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ORIGINAL ARTICLE

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In vivo and in vitro antitumor activity of oxaliplatin in combination with cetuximab in human colorectal tumor cell lines expressing different level of EGFR

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Abstract This study aimed to assess the effect of cetuximab (C225, Erbitux®, a chimeric anti-epidermal growth factor receptor (EGFR) monoclonal antibody) in combination with oxaliplatin in vitro and in vivo on four colon cancer cell lines (HCT-8; HT-29, SW620, HCT-116) expressing different levels of EGFR. In vitro, cetuximab combined with oxaliplatin significantly decreased the IC_{50} values of oxaliplatin in HCT-8 (EGF-R moderate) and HT-29 (EGF-R weak) cell lines, while SW620 (EGF-R negative) and HCT-116 (EGFR strong) cell lines remained unresponsive. This combination was synergistic in HCT-8 and HT-29 cell lines while cetuximab induced no major modification of the IC_{50} of oxaliplatin in HCT-116 or SW620 cell lines. We then determined the effect of cetuximab on the EGF-induced EGFR phosphorylation and we highlight a correlation between the basal level of phospho-EGFR and the response to the combination. In vivo, the combination of cetuximab plus oxaliplatin significantly inhibited tumor growth of HCT-8 and HT-29 (tumor delay or Td = 21.6 ± 2.9 and 18.0 ± 2.9 days respectively, synergistic effect) compared to either oxaliplatin (Td = 12.6 ± 2.3 and 14.4 ± 3.2 days respectively) or cetuximab (Td = 13.4 ± 2.9 and 14.5 ± 2.4 days, respectively) alone in xenograft models. The combination had no effect on HCT-116 and SW-620 cell lines. The observed responses are strictly dependent on the cell type, and are not correlated with the level of EGFR expression but related to the basal level of phospho-EGFR. This study provides promising preclinical results for a possible clinical investigation of the combination of oxaliplatin plus cetuximab in chemorefractory colorectal tumors.

Keywords EGFR · Inhibition · Combination · Oxaliplatin · Cetuximab · Xenograft

Abbreviations EGFR: Epidermal growth factor receptor · L-OHP: Oxaliplatin · FCS: Fetal calf serum · MAPK: Mitogen-activated protein kinases · AU: Arbitrary unit · NER: Nucleotide excision repair · Td: Tumor delay

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Introduction

Colorectal carcinoma is the fourth most common form of cancer occurring worldwide and a major cause of morbidity and mortality in Western countries. In the year 2000, an estimated 900,000 new cases of colorectal cancer were diagnosed worldwide, with an estimated 490,000 deaths [17]. Colorectal cancer accounts for an estimated 10–15% of newly diagnosed cancer cases [16]. While early stage colorectal cancer is frequently curable with surgery, unresectable metastatic disease is uniformly fatal. Adjuvant and palliative treatments for colorectal cancer mainly involve fluorouracil-based chemotherapy and irinotecan, and then, more recently, oxaliplatin [24]. Oxaliplatin (L-OHP), a third generation platinum analog, has shown significant single-agent activity in advanced colorectal cancer, with relative absence of cross-resistance with cisplatin and carboplatin [4]. However, when patients become refractory to these cytotoxic agents, there are essentially no established treatment options with demonstrated efficacy. There is a

clear need for new and improved therapies for this disease.

The last few years have witnessed growing interest and significant advances in the use of targeted therapy for colorectal cancer. Cetuximab (C225, Erbitux®) is a chimeric monoclonal antibody directed against epidermal growth factor receptor (EGFR or *erb*B-1 or HER1) [23]. EGFR is a member of HER tyrosine kinase growth factor receptor family, involved in signaling pathways affecting cellular growth, differentiation and proliferation [6]. Colorectal cancer is frequently associated with high expression levels of EGFR (25–80%), resulting in a more aggressive disease and a poor prognosis [14, 18]. Activation of EGFR leads to downstream signaling that activates mitogenic and survival pathways, such as mitogen-activated protein kinases (MAPK) and phosphatidyl-inositol-3 kinase (PI3-K/AKT) pathways [2]. Inhibition of these pathways by EGFR antagonists, such as cetuximab, can lead to induction of BAX activation of caspase-8, and downregulation of BCL-2 and NFκB [21]. These alterations may render cells more sensitive to apoptotic stimuli, such as chemotherapy.

Cetuximab binds the EGFR with high affinity, competes for ligand binding and downregulates receptor expression on the cell surface. Numerous studies have shown that cetuximab or its parental murine antibody (MAb 225) inhibits the proliferation of EGFR-expressing tumor cells in vitro and induces inhibition of tumor growth in vivo against human tumor xenografts in nude mice [15, 11]. Moreover, synergistic effects of cetuximab or MAb 225 have been demonstrated in combination with cytotoxic agents such as cisplatin [9, 25], doxorubicin [3], gemcitabine [5], paclitaxel [13] or topotecan [7], or with radiotherapy [12]. Recently, cetuximab combined with irinotecan have shown a marked synergy in irinotecan-resistant human colorectal tumor xenografts, suggesting that cetuximab could reverse chemoresistance to irinotecan [19].

These findings led us to investigate a new strategy, combining cetuximab and oxaliplatin treatment in colorectal cancer. This study was performed on four human colorectal tumor cell lines (HCT-116, HCT-8, HT-29 and SW620) expressing different levels of EGFR. We evaluated the in vivo and in vitro effects of cetuximab or oxaliplatin alone and the combination of both agents.

Materials and methods

Drugs

Cetuximab (2 mg/mL) was kindly provided by Merck (Darmstadt, Germany) and oxaliplatin (5 mg/ml) was purchased from Sanofi Synthelabo (Gentilly, France). For in vivo experiments, stock solution of oxaliplatin was diluted five times in 5% glucose solution for injection, as recommended by the supplier.

Cell lines

Human colon carcinoma cell lines HCT-116, HCT-8, HT-29 and SW620 (ATCC, Rockville, MD, USA) were routinely maintained in RPMI-1640 medium containing 5% fetal calf serum (FCS), supplemented with 2 mM L-glutamine (Cambrex). Before any in vitro study, cells were cultured for 1 week in phenol red-free medium.

Western blotting

HCT-116, HCT-8, HT-29 and SW620 cells were plated at 8.10^5 cells in 100 mm Petri dishes. Cells were harvested and lyzed in specific buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X, 0.1% SDS, 5 mM $MgCl_2$, 50 mM NaF, 2 mM PMSF, 10 mM DTT, 2 mM orthovanadate, 5 mg/ml sodium deoxycholate, 6.4 mg/ml phosphate substrate (Sigma 104®)). For HER-1–2, MAPK, AKT and beta-tubulin analysis, 100 µg of cleared lysates were separated on a 7.5, 12.5 or 10.0% SDS-polyacrylamide gels, respectively, blotted to PVDF membranes (Amersham) and incubated with specific antibodies. Detection was performed using peroxidase-conjugated secondary antibodies (Bio-Rad) and an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech). The Blots were scanned and analyzed with the Molecular Dynamics densitometer and ImageQuant software. Each value is shown as an arbitrary unit (AU) and representative of at least two independent experiments.

Cytotoxicity assay

For cell growth assay and IC_{50} determination, HCT-8, HT-29, HCT-116 and SW620 cells were seeded, respectively, at 1,500, 2,250, 3,000 and 5,250 cells/well in a 96-well plate on day 0 in the previously described medium. On day 1, cells were treated with either vehicle, cetuximab, L-OHP or the combination of cetuximab plus L-OHP. The antiproliferative effect of cetuximab alone was evaluated at concentrations ranging from 0.1 to 100 µg/ml according to three different schedules, i.e., once on day 1, every 2 days, and daily until 96 h from the cells plating. The combination effect was evaluated using a fixed cetuximab concentration of 20 µg/ml every day, combined with L-OHP concentrations ranging from 0.0625 to 2.5 µM for 24 h. Three schedules of exposure were tested, i.e., (1) sequential: 24-h exposure to L-OHP followed by cetuximab everyday (named "sequential"), (2) simultaneous: 24-h exposure to L-OHP and cetuximab followed by cetuximab everyday (named "simultaneous") and (3) cetuximab for 48 h followed by 24-h exposure to L-OHP and cetuximab simultaneously (named "pretreatment"). The inhibition of cell growth was evaluated using sulforhodamine B (SRB) colorimetric assay as described by Skehan et al. [26]. For each drug, results are expressed as the relative percentage of absorbance compared to controls without drug. Experi-

mental conditions were performed in sextuplicate (6 wells of the 96-well plate per experimental condition). All of the experiments were performed at least three times (five experiments for combination studies).

Phosphorylated EGFR quantification

The concentration of phosphorylated EGFR was measured using an active EGFR EIA Kit (Takara Biomedicals, Tokyo, Japan) following the supplier's instructions and expressed as the amount of phosphorylated EGFR (fmol/ml). Briefly, 1.10^7 of colon cancer cells were plated in RPMI 1640 phenol red-free medium supplemented with 5% FCS and 2 mM L-glutamine in 100-mm Petri dishes. 24 h later, cells were serum starved for 24 h and then treated with either cetuximab or vehicle (serum-free medium) for 1, 6 and 24 h. When indicated, EGF stimulation (20 ng/mL) was performed during the last 15 min of each treatment time.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm formalin-fixed paraffin-embedded sections of SW620, HCT-8, HT-29 and HCT-116 xenograft tumors from nude mice. Immunostaining was performed with a DAKO EGFR pharmDx™ kit using a DAKO autostainer™ slide processor according to the manufacturer's protocol. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated in serial ethanol washes (100, 80 and 50%). Antigen retrieval-based proteinase K digestion was performed before immunostaining. Antibody binding was revealed using a two-step peroxidase-conjugated polymer backbone visualization system (EnVision™, DAKO, Glostrup, Denmark). The chromogenic substrate used was DAB (3,3′-diaminobenzidine). Slides were counterstained with hematoxylin. Immunostaining assessment was performed regarding the staining intensity using a four points scale (i.e.: 0 = negative; + = weak; + + = moderate; + + + = strong) and the percentage of labeled cells.

Animals

Female Swiss athymic nude mice (Charles River, France), 4–5 weeks old, were housed in filter-capped cages kept in sterile facility and maintained in accordance with the FELASA standards. Following a 2-week quarantine, mice were included in the protocols.

Treatment of colorectal carcinoma xenografts

Preliminary studies performed with tumor xenograft generated in single flank of mice were identical to those in both flanks, leading us to implant mice in both flanks to extend our data. Colon carcinoma xenografts were established by subcutaneous injection of 10.10^6 cells of HT-29, SW620, HCT-8 or HCT-116 in both mouse flanks. When the tumor size reached around 200 mm^3 (day 0), mice were pooled and randomly assigned to four groups of five to six animals each. Optimal doses for L-OHP and cetuximab treatments were chosen according to previous studies from our laboratory [10] and others [7, 19]. A single dose of L-OHP at 10 mg/kg was administered intravenously (i.v.) into the caudal vein on day 1. Cetuximab was administered by intraperitoneal (i.p.) injection at 1 mg per dose every 3 days (q3d). This treatment was continued until tumor volume reached five times the initial volume (about 20 days). Two perpendicular diameters of tumors were measured every 2 days with a caliper square, by the same investigator. Tumor volume was calculated according to the following formula: $V\ (mm^3) = d^2\ (mm^2) \times D(mm)/2$, where d and D are respectively the smallest and the largest tumor diameters. In each group, the relative tumor volume was expressed as V_t/V_0 ratio where V_t is the mean tumor volume on a given day during the treatment and V_0 is the mean tumor volume at the beginning of the treatment. The antitumor activity was evaluated by the time to reach a tumor volume that was five times greater than the initial volume [tumor delay (Td)].

The expected Td of the combined treatment was calculated according to the following formula:

$$\begin{aligned}\text{Expected Td} = {} & \text{Mean control} \\ & + (\text{mean cetuximab} - \text{mean control}) \\ & + (\text{mean oxaliplatin} - \text{mean control})\end{aligned}$$

The effect of the combined treatment (synergistic/additive/antagonist) was assessed by calculating the ratio of the observed Td divided by that of the expected Td. If the ratio observed: expected is >1, the combination is synergistic; <1 the combination is antagonist; and $=1$ the combination is additive.

The maximum body weight loss and the number of toxic deaths were monitored as index of toxicity. Mice with tumor were inspected daily for assessment of overall clinical conditions and assessment of food and water intake. When tumor volume reached 1,000 mm^3, mice were inspected twice a day and sacrificed before occurrence of poor health conditions. At the end of the experiment, tumors were removed for immunohistochemical analysis.

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). In vitro results were analyzed using Student's t test and P value <0.05 were accepted as statistically significant.

In vivo data were statistically evaluated by means of one-way analysis of variance (ANOVA). Significant treatment effects were subsequently delineated by using

Dunnett's posthoc test. According to the homogeneity of variances (Hartley's) test, the Mann–Whitney *U* test was used for the comparison of L-OHP, cetuximab alone and combined treatments to control.

Results

Status of tumor cell lines

As shown in western blot (Fig. 1), different levels of EGFR expression were detected in the colorectal tumor cell lines. HCT-116 and HCT-8 expressed respectively a high and a moderate level of EGFR (respectively 19.6 AU: arbitrary unit, + + + and 16.4 AU, + +), while HT-29 had a weak expression (10.1 AU, +) and SW620 a very low level of EGFR expression considered as negative (0.6 AU, −). All cell lines expressed HER-2 in different proportions and high levels of total Erk-1, Erk-2 and total AKT (Fig. 1). Western blotting data were confirmed by immunohistochemical analysis (Fig. 2) on tumor xenografts showing a similar EGFR expression panel.

In vitro results

Effect of cetuximab alone

Cetuximab alone at concentrations up to 100 μg/ml was neither cytotoxic nor cytostatic, whatever the schedule used or the EGFR status, for all colorectal tumor cell lines (data not shown). The most sensitive cell line was HCT-8 with an IC_{10} between 10 and 30 μg/ml according to the daily treatment with cetuximab. Consequently, the fixed concentration of 20 μg/ml of cetuximab administered every day was chosen for combination experiments.

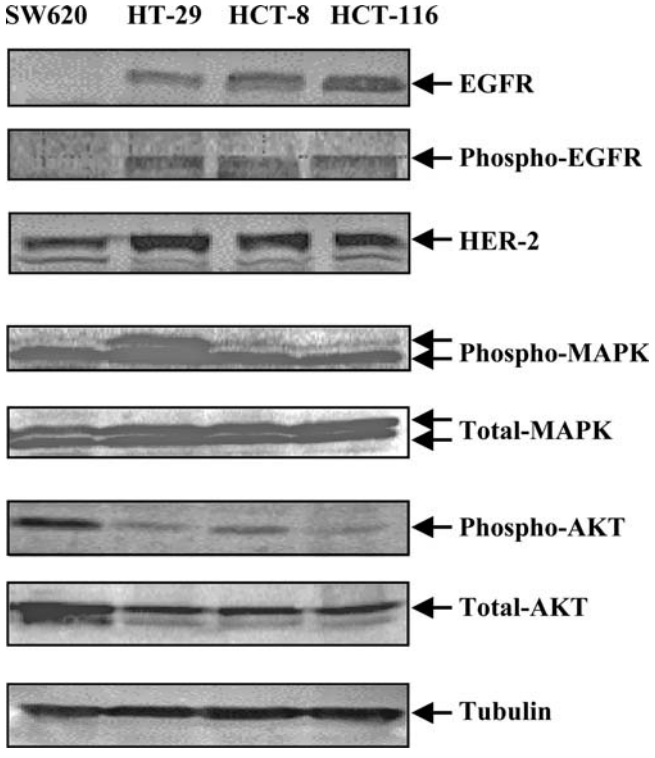

Fig. 1 Western blot analysis of EGFR, phospho-EGFR, HER-2 expression, MAPK and AKT status in colon cancer cell lines HCT-8, HT-29, HCT-116 and SW620

EGFR phosphorylation

The level of phosphorylated EGFR was measured using an active EGFR EIA Kit (Takara Biomedicals, Tokyo, Japan), and cells cultured for 24 h in a medium containing 0% FCS show differences of their EGFR basal phosphorylation level (Fig. 3a). The HCT-8 cell line exhibited the highest level of phospho-EGFR (0.51 ± 0.05 fmol/mg proteins), whereas the HT-29 cell line showed a moderate level (0.26 ± 0.02 fmol/mg proteins). Despite its high level of EGFR expression in western blot (Fig. 1), the HCT-116 cell line showed a very low level of basal phospho-EGFR using the active EGFR EIA Kit (0.11 ± 0.03 fmol/mg proteins) similar to the level observed on SW620 (0.07 ± 0.04 fmol/mg protein) with poor expression of EGFR in western blot (Fig. 1). EGF treatment (20 ng/ml for 15 min) induced an expected and important upregulation of EGF phosphorylation in all the cell lines.

Moreover, we observed that the cetuximab treatment induced a dramatic inhibition of the phospho-EGFR level EGF-induced in the four cell lines (Fig. 3b) showing that cetuximab binds to the extracellular domain of EGFR and inhibits the EGF-induced phosphorylation of the receptor. In addition, on HCT-8 cells, we showed that cetuximab decreased the level of phospho-EGFR under the basal level.

Cytotoxic effect of L-OHP combined with cetuximab

L-OHP alone: The less sensitive cell lines to L-OHP were HCT-8 ($IC_{50} = 0.87 \pm 0.05$ μM) and HT-29 ($IC_{50} = 0.88 \pm 0.03$ μM). The most sensitive cell lines were SW620 with an IC_{50} of 0.27 ± 0.05 μM and HCT-116 with an IC_{50} of 0.53 ± 0.02 μM (Fig. 4a, b).

L-OHP combined with cetuximab

Sequential: For HCT-8 and HT-29 cell lines, the sequential exposure to L-OHP plus cetuximab decreased the L-OHP IC_{50} of 27% (IC_{50}L-OHP $= 0.64 \pm 0.06$ μM, $P < 0.05$ compared to IC_{50} of L-OHP alone) and 41 % (IC_{50}L-OHP $= 0.52 \pm 0.11$ μM, $P < 0.01$ compared to IC_{50} of L-OHP alone), respectively. Cetuximab did not enhance the cytotoxic effect of LOHP on either HCT-116 or SW-620.

Simultaneous: The simultaneous schedule of exposure to LOHP and cetuximab was synergistic on HCT8 and HT-29 cell lines and decreased the IC_{50} of LOHP by

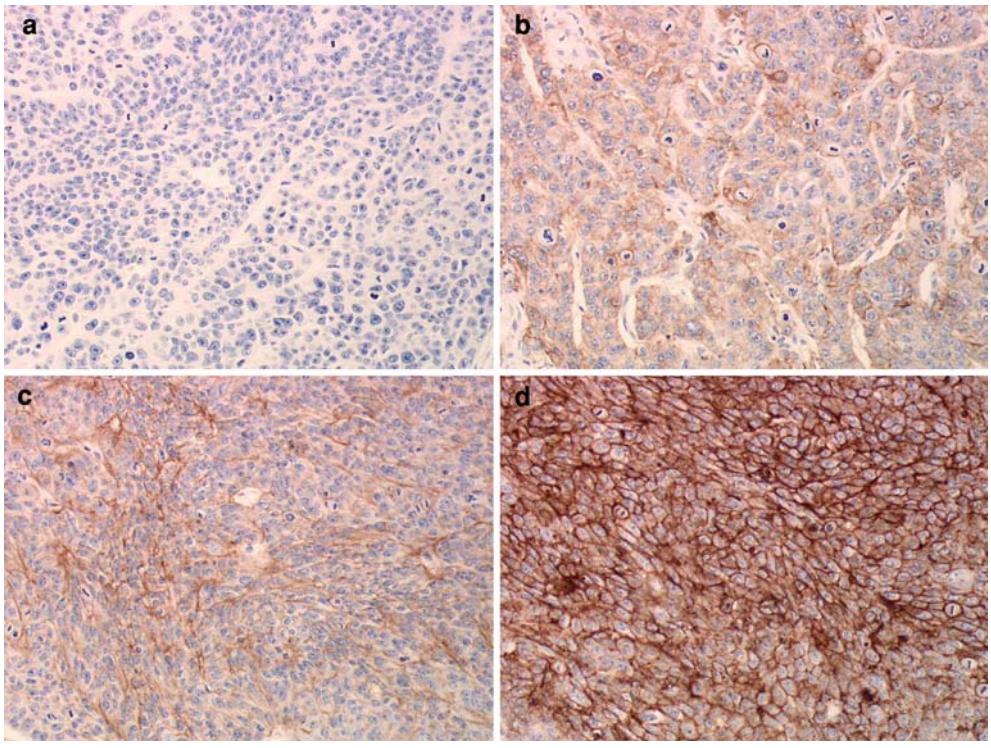

Fig. 2 Expression of EGFR in tumor xenografts established in nude mice (A = SW620 −, B = HT-29 +, C = HCT-8 ++, D = HCT-116 +++)

39% (IC_{50}L-OHP = 0.53 ± 0.07 μM, $P < 0.01$ compared to LOHP alone) and by 45% (IC_{50}L-OHP = 0.49 ± 0.06, $P < 0.005$ compared to LOHP alone), respectively. HCT-116 and SW-620 were still unresponsive to this combination.

Pretreatment: The pretreatment schedule decreased the IC_{50} of L-OHP on HCT-8 and HT-29 by 53% (IC_{50}L-OHP = 0.41 ± 0.06 μM, $P < 0.005$ compared to IC_{50} of L-OHP alone) and by 42% (IC_{50}L-OHP = 0.51 ± 0.22 μM, $P < 0.01$ compared to IC_{50} of L-OHP alone), respectively. HCT-116 and SW-620 remained unresponsive to this schedule of treatment. HCT-8 was the only cell line showing a sequence-dependance to the combined treatment of L-OHP/cetuximab. The "simultaneous" and "pretreatment" schedules were the most efficient exposure (significantly different from "sequential", $P < 0.05$) but these two schedules were not significantly different. Consequently, the "simultaneous" schedule of treatment was chosen to perform the in vivo experiments.

In vivo results

As shown in Fig. 5 and Table 1, the four colorectal tumor xenografts were poorly responsive to cetuximab or L-OHP therapy alone, with no positive effect observed in these cell lines except in HCT-8 cell line. On the other hand, in this cell line, treatment with a single i.v. dose of L-OHP combined with i.p. cetuximab every 3 days induced a dramatic reduction in the tumor growth (Td = 21.6 ± 2.9 days) compared to L-OHP alone (Td = 12.6 ± 2.3 days, $P < 0.001$) and cetuximab alone (Td = 13.4 ± 2.9 days, $P < 0.001$). The ratio observed:expected Td is 1.34. The in vivo combination of oxaliplatin/cetuximab is synergistic in HCT-8 xenografts.

In HT-29 xenograft, resistant to single treatments, the combination of L-OHP plus cetuximab also significantly enhanced (Td = 18.0 ± 2.9 days), but to a lesser extent, the antitumor effect of L-OHP alone (Td = 14.4 ± 3.2 days, $P < 0.001$) and cetuximab alone (Td = 14.5 ± 2.4 days, $P < 0.001$). The ratio observed:expected Td is 1.12. The in vivo combination of oxaliplatin/cetuximab is weakly synergistic in HT-29 xenografts.

However, the combination of cetuximab plus L-OHP was ineffective on HCT-116 (EGFR +++) and SW620 (EGFR −) xenografts, as was with each of the compound alone. In addition, no toxic death was registered and the weight curves of the L-OHP and L-OHP plus cetuximab groups were similar (data not shown), suggesting the absence of additive toxicity when combining cetuximab with L-OHP.

Discussion

The role of EGFR signaling pathway in colorectal cancer has led to considerable interest in the potential therapeutic use of agents that block EGFR signal transduction pathway and/or induce EGFR expression

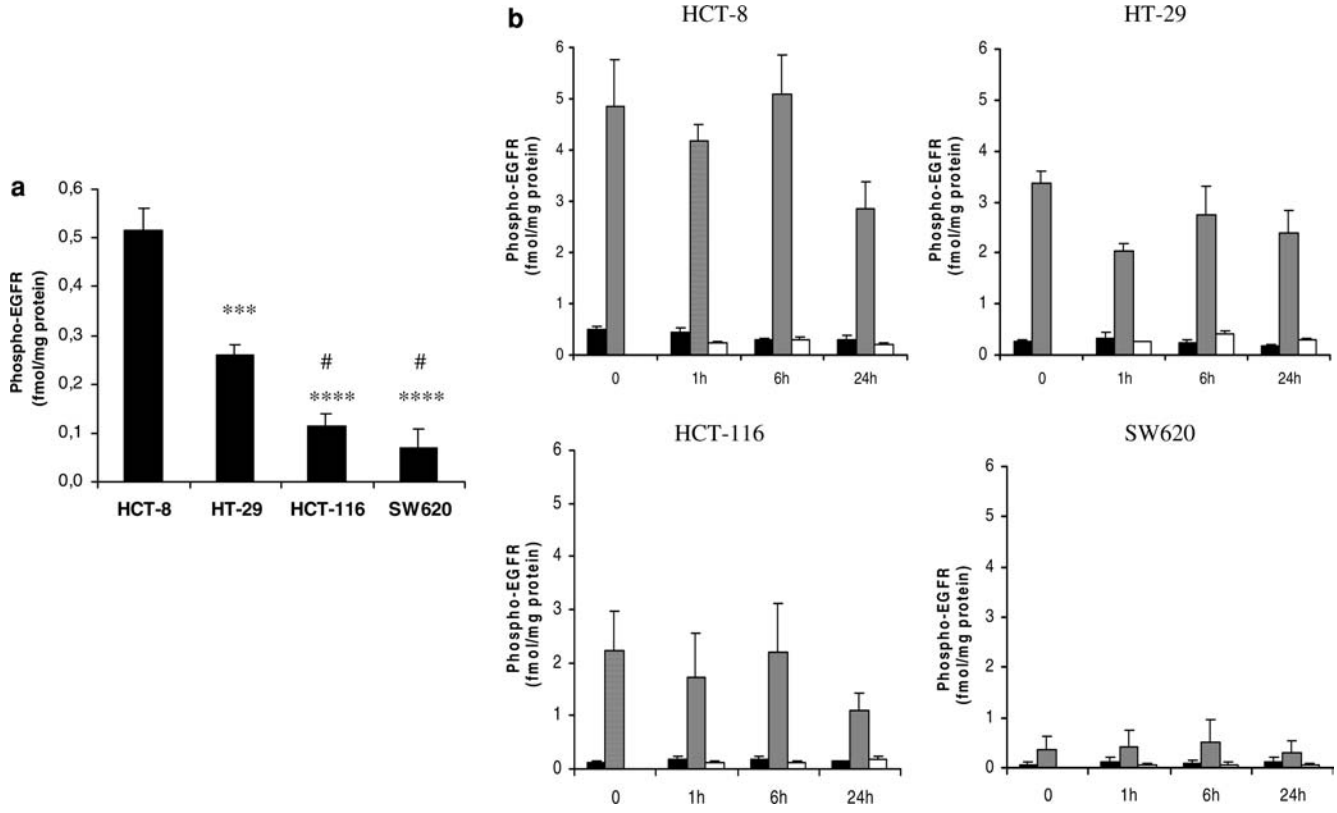

Fig. 3 **a** Basal levels of EGFR phosphorylation in the four cell lines cultured in a FCS-free medium. **b** Effect of cetuximab on EGF-induced EGFR phosphorylation in colon cancer cell lines. Tumor cells plated in 100-mm Petri dishes were serum starved 24 h before experiments. After different incubation time from 0 to 24 h with either serum-free medium (*dotted square*) or 20 μg/ml cetuximab (*open square*), cells were stimulated 15 min with 20 ng/ml EGF. Control cells (*filled square*) were cultured in serum-free medium and not exposed to EGF. The level of phosphorylated EGFR (fmol/mg proteins) was quantified using TaKaRa Kit as described in Material and methods. Data represent the average of three independent experiments, each performed in duplicate. Data are expressed as mean ± SEM. $^{***}P < 0.005$; $^{****}P < 0.0005$ Student's *t* test compared to the basal level of phospho-EGFR of HCT8 cells. $^{\#}P < 0.005$ Student's *t* test compared to the basal level of phospho-EGFR of HT29 cells

down regulation [20, 22]. Previous studies reported that blockade of EGFR pathway can enhance antitumor activity of cytotoxic drugs such as topoisomerase I and II inhibitors [7, 19] and irradiation [12] in various human tumor model, including colorectal xenografts [19]. Consequently, we hypothesized that cetuximab/L-OHP combination would enhance the antitumor activity of L-OHP on a panel of four colon carcinoma cell lines expressing different levels of EGFR that are poorly responsive to either drug alone. Therefore, this panel provides us the opportunity to explore the drug effects regarding the level of EGFR expression.

In the present study, we demonstrated that combination response is cell-line dependent, not correlated with the level of EGFR expression and correlated with the basal level of phospho-EGFR. Overall, the results show that cetuximab treatment produces, in vivo and in vitro, synergistic interaction with L-OHP in both HT-29 and HCT-8 cell lines.

While cell lines are sensitive to L-OHP in vitro with IC_{50} ranging from 0.25 to 1 μM, in vivo findings show that cell lines were completely resistant to L-OHP alone except one, HCT-8 (EGFR moderate), exhibiting a moderate sensitivity. Moreover, in vivo and in vitro cetuximab treatment had a slight effect on HCT-8 while the three other cell lines remained resistant to cetuximab when used as single agent. When cetuximab was associated to L-OHP treatment, the tumor growth of HCT-8 xenografts was significantly ($P < 0.0001$ compared to L-OHP alone) reduced, suggesting that cetuximab synergized the effect of oxaliplatin. It is interesting to note that the combination of both drugs can moderately slow the growth of HT-29 (EGFR weak) xenografts which were insensitive to either cetuximab or oxaliplatin alone. The two other cell lines, SW620 (EGFR negative) and HCT-116 (EGFR strong), were insensitive to L-OHP, cetuximab or combination of both, suggesting that a highly positive expression of EGFR is not required for response to treatment.

These results are consistent with the numerous studies [3, 5, 7, 9, 11, 12, 13, 19, 25] demonstrating that the antitumor activity of several anticancer agents increases when combined with cetuximab. In our study, EGFR expression is necessary to obtain a positive response to the combination of L-OHP plus cetuximab, but the level of expression is not correlated with the response.

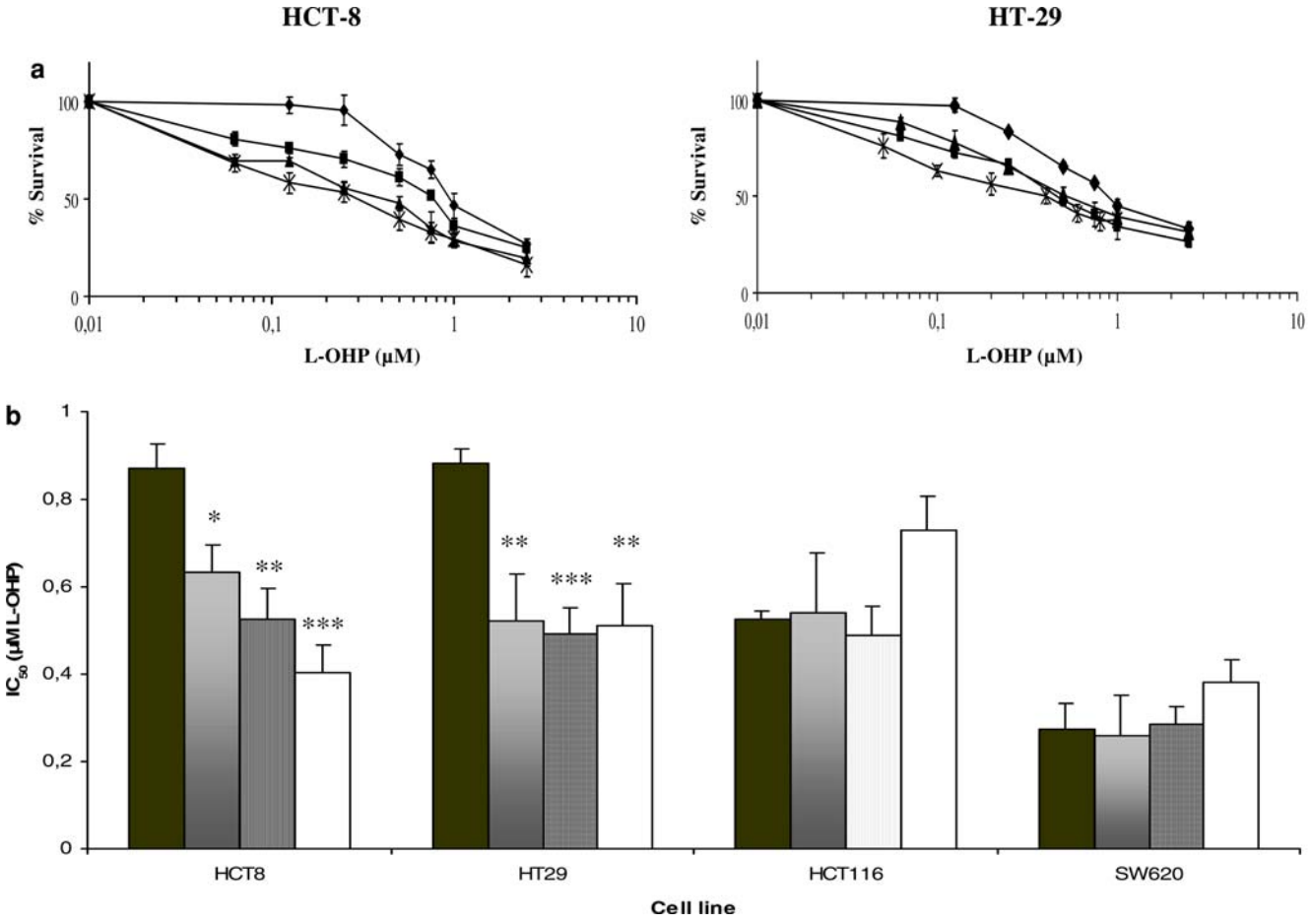

Fig. 4 In vitro effect of L-OHP combined with cetuximab in HCT-8, HT-29, HCT-116 and SW620 human colorectal carcinoma cell lines. **a** Dose-response curves of HCT-8 and HT-29 cells treated with L-OHP alone (*filled diamond*), or combined with cetuximab following different schemes of exposure: sequential L-OHP followed by cetuximab (*filled square*), simultaneous L-OHP plus cetuximab (*filled triangle*), cetuximab 48 h followed by simultaneous L-OHP plus cetuximab (*cross*). **b** Comparison of IC_{50} of L-OHP alone and that combined with cetuximab according to different sequences. Tumor cells were treated with L-OHP alone (*filled square*) from 0.0625 to 2.5 μM for 24 h or combined with a fixed cetuximab concentration of 20 μg/ml every day for 4 days. Three schedules of administration were evaluated: (1) sequential 24-h exposure to L-OHP followed by cetuximab every day (*shaded square*), (2) simultaneous L-OHP and cetuximab 24-h exposure followed by cetuximab every day (*dotted square*), (3) cetuximab 48 h followed by 24-h exposure to L-OHP and cetuximab simultaneously (*open square*). Growth inhibition was evaluated using SRB assay. Data represent the average of five different experiments, each performed in sextuplicate. Data are expressed as mean ± SEM. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ Student's *t* test compared to IC_{50} of L-OHP alone

Our in vitro findings show that cetuximab had few effects on cell proliferation (data not shown) except on HCT-8 with a maximum 10% inhibition of proliferation. This is consistent with our in vivo results where HCT-8 xenograft was also the only tumor type that showed a moderate response to cetuximab monotherapy. We concluded that the antiproliferative effect of cetuximab observed in vitro takes part but cannot fully explain its antitumor activity. Because cetuximab had a very slight antiproliferative effect on our panel of cell lines, we were unable to assess the efficacy of the combined treatment using a standard method to study synergy like Chou and Talalay [8] isobolograms. Thus, we assessed how cetuximab can improve L-OHP effect by measuring the decrease in L-OHP IC_{50} when combined to cetuximab and if the schedule of administration could improve synergistic interaction of combined treatment as described for ZD1839 by Xu et al. [28]. Three schedules of exposure to cetuximab with oxaliplatin were assessed and we showed that the schedule of treatment did not have the same impact regarding the cell type. The combination of cetuximab with L-OHP reduced IC_{50} of oxaliplatin by 53% in HCT-8 and by 39% in HT-29 cells for the simultaneous schedule. Moreover, the sequence of exposure had no impact on the HT-29 response while HCT-8 was more sensitive to simultaneous and pretreatment than sequential schedule. These findings suggest that HT-29 and HCT-8 are different regarding the synergistic mechanism of cetuximab/L-OHP combination. Hence, our in vivo and in vitro studies are similar regarding the response to combined treatment.

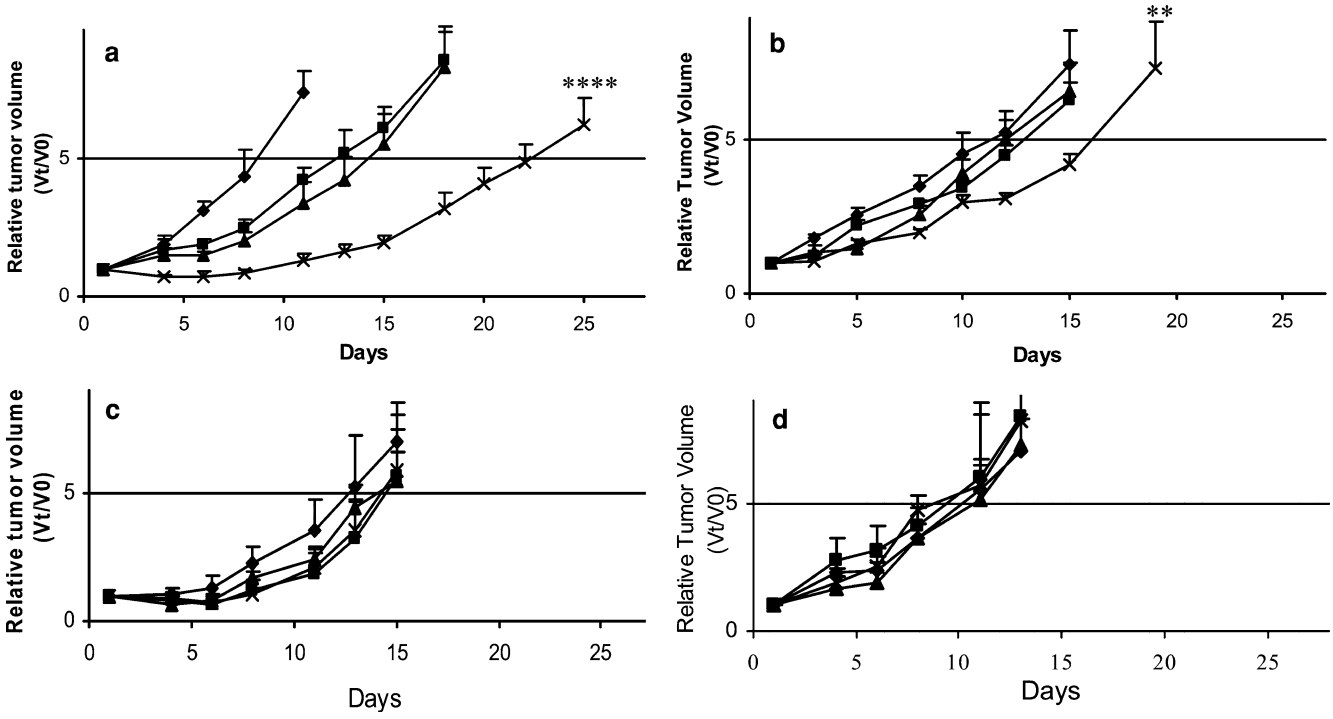

Fig. 5 Effect of L-OHP combined with cetuximab on the growth of HCT-8 (**a**), HT-29 (**b**), SW620 (**c**) and HCT-116 (**d**) colorectal tumor xenografts. Nude mice ($n = 5$–6 per group) bearing subcutaneous 200 mm^3 bilateral tumors received either saline (*filled diamond*), or cetuximab by i.p. route at 1 mg per dose/q3d during all the experiment (*filled triangle*), or L-OHP by i.v. route at 10 mg/kg as a single-injection on day 1 (*filled square*), or L-OHP i.v. at 10 mg/kg as a single injection plus cetuximab i.p. at 1 mg/dose q3d (*cross*). Data are expressed as mean ± SEM. $^{****}P < 0.0001$ and $^{**}P < 0.01$ Mann-Whitney posthoc test compared with L-OHP alone

As observed by Xu et al. [28] for ZD1839/oxaliplatin combination, the sequence of administration of oxaliplatin with cetuximab could play a role in the efficacy of this combination and would be useful to improve the clinical use of cetuximab in colorectal cancer therapeutics. Our findings led us to think that the simultaneous administration of cetuximab with oxaliplatin should be relevant.

Nevertheless, the mechanisms responsible for the combined effects of cetuximab with oxaliplatin are still unclear, strictly dependent on the cell type and require additional experiments to elucidate interactions between the EGFR pathway and oxaliplatin pharmacology.

We assessed the effect of cetuximab on EGFR phosphorylation during EGF stimulation and observed that cetuximab inhibited the EGF-induced phosphorylation, regardless of cell type. Thus, this inhibition cannot totally explain the differences observed between cell lines when cetuximab is combined with oxaliplatin. On the other hand, we showed that the basal level of phospho-EGFR, obtained in serum-starved condition, was correlated with the response to cetuximab in

Table 1 In vivo antitumor activity expressed as tumor delay (in days) in colorectal carcinoma cell lines xenograft administered with oxaliplatin, cetuximab and the combination of both drugs

	Td[a] (Days)					
	Control	Cetuximab	Oxaliplatin	Expected effect of combination[b]	Observed effect of combination	Ratio of observed: expected Td[c]
HCT-8	9.9 ± 2.5	13.4 ± 2.9	12.6 ± 2.3	16.1	21.6 ± 2.9****	1.34
HT-29	12.7 ± 3.3	14.5 ± 2.4	14.4 ± 3.2	16.2	18.0 ± 2.9**	1.12
HCT-116	11.1 ± 2.6	12.0 ± 3.6	11.6 ± 3.7	12.5	13.2 ± 3.6	0.89
SW620	9.8 ± 3.3	11.3 ± 3.8	10.6 ± 3.8	12.1	12.8 ± 4.2	1.05

$^{****}P < 0.0001$ and $^{**}P < 0.01$ Mann-Whitney post-hoc test compared with L-OHP alone. Results are expressed as the mean ± SEM of two independent experiments and tumors volume were measured three times a week ($n = 5$ mice in each group)

[a] *Td* time necessary to reach a tumor volume five times greater than the initial volume

[b] Expected Td of the combination was calculated as Mean control + (mean cetuximab-mean control) + (mean oxaliplatin-mean control)

[c] Obtained by dividing the observed Td by the expected Td of the combination. A ratio >1 indicates a more than additive effect (synergistic) and a ratio <1 indicates a less than additive effect (antagonist)

combination with L-OHP. The highly responsive cell line HCT-8 showed a level of basal phospho-EGFR significatively higher than the three other cell lines ($P < 0.005$ compared to HT29 cells and $P < 0.0005$ compared to HCT-116 and SW620 cells). The moderately responsive HT-29 cell line showed an intermediate level but significatively higher ($P < 0.005$) than the two insensitive cell lines HCT-116 and SW620. Despite its strong EGFR expression in western blot, HCT-116 cell line show a basal level of EGFR phosphorylation that is not significatively different from SW620 (EGFR negative). This basal phosphorylation of EGFR could be an indirect marker of the EGFR-dependent cell response. These data were observed in four cell lines showing a nonmutated catalytic domain of EGFR (data not shown).

The effects of cetuximab on the growth and survival of EGFR positive tumors were well documented and could be explained in preclinical models by several mechanisms. Moreover the inhibition of EGFR tyrosine kinase activation is the direct mechanism of cetuximab activity [2, 11]. EGFR pathway regulated a lot of cellular processes including proliferation, survival, cell cycle progression, which are modified by cetuximab treatment [22]. L-OHP/cetuximab synergistic effect could involve other mechanisms such as the inhibition of the EGF-dependent survival pathway and/or the enhancement of the cytotoxic effect of oxaliplatin.

We hypothesized that inhibition of survival pathway by cetuximab could result in a proapoptotic effect which may enhance the apoptotic potential of cytotoxic drugs such as oxaliplatin. On the other hand, cetuximab could increase the cytotoxic potential of oxaliplatin by decrease of its detoxication. Some studies showed that the effect of cetuximab on DNA repair mechanisms could increase radiosensitivity [11, 12, 19]. DNA damage repair system such as the Nucleotide Excision Repair (NER) plays a key role in oxaliplatin activity. An interaction between the NER system and the EGFR-mediated pathway could significantly enhance the sensitivity of some cell lines to oxaliplatin [1]. Constitutive differences regarding expression of NER proteins could explain the different rate of response between the cell lines.

Recently, an international phase II clinical investigation [27] shows that 5-fluorouracil plus folinic acid plus oxaliplatin (FOLFOX-4) combined with cetuximab may be effective and safe as first line treatment of patients with metastatic colorectal cancer. These findings and the results of our study drive us to think that further experiments should be useful to explain the mechanisms of this synergistic effect.

In conclusion, our results demonstrated that combination of cetuximab with L-OHP could enhance L-OHP antitumor activity. This synergy is dependent on the cell line, basal phospho-EGFR level and independent of the EGFR status. The combination of cetuximab with oxaliplatin could be a promising therapeutic strategy in some oxaliplatin refractory colorectal carcinoma.

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