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ORIGINAL ARTICLE

Antitumor activity of HER1/EGFR tyrosine kinase inhibitor erlotinib, alone and in combination with CPT-11 (irinotecan) in human colorectal cancer xenograft models

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Abstract Erlotinib (Tarceva®, OSI-774) is a potent, orally available, small-molecule inhibitor of HER1/EGFR tyrosine-kinase activity. In this study, the antitumor activity of erlotinib was evaluated in two human colorectal tumor xenograft models (LoVo and HCT116) in athymic mice. When erlotinib was administered as monotherapy, significant tumor growth inhibition (TGI) was seen in the LoVo model at both 100 mg/kg [TGI > 100%, $P < 0.001$; 6/10 partial regressions (PRs)] and 25 mg/kg (TGI = 79%, $P < 0.001$) doses. However, the HCT116 xenograft model was not responsive to any dose of erlotinib tested. The differential response to erlotinib of these two tumor models was not a result of differences in HER1/EGFR expression levels since these were similar in both cell lines. However, it was demonstrated that resistance to erlotinib in the HCT116 model may be a result of persistent activation of ERK in these tumors. Based on the single agent activity of erlotinib in LoVo tumors, a combination study with CPT-11 (Camptosar®, irinotecan) was performed. CPT-11 at the optimal dose of 60 mg/kg or a lower dose of 15 mg/kg resulted in significant TGI (TGI > 100%, $P < 0.001$, and TGI = 93%, $P < 0.001$, respectively) in LoVo-bearing mice. Combination treatment with erlotinib (25 mg/kg) and CPT-11 (15 mg/kg) produced significantly greater antitumor activity (TGI > 100%, $P < 0.001$; 10/10 PRs) than either agent alone ($P < 0.05$), with no increase in toxicity. These data indicate that erlotinib can enhance the antitumor activity of CPT-11, without enhanced toxicity, in the LoVo human colorectal tumor xenograft model.

Keywords Erlotinib · Tarceva · HER1/EGFR · Irinotecan · Colorectal cancer · Xenograft

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Introduction

Dysregulation of intracellular signaling via HER1/EGFR is common in a wide spectrum of solid tumors, including non-small-cell lung carcinoma (NSCLC), squamous-cell carcinoma of the head and neck, and colorectal cancer [26]. Furthermore, overexpression of HER1/EGFR has been correlated with disease progression and poor clinical outcome in many tumors [3, 6, 21].

Colorectal cancer is the third most common cancer worldwide [13], and the second-leading cause of death from cancer in North America [11], largely because of the poor clinical response of colorectal tumors to conventional chemotherapeutics. CPT-11 (irinotecan) is a semi-synthetic derivative of camptothecin, a natural alkaloid isolated from the Chinese tree *Camptotheca acuminate* [14]. Camptothecin and its analogues target topoisomerase I, an enzyme involved in DNA strand dissociation during DNA replication and repair. CPT-11 is routinely used in the treatment of metastatic colorectal cancer [27–29], either as a single agent, or in combination with other chemotherapeutic agents. The increased number of therapy options now available is

thought to have contributed to clinical improvement in the treatment of metastatic colorectal cancer [1]. Despite these developments, colorectal cancer remains a major cause of cancer morbidity and mortality, highlighting the huge demand for novel treatment strategies. HER1/EGFR overexpression may have a role in tumor proliferation and progression in colorectal cancer [5, 18], thus providing a rationale for HER1/EGFR inhibition in this disease.

A broad series of preclinical and clinical studies have shown the value of targeting HER1/EGFR in cancer treatment. Erlotinib hydrochloride (Tarceva®, OSI-774) is a potent, selective, orally available small-molecule inhibitor of the HER1/EGFR tyrosine kinase (TK) domain [9, 23, 25]. Erlotinib was approved by the US FDA in November 2004 for the treatment of advanced NSCLC after failure of at least one prior chemotherapy regimen. It is the first such drug to demonstrate a significant increase in survival in phase III trials in patients with advanced NSCLC [30]. Erlotinib, in combination with gemcitabine, is also approved for the first-line treatment of locally advanced, unresectable or metastatic pancreatic cancer. Potent single agent antitumor activity has also been demonstrated in several preclinical animal models including head and neck, vulval, and NSCLC models [9, 23]. This has led to clinical investigations in a variety of tumor types, with erlotinib used either as monotherapy or in combination with chemotherapy and/or radiation [7, 8, 17, 31].

In this study, we investigated the in vivo activity of erlotinib, either as monotherapy or when combined with CPT-11, using two models of human colorectal carcinoma (HCT116 and LoVo) in nude mice. We also assessed four potential biomarkers for erlotinib response in each model, namely basal levels of key proteins in HER1/EGFR-mediated signaling pathways (HER1/EGFR, ERK, Akt) and expression of the cell cycle inhibitor p27 after treatment. Our goal was to determine the potential therapeutic utility of erlotinib alone and combined with CPT-11 therapy to inhibit growth of human colon carcinoma tumor xenografts in a nude mouse model.

Materials and methods

Animals

Female, athymic, nu/nu-nuBR nude mice (Charles River, Wilmington, DE) aged approximately 6–7 weeks and weighing 23–25 g were used. The health of the mice was assessed daily by observation, and analysis of blood samples taken from sentinel animals on shared shelf racks. All animals were allowed to acclimatize and recover from shipping-related stress for 1 week.

Autoclaved water and irradiated food [5058-ms Pico Lab (mouse) breed chow, Purina Mills, Richmond, IN] were provided ad libitum, and the animals were kept in a 12 h light and dark cycle. Cages, bedding and water bottles were autoclaved before use and changed weekly. All animal experiments were performed in accordance with protocols approved by the Roche Animal Care and Use Committee, and in accordance with local regulations.

Materials

Erlotinib (OSI Pharmaceuticals, Melville, NY) was formulated as a fine suspension with sodium carboxymethylcellulose and Tween 80 in water for injection. Erlotinib (0.2 ml/animal) was given orally once daily for 3 weeks using a 1 ml syringe and an 18-gauge gavage needle. CPT-11 (Pharmacia & Upjohn, Kalamazoo, MI) was provided in a stock sterile saline solution of 20 mg/ml, which was diluted as required with sterile saline. CPT-11 was administered i.p. every 4 days for 3 weeks using a 1 ml syringe and a 26-gauge needle (0.2 ml/animal).

Cell culture and animal studies

Both LoVo and HCT116 cells were from American Type Culture Collection (Rockville, MD). LoVo cells were grown in F12K medium supplemented with 20% fetal bovine serum (FBS) and 2 mM L-glutamine. HCT116 cells were grown in McCoy's 5A medium with 10% heat-inactivated FBS and 2 mM L-glutamine.

Cells were suspended in calcium- and magnesium-free phosphate-buffered saline pH 7.35–7.45, and implanted subcutaneously in the right flank of each mouse. The cell concentrations for implantation were 5×10^6 cells/0.2 ml per mouse for LoVo and 3×10^6 cells/0.2 ml per mouse for HCT116. Once palpable tumors were established, tumor volumes were measured by caliper and animals were randomized so that all groups (10 animals per group for all experiments) had similar starting mean tumor volumes of 100–150 mm^3. Tumor measurements and mouse weights were taken three times per week. Animals were individually monitored throughout the experiment.

Calculations and statistical analysis

Treatment efficacy was assessed by tumor growth inhibition (TGI), based on the last measurement of

tumor volume. Tumor volumes of treated groups were given as percentages of tumor volumes of the control groups (%*T/C*), using the formula:

$$100 \times [(T - T_0)/(C - C_0)]$$

where '*T*' represents mean tumor volume of a treated group on a specific day, 'T_0' represents mean tumor volume of the same group on the first day of treatment, *C* represents mean tumor volume of a control group on a specific day, and C_0 represents mean tumor volume of the same group on the first day of treatment.

TGI was calculated using the formula:

$$100 \times \%T/C$$

Tumor volume (mm^3) was calculated using the ellipsoid formula:

$$[D \times (d^2)]/2$$

where '*D*' represents the largest diameter of the tumor, and '*d*' represents the smallest diameter. In some cases, tumor regression and/or percentage change in tumor volume was calculated using the formula:

$$[(T - T_0)/T_0] \times 100$$

where '*T*' represents mean tumor volume of the treated group on a specific day, and 'T_0' represents mean tumor volume of the same treated group at the start of treatment. At the end of an experiment, partial regression was defined as any reduction in tumor volume from that at randomization.

Statistical analysis was performed using a two-tailed student *t*-test or Mann-Whitney U-test when simple comparison between two groups was required (change in tumor volume at the end of the study). One-way analysis of variance (ANOVA) tests were used to assess overall differences between combination treatment groups and single-agent treatment groups. The significance level was set at $P \leq 0.05$. Statistical analysis was done using SigmaStat software version 2.03. (Jandel Scientific, San Francisco, CA).

Histopathology/necropsy

At the end of the study, five mice per treatment from all groups were given a full necropsy. Tumor samples were fixed by immersion in 10% zinc formalin, processed in a Tissue-Tek© VIP (Sakura Finetek, Torrance, CA) and embedded in paraffin. Sections for immunohistochemistry were cut at 5 μm.

For HER1/EGFR and p27 detection, antigen retrieval was performed by immersing sections in Target Retrieval Solution pH 6.0 (DakoCytomation, Carpinteria, CA) and heating to 94°C in a steamer (Black & Decker, Towson, MD) for 20 min. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in methanol for 15 min. To block non-specific binding sites, sections were treated for 20 min using 10% normal serum from the species in which the secondary antibody was raised, prepared in Ultra V Block (Lab Vision, Fremont, CA). For detection of total HER1/EGFR, sections were incubated overnight at room temperature with a rabbit polyclonal anti-HER1/EGFR IgG (BioGenex, San Ramon, CA), diluted 1:50 in Dako Antibody Diluent (DakoCytomation). For detection of p27, sections were incubated for 1 h at room temperature with a rabbit polyclonal anti-p27 IgG (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:600 in Dako Antibody Diluent. Primary antibodies were detected using a rabbit IgG VECTASTAIN Elite ABC peroxidase kit, with Vector NovaRED (Vector Laboratories, Burlingame, CA) as the substrate. The sections were then counterstained with hematoxylin.

For the detection of total and phosphorylated ERK and AKT, immunohistochemistry was performed using the Ventana Discovery automated slide processing system and the Ventana DAB MAP kit (Ventana Medical Systems, Tucson, AZ). For antigen retrieval, sections were treated with mild cell conditioning for phospho-ERK and phospho-AKT, or standard cell conditioning for total ERK and total AKT. To block non-specific binding sites, sections were incubated for 30 min with 20% normal serum from the species in which the secondary antibody was raised, prepared in Ventana Reaction Buffer (Ventana Medical Systems). Phosphorylated ERK and phosphorylated AKT were detected using a rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA), and a rabbit polyclonal anti-phospho-AKT (Ser473) antibody (Cell Signaling Technology), respectively, each diluted 1:50 in Ventana Antibody Diluent (Ventana Medical Systems). Total ERK was detected using a rabbit polyclonal ERK1 antibody (Santa Cruz Biotechnology) diluted 1:2,000 in Ventana Antibody Diluent. Total AKT was detected using a goat polyclonal AKT 1/2 antibody (Santa Cruz Biotechnology) diluted 1:2,000 in Dako Antibody Diluent. Sections were incubated with primary antibodies for 1 h at 37°C. Rabbit polyclonal antibodies were detected using a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) diluted 1:100 in Ventana Antibody Diluent. Goat polyclonal antibodies

were detected using a biotinylated rabbit anti-goat IgG secondary antibody (Vector Laboratories) diluted 1:400 in Dako Antibody Diluent. The sections were then counterstained using hematoxylin.

Results

Monotherapy efficacy

The antitumor effects of erlotinib were studied using HCT116 cell and LoVo cell subcutaneous xenograft models in athymic nude mice. Pilot studies determined that the maximum tolerated dose (MTD) dose for erlotinib in mice was 100 mg/kg when given on a daily basis (data not shown). Mice with established tumors (approximately 150 mm^3) were treated for 3 weeks with two different doses of erlotinib: 100 mg/kg (the MTD), and 25 mg/kg (a suboptimal dose) (Fig. 1a and b). Erlotinib 100 mg/kg produced significant antitumor activity in the LoVo xenograft model (TGI > 100%, $\%T/C = -2\%$, $P < 0.001$), with 60% of the tumors partially regressed. Erlotinib 25 mg/kg also produced antitumor activity in LoVo xenografts (TGI 79%, $\%T/C = 21\%$, $P < 0.001$), although no tumor regression was seen in this group. In contrast, erlotinib was relatively inactive against HCT116 cell xenografts. In the HCT116 xenograft model, erlotinib 100 mg/kg produced 1% TGI ($P = 0.910$) and erlotinib 25 mg/kg produced 27% TGI ($P = 0.104$).

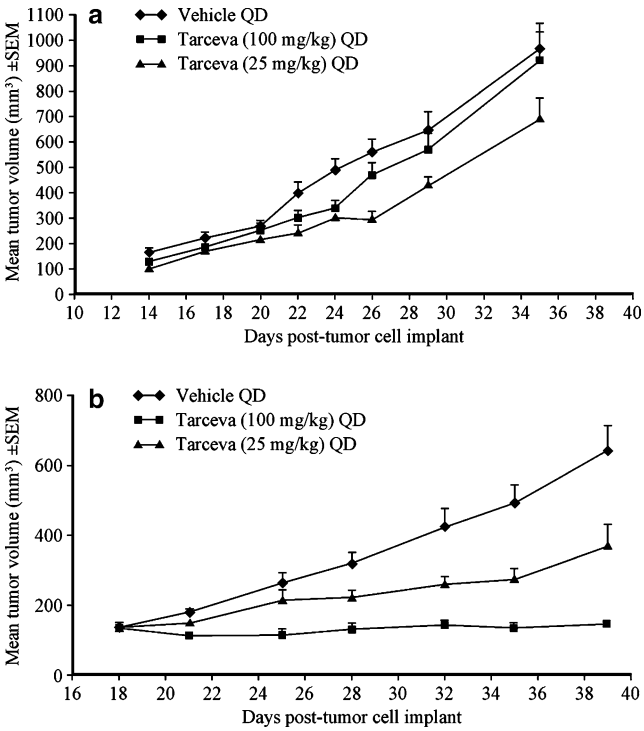

Fig. 1 **a, b** Effect of erlotinib on mean tumor volume in (**a**) the HCT116 and (**b**) the LoVo colorectal cancer xenograft models. Mice were treated with daily oral doses of erlotinib at 0, 25 and 100 mg/kg for 21 days. Tumor size was measured 2–3 times per week. Volumes are means $n = 10$

Basal levels of HER1/EGFR expression in HCT116 and LoVo xenografts

We sought to understand the biological basis of the observed differential antitumor activity of erlotinib in HCT116 and LoVo xenografts. We hypothesized that HER1/EGFR expression may be higher in responding LoVo tumors than in non-responding HCT116 tumors. However, immunohistochemical analysis showed that both colorectal cancer models demonstrated similar moderate expression of HER1/EGFR (data not shown).

Analysis of ERK and Akt levels in HCT116 and LoVo xenografts

We also considered whether the differential activation of the Ras/Raf/MEK/ERK or phosphatidyl inositol (PI) 3-kinase/AKT signaling pathways downstream of HER1/EGFR might account for the difference in response to erlotinib between LoVo and HCT116 tumors. The basal level of total ERK, detected using immunohistochemistry, was similar in HCT116 and LoVo cells (Fig. 2). However, activated (phosphorylated) ERK was only detected in HCT116 tumors, with little or no staining in the LoVo tumors. Comparable levels of both total and activated AKT were observed in both colorectal cancer models (Fig. 3).

Comparison of basal and post-erlotinib levels of p27 in HCT116 and LoVo xenografts

At the end of the 3 week treatment period, p27 levels were analyzed using immunohistochemistry in tumor samples from vehicle-treated and erlotinib-treated groups. The results showed marked differences with erlotinib treatment in these two models (Fig. 4). Levels of p27 were upregulated in response to erlotinib monotherapy (100 mg/kg) in the LoVo xenografts but were unchanged in the HCT116 xenografts, relative to vehicle-treated tumors.

Combination efficacy, pathology, tumor histology in LoVo model

Having demonstrated that erlotinib produced significant antitumor efficacy against LoVo xenografts,

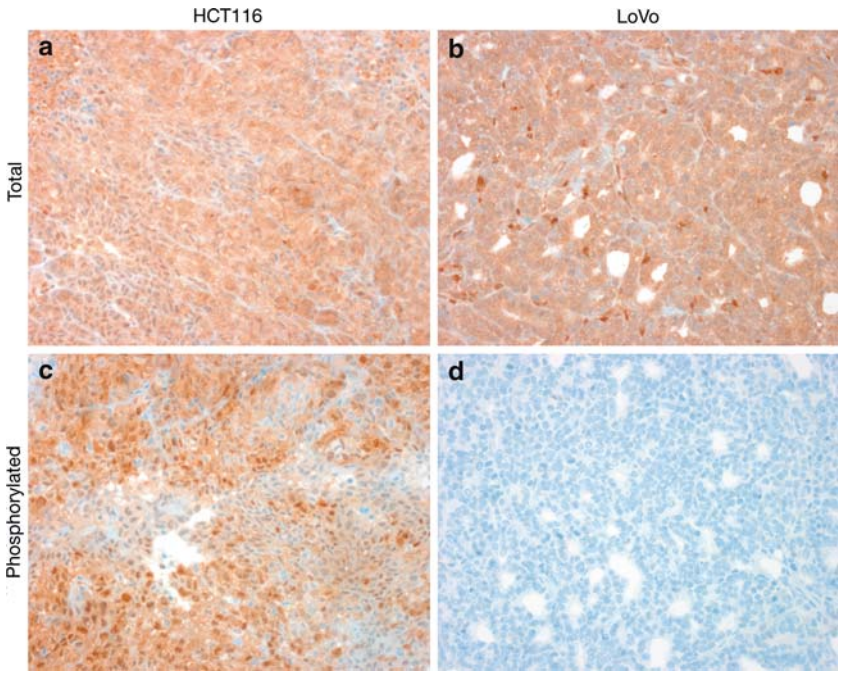

Fig. 2 Expression of total and phosphorylated ERK in HCT116 and LoVo xenografts. Untreated tumors of HCT116 and LoVo were collected and analyzed using immunohistochemistry for total (**a** HCT116, **b** LoVo) and phosphorylated (**c** HCT116, **d** LoVo) ERK. Similar levels of total ERK were present in the two colorectal cancer models. In contrast, ERK appeared to be differentially activated (phosphorylated) in the HCT116 xenograft model, with little or no staining of phosphorylated ERK in the LoVo xenograft model

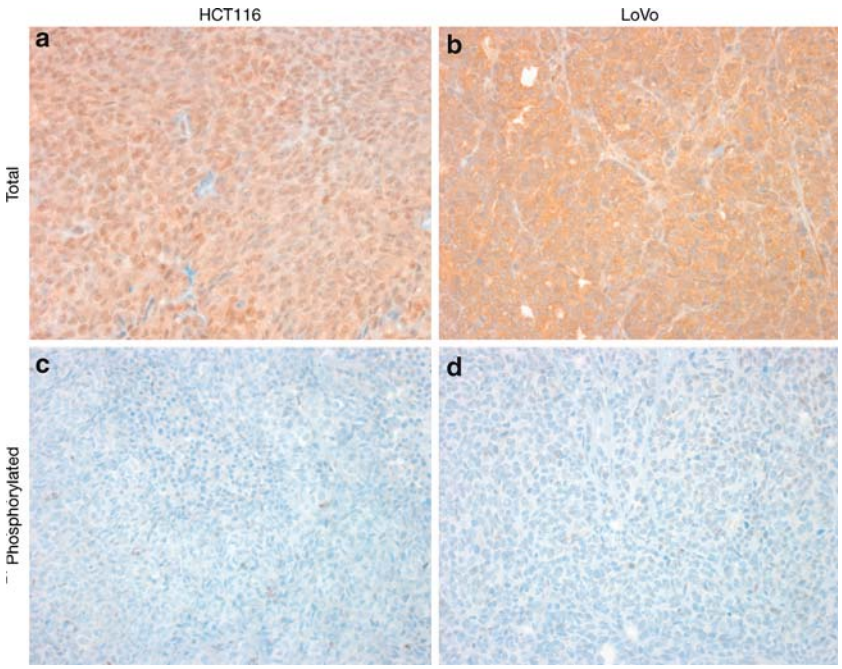

Fig. 3 Expression of total and phosphorylated Akt in HCT116 and LoVo xenografts. Untreated tumors of HCT116 and LoVo were collected and analyzed using immunohistochemistry for total (**a** HCT116, **b** LoVo) and phosphorylated (**c** HCT116, **d** LoVo) Akt. Similar levels of both total Akt and activated (phosphorylated) Akt were present in the two colorectal cancer models

we investigated whether additional activity was obtained in this model when erlotinib was combined with CPT-11.

In previous studies, the MTD of CPT-11 was determined as 66 mg/kg i.v. every 4 days (data not shown); in this study, we chose a dose of 60 mg/kg i.v. every 4 days. Significant TGI was observed using this dose (TGI > 100%, $\%T/C = -5\%$, $P < 0.001$), and partial regression was observed in 90% of the tumors. A suboptimal dose of CPT-11 (15 mg/kg q4d i.v.; approximately 1/4 MTD) was also studied; this gave 93% TGI ($\%\ T/C = 7\%$, $P < 0.001$) (Fig. 5; Table 1).

When CPT-11 and erlotinib were combined at the high doses of 60 mg/kg q4d i.v. and 100 mg/kg qd p.o., respectively, gross toxicity appeared as early as 5 days post-study initiation. However, the combination of CPT-11 15 mg/kg q4d i.v. and erlotinib 25 mg/kg qd p.o. was well tolerated, with no signs of gross toxicity.

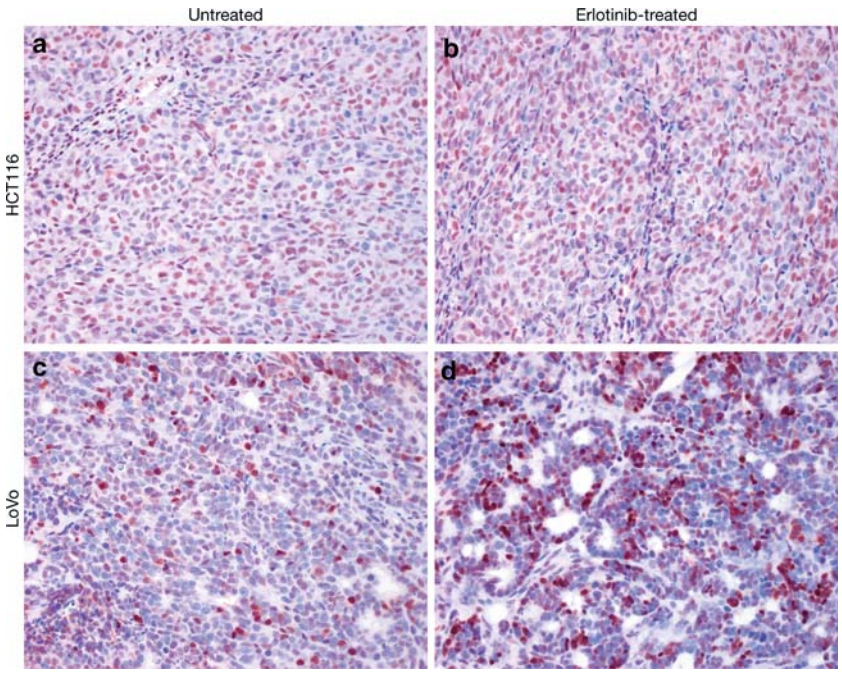

Fig. 4 p27 levels assessed by positive nuclei staining in untreated (**a** HCT116, **c** LoVo) and erlotinib 100 mg/kg treated (**b** HCT116, **d** LoVo) xenografts. Similar p27 levels were observed in untreated and erlotinib-treated HCT116 xenografts. In contrast, an increase in p27 was seen in erlotinib-treated LoVo tumors, compared with untreated LoVo tumors

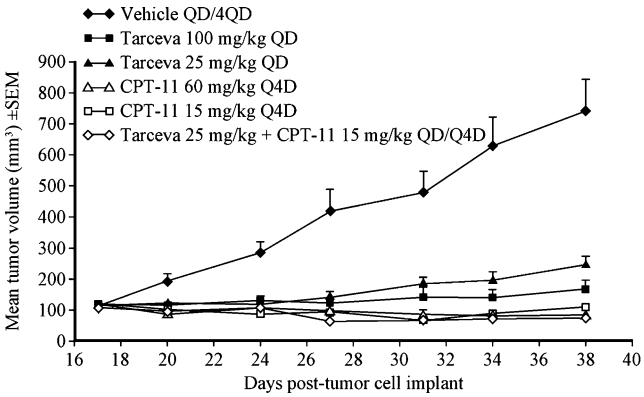

Fig. 5 Effect of erlotinib and CPT-11 alone and in combination on mean tumor volume in the LoVo colorectal xenograft model. Mice were treated with vehicle, oral erlotinib alone (25 or 100 mg/kg/day for 3 weeks), i.p. CPT-11 alone (15 or 60 mg/kg every 4 days for 3 weeks), or combination treatment with erlotinib 25 mg/kg/day plus CPT-11 15 mg/kg every 4 days for 3 weeks. Tumor size was measured 2–3 times per week. Volumes are means $n = 10$

This combination inhibited tumor growth by more than 100% (%$T/C = -5\%$, $P < 0.001$), with partial regression observed for all 10 tumors (100%). The TGI produced by the combination was significantly greater than that produced by either CPT-11 15 mg/kg q4d alone ($P < 0.05$) or erlotinib 25 mg/kg qd alone ($P < 0.05$) (Fig. 5; Table 1).

At the end of the study, necropsy was performed in five mice from each treatment group; no overt signs of gross toxicity were seen in any case. Histopathology revealed no treatment-related microscopic findings in heart, cecum, colon, duodenum, jejunum, kidney, liver, lung, stomach or spleen.

Histological staining revealed that treatment with erlotinib, CPT-11 or the combination tended to cause tumor cell atrophy, with decreased necrosis, increased interstitial fibrosis and differentiation of tumor cells to form tubular morphology. A clear treatment-related effect was a decrease in mitotic figures, indicative of the antiproliferative effects of combined treatment with erlotinib 25 mg/kg and CPT-11 15 mg/kg in the LoVo model (Fig. 6).

Discussion

In this investigation, we demonstrate that the HER1/EGFR inhibitor erlotinib produces significant TGI in the LoVo human colorectal tumor xenograft model, but not in the HCT116 model. We investigated molecular differences that may underlie the variable sensitivity to erlotinib of these two models. It has been reported that cells with high expression of HER1/EGFR are particularly sensitive to HER1/EGFR inhibition [2, 24, 19], suggesting that high expression of the receptor is required to modulate sensitivity. However, despite the different sensitivity of the LoVo and HCT116 models to erlotinib, both of these cell lines expressed similar, moderate levels of HER1/EGFR. Therefore, HER1/EGFR expression levels are not correlated with sensitivity to erlotinib in LoVo or HCT116 colorectal cancer models.

Table 1 LoVo colorectal xenograft model: tumor growth inhibition (TGI) for erlotinib and irinotecan, alone and in combination

	TGI (%)	Number of tumor regressions
Erlotinib		
100 mg/kg	>100[a]	6
25 mg/kg	79[a]	0
Irinotecan		
60 mg/kg	>100[a]	9
15 mg/kg	93[a]	4
Combination		
25 mg/kg erlotinib + 15 mg/kg irinotecan	>100[b]	10

[a] $P < 0.001$ versus control

[b] $P < 0.05$ versus corresponding doses of either single agent

In NSCLC, certain mutations in the TK domain of HER1/EGFR (most commonly located in exons 19 and 21 of the HER1/EGFR gene) are associated with increased sensitivity to HER1/EGFR TK inhibitors such as erlotinib [22]. However, survival in patients with NSCLC receiving erlotinib does not appear to be significantly associated with HER1/EGFR TK mutation status [32, 33]. HER1/EGFR TK mutations are rare in colorectal cancer [15, 4] and previous reports suggest that both HCT116 and LoVo colorectal carcinoma cell lines possess wild-type HER1/EGFR TK domains [15, 20]. Therefore, we believe that it is unlikely that the differential sensitivity of LoVo and HCT116 cells to erlotinib is determined by HER1/EGFR mutation status.

HER1/EGFR is part of a large signaling network [34], so a number of factors, rather than expression levels or mutation status of the receptors alone, may determine the sensitivity of a cell to HER1/EGFR inhibitors. We studied the activation status of the Ras/Raf/MEK/ERK and PI3-kinase/AKT pathways, two major intracellular signaling pathways activated by HER1/EGFR. There were no differences in total or activated AKT, or total ERK between the two cell lines. However, activated ERK was only detected in HCT116 cells. These data are consistent with those of previous studies showing that intrinsic activation of ERK correlates with the reduced efficacy of HER1/EGFR inhibitors in several epithelial tumor cell lines [10, 16]. Therefore, our data suggest that persistently phosphorylated ERK may account for the lack of efficacy of erlotinib in some tumor cell lines.

We further investigated the nuclear expression of p27, the upregulation of which has been suggested as a promising biomarker of erlotinib activity [17]. An increase in p27 was seen in LoVo tumors treated with the higher dose of erlotinib (100 mg/kg); this dose also produced significant TGI in the LoVo model. However, erlotinib 100 mg/kg was not active against HCT116 tumors and had no effect on p27 levels. This finding, together with the well-established and relatively simple methodology for quantitation (nuclei with positive staining [12]), make p27 an attractive biomarker that is currently being incorporated into disease-oriented clinical studies to explore its value as a surrogate of activity.

The potent antitumor activity produced by erlotinib 25 mg/kg in the LoVo human colorectal xenograft model was enhanced significantly ($P < 0.05$) by combination with CPT-11 15 mg/kg, with partial regression observed in all 10 tumors in the group receiving combination treatment. This combination was well tolerated, with no treatment-related toxicity or body weight loss observed.

In conclusion, our results suggest the existence of complementary mechanisms of action with the HER1/

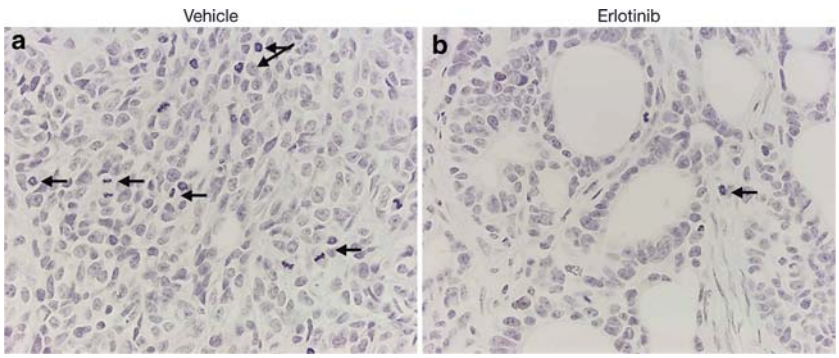

Fig. 6 Representative images of tumors from mice (**a**) treated with vehicle and (**b**) treated with erlotinib 25 mg/kg plus CPT-11 15 mg/kg. Treatment with the combination of erlotinib plus CPT-11 caused tumor cell atrophy with decreased necrosis, increased interstitial fibrosis, and differentiation of tumor cells to form tubular morphology. A clear treatment-related anti-tumor effect was a decrease in mitotic figures (*arrows*) indicative of the antiproliferative effects of combined therapy with erlotinib 25 mg/kg plus CPT-11 15 mg/ kg in this LoVo xenograft model

EGFR inhibitor erlotinib and topoisomerase I inhibitor CPT-11 that could be the basis for the observed improvement in activity seen with the combination. Although further research is needed to fully evaluate this promising new application of erlotinib in colorectal cancer treatment, the concept of combined molecular targeting of HER1/EGFR seems to present a rewarding strategy in the field of molecular therapeutics.

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