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*BASIC RESEARCH*

# Signaling pathways involved in the inhibition of epidermal growth factor receptor by erlotinib in hepatocellular cancer

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## Abstract

**AIM:** To examine the underlying mechanisms of erlotinib-induced growth inhibition in hepatocellular carcinoma (HCC).

**METHODS:** Erlotinib-induced alterations in gene expression were evaluated using cDNA array technology; changes in protein expression and/or protein activation due to erlotinib treatment as well as IGF-1-induced EGFR transactivation were investigated using Western blotting.

**RESULTS:** Erlotinib treatment inhibited the mitogen activated protein (MAP)-kinase pathway and signal transducer of activation and transcription (STAT)-mediated signaling which led to an altered expression of apoptosis and cell cycle regulating genes as demonstrated by cDNA array technology. Overexpression of proapoptotic factors like caspases and gadds associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-$X_L$ or jun D accounted for erlotinib's potency to induce apoptosis. Downregulation of cell cycle regulators promoting the $G_1$/S-transition and overexpression of cyclin-dependent kinase inhibitors and gadds contributed to the induction of a $G_1/G_0$-arrest in response to erlotinib. Furthermore, we displayed the transactivation of EGFR-mediated signaling by the IGF-1-receptor and showed erlotinib's inhibitory effects on the receptor-receptor cross talk.

**CONCLUSION:** Our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the design of combination therapies that act additively or synergistically. Moreover, our data on the pathways responding to erlotinib treatment could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.



**Key words:** Epidermal growth factor receptor; Insulin-like growth factor receptor; Tarceva™; Signal transducer of activation and transcription; Extracellular regulated kinase; Gene expression

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause half a million deaths annually. The incidence of HCC is dramatically increasing in the USA, Europe and Asia, due to high prevalence of chronic hepatitis B and hepatitis C virus infections, alcohol disease, diabetes and obesity[1]. Unfortunately, the majority of patients suffer from advanced disease at presentation. Therefore curative local ablation, surgical resection of HCC, or liver transplantation can be achieved only in a minority of patients. Local tumor destruction, chemoembolization or systemic chemotherapy are the treatment options of advanced HCC. However, overall survival is poor. Apart from chemoembolization, which improves survival in well-selected patients with unresectable HCC, palliative treatment options do not appear to greatly improve overall survival[2]. Therefore, innovative treatment approaches are urgently needed. Recently, evidence has been accumulated that the epidermal growth factor receptor (EGFR) is a promising target for cancer therapy. A great variety of tumors show abnormal, enhanced and/or constitutive expression of EGFR. Several reports indicate that EGFRs are expressed frequently in human HCC, most likely contributing to the aggressive growth characteristics of these tumors[3,4]. Especially in poorly differentiated HCCs, EGFR overexpression has been demonstrated to be a negative prognostic factor, since it positively correlated with early

tumor recurrence and the occurrence of extrahepatic metastasis[3,5]. Hence, the EGFR is a promising target for innovative treatment strategies in HCC.

The EGFR is a member of a family of four closely related receptors: EGFR (ErbB-1), HER-2/*neu* (ErbB-), HER-3 (ErbB-3), and HER-4 (ErbB-4). Upon ligand binding the EGFR becomes activated by dimerization which leads to subsequent activation of EGFR tyrosine kinase (TK) activity, initiating receptor-mediated signal transduction, cell mitogenesis and cell transformation[6]. The EGFR downstream intracellular signal transduction pathways include components of Ras/mitogen-activated protein kinase (MAPK), phosphatidyl inositol 3-kinase, signal transducer and activator of transcription (STAT), downstream protein kinase C and phospholipase D pathways[7]. The Ras/MAPK cascade is supposed to be one of the major signaling routes of the EGFR system[8].

Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine] is a novel orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. The mechanism of action of erlotinib is competitive inhibition of the binding of ATP to the TK domain of the receptor, resulting in inhibition of EGFR autophosphorylation[9]. Single agent activity was observed in pretreated patients with non-small-cell lung cancer(NSCLC), head and neck carcinoma and ovarian cancer[10]. Recently, the results of the BR.21 phase III trial showed a significant 42.5% improvement in median survival compared to placebo in patients with advanced NSCLC[11] and the US Food and Drug Administration (FDA) has approved erlotinib for this indication in November 2004.

In a previous study we have shown that EGFR-TK-inhibition by erlotinib potently suppresses the growth of human EGFR-expressing HCC cells by inducing both apoptosis and cell cycle arrest at the $G_1$/S-transition[12].

The objective of the current study was to examine the underlying mechanisms of erlotinib-induced growth inhibition in HCC cells. For this purpose we studied the effects of erlotinib on downstream signaling molecules of the EGFR. We used cDNA array technology to investigate the EGFR-TKI-induced modulation of apoptosis- and cell cycle-related genes and Western blot analysis to evaluate changes in the activation of the mitogenic MAP-kinase- and Jak-STAT-pathways as well as changes in the expression of cell-cycle regulating and antiapoptotic proteins. Additionally, we investigated the influence of IGF-1R-activation on EGFR-mediated signaling and erlotinib's effects on the IGF-1R/EGFR-network.

## MATERIALS AND METHODS

### Materials

The highly differentiated human hepatocellular carcinoma cell line Huh-7 and the well differentiated hepatoblastoma cell line HepG2 were cultured in RPMI 1640 medium containing 100 mL/L fetal bovine serum and 100 kU/L penicillin and 100 mg/mL streptomycin. Erlotinib hydrochloride was a kind gift from Roche (Penzberg, Germany), cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma (München, Germany), if not stated otherwise. Stock solutions were prepared in DMSO and stored at -20℃ and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 5 g/L, thus not affecting cell growth. To evaluate the effects of erlotinib, cells were incubated with either control medium or medium containing rising concentrations of erlotinib.

### Drug combination studies

To check for possible additive or synergistic effects, combination treatment of erlotinib plus AG1024 (Calbiochem, Bad Soden, Germany) was studied. The 5 μmol/L or 10 μmol/L of the tyrphostine AG1024 was combined with 10 μmol/L erlotinib (e.g. approximately its $IC_{50}$ value). The antineoplastic activities of the combinations were compared to those of each drug alone. For all experiments cell number was evaluated by crystal violet staining as described[12]. In brief, cells in 96-well plates were fixed with 10 g/L glutaraldehyde, then cells were stained with 1 g/L crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 g/L Triton-X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-reader.

### Western blot analysis

Western blotting was performed as described[13]. Blots were blocked in 2.5% BSA and then incubated at 4℃ overnight with the following antibodies: ERK1/2 (1:500), p-ERK1/2 (1:500), cyclin D1 (1:100), Bcl-$X_L$ (1:200), STAT1 (1:1000), STAT3 (1:1000), STAT5 (1:1000), β-IGF-1R (1:1000), $p21^{Waf1/CIP1}$ (1:200; all from Santa Cruz Biotechnology, CA), $p27^{KIP1}$ (1:2500; Becton-Dickinson, Heidelberg, Germany), p-EGFR, p-STAT1(TYR701), p-STAT3(TYR705), p-STAT5 (TYR694) (all 1:500 and all from Cell Signaling, MA) and p-IGF-1R (1:1500; Biomol, Hamburg, Germany). β-actin (1:5000; Sigma, Deisenhofen, Germany) served as loading control. One representative out of three independent experiments was shown for each Western blot.

### RNA extraction and poly(A)$^+$ mRNA preparation

Total RNA was extracted from cultured HepG2 cells with RNAClean according to the munufacturer's recommendations (Hybaid, London, UK). Polyadenylated (poly(a)$^+$) mRNAs were enriched using magnetic Dynabeads according to the instructions of the supplier (Dynal, Oslo, N). The quality of poly(A)$^+$ and total RNA was assessed by agarose gel electrophoresis.

### cDNA array

HepG2 cells were treated with 10 μmol/L erlotinib for 48 h to determine erlotinib-induced differential gene expression. Untreated cells served as controls. We used the Atlas Human Apoptosis cDNA array with 205 human cDNAs spotted in duplicate on a nylon membrane (Clontech, Palo Alto, CA) as previously described[14]. A complete list of the cDNAs and controls as well as their accession numbers is available on the web (http://www.clontech.com/clontech/atlas/genelists/7743-1_HuApop.pdf). The hybridization signals were

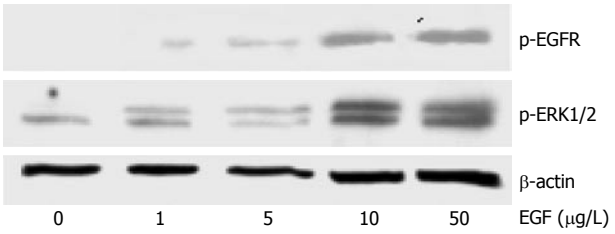


**Figure 1** EGF-induced EGFR- and ERK1/2-activation in HCC cells.

photometrically evaluated using TINA software (Raytest Isotopenmessgeräte, Straubenhardt, D). Altered expression of a respective gene is given as a fold increase or decrease compared to the signal of the control. Data analysis was performed as described[14].

### *Statistical analysis*

The means of three independent experiments of the cDNA expression arrays and the drug combination studies ± SD are shown. Significance between AG1024 treated samples and AG1024 plus erlotinib treated samples was calculated by Student's two sided *t*-test. $P < 0.05$ was regarded as significant.

## RESULTS

### *EGF-induced EGFR- and ERK1/2-activation*

To shed light on the signaling pathways modulated by EGFR-TK inhibition in HCC cells, we investigated the phosphorylation of ERK1/2 known to be involved in EGFR-mediated mitogenic and antiapoptotic signaling. In order to demonstrate the influence of the epidermal growth factor (EGF) on the activation of the EGFR and ERK1/2, serum-starved HepG2 cells were incubated for 15 min with increasing concentrations of EGF (1-50 μg/L). Serum-starved cells were chosen to exclude the influence of growth factors contained in the fetal calf serum (FCS) of the cell medium. Western blotting of whole cell lysates revealed a dose-dependent increase of activated EGFR and ERK1/2 in response to EGF incubation (Figure 1).

### *Inhibitory action of erlotinib on EGF-induced EGFR- and ERK1/2 activation*

EGF-induced activation of the EGFR and the mitogenic ERK1/2 was blocked by pretreating the cells with erlotinib. HepG2 cells were incubated for 30 min with escalating concentrations of erlotinib (0.1, 1, 10 μmol/L) and subsequently stimulated with EGF (10 μg/L). Again, activation of EGFR and ERK1/2 was determined by Western blotting. Erlotinib-untreated cells (control) displayed a pronounced activation of EGFR and ERK1/2 due to EGF-stimulation. Low concentrations of erlotinib (0.1 μmol/L) completely blocked EGFR-phosphorylation and ERK1/2-activation decreased dose-dependently (Figure 2A).

To mimic *in vivo* conditions, we finally examined the influence of erlotinib (10 μmol/L) on the activation of ERK1/2 in HepG2 cells stimulated by growth factors contained in the medium's FCS. We revealed a time-dependent decrease in phosphorylation of ERK1/2 (Figure 2B) in HCC cells due to erlotinib treatment (up to 72 h).

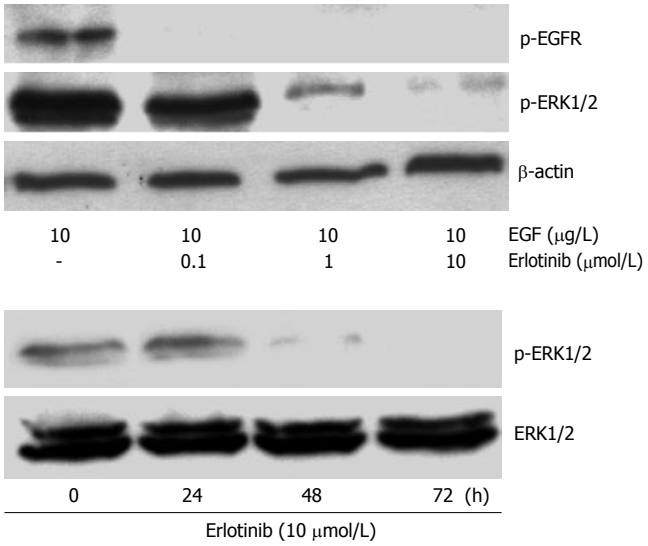


**Figure 2** Erlotinib inhibited EGFR (**A**) and ERK1/2 (**B**) activation in HCC cells.

### *STAT expression and EGF-induced activation*

EGFR activation is supposed to result in autophosphorylation of Janus-kinases (Jaks) with subsequent activation of mitogenic STATs but has not been investigated in HCC so far. Western blot analyses revealed the expression of STATs 1, 3 and 5 in Huh-7 cells (Figures 3A-C). Moreover, EGF induced STAT activation could be shown for STATs 1 and 3 (Figures 3A and B) but not in the case of STAT5 (Figure 3C). Serum-starved Huh-7 cells were stimulated with EGF (10 or 50 μg/L, 15 min) and whole cell lysates were used for determinations.

### *Inhibitory effect of erlotinib on STAT-activation*

EGF-induced STAT activation was blocked by pretreating Huh-7 cells with erlotinib. Erlotinib-untreated cells (control) displayed a pronounced activation of STATs 1 and 3 in response to EGF-incubation (10 μg/L, 15 min). Erlotinib pretreatment (0.1, 1, 10 μmol/L; 30 min) dose-dependently inhibited the phosphorylation of STATs 1 and 3, whereas the total quantity of STAT 1 and 3 remained unchanged (Figure 4; +: addition of the respective substance, -: absence of the respective substance).

### *Differential gene expression induced by erlotinib*

To further characterize the underlying molecular mechanisms of erlotinib-induced apoptosis and cell cycle arrest, the differential expression of genes related to cell cycle and apoptosis control was investigated using cDNA array technology. HepG2 cells were incubated for 48 h with 10 μmol/L erlotinib, as a significant arrest of both the cell cycle and apoptosis-induction had been observed under these conditions[12]. Erlotinib modulated the expression of 25 genes (Table 1). We found an overexpression of genes encoding apoptosis-related cysteine proteases (caspases) 3 and 7, both known to be important enzymes of the apoptotic process. Moreover, growth arrest and DNA-damage inducible (gadd-) genes encoding gadds 45 and 153 both associated with induction of apoptosis and growth arrest[15,16] as well as the insulin-like growth factor

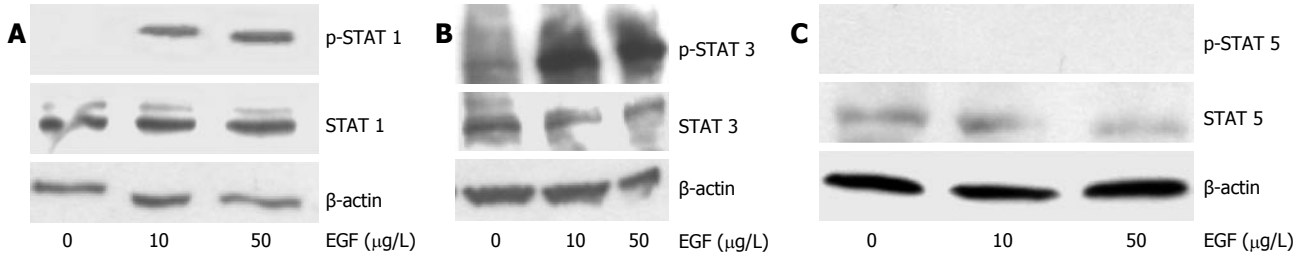


**Figure 3** STAT-expression and EGF-induced activation in HCC cells. EGF induced STAT activation could be shown for STATs 1 and 3 (**A** and **B**) but not in the case of STAT5 (**C**).

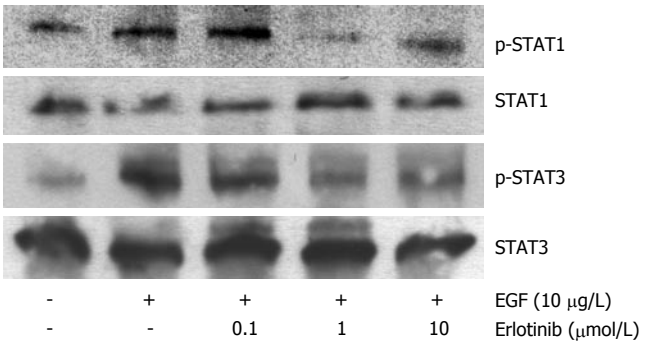


**Figure 4** Erlotinib inhibited STAT-activation in HCC cells.

binding protein 6 (IGFBP-6) and cyclin B1, an important molecular regulator of the $G_1$/S and $G_2$/M cell cycle transitions[17] were markedly overexpressed. Genes encoding cell-cycle progression promoting proteins were found to be suppressed (particularly those proteins promoting the transition from the $G_1$ to the S-phase, e.g. CDK4 or cyclin A2). Additionally, a suppression of anti-apoptotic genes like Bcl-2 or the jun D proto-oncogene was detected as well as of the DNA-replication promoting proliferating cell nuclear antigen (PCNA).

### *Modulatory effect of erlotinib on expression of cell cycle regulators and antiapoptotic members of the Bcl-2 family*

Changes in the expression of important cell cycle and apoptosis regulating proteins due to EGFR-inhibition were assessed by Western blotting. Treating HepG2 cells for up to 48 h with erlotinib (10 μmol/L) resulted in an increase of the cyclin-dependent kinase inhibitors (CDKIs) $p21^{Waf1/CIP1}$ and $p27^{Kip1}$. The expression of cyclin D1, a protein regulating the transition from the $G_1$ to the S-phase remained unchanged. Alterations in protein expression of the respective cell cycle regulators occurred within 24 h (Figure 5A). Additionally, a 48-h-treatment of HepG2 cells with 10 μmol/L erlotinib led to a significant suppression of the antiapoptotic Bcl-2 family members Bcl-$X_L$ (Figure 5B) and Bcl-2 (Figure 5C).

### *Modulation of the MAP-kinase pathway and transactivation of EGFR induced by IGF-1*

Comparable to the EGFR, the IGF-1-receptor is associated with carcinogenesis and tumor growth. Thus, we evaluated the mitogenic effects of IGF-1 on Huh-7 and HepG2 cells, both cell lines strongly expressing the IGF-1R[18]. Besides, we focused on a possible transactivation of the EGFR in response to IGF-1 treatment. Serum-starved HepG2 cells were treated for 15 min with IGF-1 (50-250 μg/L) followed by the determination of changes in EGFR- and ERK1/2-activation by Western blot. IGF-1 dose-dependently activated the mitogenic MAP-kinase pathway, moreover we could show that IGF-1 treatment in the absence of EGF or other growth factors resulted in an activation of the EGF-receptor, indicating a possible IGF-1R/EGFR cross talk in hepatocellular carcinoma cells (Figure 6).

**Table 1 Transcripts differentially regulated in HepG2 cells in response to erlotinib**

| GenBankID | Gene name | Mean[1] | SD |
|---|---|---|---|
| M62402 | Insulin-like growth factor binding protein 6 | 5.85 | 0.73 |
| S66431 | Retinoblastoma-binding protein 2 | 3.22 | 0.39 |
| U13737 | Caspase 3. apoptosis-related cysteine protease | 3.18 | 0.52 |
| Y09392 | Tumor necrosis factor receptor superfamily. member 12 (translocating chain-association membrane protein) | 3.04 | 0.61 |
| M60974 | Gadd45 | 2.37 | 0.34 |
| S40706 | Gadd153 | 2.31 | 0.09 |
| M25753 | Cyclin B1 | 2.21 | 0.47 |
| U37448 | Caspase 7. apoptosis-related cysteine protease | 2.10 | 0.34 |
| L16785 | Non-metastatic cells 2. protein (NM23B) | 2.03 | 0.22 |
| U23765 | BCL2-antagonist/killer 1 | 0.53 | 0.13 |
| X51688 | Cyclin A2 | 0.50 | 0.32 |
| M34065 | Cell division cycle 25C | 0.49 | 0.06 |
| L22005 | Cell division cycle 34 | 0.48 | 0.17 |
| X85134 | Retinoblastoma-binding protein 5 | 0.38 | 0.12 |
| L25676 | Cyclin-dependent kinase 9 (CDC2-related kinase) | 0.38 | 0.05 |
| U66879 | BCL2-antagonist of cell death | 0.36 | 0.05 |
| D25216 | KIAA0014 gene product | 0.35 | 0.14 |
| X86779 | Fas-activated serine/threonine kinase | 0.30 | 0.06 |
| L29220 | CDC-like kinase 3 | 0.28 | 0.03 |
| X74262 | Retinoblastoma-binding protein 4 | 0.27 | 0.09 |
| M14505 | Cyclin-dependent kinase 4 | 0.25 | 0.07 |
| AF010312 | LPS-induced TNF-alpha factor | 0.24 | 0.06 |
| U25265 | Mitogen-activated protein kinase 5 | 0.22 | 0.03 |
| M15796 | Proliferating cell nuclear antigen | 0.17 | 0.03 |
| X56681 | Jun D proto-oncogene | 0.13 | 0.06 |

[1] Arithmetic means of ratios (treated:untreated) from three separate array measurements.

### *Inhibitory action of erlotinib on mitogenic effects of EGF and/or IGF-1 and EGFR/IGF-1R receptor cross-talk*

Erlotinib-pretreated (30 min, 10 μmol/L) serum-starved

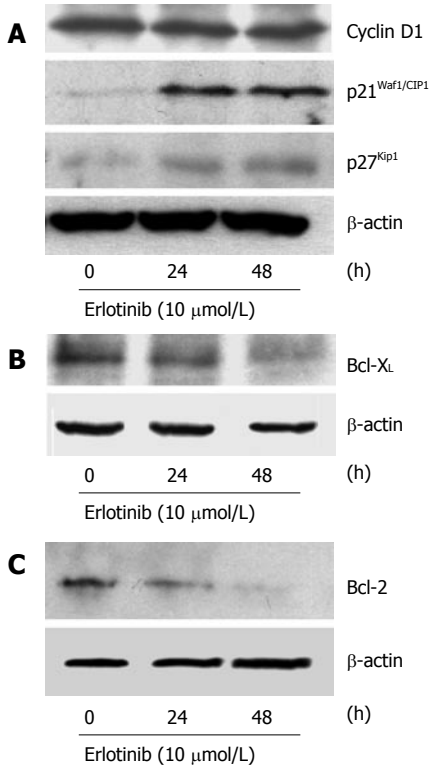


**Figure 5** Erlotinib modulated the expression of cell cycle regulators (**A**) and antiapoptotic members of the Bcl-2 family (**B** and **C**).

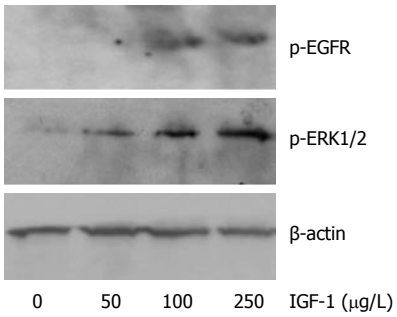


**Figure 6** IGF-1-induced activation of ERK1/2 and transactivation of the EGFR.

HepG2 cells were incubated with EGF (10 μg/L) and/or IGF-1 (100 μg/L) for 15 min. Growth factor untreated cells served as control. Comparable to the experiments described above, EGF and/or IGF-1-treatment resulted in the activation of both EGFR and ERK1/2. Erlotinib pretreatment completely inhibited growth factor-induced EGFR activation and reduced p-ERK1/2 levels below control values. Thus, in addition to the blockade of EGF and IGF-1-induced mitogenic signaling, erlotinib potently suppressed IGF-1 induced IGF-1R/EGF-receptor transactivation (Figure 7). In order to exclude unspecific effects of erlotinib on the activation of the IGF-1R by its ligands, we additionally investigated the effects of the EGFR-blocker on the expression and activation of IGF-1R. HepG2 cells were cultured up to 48 h in medium containing 100 mL/L FCS and 10 μmol/L erlotinib. Western blot analysis showed neither changes in the expression of the β-chain of IGF-1R or the IGF-1R

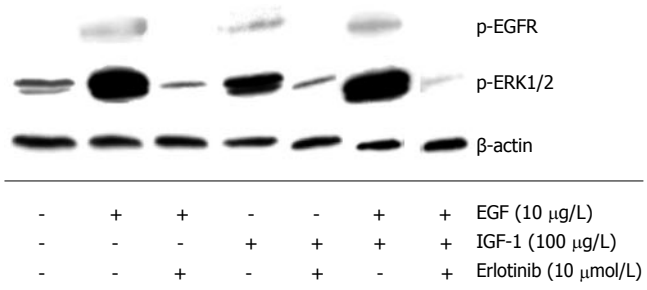


**Figure 7** Erlotinib inhibited EGF and/or IGF-1-induced ERK1/2-activation and EGFR/IGF-1R cross talk.

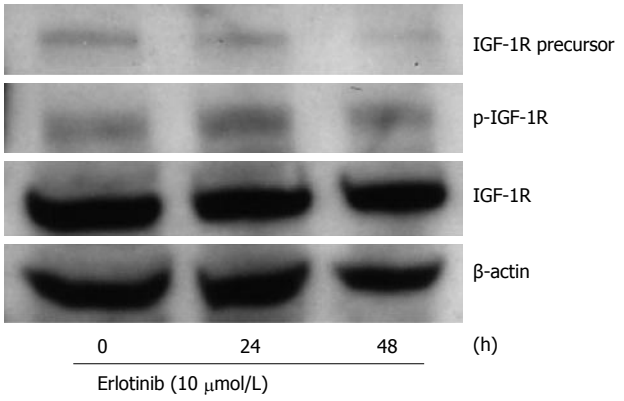


**Figure 8** Erlotinib did not influence IGF-1R expression or activation.

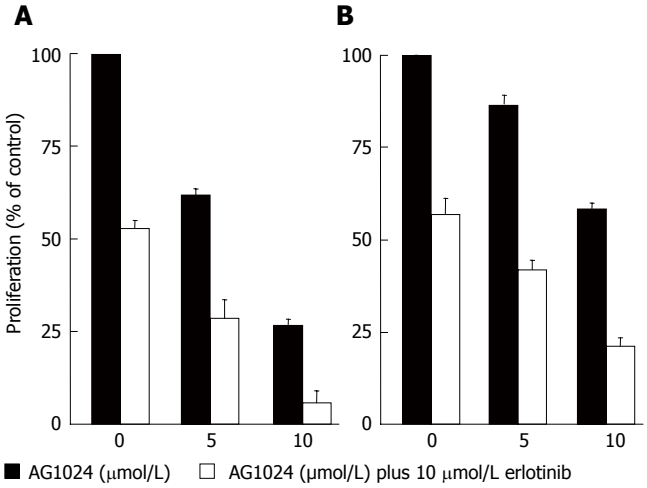


**Figure 9** Antiproliferative effects of erlotinib plus AG1024. **A**: HepG2; **B**: Huh-7.

precursor nor in the phosphorylation of the receptor as compared to control (Figure 8).

### *Growth inhibitory effects of simultaneous blockade of EGFR and IGF-1R*

On the basis of the results obtained in our investigations on EGFR/IGF-1R receptor signaling, we evaluated the growth inhibitory effects of a simultaneous blockade of the EGFR by erlotinib and the IGF-1R by AG1024. The tyrphostine AG1024 is a specific inhibitor of the IGF-1R-tyrosine kinase activity. Cells were treated with 5 or 10 μmol/L AG1024 and 10 μmol/L erlotinib for

72 h. Upon treatment with the IGF-1R-TKI alone both HepG2 (Figure 9A) and Huh-7 cells (Figure 9B) displayed a pronounced reduction of cell numbers (Figure 9, black bars). When combining AG1024 with erlotinib, synergistic antineoplastic effects were observed in both cell lines (Figure 9, hatched bars).

## DISCUSSION

Treatment options of advanced hepatocellular cancer (HCC) are unsatisfactory, and the prognosis of patients suffering from advanced HCC is poor. New, effective and well-tolerated therapy strategies are urgently needed. The EGF/EGFR system is known to have strong stimulatory effects on the growth of hepatoma cells. Several studies have demonstrated EGFR expression to be a common feature of HCCs[4,5], underlining the role of the EGFR-TK as a rational target for future hepatocellular cancer treatment. We recently demonstrated that the EGFR-TK-inhibitor erlotinib potently inhibited the growth of EGFR-expressing human hepatocellular cancer cells by a time- and dose-dependent induction of apoptosis and an arrest at the $G_1$-to-S-transition of the cell cycle[12]. In accordance, preliminary results of a phase II trial of erlotinib in patients with HCC suggest a clinical benefit by erlotinib[19]. However, the underlying mechanisms and corresponding molecular events by which erlotinib mediates apoptosis and cell cycle arrest are not yet understood. In this study, we identified signaling molecules involved in erlotinib-mediated apoptosis and cell cycle modulations and showed the functional involvement of the identified pathways.

We demonstrate that EGFR is activated upon EGF binding in HCC cells with a subsequent activation of ERK1/2, a key protein of the mitogen-activated protein kinase (MAPK)-pathway and that erlotinib potently inhibits EGFR activation associated with an inhibition of the mitogenic downstream signaling. MAPKs are important regulators of apoptosis, proliferation and differentiation[20]. Once activated, ERK1/2 translocates to the nucleus where it acts as a regulator of gene expression of various proteins, e.g. activated ERK1/2 inhibits the expression of the cell cycle inhibitors $p21^{Waf1/CIP1}$ [21,22] and $p27^{Kip1}$ [23]. Using Western blotting we could demonstrate an increase of expression of both proteins in response to erlotinib treatment within 24 h. Moreover, ERK1/2-inhibition is known to up-regulate the expression of gadd45[24] which could be confirmed in this work by cDNA expression arrays. The expression of the gadd45 gene has been correlated with the presence of a strong growth arrest as it interacts with $p21^{Waf1/CIP1}$ to induce cell cycle arrest[25]. Overexpression of gadd genes causes growth inhibition and/or apoptosis, and combined overexpression of gadd genes leads to synergistic suppression of cell growth[26]. Furthermore, activated ERK1/2 has been reported to inhibit the apoptotic process by inhibiting caspase activation[27] and the expression of several antiapoptotic proteins[20]. In accordance, our previous investigations demonstrated the increase of caspase-3 activity due to EGFR-blockade[12,28]. In this work cDNA expression arrays revealed that gene expression of caspases 3 and 7 is up-regulated due to erlotinib treatment. In addition to the activation of the proapoptotic caspase network a decrease of gene and protein expression of antiapoptotic members of the Bcl-2 family as demonstrated by cDNA expression arrays and Western blotting may account for erlotinib's apoptosis inducing capabilities.

Comparable to the MAPK-pathway, Jak-STAT-signaling is involved in cell proliferation and cell cycle progression[29]. STATs are latent in the cytoplasm and become activated through tyrosine phosphorylation which typically occurs through JAKs or growth factor receptor-TKs. Phosphorylated STATs form homo- or hetero-dimers, enter the nucleus and function as transcription factors. Transcriptional changes concerning apoptosis and cell cycle related genes are similar to those described for ERK1/2[30]. In normal cells, ligand dependent activation of STATs is a transient process but in tumors the STAT proteins (in particular STATs 1, 3 and 5) are often constitutively activated[29]. STATs 3 and 5 are noted for the proliferative effects and inhibition of apoptosis whereas the role of STAT1 in oncogenesis and tumor progression is controversial[31]. Effects of EGFR-TK-inhibition on Jak-STAT-signaling has not been investigated so far. We demonstrated the expression of STATs 1, 3 and 5 in HepG2 cells and the activation of STATs 1 and 3 but not STAT5 due to EGF-stimulation. Erlotinib-treatment inhibited STAT-activation thus contributing to cell cycle arrest and apoptosis-induction.

To shed light on transcriptional changes in response to EGFR-TK-inhibition by erlotinib we performed cDNA expression arrays. As described above, erlotinib increased the expression of genes encoding proapoptotic factors like caspases and gadds whereas the expression of genes encoding antiapoptotic proteins like Bcl-2 or the jun D proto-oncogene was found to be decreased. At the same time we found a different expression of a variety of genes encoding cell cycle regulators: Cell-cycle promoters like CDK4 or cyclin A2 were suppressed, the important molecular regulator of the $G_2$/M cell cycle transition cyclin B1[17] was markedly overexpressed probably accounting for a partial $G_2$/M-block we observed in response to EGFR-blockade in previous investigations[12,28]. Interestingly, the insulin-like growth factor binding protein 6 (IGFBP-6) was found to be the gene with the strongest overexpression. IGFBPs are a family of six homologous proteins with high binding affinity for IGF-1 and IGF-2. In addition to functioning as simple carrier proteins, IGFBPs in serum regulate the endocrine actions of IGFs by changing the amount of IGF available to activate IGFRs, and locally produced IGFBPs act as autocrine/paracrine regulators of IGF action[32]. Furthermore, recent *in vitro* and *in vivo* findings show that IGFBPs may function independently of the IGFs as growth modulators[32]. IGFBP-6 differs from the other IGFBPs because it has a markedly higher affinity for IGF-2 than for IGF-1, whereas the other IGFBPs bind the two IGFs with similar affinities[33]. IGF-2 overexpression is described in several tumor-xenograft models and in human HCCs[34]. Additionally, a correlation of IGF-2 overexpression with HepG2 and Huh-7 cell growth has been shown[35] as well as a modulation of IGFBP-expression through the EGFR signaling pathway[36]. It may be speculated that reduced amounts of bioavailable IGF-2 as a result of EGFR-blockade-induced IGFBP-overexpression contribute to the growth inhibition of hepatocellular can-

cer cells by a further reduction of mitogenic stimuli. However, additional investigations on the complex network of IGF-2R, IGF-2 and IGFBPs have to be undertaken in order to explain our observations and their relevance with anti-EGFR based therapy strategies.

Some of the molecular targets investigated in this study may be used as surrogate biomarkers for anti-EGFR-based therapeutic strategies. The rational selection of cancer patients for EGFR inhibition therapies remains a major challenge because there is no clear correlation between EGFR overexpression and response to EGFR inhibitors[10]. Thus, the finding of new biomarkers is mandatory. Interestingly, using gadd153 induction as a predictor of clinical response has already been evaluated for paclitaxel treatment of cancer patients[37].

Like the EGFR, the insulin-like growth factor receptor 1 (IGF-1R) contributes to the growth, survival, adhesion and motility of cancer cells. The IGF-1R is a tetrameric tyrosine kinase receptor which can be activated by either IGF-1 or IGF-2. IGF-1R signaling is mediated through MAPK, phosphatidylinositol-3-kinase (PI3K) and stress-activated protein kinase (SAPK)[38]. In the present study we showed that the MAPK-pathway was activated by IGF-1 in HCC cells. Moreover, our results revealed an IGF-1R mediated transactivation of the EGFR in HCC cells. Several modes of indirect EGFR activation have been described so far[39]. As compelling evidence demonstrates the significance of EGFR signal transactivation in human disorders, the components of this signaling mechanism represent promising targets for therapeutic intervention. EGFR transactivation induced by activation of G-protein-coupled receptors, cytokine receptors and voltage-dependent $Ca^{2+}$-channels has been described. Though the exact mechanisms of the receptor-receptor cross talk are not known yet, RTKs are supposed to be important mediators of the transactivation process[40]. In accordance, our results exhibited erlotinib's potency to inhibit IGF-1 induced transactivation without affecting the activation of the IGF-1R by its ligands. This finding may explain results of previous *in-vitro* studies showing greater antineoplastic activity for EGFR-TK-inhibition in HepG2 and Huh-7 cells[12,18] than for inhibition of endogenous ligand binding by cetuximab[28].

In addition to the induction of EGFR-transactivation, IGF-1R is known to be involved in resistance towards anti-EGFR-based therapeutic approaches. This arises from the fact that alternative signaling pathways of the IGF-1R can compensate for a blocked primary EGFR pathway. As IGF-1R is strongly expressed in HCC cells[34], co-targeting of IGF-1R and EGFR in HCC cells may be a way to avoid or overcome resistance towards EGFR blockade. Combining erlotinib with the IGF-1R-TKI AG1024 resulted in synergistic effects in HepG2 and Huh-7 cells. These results suggest that combination regimens targeting both EGFR and other growth factor receptors such as IGF-1R may simultaneously yield greater anticancer activity than approaches that address only a single receptor and should be investigated more extensively in future studies.

In summary, our data suggest that EGFR-TK-blockade by erlotinib leads to an inhibition of the mitogenic MAP-kinase pathway as well as to an interruption of STAT-mediated signaling, resulting in a different expression of apoptosis and cell cycle regulating genes in HCC cells. Overexpression of proapoptotic factors like caspases and gadds associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-$X_L$ or jun D may account for erlotinib's action to induce apoptosis. Downregulation of cell cycle regulators promoting the G1-to-S-transition and overexpression of cyclin-dependent kinase inhibitors and gadds contribute to the induction of a $G_1/G_0$-arrest in response to EGFR-TK-inhibition by erlotinib. Moreover, our results point at the transactivation of EGFR-mediated signaling by the IGF-1R and show erlotinib's inhibitory effects on the receptor-receptor cross talk mechanisms. Finally we demonstrate that synchronous targeting of EGFR and IGF-1R yields greater antineoplastic effects than approaches that address only a single receptor. To conclude, our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the finding of combination therapies that act additively or synergistically. Moreover, our data on the pathways activated by erlotinib could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.

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