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EGFR blockade by cetuximab alone or as combination therapy for growth control of hepatocellular cancer

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancer-related causes of death worldwide. In light of the very poor 5 year survival new therapeutic approaches are mandatory. Several reports indicate that the epidermal growth factor receptor (EGFR) is expressed frequently in HCC, most likely contributing to the aggressive growth characteristics of these tumors. Cetuximab, a chimeric monoclonal IgG1 antibody directed against the EGFR, potently suppresses the growth of various cancers but its effect on HCC remains to be explored. We therefore studied the antineoplastic potency of cetuximab in human HCC cells alone and in combination with growth factor tyrosine-kinase inhibition (TKI) or HMG-CoA-reductase inhibiton or conventional cytostatics.

Cetuximab inhibited growth of p53 wild-type HepG2 hepatocellular cancer cells in a time- and dose-dependent manner. Cetuximab treatment resulted in arresting the cell cycle in the G_1/G_0-phase due to an increase of expression of the cyclin-dependent kinase inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ and a decrease in cyclin D1 expression. Additionally, we observed a moderate increase in apoptosis as demonstrated by caspase-3 activation. Combining cetuximab with TKIs (erlotinib or AG1024) or the HMG-CoA-reductase inhibitor fluvastatin or doxorubicin resulted in synergistic antiproliferative effects. In contrast, p53 mutated Huh-7 hepatocellular cancer cells proved to be less sensitive towards cetuximab, but when combined with TKIs or fluvastatin or doxorubicin a pronounced reduction of cell growth was observed. To conclude, our study may provide a rationale for future clinical investigations of cetuximab combination therapy for growth control of hepatocellular cancer.

Keywords: Epidermal growth factor receptor; Insulin-like growth factor receptor; EGF; IGF; Statins; Chemoprevention; Erbitux™; Tarceva™; Doxorubicin; Cisplatin

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide 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 liver cirrhosis, chronic hepatitis B and chronic hepatitis C infections, alcohol disease and obesity [1,2]. Unfortunately, the majority of patients suffer from advanced HCC at presentation. Therefore, curative local ablation, surgical resection or liver transplantation can be achieved in only a minority of HCC patients. Local tumor destruction, chemoembolisation or systemic therapy are the treatment options of advanced HCC. However, overall survival is poor [3]. Apart from chemoembolisation, which improves survival in well-selected patients with unresectable HCC, palliative treatment options do not appear to improve overall survival [4]. Therefore, innovative treatment approaches are urgently needed.

Abbreviations: AG1024, 3-bromo-5-*t*-butyl-4-hydroxy-benzylidene-malonitrile; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; erlotinib, (*N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine); HCC, hepatocellular carcinoma; HMG-CoA-reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A-reductase; IGF-1R, insulin-like growth factor 1 receptor; Mab, monoclonal antibody; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor

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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 [5–7], most likely contributing to the aggressive growth characteristics of these tumors [5,7]. Especially, in poorly differentiated HCCs, EGFR overexpression has been demonstrated to be a negative prognostic factor, since it positively correlated with early tumor recurrence [5,8] and the occurrence of extrahepatic metastasis [6]. Hence, the EGFR is a promising target for innovative treatment strategies in HCC.

Moreover, EGFR-expression appears to play an important role in hepatocellular carcinogenesis. Thus, pre-malignant liver nodules display EGFR-overexpression. In contrast, advanced and differentiated HCCs tend to loose EGFR-overexpression [9].

The EGFR is a member of a family of four 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 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 [10]. Inhibiting EGFR and its specific TK activity is regarded as a promising approach for innovative therapeutic strategies in cancer treatment [11].

Cetuximab is a chimeric monoclonal IgG1 antibody (Mab) that is directed against the EGFR. Cetuximab binds specifically to the EGFR with an affinity that is approximately 5–10-fold higher than that of endogenous ligands. Thus, cetuximab blocks binding of endogenous EGFR ligands (particularly of EGF and TGF-α) resulting in an inhibition of the function of the receptor. Furthermore, cetuximab induces an internalization of the receptor leading to down-regulation of EGFR [12,13] and it targets cytotoxic immune effector cells towards EGFR-expressing tumor cells (antibody dependent cell-mediated cytotoxicity [14]).

Cetuximab has been shown to inhibit the proliferation of a variety of human cancer cell lines including breast, colon, lung, kidney and prostate [12,13]. Additionally, a series of clinical studies (phases I–III) have tested cetuximabs' antineoplastic potency in cancer patients [15]. Most encouraging results were seen in advanced, EGFR-positive non-small-cell lung cancer with cetuximab plus cisplatin or vinorelbine, cetuximab plus irinotecan/5-FU/folinic acid in metastatic EGFR positive colorectal cancer and in advanced head and neck cancer for cetuximab combined with radiation [15]. Recently, cetuximab has received approval in the US and Europe for EGFR-expressing metastatic colorectal cancer after failure of irinotecan-including cytostatic therapy.

So far, EGFR inhibition by cetuximab has not been evaluated for the treatment of human HCC. Hence, in the present study we characterized the antineoplastic potency of cetuximab in the two human HCC cell lines Huh-7 and HepG2 alone and in combination with the EGFR-TKI erlotinib or the IGF-1R-TKI AG1024 or the HMG-CoA-reductase inhibitor fluvastatin or conventional cytostatics (doxorubicin and cisplatin).

2. Material and methods

2.1. Cell lines

The well differentiated wild-type p53 hepatoblastoma cell line HepG2 [16] and the highly differentiated human hepatocellular carcinoma cell line Huh-7 [17] with mutated p53 were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin.

Both cell lines are supposed to be particularly indicative of clinical HCC activity as they have preserved differentiated liver characteristics, as evidenced by production of many human plasma proteins [16,17].

2.2. Drugs

Cetuximab (Erbitux™) was bought from Merck Pharma GmbH (Darmstadt, Germany), AG1024 and fluvastatin were from Calbiochem (Bad Soden, Germany) and erlotinib hydrochloride (Tarceva™) was a kind gift from Roche (Penzberg, Germany). Cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma (Deisenhofen, Germany), if not stated otherwise. Stock solutions were prepared in DMSO and stored at −20 °C and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 0.5%, thus not affecting cell growth. To evaluate the effects of cetuximab, cells were incubated with either control medium or medium containing rising concentrations of cetuximab.

2.3. Measurement of growth inhibition

Cell number was evaluated by crystal violet staining, as described in Ref. [18]. In brief, cells in 96-well plates were fixed with 1% glutaraldehyde, then cells were stained with 0.1% crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton-X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-Reader.

2.4. Drug combination studies

To check for possible additive or synergistic effects, combination treatment of cetuximab plus the EGFR-TKI erlotinib or the IGF-1R-TKI AG1024 or the HMG-CoA-

reductase inhibitor fluvastatin or conventional cytostatics (cisplatin and doxorubicin) was studied. 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 above.

2.5. Detection of apoptosis

Changes in caspase-3 activity were assessed by measuring the cleavage of the fluorogenic substrate AC-DEVD-AMC (Calbiochem–Novabiochem, Bad Soden, Germany), as described previously [19]. In brief, cell lysates were incubated for 1 h at 37 °C with a substrate solution containing 20 μg/mL AC-DEVD-AMC, 20 mM HEPES, 10% glycerol, 2 mM DTT with a pH adjusted to 7.5. Substrate cleavage was measured fluorometrically using a Versa-Fluor fluorometer (filter wavelengths: excitation, 360/40 nm; emission, 460/10 nm) from Biorad, Munich, Germany.

2.6. Determination of cytotoxicity

Cells were seeded into 96-well microtiter plates and incubated with 10–1000 μg/mL cetuximab for 3, 6, 24, 48 or 72 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured by using a colorimetric kit from Roche (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer.

2.7. Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen [20]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson).

2.8. Measurement of DNA synthesis

Cells were seeded into 96-well microtiter plates and treated with escalating concentrations of cetuximab (10–1000 μg/mL). After 72 h, cells were incubated for 2 h with the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU). BrdU is incorporated instead of thymidine into the DNA of proliferating cells and subsequently detected by an immunoassay (Cell Proliferation Elisa, BrdU (colorimetric), Roche Diagnostics) as described by the manufacturer.

2.9. Western blot analysis

Western blotting was performed as described [21]. Blots were blocked in 2.5% BSA and then incubated at 4 °C overnight with the following antibodies: monoclonal mouse-anti-human cyclin D1 (1:1000), polyclonal rabbit-anti-human $p21^{Waf1/CIP1}$ (1:400; both from Santa Cruz Biotechnology, CA, USA), $p27^{KIP1}$ (1:2500; Becton Dickinson) and β-actin (1:5000; Sigma) served as loading control.

2.10. Statistical analysis

If not stated otherwise, means of at least three independent experiments ±S.E.M. are shown. Significance between controls and treated samples was calculated by Student's two-sided *t*-test. Caspase-3 measurements were evaluated using the two-sided Welch *t*-test. *P*-values were considered to be significant at <0.05.

3. Results

3.1. Growth inhibitory effects of cetuximab on HCC cells

Growth inhibition by cetuximab was studied by measuring cell proliferation. Cetuximab time- and dose-dependently inhibited the proliferation of HepG2 cells (Fig. 1A and C) by up to 57% as compared to controls. Huh-7 cell proliferation was less sensitive to cetuximab (Fig. 1B and C), except for the treatment with concentrations as high as 1000 μg/mL which resulted in ∼20% growth inhibition after 72 h.

3.2. Cetuximab and DNA synthesis

Determination of DNA-BrdU-incorporation 72 h after the start of incubation with cetuximab revealed a dose-dependent decrease in DNA synthesis of up to 25% in HepG2 cells. Again, the response in Huh-7 cells was less pronounced reaching a maximal reduction of 11% for the highest concentration (1000 μg/mL) compared to control (Fig. 2).

3.3. Cetuximab and cell cycle regulation

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of cetuximab in hepatocellular cancer cells, we performed cell cycle analyses. Incubating HepG2 with rising cetuximab concentrations (10–1000 μg/mL) for 24 h led to a dose-dependent arrest of the cells in the G_1/G_0-phase of the cell cycle (up to +30% compared to control), thereby decreasing the proportion of cells in the S-phase. G_1/G_0-arrest of HepG2 cells was significant above 10 μg/mL of cetuximab. The proportion of cells in the G_2/M-phase of the cell cycle remained nearly unaffected indicating a partial additional block in the G_2/M-phase (Fig. 3A). Huh-7 cells (Fig. 3B) required a high concentration of cetuximab (1000 μg/mL)

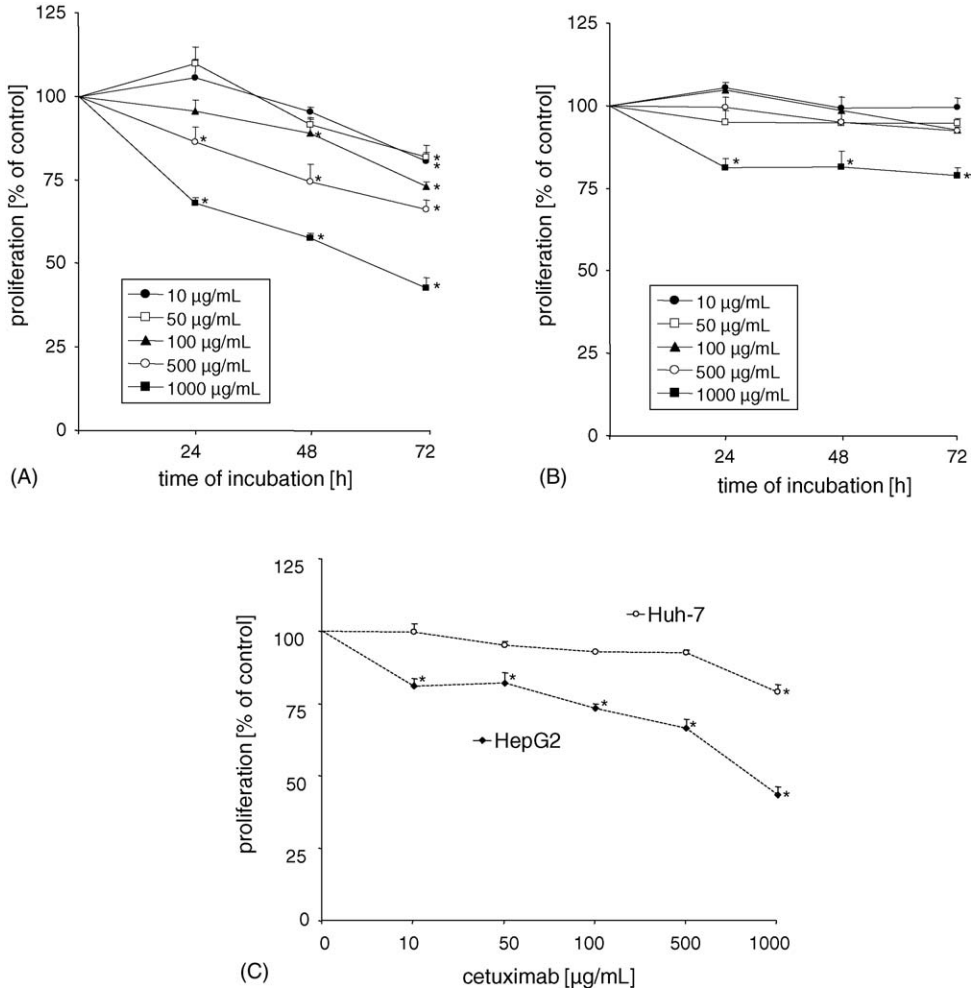

Fig. 1. Antiproliferative effects of cetuximab. Cetuximab caused a time- and dose-dependent growth inhibition in hepatocellular HepG2 carcinoma cells (A) whereas the growth of Huh-7 (B) was less sensitive to cetuximab. After 72 h of continuous incubation with rising concentrations of cetuximab cell numbers of HepG2 (A) and Huh-7 cells (B) decreased by 57 and 20% as determined by crystal-violet staining. (C) Displays the dose–response curve in HepG2 and Huh-7 cells after 72 h of continuous incubation with cetuximab. Data are given as percentage of controls (means ± S.E.M. of four independent experiments). Asterisk (*) statistical significance ($P < 0.05$) of growth inhibition compared to controls.

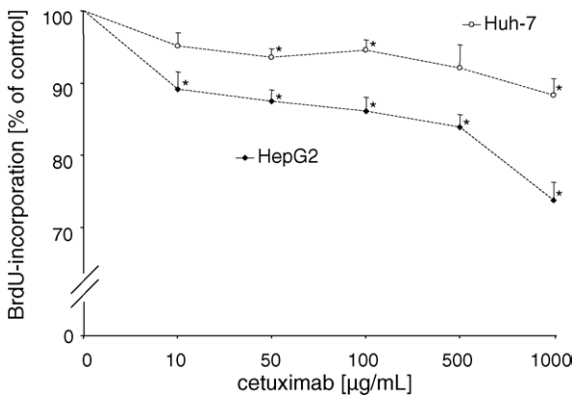

Fig. 2. Decrease of DNA synthesis in response to cetuximab. After 3 days of incubation with different doses of cetuximab (10–1000 μg/mL) DNA synthesis markedly dropped in HepG2 cells but less so in Huh-7 cells as indicated by the decrease of incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) into cellular DNA. Data of three independent experiments ± S.E.M. are shown. Asterisk (*) statistical significance ($P < 0.05$) compared to controls which were set at 100%.

for significantly arresting in the G_1/G_0-phase, and the effect observed was less pronounced (+11.5% compared to control).

3.4. Cetuximab modulates the expression of cell cycle regulators

To further characterize cetuximab's effects on the cell cycle, we performed Western blots to reveal the underlying molecular mechanisms. Treating HepG2 cells for up to 48 h with a sub-IC_{50}-concentration of cetuximab (250 μg/mL) resulted in a suppression of cyclin D1, which is essential for the transition from the G_1 to the S-phase. At the same time, expression of the cyclin-dependent kinase inhibitors (CDKI) $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ markedly increased. Alterations in protein expression of the respective cell cycle regulators occurred within 24 h (Fig. 4).

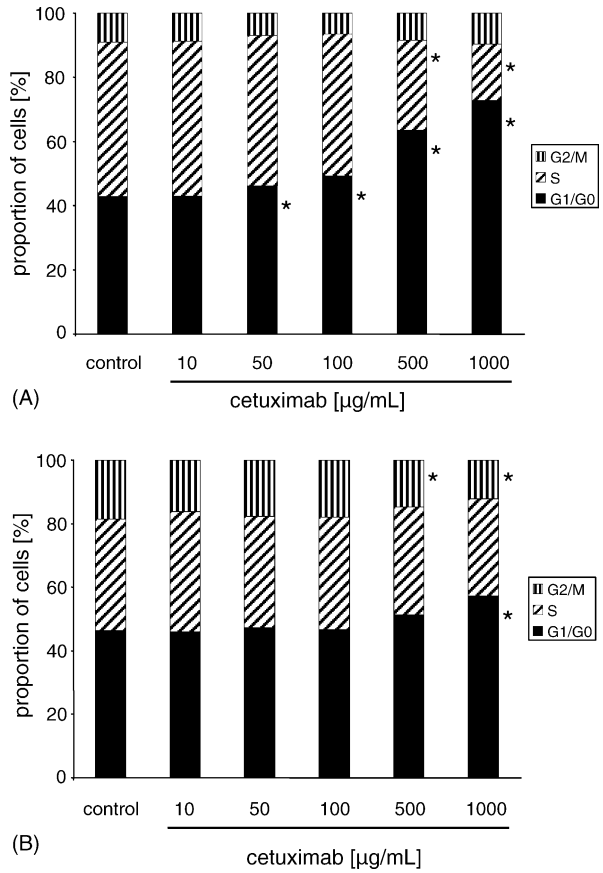

Fig. 3. Induction of cell cycle arrest in the G_1/G_0-phase by cetuximab. Twenty-four hours of incubation of HepG2 (A) and Huh-7 (B) cells with cetuximab led to a dose-dependent accumulation of the cells in the G_1/G_0-phase of the cell cycle that was more pronounced in HepG2 cells. Accordingly, the number of cells in the S-phase decreased. The proportion of cells in the G_2/M-phase remained nearly unchanged indicating a partial additional block in the G_2/M-phase. Means of three independent experiments for each cell line are shown. Asterisk (*) statistical significance ($P < 0.05$) compared to untreated controls.

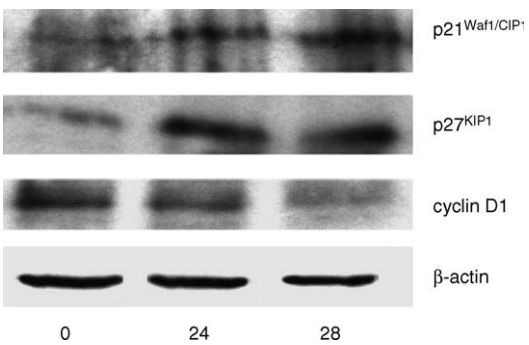

Fig. 4. Modulation of the expression of cell cycle regulators by cetuximab. Modulation of protein expression by cetuximab was analyzed by Western blotting. HepG2 cells were treated with cetuximab (250 μg/mL) for 24 and 48 h. The cell cycle promoter cyclin D1 was down-regulated by cetuximab, whereas the cell cycle inhibitors $p21^{Waf1/CIP1}$ and $p27^{KIP1}$ were up-regulated. One representative out of three independent experiments is shown for each protein investigated.

3.5. Induction of apoptosis by cetuximab

To study the potency of cetuximab to induce apoptosis in HCC cell lines we investigated cetuximab-induced activation of caspase-3, a key enzyme of the apoptotic pathway. Cells were treated with rising cetuximab concentrations (10–1000 μg/mL) and caspase-3 activity was determined after 24, 48 and 72 h of incubation. In HepG2 cells caspase-3 activity increased dose-dependently (Fig. 5A) to 215%, as compared to controls. Regardless of the duration of incubation no significant caspase-3 activation could be observed at concentrations below 100 μg/mL of the Mab. After 72 h of continuous incubation caspase-3 activity dropped. Huh-7 cells only displayed a slight increase in caspase-3 activity in response to cetuximab treatment (Fig. 5B).

3.6. LDH release from HCC cells after cetuximab treatment

Cytotoxicity was evaluated by LDH release. Incubating HepG2 cells with 10–1000 μg/mL cetuximab for up to 6 h did not result in a pronounced increase of LDH release (Fig. 6A). This indicates that cetuximab does not directly affect cell membrane integrity and does not have immedi-

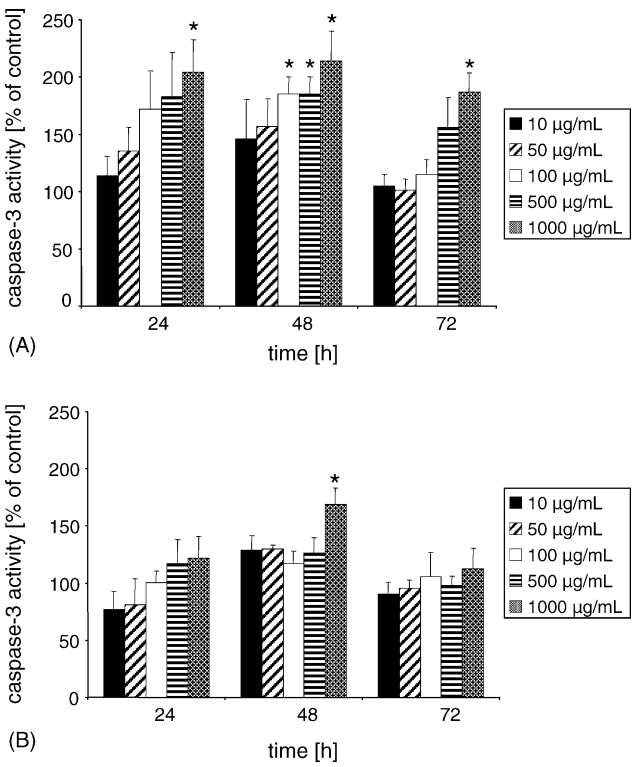

Fig. 5. Cetuximab-induced caspase-3 activation. Cetuximab time- and dose-dependently induced caspase-3 activation in HepG2 (A) cells but less so in Huh-7 cells (B). Cells were treated with escalating concentrations (10–1000 μg/mL) of cetuximab for 24, 48 and 72 h. Data are shown as means ± S.E.M. of at least four independent experiments for each cell line. Asterisk (*) statistical significance ($P < 0.05$) compared to controls which were set at 100%.

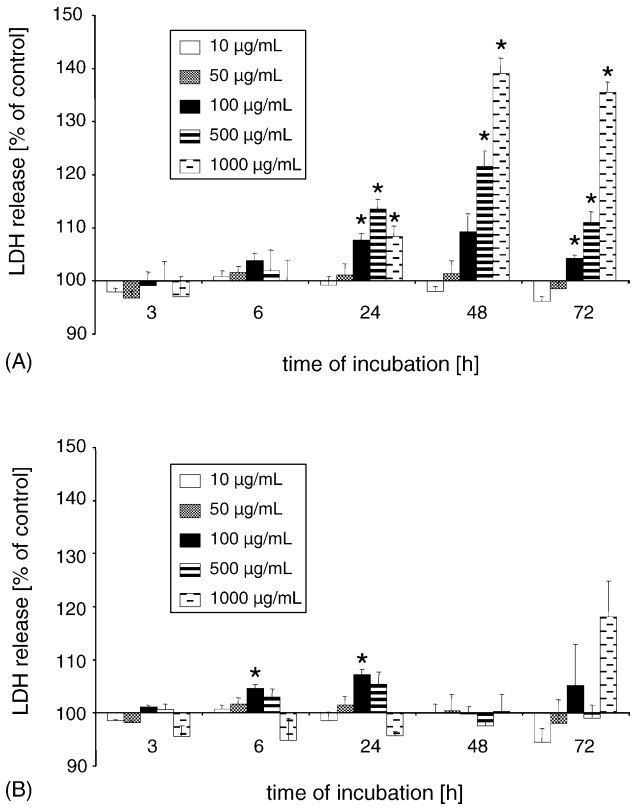

Fig. 6. Cetuximab-induced cytotoxicity. LDH release into the supernatant of HepG2 (A) and Huh-7 cells (B) was determined after incubation with rising concentrations (10–1000 μg/mL) of cetuximab. No significant LDH release was observed during the first hours of incubation. Data of at least three independent experiments ± S.E.M. are shown. Asterisk (*) statistical significance ($P < 0.05$) compared to controls which were set at 100%.

ate necrotic effects even at high concentrations. Moreover, it underlines the specificity of the Mab. On the other hand, after 24 h LDH release increased dose-dependently up to 40% above control levels. Most probably this was due to loss of cell membrane integrity of cells in late apoptotic stages. This is an in vitro phenomenon, in vivo apoptotic cells are finally eliminated by macrophages. In Huh-7 cells no pronounced increase of LDH activity was detectable corresponding to the results of the caspase-3 experiments (Fig. 6B).

3.7. Antineoplastic potency of cetuximab in combination with the EGFR-TK inhibitor erlotinib

In light of encouraging results we obtained with erlotinib in previous studies [22] and reports on combination therapies of cetuximab with EGFR-TK inhibitors, such as erlotinib in other tumor entities, we studied possible (over-)additive antineoplastic effects of cetuximab plus erlotinib in HCC cells. Cells were treated with erlotinib (10 μM) alone or in combination with rising concentrations of the Mab (again 10–1000 μg/mL) for 72 h.

Upon treatment with erlotinib alone a strong growth inhibitory effect of 49% (HepG2) and 40% (Huh-7) was observed after 72 h of continuous exposure to the drug. In HepG2 cells erlotinib's antineoplastic effects doubled when the EGFR-TK inhibitor was combined with cetuximab, reducing cell proliferation between 56% (10 μg/mL cetuximab) and almost 80% (1000 μg/mL cetuximab) after 72 h (Fig. 7A). No such effect could be detected in Huh-7 cells. Although erlotinib alone displayed a pronounced reduction of cell number, this effect was only augmented when 10 μM erlotinib was combined with 1000 μg/mL of the Mab, resulting in a growth inhibition of 57% (Fig. 7B).

3.8. Antineoplastic potency of cetuximab in combination with the IGF-1R-TK inhibitor AG1024

To check the potency of simultaneous targeting multiple growth factor receptor pathways for more efficacious treatment we combined EGFR blockade by cetuximab

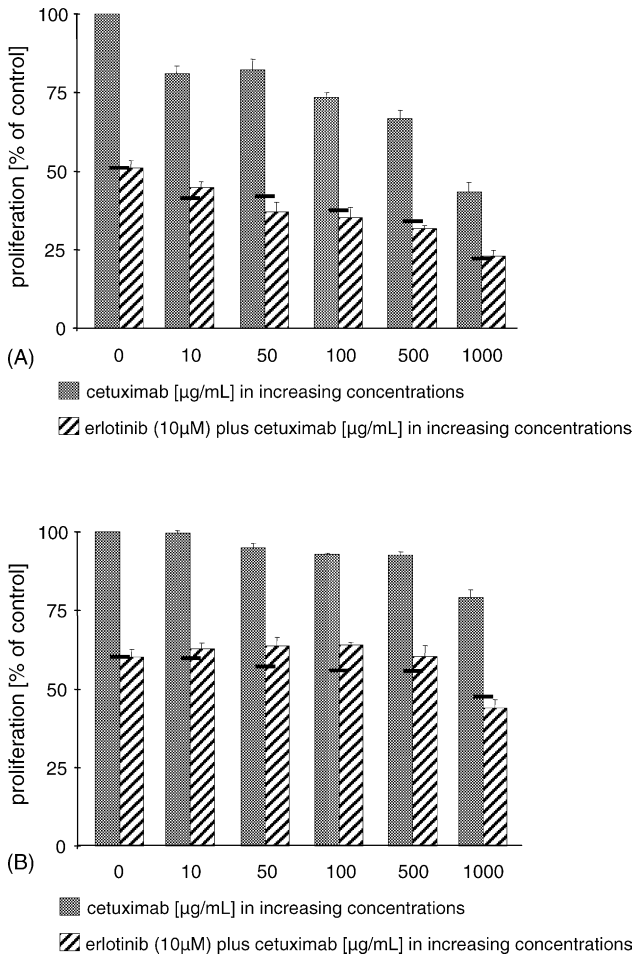

Fig. 7. Antiproliferative effects of cetuximab plus erlotinib, HepG2 (A) and Huh-7 hepatocellular carcinoma cells (B) were treated for 72 h with 10 μM erlotinib plus rising concentrations of cetuximab (10–1000 μg/mL). Grey bars show the effects of different concentrations of cetuximab on its own. Hatched bars indicate the antiproliferative effects of combination treatment (10 μM erlotinib plus different concentrations of cetuximab) obtained by crystal violet staining. Horizontal black lines indicate the values of the calculated additive growth inhibition. Data are given as percentage of controls which were set at 100%. Means ± S.E.M. of three independent experiments.

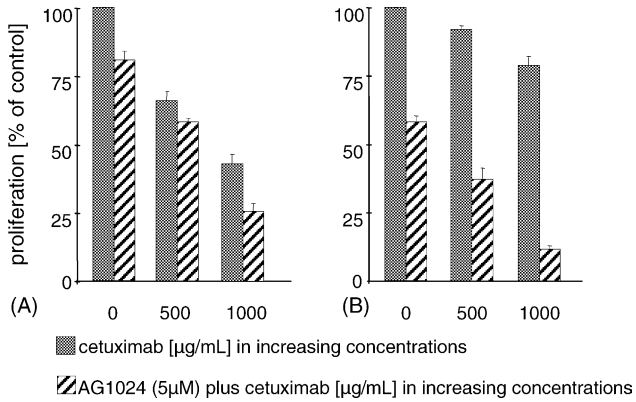

Fig. 8. Antiproliferative effects of cetuximab plus AG1024. HepG2 (A) and Huh-7 hepatocellular carcinoma cells (B) were treated for 72 h with 5 μM of the IGF-1R-TK inhibitor AG1024 plus cetuximab (500 or 1000 μg/mL). Grey bars show the effects of cetuximab on its own. Hatched bars indicate the antiproliferative effects of combination treatment (5 μM AG1024 plus cetuximab) obtained by crystal violet staining. Horizontal black lines indicate the values of the calculated additive growth inhibition. Data are given as percentage of controls, which were set at 100%. Means ± S.E.M. of three independent experiments.

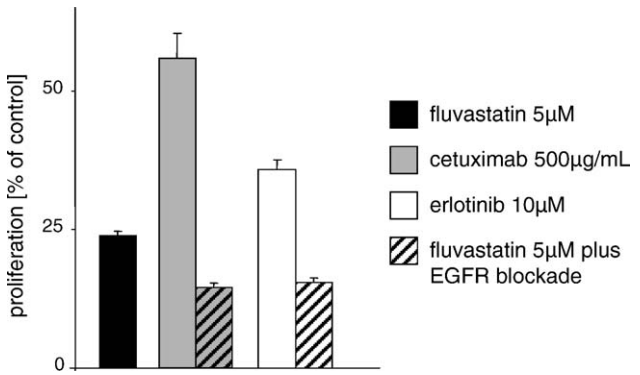

Fig. 9. Antiproliferative effects of fluvastatin plus EGFR blockade. HepG2 cells were treated for 72 h with 5 μM of the HMG-CoA-reductase inhibitor fluvastatin alone or in combination with the anti-EGFR monoclonal antibody cetuximab or the EGFR tyrosine kinase inhibitor erlotinib. Data are given as percentage of controls, which were set at 100%. Means ± S.E.M. of three independent experiments.

(500 and 1000 μg/mL) with IGF-1R-TK inhibition by AG1024 (5 μM). We observed additional or synergistic effects for HepG2 cells (Fig. 8A) and even a strong synergism for the Huh-7 cell line (Fig. 8B) after 72 h of continuous exposure to the drugs.

3.9. Antineoplastic potency of EGFR inhibition in combination with HMG-CoA-reductase inhibition by fluvastatin

Next, we investigated fluvastatin's (5 μM) influence on the growth of HepG2 cells alone or when combined with cetuximab (500 μg/mL) or erlotinib (10 μM). Fluvastatin treatment resulted in a strong growth inhibition of ~75% after 72 h of treatment. The combination of fluvastatin with either EGFR blocker resulted in an augmented inhibition of cell proliferation (Fig. 9).

3.10. Antineoplastic potency of cetuximab in combination with cytostatics

In light of encouraging results of combination therapies of EGFR-inhibition by cetuximab with cytostatics in other tumor entities [15], we evaluated possible synergistic antineoplastic effects of cetuximab plus cytostatics in HCC. Cells were treated with cetuximab and rising concentrations of either doxorubicin (1–500 nM) or cisplatin (1–50 μM) for 72 h.

Upon treatment with doxorubicin a pronounced dose-dependent growth inhibition up to 86% (500 nM) was observed after 72 h in HepG2 (Fig. 10A) and Huh-7 cells (Fig. 10B). When combining doxorubicin with cetuximab, both cell lines displayed synergistic antineoplastic effects of almost 100%.

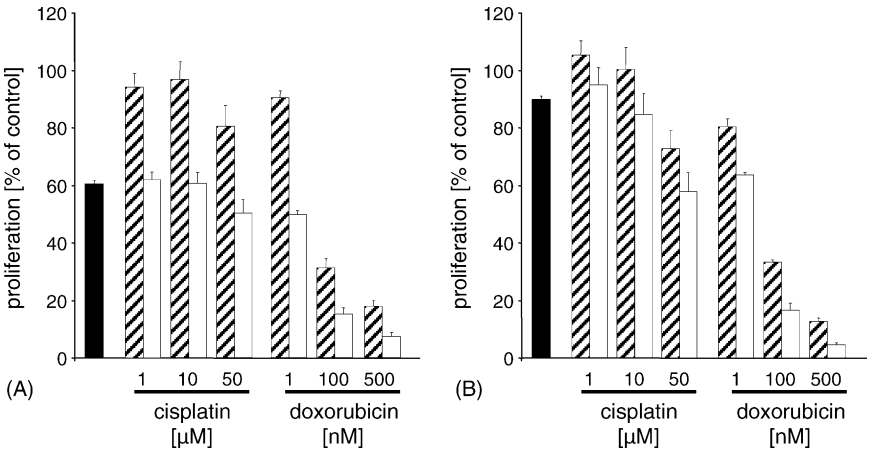

Fig. 10. Antiproliferative effects of cetuximab plus cytostatic drugs, HepG2 (A) and Huh-7 (B) carcinoma cells were treated for 72 h with rising concentrations of doxorubicin or cisplatin alone (hatched bars), or in combination with 500 μg/mL cetuximab (white bars). Black bars show the effects of 500 μg/mL cetuximab alone. Data are given as percentage of controls, which were set at 100%. Means ± S.E.M. of three independent experiments.

By contrast, Huh-7 and HepG2 cell growth was nearly unaffected by cisplatin (except for the very high concentration of 50 μM cisplatin). The growth inhibitory effect of cetuximab was unaffected by the addition of the platin compound.

4. Discussion

Treatment options of advanced hepatocellular cancer are unsatisfactory, and the prognosis of patients suffering from advanced HCC is poor. Thus, novel therapeutic approaches are much needed.

The specific EGFR monoclonal antibody cetuximab inhibits the growth of a variety of human cancer cells [15] but has not been tested for the treatment of HCC so far. Hence, in this study we provide evidence that cetuximab alone or in combination with the EGFR-TK inhibitor erlotinib or IGF-1R-TK inhibition or HMG-CoA-reductase inhibition might be a promising anticancer agent for HCC. Moreover, we show that cetuximab enhances the efficacy of conventional cytostatics.

The EGF/EGFR system is known to have strong stimulatory effects on the growth of hepatoma cells [23]. Several studies have demonstrated EGFR expression to be a common feature of HCCs [7,8], underlining the role of the EGFR-TK as a rational target for innovative treatment strategies in hepatocellular cancer. Moreover, we recently demonstrated both mRNA and protein expression of EGFR in Huh-7 and HepG2 carcinoma cells [24].

In HepG2 cells, cetuximab inhibited HCC growth in a time- and dose-dependent manner. Cetuximab dose-dependently led to a strong arrest in the G_1/G_0-phase of the cell cycle and time- and dose-dependently induced an increase in apoptosis as demonstrated by caspase-3 activation. Accordingly, a significant decrease in DNA synthesis was observed due to cetuximab treatment. In contrast, Huh-7 cell growth was inhibited only by high doses of cetuximab. In Huh-7 cells, we observed a slight arrest in the G_1-phase of the cell cycle but no apoptosis induction. DNA synthesis decreased slightly as demonstrated by DNA-BrdU-incorporation.

Despite both cell lines strongly expressing the EGFR [24], HepG2 and Huh-7 cells respond differently towards cetuximab treatment. Our data are in line with a series of recent reports [25,26] that show there is no correlation between EGFR (over-)expression and the extent of response or resistance towards EGFR blockade. Therefore, new surrogate markers have to be identified that predict tumors, which will respond to EGFR blockade.

It is known that EGFR blockade can lead to cell cycle arrest and/or apoptosis induction. In general, the respective contribution of either cell cycle arrest and/or apoptosis to the antitumor activity of EGFR inhibition differs among various tumor types [15], but the exact mechanisms of EGFR-inhibition induced cell cycle arrest and apoptosis are not known yet.

Here, we demonstrate that cetuximab's antineoplastic action is mainly due to changes in the expression of cell cycle regulators resulting in a strong G_1/G_0-arrest in HepG2 but less so in Huh-7 cells. In a previous study, we have characterized the antiproliferative effects of the EGFR-TKI erlotinib in HCC cells [22]. Erlotinib treatment caused both an induction of apoptosis and G_1/G_0-arrest. The cell cycle arrest elicited by erlotinib was more evident in HepG2 cells, whereas the induction of apoptosis was the prevalent mode of the antineoplastic effect of erlotinib in Huh-7 cells.

It is intriguing to speculate that the different sensitivity towards cetuximab treatment is due to differences of the p53-status of the two cell lines. Whereas HepG2 cells express wild-type p53, Huh-7 cells have a mutated p53 protein [27]. Functionally intact p53 is known to be a prerequisite for G_0/G_1-arrest [27,28]. The tumor suppressor p53 is a key component of cell cycle checkpoints activated in response to a variety of cellular stresses [29]. This is due to its ability to integrate different signals and to induce arrest at different points of the cell cycle [30]. Activated p53 functions as a transcription factor and up-regulates the expression of a number of genes including $p21^{Waf1/CIP1}$ and gadd45 whose products help to arrest the cell cycle [31,32]. Here, we demonstrate that cetuximab treatment results in an up-regulation of the cell cycle inhibitors $p21^{Waf1/CIP1}$ and $p27^{KIP1}$ in HepG2 cells but not in Huh-7 (data not shown); correspondingly cyclin D1 was found to be down-regulated. We hypothesize that the mutated p53-status of Huh-7 cells may be responsible for their resistance towards cetuximab. According to this notion a tumors p53-status might correlate with the response to cetuximab treatment. Further studies on the influence of p53-status and EGFR mutations and the response to EGFR blockade are needed.

So far, few studies have examined mechanisms of resistance to cetuximab. Several deletions and mutations of EGFR have been described. The most common is the type III mutation (EGFRvIII [33]), which leads to a deletion of the extracellular domain of EGFR thereby influencing cetuximab binding. However, a previous study on the presence of EGFRvIII in HepG2 or Huh-7 cells showed negative results [24]. Resistance to EGFR targeting may arise from the fact that alternative signaling pathways of the growth factor receptors can compensate for a blocked primary EGFR pathway. Thus, the insulin-like growth factor 1 receptor (IGF-1R) is known to be involved in EGFR resistance. As IGF-1R is strongly expressed in HCC cells [34,35 unpublished work], co-targeting of IGF-1R and EGFR may be a way to avoid or overcome resistance towards EGFR blockade [36]. Combining cetuximab with the IGF-1R-TKI AG1024 resulted in additive and especially in Huh-7 cells in (strong) synergistic effects. These results suggest that a combination regimen targeting both EGFR and other growth factor receptors simultaneously,

such as IGF-1R, may yield greater anticancer activity than approaches that address only a single receptor.

To expand the concept of combined molecular targeted therapeutic approaches in HCC, we studied possible (over-) additive effects of combinations of cetuximab with the EGFR-TK inhibitor erlotinib. We recently demonstrated the in vitro activity and potential of erlotinib in the treatment of HCC [22]. Moreover, encouraging results from a phase II study suggest a benefit for EGFR blockade with erlotinib [37]. For non-hepatocellular cancers synergistic antineoplastic effects have been described for cetuximab combined with an EGFR-TK inhibitor [38]. Here, we demonstrate for the first time for human HCC cells that combining an EGFR monoclonal antibody with erlotinib may augment the efficacy of either monotherapy. In HepG2 cells dual-agent molecular targeting resulted in doubling cetuximab's antiproliferative potency and reduced cell proliferation by 78% compared to control. Huh-7 displayed a pronounced reduction of cell number upon erlotinib treatment, but combining the Mab with erlotinib did not result in significantly enhanced antineoplastic effects. Augmented antitumor effectiveness and overcoming of non-response may be explained by the different ways of action of the Mab and the TKI: the antibody cetuximab is highly specific for the EGFR and targets the extracellular ligand-binding domain of the receptor. Erlotinib inhibits the intracellular receptor-tyrosine kinase domain of the EGFR with high affinity. Nevertheless, TKIs are known not to be absolute in their specificity for the EGFR tyrosine kinase [39]. Inhibition of further enzymes of the kinome may play a favorable role in exerting erlotinib's antineoplastic effects. Our findings underline and expand findings of others [38] that simultaneous administration of anti-EGFR-Mab and TKIs is a promising novel approach for optimizing EGFR signaling inhibition.

Recently, evidence has been accumulated that inhibition of the HMG-CoA-reductase by statins not just reduces elevated levels of serum cholesterol but also inhibits tumor growth [40,41]. Thus, we here investigated the antineoplastic effect of fluvastatin alone and in combination with cetuximab or erlotinib. Fluvastatin is an effective antiproliferative agent for the treatment of HCC cells [42] but combination of fluvastatin with EGFR blockade was superior to monotherapy. Clinical studies are needed to evaluate the potency and safety of these innovative approaches, not only in the palliative situation but also for chemoprevention of HCC in high-risk patients [43].

To expand the concept of an anti-EGFR-based therapeutic approach, the present study focused on possible synergistic effects of combinations of cetuximab with cytostatics. For non-hepatocellular carcinomas a potentiation of antineoplastic effects has already been described for cetuximab combined with cytostatics [15] and a series of clinical trials is being conducted at the moment (www.clinicaltrials.gov). Here, doxorubicin and cisplatin, two cytostatics commonly used for HCC-chemoembolization [44], were chosen for the evaluation of combination effects in HCC.

Pronounced synergistic effects were seen when combining doxorubicin with cetuximab. Although the exact mechanisms of the enhanced antineoplastic efficacy of cytostatics plus EGFR-inhibition are not clear yet, EGFR ligands, such as EGF or TGF-α are thought to be important survival factors in chemotherapy treated cancer cells. Thus, interruption of EGFR signaling leads to irreparable cancer cell damage, induction of apoptosis and cell cycle arrest [45]. Potentiation of antitumor effects may have important clinical implications. Instead of increasing cytostatics to supratoxic levels it may be possible to enhance antitumor activity by addition of cetuximab.

Even in cisplatin-insensitive Huh-7 and HepG2 cells, cetuximab alone exerted its antiproliferative effects. Thus, cetuximab might be a promising agent in the treatment of HCC patients who failed on conventional chemotherapy.

To conclude, our study provides first evidence that the growth of human hepatocellular HepG2 cells can be potently suppressed by EGFR inhibition with cetuximab. A strong cell cycle arrest in the G_1/G_0-phase associated with a decrease in DNA synthesis and a slight increase in apoptosis accounted for the effects observed. Possibly due to their p53-mutation, Huh-7 cells failed to arrest in the G_1-phase of the cell cycle and proved to be less sensitive to cetuximab treatment. This study may provide a rationale for future clinical investigations of cetuximab combination therapy: combining cetuximab with the TKIs erlotinib and AG1024 or the HMG-CoA-reductase inhibitor fluvastatin or doxorubicin resulted in augmentation of growth inhibition in HCC cells. Dual-agent targeting of EGFR or simultaneously targeting EGFR and IGF-1R yields greater antineoplastic effects than approaches that address only a single receptor and warrants further investigations. Cetuximab-insensitive Huh-7 cells still responded to TKI treatment thereby underlining the different pharmacodynamics of Mabs and TKIs.

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