Purpose: To investigate the effect and related molecular mechanisms of lapatinib/celastrol combination or single-agent treatment in HER2/neu-overexpressing MDA-MB-453 breast cancer cells.

Methods: The effects of treatment with lapatinib, celastrol or their combination on cell growth were determined using MTT assay. Drug synergy was determined using the combination index (CI) methods derived from Chou-Talalay equations using CalcuSyn software. Apoptotic morphology was observed by fluorescence microscope with Hoechst 33258 staining. Changes of apoptotic and growth pathways-related proteins were analysed by Western blot. The expression of HER2 of cell surface was performed by flow cytometry. Subcellular distribution of HER2 was observed by immuno-fluorescence study.

Results: Combination celastrol and lapatinib produced strong synergy in growth inhibition and apoptosis in comparison to single-agent treatment in HER2/neu-overexpressing MDA-MB-453 cells. Interestingly, compared with celastrol treatment alone, lapatinib/celastrol combination induced more HER2 membrane protein downregulation and ectopic to cytoplasm and nucleus in MDA-MB-453 cells.

Conclusion: The combination of celastrol and lapatinib could be used as a novel combination regimen which provides a strong anticancer synergy in the treatment of HER2/neu-overexpressing cancer cells.

Key words: celastrol, HER2, lapatinib, MDA-MB-453 cells, synergy

Introduction

It is well known that overexpression of HER2/neu is associated with a poor prognosis in many malignancies, including breast, ovarian, lung and prostate cancer, etc [1-4]. During the last decade, HER2/neu has been the focus for development of novel anticancer drugs in the form of small molecules (e.g. lapatinib) or monoclonal antibodies (e.g. herceptin), which have shown promising results [5,6]. However, their efficacy and long-term use in patients are quite limited due to resistance to these inhibitors or severe side effects. Therefore, novel therapeutic strategies in this field are still required.

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

Our previous data have shown that celastrol enhanced the anticancer effect of lapatinib in HepG2 human hepatocellular carcinoma cells in vitro [11]. In this study, we further investigated the effect and related molecular mechanisms of lapatinib/celastrol combination in HER2/neu-overexpressing MDA-MB-453 cells.

Methods

Chemicals and Reagents

Lapatinib was purchased from Glaxo Smith Klein Co (Philadelphia, PA, USA). Celastrol was purchased from PayPay Technologies (Guangdong, China). Anti-mouse IgG-fluorescein isothiocyanate (FITC) (for immunofluorescence assay), 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-9, caspase-3 and p-HER2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against HER2 for flow cytometry and western blot were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against 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 purchased from Whiga Biotechnology (Guangzhou, China).

Cell lines and cell culture

Human breast cancer cell lines MDA-MB-453 obtained from cell bank of Chinese Academy of Medical Sciences (Beijing, China) were grown in DMEM medium containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS). Cells were cultured in a humidified atmosphere incubator of 5% CO₂ and 95% air at 37°C.

Cytotoxicity assays and analysis of drug synergy

The effects of treatment with lapatinib and/or celastrol on cell growth were determined using MTT assay [12]. After 68-h treatment, 20 μL MTT (5 mg/mL stock solution of saline) were added to each well for 4 hrs. 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, Hercules, CA, USA) 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 combination index (CI) methods derived from Chou-Talalay equations [13] using CalcuSyn software (Biosoft, Cambridge, UK). A CI value of 1 indicates an additive effect between two agents, whereas a CI value of <1 indicates synergy.

Assessment of apoptosis morphology by Hoechst 33258 staining

MDA-MB-453 cells were treated with the indicated concentrations of lapatinib and/or celastrol for 48 hrs. 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.

**Whole-cell lysates and Western blot analysis**

After MDA-MB-453 cells were exposed to the indicated concentrations of lapatinib and/or celastrol for 48 hrs, whole cells were harvested and washed twice with ice-cold PBS, the pellet was vortexed and 1× 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 μL/5×10^6 cells. After heated at 95°C for 20 min, the lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The protein concentration was determined by nucleic acid-protein analyzer (Beckman, CA, USA). Equal amount of lysate protein was separated on 8% to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Pall). The nonspecific binding sites were blocked with Tris-Buffered Saline and Tween 20 (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 hrs. The membranes were incubated overnight at 4°C with specific primary antibodies. Then, the membranes were washed three times with TBST buffer and incubated at room temperature for 1 hr with horseradish peroxidase-conjugated secondary antibody. After three 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 (Rochester, NY, USA) [14].

**Expression of HER2 protein analysed by flow cytometry**

Determination of the expression of HER2 of cell surface was performed according to manufacturer’s instruction. MDA-MB-453 cells (6×10⁵) were seeded in 25 cm² flasks and allowed to attach. After treated with the indicated concentrations of lapatinib and/or celastrol for 48 hrs, 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 three washes with an isotonic PBS buffer [supplemented with 0.5% bovine serum albumin (BSA)]. Then, MDA-MB-453 cells (100μL) were incubated at 4°C for 45 min with 20μL of R-Phytoerythrin (R-PE)-conjugated anti-human HER2 reagent. Follow ing this incubation, cells were washed twice with PBS buffer (supplemented with 0.5% BSA) and the supern tant was discarded. Finally, the cells were resuspended in 400 μL PBS buffer for flow cytometric analysis. Isotype control samples were treated in an identical manner with PE-labeled mouse IgG2bκ antibody [15].

**Immunofluorescence assay**

After MDA-MB-453 cells grown on coverslips were treated with the indicated concentrations of lapatinib and/or celastrol for 48 hrs, cells were fixed with 4% polyoxymethylene for 20 min, and Triton X-100 was added for 10 min at room temperature. Then, the cells were rinsed with PBS for three times, and the non-specific binding sites were blocked in PBS with 1% BSA for 1 hr. The cells were incubated overnight at 4°C with HER2 antibody followed by FITC-conjugated secondary antibody at room temperature for 1 hr. Subcellular distribution of HER2 protein was observed under fluorescence microscopy with standard excitation filters (Leica Dmirb) in random microscopic field at ×400 magnification [14].

**Statistics**

For each protocol, three independent experiments were performed. The results were expressed as 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 and p<0.01 was indicative of very significant statistical difference.

**Results**

**Lapatinib/celastrol synergistically inhibit cell proliferation in MDA-MB-453 cells**

The growth inhibitory effects on MDA-MB-453 cells of lapatinib and/or celastrol were determined using MTT assay. Our results showed that the combination treatment with lapatinib/celastrol yielded significantly greater growth inhibition than 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, 0.234375-30μmol/L), indicating that celastrol (range, 0.234575-50μmol/L) was synergistic with lapatinib across a broad range of concentrations.

**Lapatinib/celastrol combination synergistically induced MDA-MB-453 cells apoptosis**

To assess the ability of celastrol to sensitize MDA-MB-453 cells to lapatinib, we observed the morphologic characteristics of apoptosis. Control cells showed even distribution of the stain and round homogeneous nuclei features. 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 3). Western blot further
revealed that when the cells were treated with the combination of celastrol (5μmol/L) and lapatinib (5μmol/L) a strong synergy in growth inhibition and apoptosis in vitro in comparison to single treatments was shown, indicating more activation of apoptotic related proteins (caspase-9 and caspase-3) and downregulation of growth pathway related proteins (HER2, P-HER2, p-Akt, p-ERK1/2) (Figure 4).

**Lapatinib enhanced the ability of celastrol to decrease HER2 membrane protein expression in MDA-MB-453 cells**

To further investigate the mechanism of lapatinib/celastrol combination that synergistically induced MDA-MB-453 cell apoptosis, we also determined the expression of HER2 of cell surface. Flow cytometry revealed that treating the cells with the combination of celastrol (5μmol/L) and lapatinib (5μmol/L) produced strong synergy in decreasing the expression of HER2 of the cell surface compared with celastrol or lapatinib alone (Figure 5). Isotype control samples were treated in an identical manner with PE-labeled mouse IgG2bκ antibody. The expression levels of HER2 of the cell surface were 94.77±1.15% for the control, 87.13±1.51% for lapatinib alone, 30.23±0.93% for celastrol alone, and 20.57±1.72% for lapatinib plus celastrol combination, respectively.

**More changes of subcellular distribution of HER2 induced by lapatinib/celastrol combination in MDA-MB-453 cells**
To further investigate the mechanism of the downregulation of HER2 protein, immunofluorescence study with anti-HER2/neu antibody was also performed. As shown in Figure 6, the control cells had strong immunofluorescence at the plasma membrane. After celastrol treatment, the immunofluorescence at the plasma membrane was attenuated and replaced with diffuse cytoplasmic and nuclear punctate staining. More changes of subcellular distribution of HER2 were observed by lapatinib/celastrol combination than celastrol treatment alone in MDA-MB-453 cells.

Discussion

Molecular targeting therapy for cancer treatment is a fast-growing research field in oncology [6]. Lapatinib is an inhibitor of the intracellular tyrosine kinase domains of both the EGFR and HER2 receptors. Mutations or dysregulation in these receptors has been shown to play a role in the development of certain cancers. Lapatinib was approved for use in combination with capecitabine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpressed HER2 and who had received prior therapy with anthracycline, taxane, and trastuzumab. As the new tyrosine kinase inhibitors (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 [14]. 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 TKIs. One particularly promising approach is the modulation of TKI pathways by inhibiting the expression of HER2. To further explore the versatility of this idea, we proposed a novel strategy with celastrol to improve lapatinib therapy in HER2/neu-overexpressing MDA-MB-453 cells.

Celastrol is a natural product used in traditional Chinese medicine that has demonstrated ability to inhibit cancer progression and down-regulate NF-kappa B activity in prostate cancer and leukemia cells [16-18]. Our previous data has shown that celastrol enhanced the anticancer effect of lapatinib in HepG2 human hepatocellular carcinoma cells in vitro [10]. In this study, we further investigated the effect and related molecular mechanisms of lapatinib/celastrol combination in HER2/neu-overexpressing MDA-MB-453 cells.
Our results showed for the first time that the combination of lapatinib and celestrol synergistically inhibited the proliferation and induced apoptosis in MDA-MB-453 cells in vitro. Treatment with celestrol plus lapatinib was synergistic in inhibiting MDA-MB-453 cell growth across a broad range of concentrations (Figure 2). More apoptotic bodies were observed in the lapatinib/celastrol combination compared with lapatinib or celestrol treatment alone (Figure 3). Moreover, combination treatment with lapatinib/celastrol resulted in lower levels of HER2 of the cell surface compared with lapatinib/celastrol combination treatment, n=3.

Figure 5. Combination of celestrol and lapatinib further induced decrease of HER2 membrane protein in MDA-MB-453 cells. A: Determination of the expression of HER2 of cell surface by flow cytometry: isotype control samples were treated in an identical manner with PE-labeled mouse IgG2bκ antibody. B: Statistical analysis of the expression of HER2. Mean±SD of three assays. ** p<0.01 compared with lapatinib/celastrol combination treatment, n=3.
HER2 distribution induced by lapatinib/celastrol combination

with either agent alone (Figure 5). Interestingly, more changes of subcellular distribution of HER2 were observed by lapatinib/celastrol combination than celastrol treatment alone in MDA-MB-453 cells (Figure 6). Western blotting further revealed that the combination of celastrol and lapatinib produced strong synergy in cell growth inhibition and apoptosis-inducing abilities in vitro in comparison to single treatments, showing more activation of apoptotic related proteins (caspase-9 and caspase-3) and downregulation of growth pathway related proteins (HER2, P-HER2, p-Akt, p-ERK1/2) (Figure 4), implicating activation of mitochondrial-dependent cell apoptosis pathway and inhibition of the PI3K/Akt and MAPK proliferation pathway maybe involved in the observed synergistic effects. These results also indicated that lapatinib’s antitumor effect (inhibiting HER2 activity) could be amplified by celastrol (downregulating the expression of HER2) in MDA-MB-453 cells.

In summary, our results indicated that celastrol could enhance the cytotoxicity and apoptosis-inducing effect of lapatinib in HER2/neu-overexpressing MDA-MB-453 cells. Mechanistic studies consistently showed that celastrol not only directly inhibited the expression of HER2 and changed its redistribution, 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 enhance HER2/neu-overexpressing cancer therapy.

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Conflict of interests

The authors declare no conflict of interests.

References