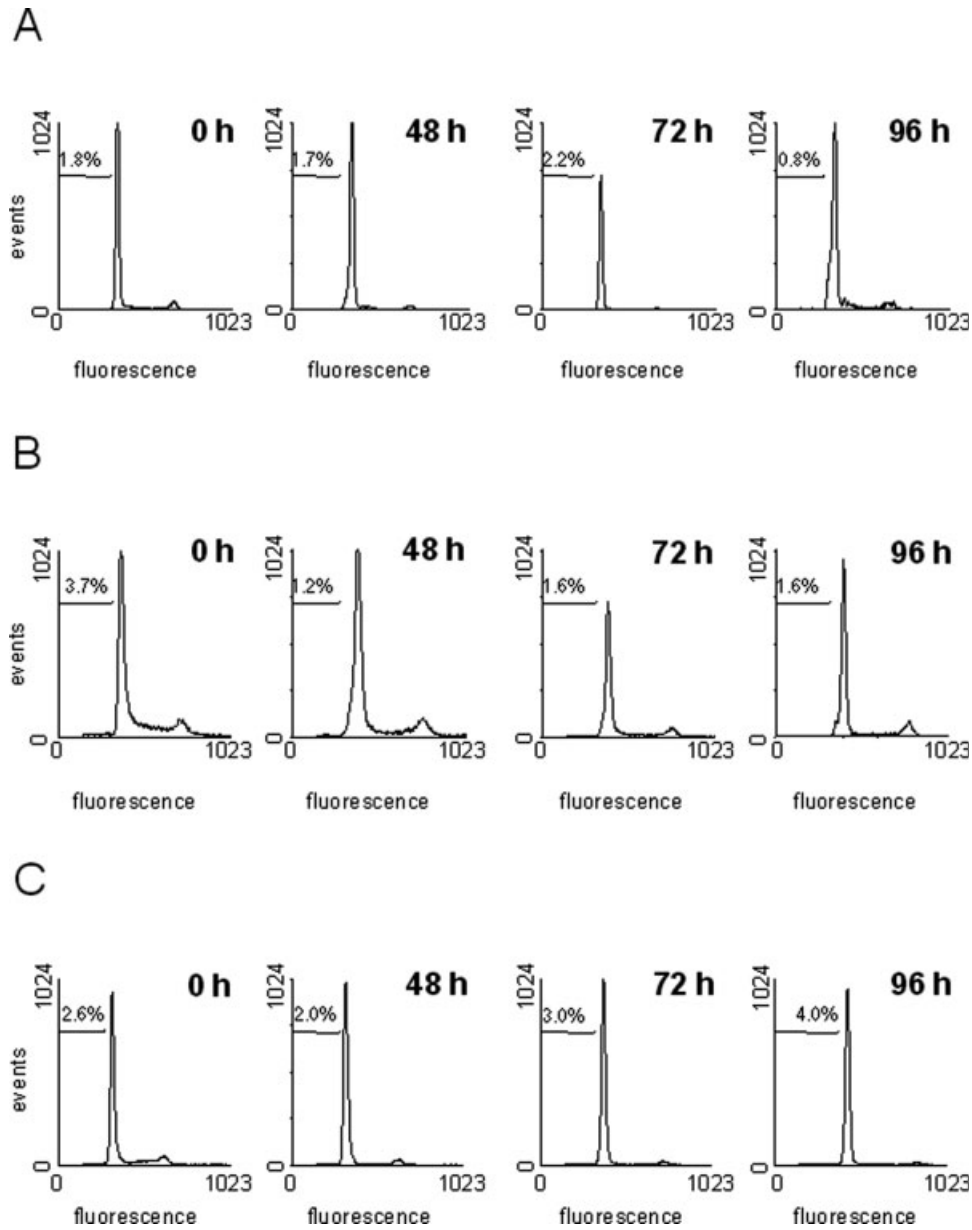

FIGURE 6 – Erlotinib does not induce apoptosis in esophageal cancer cells. Kyse-30 ((*a*), 25 μM), Kyse-70 ((*b*), 25 μM) or Kyse-140 ((*c*), 5 μM) were incubated with erlotinib for 48–96 hr. The respective percentage of sub-G1 apoptotic cells is given in each histogram. Representative data from 3 independent experiments are shown.

Erlotinib modulates the expression of cell cycle regulators

The expression of cell cycle-related proteins were investigated to shed light on the pathways downstream of erlotinib-induced dephosphorylation of EGFR and ERK1/2 leading to cell cycle arrest. Erlotinib dose-dependently decreased the expression of cyclin D1 but increased the expression of the CDK inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ (Fig. 7*b*). These data suggest that erlotinib-induced cell cycle arrest is mediated by $p21^{Waf1/CIP1}$ and $p27^{Kip1}$, resulting in a decrease of cyclin D1.

Discussion

Medical therapy of advanced esophageal cancer is still unsatisfactory. Moreover, many patients with esophageal cancer are in bad clinical condition precluding aggressive chemotherapy. Thus, there is a strong need for new, effective and well-tolerated treatment approaches. The EGF/EGFR system is known to have strong mitogenic effects in esophageal cancer cells.[11] Moreover, EGFR is generally overexpressed in esophageal cancer.[4,11,25] The esophageal cancer cells studied by us expressed both EGFR and IGFR. IGFR is overexpressed in esophageal cancer, and regulation of cell growth and apoptosis of esophageal cancer cells is tightly associated with IGFR signaling.[10,11] As IGFR signaling may depend on the transactivation of the EGFR-TK,[14,15] the antineoplastic effects of erlotinib in esophageal cancer cells may at least in part be due to the inhibition of IGFR signaling. Thus, the link between EGFR-TK inhibition and IGFR signaling in esophageal cancer warrants further investigation.

Erlotinib inhibits the growth of a variety of human cancer cells.[6,26] Disease stabilization has been reported upon erlotinib treatment in patients with nonsmall cell lung cancer and head and neck carcinomas.[7,8] Nevertheless, erlotinib has not been evaluated for its suitability and efficacy in esophageal cancer disease so far. Here, we provide evidence that erlotinib inhibits the growth both of esophageal cancer cell lines and of primary esophageal cancer cells. This effect was observed in the presence of growth factors like EGF, IGF and TGF-α, which are also present *in vivo*, thus qualifying erlotinib as a promising novel antineoplastic drug to be

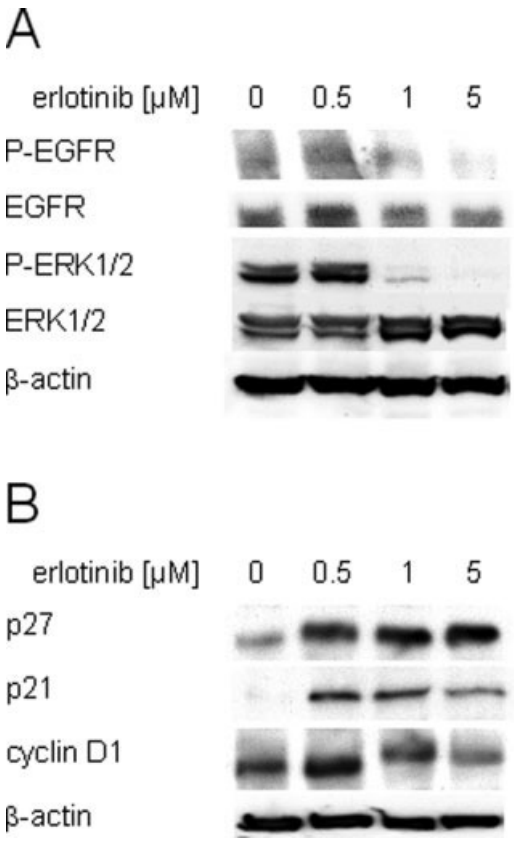

FIGURE 7 – Effects of erlotinib on EGFR and ERK1/2 activity and on the expression of cell cycle regulators. Modulation of protein phosphorylation or protein expression by erlotinib treatment was analyzed by Western blotting. Kyse-140 cells were treated with erlotinib (0–5 μM) for 24 hr. (*a*) Erlotinib treatment induced a dose-dependent dephosphorylation of mitogenic EGFR and ERK1/2. (*b*) The cell cycle promoter cyclin D1 was downregulated by erlotinib treatment, whereas the cell cycle inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ were upregulated. One representative out of 3 experiments is shown for each investigated protein.

tested in esophageal cancer patients in the future. Differences in the sensitivity of esophageal cancer cells to erlotinib may be due to mutations in the TK domain of EGFR, which has already been proposed for other tumor entities.[27]

Primary cell cultures of human esophageal cancers were established as a tool to design a rational individual medical treatment of an individual patient. The primary goal was to study erlotinib's antineoplastic potency in a bench to bedside approach, as commercially available cell lines may represent well-suited but nevertheless nonrepresentative models of esophageal carcinomas. Moreover, chemosensitivity testing of primary cultures was performed to establish a new method for predicting the response of an individual patient to a certain drug. Attempts to predict individual responses have already been undertaken for breast cancer[28] and colorectal cancer,[29] respectively. Such an approach may pave the way to an individualized medical treatment of cancer patients.

In 4 out of 5 tested primary cell cultures, erlotinib exhibited antineoplastic potency. The failure of erlotinib to inhibit the growth of one esophageal adenocarcinoma further strengthens the approach of pretreatment *in vitro* testing of individual chemosensitivity. It also shows that targeting growth factor receptor pathways by one single agent may not be sufficient and thus argues for combination treatment approaches. Therefore, we evaluated the combinations of erlotinib plus cetuximab, a humanized monoclonal antibody against EGFR, or of erlotinib plus the IGFR-TK inhibitor AG1024. Both combinations resulted in additive or even overadditive antiproliferative effects. Especially, the combination treatment of Kyse-70 cells with erlotinib plus cetuximab resulted in a strong synergistic effect, exceeding the extrapolated growth inhibition of the single agents by ~20%. These findings argue for combination therapies of erlotinib with cetuximab or IGFR-TK inhibitors in esophageal cancer patients. In line with our study, maximizing EGFR signaling inhibition by combining anti-EGFR monoclonal antibody with EGFR-TKI has been shown to augment the potency of anti-EGFR targeted therapy.[30] Moreover, sustained EGFR-TK inhibition has been shown to cause resistance associated with increased IGF-1R signaling, which can be overcome by IGF-1R-TK inhibition.[31] We therefore conclude that the simultaneous inhibition of both EGFR-TK and IGFR-TK, shown to additively inhibit the growth of esophageal cancer cells, may increase the efficacy and duration of clinical response to erlotinib in esophageal cancer patients. In summary, potentiation of the antitumor efficacy of distinct classes of growth factor receptor inhibitors by erlotinib may have important clinical implications for the treatment of esophageal cancer.

Drug resistance is one of the major problems of chemotherapy. Potential mechanisms of drug resistance include the activation of Ras/Raf/Mek/ERK signal transduction cascade and the increase of cholesterol levels in cancer cells, both being controlled by isoprenoids.[32] The production of isoprenoids is catalyzed by HMG-CoAR which may therefore be a rational molecular target for innovative antineoplastic treatment of esophageal cancer. Fluvastatin is an effective inhibitor of HMG-CoAR and has already been shown to inhibit tumor cell growth.[33] Here we demonstrate the antineoplastic effect of fluvastatin alone and in combination with erlotinib in esophageal cancer cells. Moreover, fluvastatin synergistically enhanced the antiproliferative effect of erlotinib. Hence, combining erlotinib and fluvastatin is a promising approach for dual targeting treatment strategies in esophageal cancer disease. Further investigations will be conducted to clarify the exact mechanisms underlying the observed overadditive growth inhibitory effects.

The mechanisms underlying erlotinib's antiproliferative action in esophageal cancer cells were further characterized. Erlotinib induced cell cycle arrest in the G1/G0-phase, suggesting that erlotinib acts at the G1/S checkpoint. G1/S cell cycle arrest induced by EGFR-TK inhibition has already been described in neuroendocrine gastrointestinal tumor cells[12] and hepatocellular carcinoma cells,[13] head and neck cancer[34] and malignant pleural mesothelioma.[35] Although the induction of apoptosis by EGFR-TK inhibition has recently been reported,[13,26,36] the respective contribution of either cell cycle arrest and/or apoptosis to the antitumor activity of EGFR-TK inhibition differs among various tumor types.[26] As erlotinib neither induced mitochondrial alterations, nor caspase activation, nor DNA fragmentation in esophageal cancer cells, we suggest that apoptosis induction does not account for the antineoplastic effects of erlotinib observed in this study. Finally, a potential induction of necrotic cell death by erlotinib was excluded. Thus, the erlotinib-induced inhibition of esophageal cancer growth is due to cell cycle arrest.

Activated EGFR induces the Ras-Raf-MEK-ERK pathway, leading to the induction of cyclin D1 expression.[37,38] Thus, we analyzed erlotinib-induced changes of EGFR and ERK1/2 acitivity, and $p21^{Waf1/CIP1}$, $p27^{Kip1}$ and cyclin D1 expression to elucidate the pathways underlying erlotinib-induced cell cycle arrest. Erlotinib induced an inactivation of both EGFR and ERK1/2, in agreement with observations in other tumor entities.[39–41] Defective function of cell cycle regulators is a main cause for tumor development and progression. For example, the cell cycle promoter cyclin D1 is frequently overexpressed in esophageal cancer.[4] Successful therapeutic strategies have to balance or bypass impaired signaling. We herein demonstrate that erlotinib treatment increased the expression of the cell cycle-inhibiting molecules $p21^{Waf1/CIP1}$ and $p27^{Kip1}$, but decreased the expression of cyclin

D1. The suppression of cyclin D1 expression by erlotinib may be a suitable surrogate marker of response to erlotinib treatment.[39]

To conclude, our study provides first evidence that EGFR-TK inhibition by erlotinib potently inhibits the growth of human esophageal cancer cells by inducing cell cycle arrest. Thus, erlotinib, alone or in combination with other targeted agents, with radiotherapy or cytostatics, qualifies for the development of tailored therapies of esophageal cancer and should be tested in future clinical trials. As EGFR is upregulated during esophageal carcinogenesis, investigations should be pursued to modulate the EGF/EGFR system (by erlotinib) as a possible means of esophageal cancer prevention in patients at high risk.[42,43]

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