

ORIGINAL ARTICLE

Celastrol enhanced the anticancer effect of lapatinib in human hepatocellular carcinoma cells *in vitro*

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Summary

Purpose: To investigate whether celastrol could show synergism combined with lapatinib in HepG2 human hepatocellular carcinoma (HCC) cell line *in vitro*.

Methods: The effects of treatment with lapatinib and/or celastrol on cell growth were determined using MTT assay. Drug synergy was determined using combination index (CI) methods derived from Chou-Talalay equations using CalcuSyn software. Apoptotic morphology was observed by fluorescence microscope with Hoechst 33258 staining. The expression of EGFR of cell surface was performed by flow cytometry. Changes of apoptotic and growth pathways-re-

lated proteins were analysed by Western blotting.

Results: The combination of celastrol and lapatinib produced strong synergy in growth inhibition and apoptosis *in vitro* in comparison to single-agent treatments. Moreover, celastrol enhanced the ability of lapatinib to down regulate EGFR protein expression in HepG2 cells.

Conclusion: These data indicate that the combination of celastrol and lapatinib could be used as a novel combination regimen which could hopefully provide strong anticancer synergy in the treatment of HCC.

Key words: celastrol, EGFR, HepG2, lapatinib, synergy

Introduction

HCC is a common and aggressive malignant tumor worldwide, with morbidity showing a rising trend [1]. Currently, the therapeutic interventions for HCC are mainly surgery and chemotherapy, but the activity of the existing chemotherapeutic drugs is not good enough and they also have numerous side-effects [2,3]. Many selective inhibitors of HER family receptor tyrosine kinases (RTK), including erlotinib, gefitinib, and lapatinib (Figure 1), have become important therapeutics against multiple solid cancers including HCC [4,5]. However, due to the molecular heterogeneity among and within tumors, their efficacy is restricted to only a small subset of patients. The efficacy of RTK inhibitors is also limited by drug resistance mechanisms that frequently emerge following treatment [6]. Several strategies

have been proposed to overcome the low response rate and acquired resistance to RTK inhibitors. One particularly promising approach is the modulation of RTK pathways by the inhibition of the EGFR expression.

Celastrol (Figure 1), purified from the plant *Tripterygium wilfordii*, also called the "Thunder of God Vine", has been used as a natural remedy in Chinese medicine for over 2,000 years. In the past decade, celastrol has become the focus of numerous preclinical studies that have shown its potential for use in a wide range of conditions, from inflammatory diseases such as arthritis and Crohn's disease to neurologic diseases such as Alzheimer's and amyotrophic lateral sclerosis [7,8]. More recently, both *in vitro* and *in vivo* studies have yielded results suggesting that celastrol may also be effective in the treatment of chemoresistant neoplasms including pancreatic cancer,

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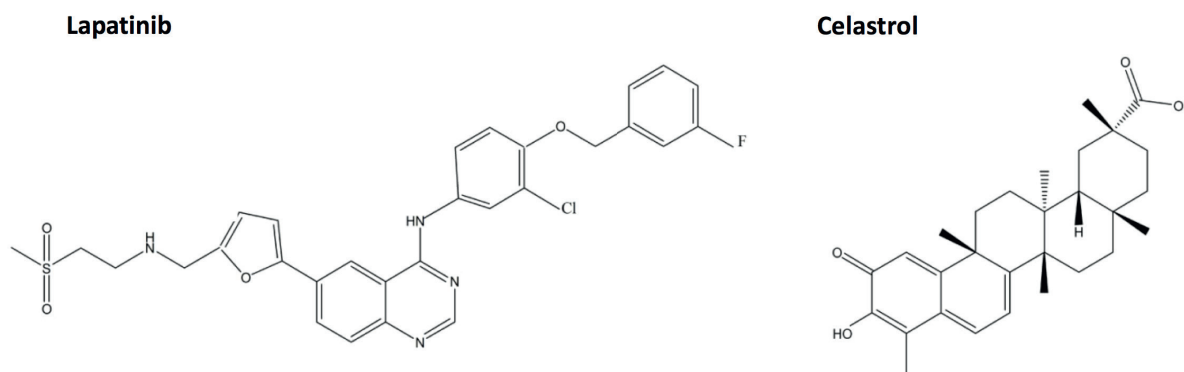


Figure 1. Chemical structure of Lapatinib and Celastrol.

glioma, and melanoma [9,10]. Preclinical studies in melanoma have shown that celastrol synergistically enhances temozolomide cytotoxicity in melanoma cells [9].

In this article we describe for the first time that the combination of celastrol and lapatinib produced strong synergy in growth inhibition and apoptosis *in vitro* in HepG2 cells in comparison to single-agent treatment.

Methods

Chemicals and reagents

Lapatinib for this study was purchased from Glaxo Smith Klein Co. (Philadelphia, PA, USA). Celastrol was purchased from PayPay Technologies (Guangdong, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). Antibodies against caspase-3, and p-EGFR were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against EGFR (for FCM and WB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt, ERK1/2, p-Akt, p-ERK1/2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-mouse IgG-horseradish peroxidase, and anti-rabbit IgG-horseradish peroxidase were purchased from KangChen Biotechnology (Shanghai, China). All tissue culture supplies were purchased from Life Technologies (Carlsbad, CA, USA). Other routine laboratory reagents of analytical or high-performance liquid chromatography grade were obtained from Whiga Biotechnology (Guangzhou, China).

Cell lines and cell culture

The human HCC cell line HepG2 was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium, which contained 100 U/mL penicillin, 100 U/mL streptomycin, and 10% fetal bovine serum. All cells were cultured

in a humidified atmosphere incubator with 5% CO₂ and 95% air at 37 °C.

Cytotoxicity assays and analysis of drug synergy

The effects of treatment with lapatinib or/and celastrol on cell growth were determined using MTT assay [11]. After 68 h of treatment, 20 µL MTT (5 mg/mL stock solution of saline) were added to each well for 4 h. Subsequently, the supernatant was removed, and MTT crystals were solubilized with 100µL anhydrous DMSO in each well. Thereafter, cell viability was measured by model 550 microplate reader (Bio-Rad) at 540 nm, with 655 nm as reference filter. The 50% inhibitory concentration (IC₅₀) was determined as the anticancer drug concentration causing 50% reduction in cell viability and calculated from the cytotoxicity curves (Bliss' software). Percent cell survival was calculated using the following formula: survival (%) = [(mean experimental absorbance) / (mean control absorbance)] × 100%. Drug synergy was determined using CI methods derived from the Chou-Talalay equations [12] using CalcuSyn software (Biosoft, Cambridge, UK). A CI value of 1 indicated an additive effect between two agents, whereas a CI value of <1 indicated synergy.

Assessment of apoptosis morphology by Hoechst 33258 staining

HepG2 cells were treated with the indicated concentrations of lapatinib or/and celastrol for 48 h; both floating and trypsinized adherent cells were collected, washed once with ice-cold PBS, fixed with 1 mL of 4% paraformaldehyde for 20 min, and washed once with ice-cold PBS. Then, the cells were incubated in 1 mL PBS containing 10 µmol/L Hoechst 33258 at 37 °C for 30 min, washed twice, and observed using fluorescence microscopy with standard excitation filters (Leica, Germany) in random microscopic fields at × 400 magnification.

Expression of EGFR protein analysed by flow cytometry

Determination of the cell surface EGFR expression

was performed according to the manufacture's instruction. HepG2 cells (6×10^5) were seeded in 25 cm^2 flasks and allowed to attach. After treatment with the indicated concentrations of lapatinib or/and celestrol for 48 h, both floating and attached cells were collected and washed with ice-cold PBS twice. Single-cell suspensions were prepared by the addition of 0.5 mmol/L EDTA followed by 3 washes with an isotonic PBS buffer [supplemented with 0.5% bovine serum albumin (BSA)]. Then, HepG2 cells ($100 \mu\text{L}$) were incubated at 4°C for 45 min with $20 \mu\text{L}$ of R-PE-conjugated anti-human EGFR reagent. Following this incubation, cells were washed twice with PBS buffer (supplemented with 0.5% BSA) and the supernatant was discarded. Finally, the cells were resuspended in $400 \mu\text{L}$ PBS buffer for flow cytometric analysis. Isotype control samples were treated in an identical manner with PE-labeled mouse IgG2bk antibody [13].

Whole-cell lysates and Western blot analysis

After HepG2 cells were exposed to the indicated concentrations of lapatinib and/or celestrol for 48 h, whole cells were harvested and washed twice with ice-cold PBS, and the pellet was vortexed and $1 \times$ lysis buffer [50 mmol/L Tris- HCl (PH 6.8), 10% glycerol, 2% SDS, 0.25% bromophenol blue, and 0.1 mol/L DTT] was added for $100 \mu\text{L}/5 \times 10^6$ cells. After being heated at 95°C for 20 min, the lysates were centrifuged at $12,000 \text{ rpm}$ for 10 min and the supernatant was collected. The protein concentration was determined by nucleic acid-protein analyzer (Beckman, USA). Equal amount of lysate protein was separated on $8\text{-}12\%$ SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Pall). The nonspecific binding sites were blocked with TBST buffer [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), and 0.4% (v/v) Tween 20] containing 5% nonfat dry milk for 2 h. The membranes were incubated over-

A The inhibitory effects of Celestrol combined with Lapatinib on proliferatin of HepG2 cells

Lapatinib		Celestrol		Lapatinib+Celestrol		
Concentration ($\mu\text{mol/L}$)	Inhibition Rate	Concentration ($\mu\text{mol/L}$)	Inhibition Rate	Inhibition Rate	Combination Index	Effect
0.25	13.27 ± 2.56	0.125	5.96 ± 0.88	17.30 ± 2.01	0.831	Synergistic
0.5	20.39 ± 0.49	0.25	12.55 ± 0.39	31.72 ± 1.46	0.560	Synergistic
1	28.99 ± 2.36	0.5	20.98 ± 1.37	42.42 ± 1.63	0.557	Synergistic
2	36.86 ± 4.00	1	29.09 ± 2.34	53.82 ± 1.15	0.560	Synergistic
4	46.28 ± 4.57	2	42.52 ± 0.37	69.23 ± 1.01	0.424	Synergistic
8	55.11 ± 3.60	4	54.10 ± 1.27	75.87 ± 1.61	0.518	Synergistic
16	67.34 ± 2.27	8	63.76 ± 0.95	86.54 ± 1.63	0.373	Synergistic

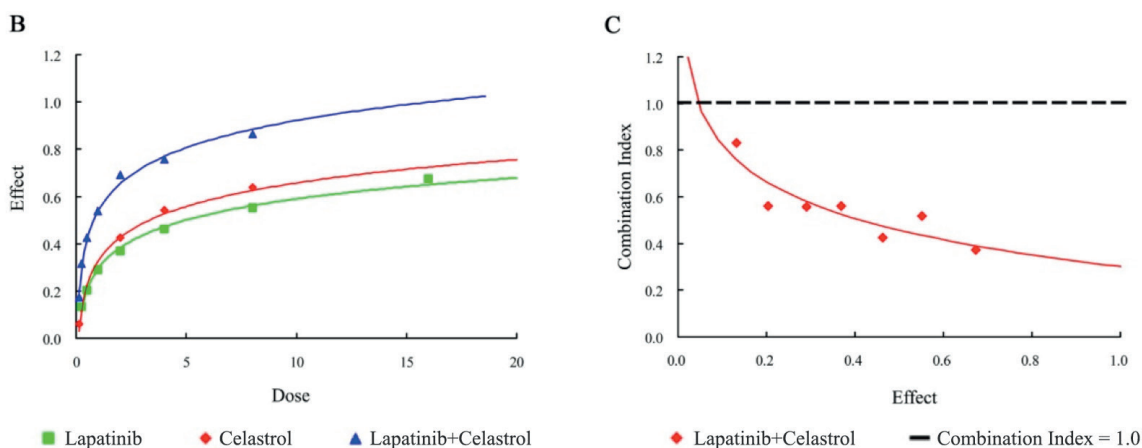


Figure 2. Lapatinib in combination with celestrol synergistically induced cytotoxicity in HepG2 cells. HepG2 cells were treated with lapatinib as a single drug or in combination with celestrol as described in the methods section. Drug combinations included serial dilution at 2:1 for lapatinib plus celestrol. Lapatinib plus celestrol combination included variable celestrol (0.125 to $8 \mu\text{mol/L}$) and lapatinib (0.25 to $16 \mu\text{mol/L}$) concentrations. Controls included untreated and DMSO treated cells. Growth inhibition of cells was then evaluated by MTT assay. Shown here is the comparison of cytotoxicity of single drug treatments or combination of lapatinib with celestrol, showing efficacy of the combinatorial treatment over single drugs. **A:** Chou-Talalay analyses for celestrol plus lapatinib combination. Combination index (CI) < 1 indicates synergy; CI = 1 indicates additive effects; CI > 1 indicates antagonism. **B:** Dose-effect plot. **C:** CI-effect plot.

night at 4 °C with specific primary antibodies. Then, the membranes were washed 3 times with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody. After 3 washes with TBST buffer, the immunoblots were visualized by the enhanced Phototope-Horseradish Peroxidase Detection Kit purchased from Cell Signaling Technology (Danvers, MA, USA) and exposed to Kodak medical X-ray processor [14].

Statistics

For each protocol, 3 independent experiments were performed. Results were expressed as the mean±standard error of the mean (SEM). Statistical calculations were performed by using SPSS16.0 software. Differences in measured variables between experimental and control groups were assessed by the Student's t-test. $p < 0.05$ was indicative of significant difference and $p < 0.01$ was indicative of very significant difference.

Results

Lapatinib/celastrol synergistically inhibit HepG2 cell proliferation

Combination treatment with lapatinib/celastrol yielded significantly greater growth inhibition than either lapatinib or celastrol treatment alone (Figure 2A and B). As shown in Figure 2A and C, CI values were < 1 at all doses of lapatinib tested (range 0.25-16 $\mu\text{mol/L}$), indicating that celastrol (range 0.125-8 $\mu\text{mol/L}$) was synergistic with lapatinib across a broad range of concentrations.

Celastrol significantly enhanced the ability of lapatinib to induce apoptosis in HepG2 cells

To assess the ability of celastrol to sensitize HepG2 cells to lapatinib, we observed the morphologic characteristics of apoptosis. Control cells showed even distribution of the stain and round homogeneous nuclei. Apoptotic cells displayed typical changes including reduction of cellular volume, staining bright and condensed or fragmented nucleus. More apoptotic bodies were observed in the lapatinib/celastrol combination compared with lapatinib or celastrol treatment alone (Figure 3A-D).

Celastrol enhanced the ability of lapatinib to down-regulate EGFR protein expression in HepG2 cells

To assess the ability of celastrol to enhance the anticancer effect of lapatinib in HepG2 cells, we also determined the expression of EGFR of cell surface. Flow cytometry revealed when cells were

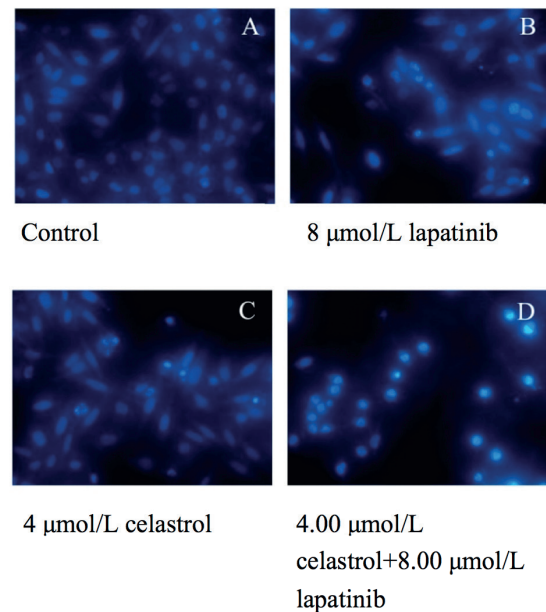


Figure 3. The combination of celastrol and lapatinib induced more markedly apoptotic morphologic changes than lapatinib or celastrol alone in HepG2 cells. The apoptotic bodies of HepG2 cells were detected by Hoechst 33258 staining and observed under fluorescence microscope in random microscopic fields at $\times 400$ magnification. **A:** Control; **B:** 8.00 $\mu\text{mol/L}$ lapatinib; **C:** 4.00 $\mu\text{mol/L}$ celastrol; **D:** 8.00 $\mu\text{mol/L}$ lapatinib + 4.00 $\mu\text{mol/L}$ celastrol.

treated with the combination celastrol (4 $\mu\text{mol/L}$) and lapatinib (8 $\mu\text{mol/L}$), a strong synergy was observed in the expression of EGFR of cell surface compared with celastrol or lapatinib alone (Figure 4A). Isotype control samples were treated in an identical manner with PE-labeled mouse IgG2b κ antibody. The expression levels of EGFR of cell surface were $93.60\% \pm 2.62$ for control, $86.46\% \pm 1.75$ for lapatinib alone, $51.53\% \pm 2.49$ for celastrol alone, and $30.53\% \pm 1.68$ for lapatinib plus celastrol combination (Figure 4B).

Western blotting further revealed that when cells were treated with the combination celastrol (4 $\mu\text{mol/L}$) and lapatinib (8 $\mu\text{mol/L}$), a strong synergy in growth inhibition and apoptosis *in vitro* in comparison to single treatments was obvious, showing more activation of apoptotic-related protein (caspase-3) and downregulation of growth pathway-related proteins (EGFR, P-EGFR, p-Akt, p-ERK1/2) (Figure 5).

Discussion

Molecular targeting therapy for cancer treatment is a fast-growing research field in oncology. EGFR is often overexpressed, dysregulated or mutated in many epithelial malignancies, and

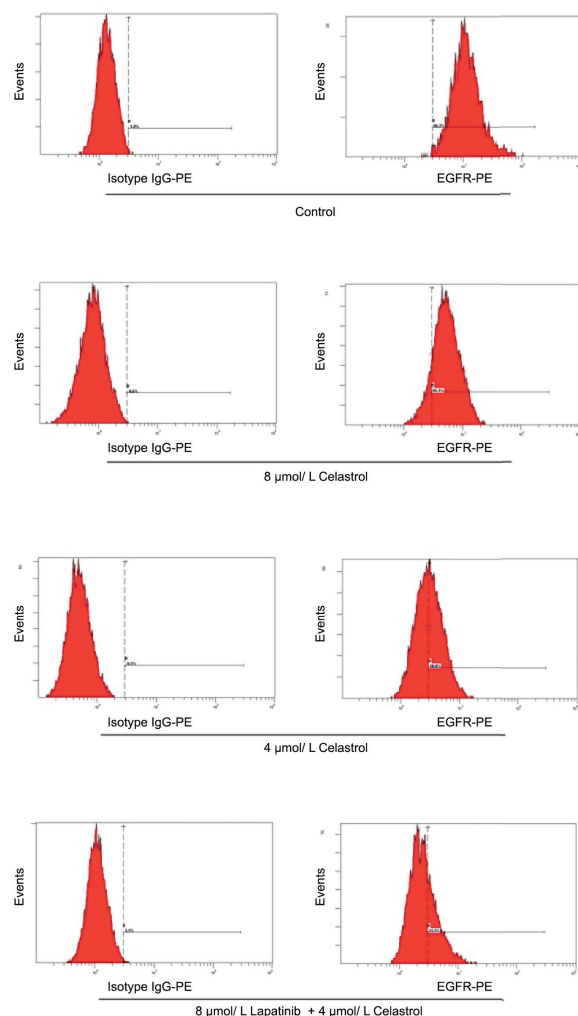
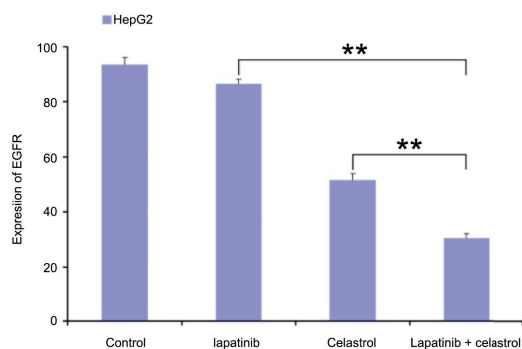
A**B**

Figure 4. The combination of celestrol and lapatinib further induced decrease of EGFR membrane protein in HepG2 cells. **A:** Determination of the expression of EGFR of cell surface by flow cytometry; isotype control samples were treated in an identical manner with PE-labeled mouse IgG2b κ antibody; **B:** Statistical analysis of EGFR expression. Mean $\pm$ SD of three assays. ** $p < 0.01$, compared with lapatinib/celestrol combination treatment, $n=3$.

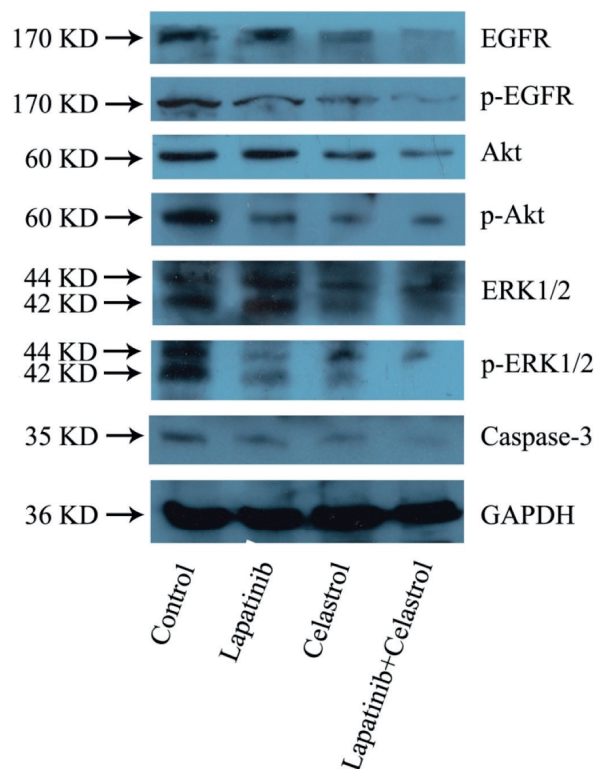


Figure 5. Celestrol enhances lapatinib-induced apoptosis and reduces tumor growth in HepG2 cells. Cells were treated with indicated concentrations of lapatinib and/or celestrol for 48 h. Equal amounts of protein from cell lysates were analyzed for changes in EGFR and p-EGFR protein level, inhibited downstream signaling pathways and induced apoptosis by Western blot. GAPDH was used as a loading control.

these abnormal alterations of EGFR activate a series of intracellular signaling pathways and play a central role in many of the processes involved in tumor growth and progression, such as proliferation, angiogenesis, invasiveness, metastasis, decreased apoptosis, and loss of differentiation [15,16]. Consequently, EGFR has been proposed as a rational molecular target for anticancer strategies. Currently, two predominant classes of anti-EGFR targeting agents have been developed for use in the clinic, including monoclonal antibodies such as cetuximab that blocks the ligand binding to the extracellular domain of EGFR and prevents receptor activation, and small molecule TKIs such as gefitinib, erlotinib and lapatinib that compete with ATP to bind to the intracellular receptor catalytic domain of EGFR [17,18].

Lapatinib is an inhibitor of the intracellular tyrosine kinase domains of both the EGFR and HER-2 receptors. Mutations or dysregulation in these receptors have been shown to play a role in the development of certain cancers. Lapatinib was approved for use in combination with capecit-

abine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpressed HER-2 and who had received prior therapy with an anthracycline, a taxane, and trastuzumab. As the new TKIs are being introduced into the clinic, a significant effort will be directed toward increasing the anticancer activity of conventional chemotherapeutic agents or restoring chemosensitivity of resistant cancer cells to conventional chemotherapeutic agents [13]. However, due to the heterogeneous and dynamic nature of tumors, the effectiveness of these agents is often hindered by poor response rates and acquired drug resistance. Several strategies have been proposed to overcome the low response rate and acquired resistance to RTK inhibitors. One particularly promising approach is the modulation of RTK pathways by inhibiting the expression of EGFR. To further explore the versatility of this idea, we proposed a novel strategy with celastrol to improve lapatinib effectiveness in HepG2 cells.

Celastrol is a natural product used in the traditional Chinese medicine that has demonstrated ability to inhibit cancer progression and downregulate NF-kappa B activity, and also to decrease NF-kappa B activity in prostate cancer and leukemia cells [19-21]. However, it had not previously been evaluated as a potential chemotherapeutic sensitizer for HCC. Our results showed for the first time that the combination of lapatinib and celastrol synergistically inhibited the proliferation and induced apoptosis of HepG2 cells *in vitro*. In addition, this combined treatment showed inhibitory capacity across a broad range of concentrations (Figure 2A). More apoptotic bodies were observed in the lapatinib/celastrol combination compared with lapatinib or celastrol treatment alone (Fig-

ure 3A-D). Moreover, the combination of lapatinib with celastrol resulted in lower levels of EGFR of cell surface compared with either agent alone (Figure 4). Western blotting further revealed that the combination of celastrol and lapatinib produced strong synergy in growth inhibition and apoptosis-inducing capabilities *in vitro* in comparison to single treatments, indicating enhanced activation of apoptotic related protein (caspase-3) and downregulation of growth pathway related proteins (EGFR, P-EGFR, Akt, p-Akt, ERK1/2, p-ERK1/2) (Figure 5). All these observations could imply involvement of the MAPK and PI3K/Akt pathway in the observed synergistic effects. These results also indicated that lapatinib's antitumor effect (inhibiting EGFR activity) could be amplified by celastrol (downregulating the expression of EGFR) in HepG2 cells.

In summary, our results indicated that celastrol could enhance the cytotoxicity and apoptotic capacity of lapatinib. Mechanistic studies consistently showed that celastrol not only directly inhibited the expression of EGFR, but also had the potential to achieve further synergy in tumor suppression when combined with lapatinib. Celastrol may also be useful in combination therapies by enhancing the efficacy of targeting agents. This work also showed a practical approach to improve HCC therapy.

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