Research Article

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Erlotinib and sorafenib in an orthotopic rat model of hepatocellular carcinoma

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Background & Aims: The combination of erlotinib with sorafenib is currently being investigated in a phase III RCT. We studied the effect of erlotinib and sorafenib on HCC in a preclinical model.

Methods: The Morris Hepatoma (MH) and HepG2 cells were treated *in vitro* with sorafenib (1–10 μM) and erlotinib (1–5 μM) and evaluated for tumor cell viability, apoptosis, and target regulation. Antiangiogenic effects were studied by measuring VEGF levels, endothelial cell viability, apoptosis, migration, and the aortic ring assay.

In vivo, MH cells were implanted into the liver of syngeneic rats and treated with vehicle, sorafenib 5–10 mg/kg, erlotinib 10 mg/kg, and respective combinations.

Results: *In vitro*, erlotinib downregulated p-ERK but showed no significant effect on tumor cell viability in MH and HEPG2 cells. Despite a similar target regulation, sorafenib significantly reduced cell viability of HCC cells by induction of apoptosis, in a dose-dependent manner (11 ± 5%; 20 ± 10%; 51 ± 5% for sorafenib 1, 5, 10 μM). No additional effect was observed upon combination with erlotinib.

Of note, erlotinib treatment resulted in endothelial cell migration and vascular sprouting of aortic rings through induction of VEGF mRNA and protein levels in endothelial and tumor cells, which was blocked by sorafenib. *In vivo*, erlotinib had no single agent antitumor activity, raised serum-VEGF levels, and lacked a synergistic effect in combination with sorafenib.

Conclusions: Erlotinib had no antitumor effect on HCC *in vitro* nor *in vivo,* but induced VEGF, which may reflect a resistance mechanism to erlotinib monotherapy. No improvement of sorafenib efficacy was observed upon combination with erlotinib.

Keywords: Hepatocellular carcinoma; Sorafenib; Erlotinib.

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Abbreviations: HCC, hepatocellular carinoma; VEGF, vascular endothelial growth factor; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; BCLC, Barcelona Liver Cancer Classification; MH, Morris Hepatoma 3924A; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

Introduction

Hepatocellular carcinoma (HCC) is arguably the most serious complication of chronic liver disease with an estimated yearly incidence of 1–4% in patients with cirrhosis, regardless of the underlying disease etiology [1,2]. Even more troubling, the incidence of liver cancer between 1992 and 2001 shows the sharpest increase in males and the second sharpest increase in women in the industrialized world, while many other malignancies show declining rates [3].

Sorafenib, a small-molecule multikinase inhibitor, is a potent inhibitor of RAF kinase *in vitro*. Sorafenib is also a strong inhibitor of vascular endothelial growth factor (VEGF)-receptor 1, 2, and 3, platelet derived growth factor-β-receptor, c-kit, and RET. Sorafenib demonstrates antitumor activity in a broad range of human xenograft animal models including colon, mammary, pancreatic, ovarian cancer [4], and hepatocellular carcinoma [5].

Sorafenib has become the standard for drug treatment of patients with unresectable HCC [6], who cannot be treated with surgery (resection/liver transplantation), interventional curative (radiofrequency ablation/percutaneous ethanol injection), or interventional palliative (TACE) therapies. Despite this significant progress in HCC treatment, further developments are needed to improve outcomes of HCC patients undergoing drug treatment.

Erlotinib acts through direct and reversible inhibition of the epidermal growth factor receptor tyrosine kinase (HER1/EGFR, hereafter referred to as EGFR), which is upregulated in the majority of HCCs [7–10]. Erlotinib is an orally active antitumor agent registered so far for the treatment of non-small cell lung cancer (NSCLC), pancreatic cancer, and other solid tumors [11].

Erlotinib inhibits the epidermal growth factor (EGF)-dependent proliferation of cells, blocks cell cycle progression at the G1 phase, and sensitizes HCC cells to chemotherapeutic agents *in vitro* [12].

Two open label uncontrolled single arm non-randomized phase 1/2 trials, testing erlotinib as single agent in patients with unresectable HCC [13,14], showed a modest antitumor activity.

The success of sorafenib in the SHARP trial and the potential therapeutic impact of EGFR targeting therapies on HCC finally led to the design of a large phase III clinical trial testing the combination of erlotinib and sorafenib in patients with HCC and Barcelona Liver Cancer Classification (BCLC)-tumor stage C (SEARCH-Study: NCT00901901). However, preclinical information on the efficacy of this combination is still lacking.

In view of the large currently ongoing phase III trial investigating erlotinib in combination with sorafenib as first line treatment in BCLC-C patients, we investigated this drug combination *in vitro* and in an orthotopic rat model of HCC.

Materials and methods

Cell lines

The rat Morris Hepatoma 3924A (MH) cell line was obtained from the German Cancer Research Center (DKFZ; Heidelberg, Germany) and was cultured in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 20% fetal bovine serum, L-glutamine, penicillin, and streptomycin (Life Technologies). The HepG2 cell line (kindly provided by Prof. Grasl-Kraupp, Institute of Cancer Research, Vienna), and the HCC cell line SNU398 (derived from a hepatitis B positive donor, ATCC – LGC Standards, Wesel, Germany) as well as the non-small cell lung cancer cell line HCC827 (ATCC) were maintained as described above but with 10% fetal bovine serum; the human HCC cell line HUH-7 (Riken Cell Bank, Tsukuba, Japan) was maintained in DMEM (Life Technologies) supplemented as mentioned above. Rat aortic endothelial cells were isolated from thoracic aorta of ACI rats by collagenase and cultured in appropriate flasks and plates coated with 1% gelatine (Sigma–Aldrich, Germany) as described in [15].

Treatments

Sorafenib was kindly provided by Bayer HealthCare (Leverkusen, Germany). Erlotinib was kindly provided by Roche (Basel, Switzerland). For *in vitro* experiments, both drugs were dissolved in pure DMSO. Controls were treated with DMSO concentrations of the highest combination groups (maximum 0.3% DMSO). For *in vitro* experiments, we used sorafenib at 1–10 μM and pharmacologically relevant doses of erlotinib at 1–5 μM corresponding to the steady state plasma concentration in patients, which can be achieved after oral dosing of erlotinib with 150 mg/dl [16].

Cetuximab (Erbitux®, Merck Serono, Darmstadt, Germany) was dissolved in aqua dest. and used for *in vitro* experiments at a concentration of 5 μg/ml.

Human EGF was purchased from Sigma Aldrich (St. Louis, MO) and used at a concentration of 50 ng/ml. Rat EGF was purchased from RnD Systems (Minneapolis, MN) and used at a concentration of 50 ng/ml.

The MEK1/2 inhibitor U0126 (Cell Signalling, Danvers, MA) was dissolved in pure DMSO and used for *in vitro* experiments at a concentration of 10 μM corresponding to the manufacturer's recommendations.

Cell viability

Cell viability (tumor and endothelial cells) was determined by the neutral red assay [17].

Cells in the logarithmic phase of growth were plated in 0.5 ml complete medium in 24-well plates and allowed to attach overnight. The following day, sorafenib, erlotinib or a combination of both was added. Cells were then incubated for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 and then analyzed. At least three independent experiments were run in triplicates.

Assessment of apoptosis

After 72-h incubation with indicated concentrations of respective treatments, tumor and endothelial cells were harvested and labeled with FITC-conjugated Annexin V and 7-AAD (Beckman Coulter, Germany) according to the manufacturer's recommendations. A flow cytometer (Beckman Coulter Cell Lab Quanta SC, Beckman Coulter, Germany) was used to quantify Annexin V and 7-AAD positive/negative apoptotic cells.

Immunoblotting

Preparation of tumor tissue and cell lysates, as well as their immunoblotting was performed according to standard methods, as previously described in detail [18,19]. Protein expression of p-EGFR (Tyr1173, Tyr1068), p-ERK (MAPK p42/p44, Thr202/Thyr204), p-AKT (Ser473) and the corresponding total protein was detected with antibodies from Cell signaling. Anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, USA) and Anti-mouse IgG HRP (Pierce, Rockford, USA) were used as secondary antibodies.

Immunohistochemistry

Paraffin-embedded tissue samples were cut into 3 μm thin slices. After deparaffinization with Neo-Clear (Merck), endogenous peroxidase was blocked by immersing slides in 2.5% H_2O_2 diluted in pure methanol. For Von-Willebrand factor staining, antigen retrieval was performed by digestion in 0.1% protease type XIV (Sigma Aldrich). Unspecific staining was blocked by incubation with goat serum diluted in Tris-buffered saline pH 7.4. Endogenous biotin was blocked with avidin/biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Slides were incubated with Von-Willebrand factor primary antibody (1:600 dilution in goat serum, Abcam, Cambridge, UK) overnight at 4 °C. Negative control slides were incubated with polyclonal rabbit IgG control antibody (RnD Systems) at corresponding dilutions. Excess antibody was removed by washing in Tris-buffered saline followed by incubation of slides with biotinylated goat anti-rabbit secondary antibody (1:300 dilution, Vector Laboratories) for 30 min at room

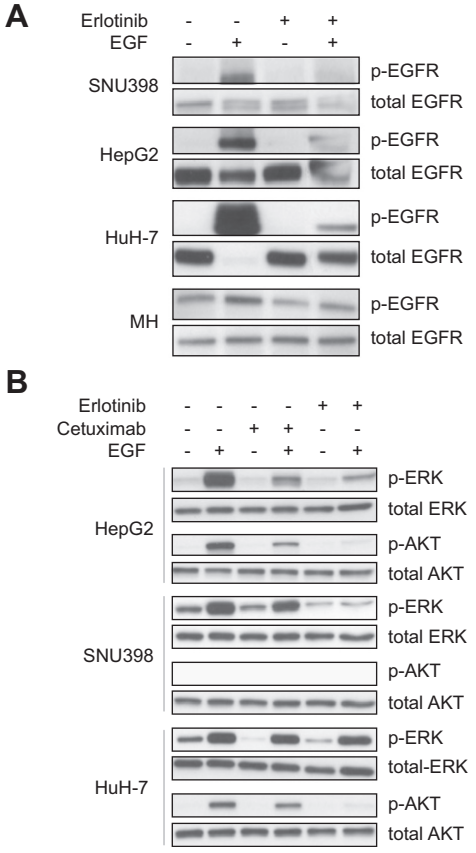

Fig. 1. Effects of erlotinib and cetuximab on target regulation, after EGF stimulation of HCC cells. (A) Indicated HCC cells were starved for 24 h, treated with erlotinib (5 μM) for 3 h, followed by EGF stimulation (50 ng/ml) for 5 min, and immunoblotted for total and p-EGFR. (B) Indicated HCC cells were starved for 24 h, treated with erlotinib (5 μM), or human cetuximab (5 μg/ml) for 3 h, followed by EGF stimulation (50 ng/ml) for 5 min, and immunoblotted for p-ERK, p-AKT, and the respective total proteins.