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Induction of necroptosis in human breast cancer drug-resistant cells by SMAC analog LCL161 after caspase inhibition requires RIP3

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LCL161 is a second mitochondrial activator of caspases (SMAC) mimetic and inhibitor of apoptosis protein (IAP) antagonist that has oral bioavailability, exhibits anti-tumor effects and improves the chemical sensitivity of many cancers. The aim of this study was to ascertain the effects and mechanisms of the SMAC analog LCL161 on breast cancer drug-resistant cells after undergoing caspase inhibition. This was achieved through use of colony formation and CCK-8 assays to detect cell proliferation. Flow cytometry, Western blot analysis, ATP assay, immunofluorescence and siRNA transfection were used to study the molecular mechanisms of LCL161-induced death of cisplatin-resistant MCF-7 cells after caspase inhibition. LCL161 exhibited an inhibitory effect on MCF-7/DDP cells including after inhibition of caspase. However, LCL161 could not on its own induce a necroptosis effect on MCF-7/DDP cells ($P < 0.01$ or $P < 0.001$). When used jointly with the caspase inhibitor z-VAD-fmk, it significantly decreased intracellular ATP levels ($P < 0.01$ or $P < 0.05$). This induction of necroptosis occurred through the activation of the RIP1-RIP3-MLKL programmed cell necrosis cascade. Knockdown of RIP3 using siRNA protected against the combined LCL161 / z-VAD-fmk-induced cell death ($P < 0.01$ or $P < 0.001$). These findings support the hypothesis that LCL161 combined with caspase inhibition can induce a necroptosis effect on MCF-7/DDP cells, suggesting that it has potential to be an effective treatment for breast cancer.

1. Introduction

Breast cancer is the most common cancer globally and the sixth leading cause of death among female patients (Ferlay et al. 2018). Doxorubicin, paclitaxel, cisplatin and cyclophosphamide are effective as first-line chemotherapy in most breast cancers. However, drugs used in chemotherapy have limited efficacy and serious side effects (Rapoport et al. 2014). For patients with advanced recurrence and metastasis, multidrug resistance of cancer cells is the leading cause of chemotherapy failure in advanced cancer patients (Tang et al. 2016). Drug-resistant cells can also accelerate tumor progression thereby leading to failure of tumor treatment through a variety of mechanisms (Jeselsohn et al. 2016). Therefore, it is imperative that a therapeutic strategy for multi-drug-resistant breast cancer is developed that has improved efficacy with lower toxicity.

Apoptosis inhibitors are proteins that control programmed cell death (Finlay et al.2017). The expression of inhibitor of apoptosis protein (IAP) is significantly elevated in breast cancer cells, thereby increasing their viability and enhancing rate of tumor growth and subsequent metastasis (Vasilikos et al. 2017). In cancer cells, pro-apoptotic factors become inhibited through anti-apoptotic proteins. For example, IAPs upregulate and promote uncontrolled cell division (Kocab et al. 2016) Apoptosis can be induced by downregulation of the expression of IAPs by RNA interference so that they are susceptible to death receptors, chemotherapeutic drugs and radiation therapy, which indicate that IAPs are an important target for cancer treatment (Silke et al. 2016). The ability of cells to prevent apoptosis is a major marker of cancer, so endogenous inhibitors of apoptosis can be regarded as promising targets for the design of an anticancer therapeutics (Rathore et al. 2017).

The second mitochondrial activator of caspases (SMAC) mimetic LCL161 acts as a synthetic IAP antagonist, and in a manner similar to the way SMAC inhibits tumors by degrading IAPs (Fig. 1A), it binds and participates in downregulating multiple IAPs and induces apoptosis by activating caspase (Philchenkov et al. 2016). Studies have shown that LCL161 significantly inhibits proliferation and promotes apoptosis in multiple solid tumors and cell lines of multiple myeloma (Chesi et al. 2016), hepatocellular carcinoma (Ren et al. 2016) and leukemia (Gerges et al. 2016). In combination chemotherapy and radiotherapy it has synergistic effects and may improve the therapeutic effect in targeted therapy (Yang et al. 2016). Ten phase II clinical trials of LCL161 have demonstrated that oral LCL161 is well tolerated, well absorbed and has low toxicity (Fulda.

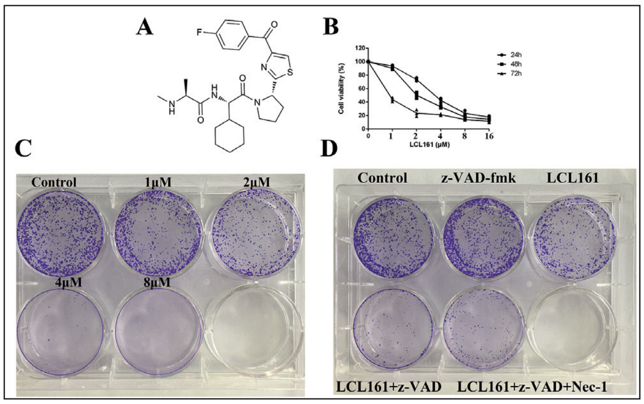

Fig. 1: LCL161 inhibits growth and induces cell death in human breast cancer MCF-7/DDP cells. (A) Chemical structure of LCL161. (B) MCF-7/DDP cells were treated with various concentrations of LCL161 (from 0 to 16 μM) for 24, 48 and 72 h. Cell viability was measured using a CCK-8 assay after treatment. (C) The capability of MCF-7/DDP cells to form colonies was assessed using a colony formation assay after treatment with various concentrations of LCL161 (from 0 to 8 μM) for 7 days. (D) Groups comprised: control; 10μM z-VAD-fmk; 2 μM LCL161; 10μM z-VAD-fmk + 2 μM LCL161; 10μM z-VAD-fmk + 2 μM LCL161 + 10 μM Nec-1 for 7 days.

et al. 2015) However, the precise mechanism of LCL161 against cancers is still unknown. In addition, there are few encouraging reports about LCL161 in human breast cancer-resistant cells. Therefore, we investigated the effect of LCL161 and a caspase inhibitor on the proliferation of breast cancer MCF-7/DDP cells.

In previous studies, we found that LCL161 can induce apoptosis in lung cancer cells through apoptosis pathways, thereby inhibiting proliferation (Jin et al. 2016), but no studies on breast cancer chemotherapy-resistant cells have been published. Thus, in this study we first employed cisplatin-resistant MCF-7 cells as the main research object, and we found that LCL161 induced the onset of breast cancer cell death demonstrating an anti-tumor effect following caspase inhibition.

In the course of this research, our results demonstrated that caspase inhibition sensitizes MCF-7/DDP cells to LCL161-induced necroptosis. This is primarily because of upregulation of necroptosis proteins on MCF-7/DDP cells. Western blot analysis and immunofluorescence assays established that after caspase inhibition, LCL161 induces MCF-7/DDP necroptosis-related protein expression. Taken together, the findings suggest that after caspase inhibition, LCL161 induces the expression of the transcription factors of RIP1 to RIP3. On this basis, we have demonstrated that we can induce necroptosis to resolve drug resistance in breast cancer.

2. Investigations and results

2.1. LCL161 reduces the viability of MCF-7/DDP cells

CCK-8 assays were used to detect the antiproliferative effects of LCL161. MCF-7/DDP cells were inoculated with LCL161 for 24, 48 and 72 h (Fig. 1B). The CCK-8 assay demonstrated that LCL161 inhibited cell proliferation as LCL161 concentration increased (P<0.01).

2.2. z-VAD-fmk enhanced LCL161-induced death of MCF-7/DDP cells

MCF-7/DDP cells were treated with various concentrations of LCL161 for 48 h. As established by CCK-8 assay, LCL161 combined with z-VAD-fmk significantly inhibited the growth of MCF-7/DDP cells (Fig. 2B). This combination treatment decreased cell viability significantly from 91.24±2.27 % to 46.94±3.31 %. Interestingly, we also studied the effect of Nec-1 (10 μM). After pretreatment with Nec-1, cell viability increased significantly from 46.94±3.31 % to 68.38±5.26 % at an LCL161 concentration of 2 μM (P<0.01). Furthermore, we observed that LCL161 combined with z-VAD-fmk inhibited MCF-7/DDP cell colony formation, in contrast with LCL161 alone (Fig. 2B).

2.3. z-VAD-fmk sensitized MCF-7/DDP cells to LCL161-induced apoptosis

Annexin-V FITC/PI staining demonstrated an increase in the proportion of apoptotic cells after addition of LCL161 in the group pretreated with 10 μM z-VAD-fmk for 2h (Fig. 2A). Compared with cells prior to treatment, the proportion of apoptotic cells increased from 0.94±0.41 % to 16.88±1.27 % after treatment with LCL161. This increased further to 44.7±2.24 % after the cells were pretreated for 2 h with 10 μM z-VAD-fmk prior to the addition of LCL161. In this latter group, a large quantity of late stage apoptotic cells could be observed in the AV+ PI+ quadrant (Fig. 2A), i.e. after z-VAD-fmk pretreatment, LCL161 induced a significant increase in necroptosis in the MCF-7/DDP cells. In addition, when Nec-1 was added to the pretreatment, the proportion of apoptotic cells decreased 30.18±1.71 %, i.e. the necroptosis caused by the combination of the two drugs was inhibited (P <0.01).

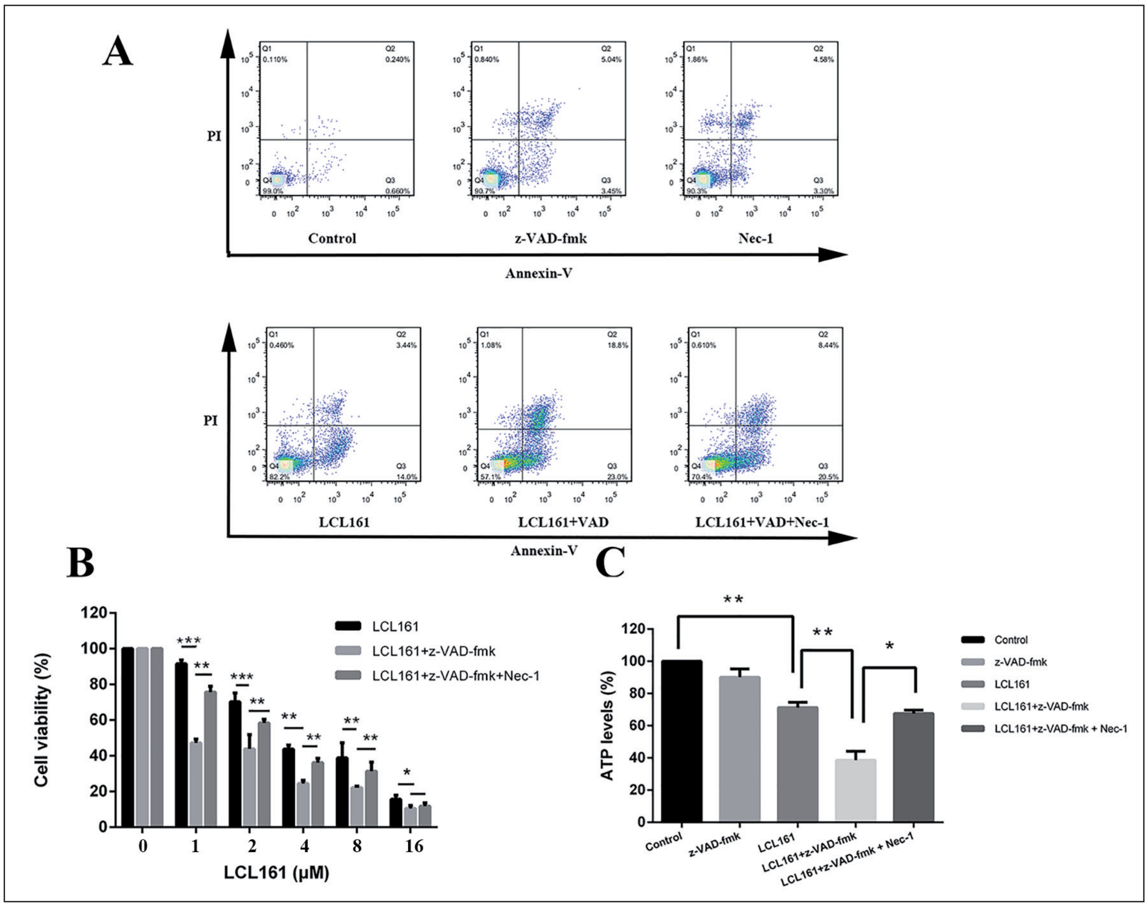

Fig. 2: LCL161 and z-VAD-fmk synergistically reduce cell viability and induce apoptosis in MCF-7/DDP cells. Groups comprised: control; 10μM z-VAD-fmk; 2 μM LCL161; 10 μM z-VAD-fmk + 2 μM LCL161; 10μM z-VAD-fmk + 2 μM LCL161 + 10 μM Nec-1 for 48 hours. (A) Flow cytometry of Annexin V / PI stained cells demonstrates that LCL161 increased programmed cell death after caspase inhibition in MCF-7/DDP cells. (B) z-VAD-fmk cooperates to produce a more effective inhibitory effect on LCL161-induced cell death. (C) Reduction in ATP production after LCL161 treatment. Cellular levels of ATP were measured after 5 h. Results represent means ± SD of three independent experiments. *P<0.05, **P < 0.01, ***P < 0.001.

2.4. LCL161 combined with z-VAD-fmk significantly decreased intracellular ATP levels in MCF-7/DDP cells

ATP performs important tasks in numerous physiological and pathological processes. In apoptosis and necrosis, the rupture of the plasma membrane causes a collapse in membrane potential and levels of ATP reduce. Compared with the control group, LCL161 reduced ATP levels in MCF-7/DDP cells after 5 h (P<0.01), but more significantly so after caspase inhibition of the MCF-7/DDP cells (Fig. 2C). This suggests that LCL161 induced cell death when caspase was inhibited, by decreasing intracellular ATP levels, interfering with cell energy metabolism. Pretreatment of cells for 2h with 40μM Nec-1 reversed the decrease in mitochondrial membrane potential.

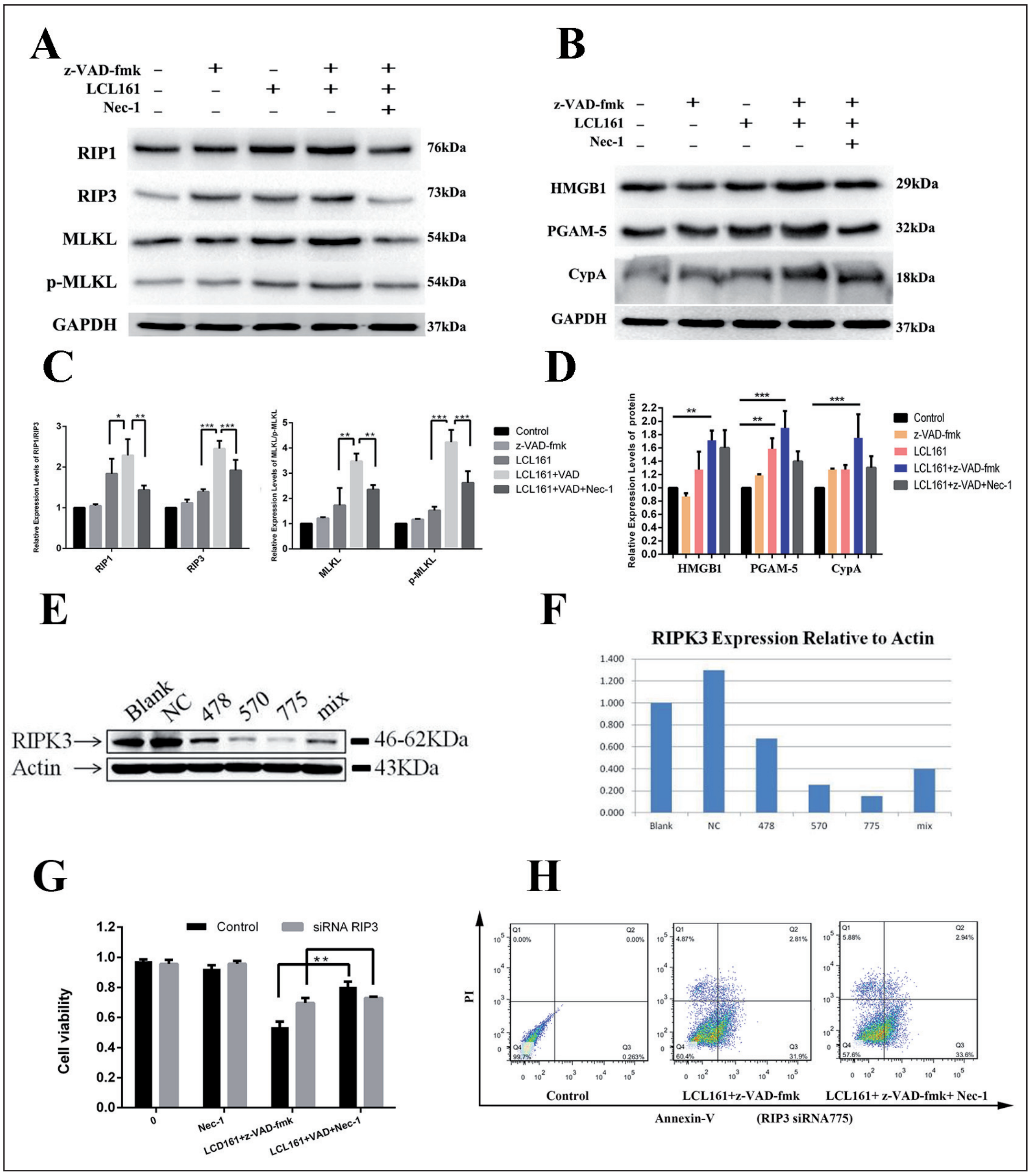

Fig. 3: LCL161 and z-VAD-fmk cooperate to activate necroptosis in MCF-7/DDP cells. Groups comprised: control; 10 μM z-VAD-fmk; 2 μM LCL161; 10 μM z-VAD-fmk + 2 μM LCL161; 10μM z-VAD-fmk + 2 μM LCL161 + 10μM Nec-1 for 48 hours. (A, B) Whole-cell lysates from the MCF-7/DDP cells treated with the different groups, subjected to western blot analysis. (C, D) Relative expression of RIP1, RIP3, MLKL, Phospho-MLKL, HMGB1, PGAM-5 and CypA. (E, F, G, H) Knockdown of RIP3 using RIP3 siRNA 775 blocked RIP3 protein expression and prevented LCL161 combined with z-VAD-fmk inducing programmed necrosis. Data represent means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001.

2.5. RIP1 is a critical mediator of LCL161- induced apoptosis

Receptor-interacting protein (RIP) 1 and RIP3 have been demonstrated to be key regulators of necroptosis. As SMAC mimetics have been shown to release RIP1 from the TRADD-TRAF2-RIP1-cIAP complex, Western blot analysis was used to detect protein expression levels (Fig. 3A). It has been previously demonstrated that LCL161 accelerates the degradation of cIAP1 and cIAP2 (Yang et al. 2016). Similarly, LCL161 results in the accumulation of RIP1. Western blot analysis demonstrated that RIP1 protein could not be activated by z-VAD-fmk individually. LCL161 significantly upregulated the expression of RIP1 after caspase inhibition. Furthermore, MCF-7/DDP cells pretreated with Nec-1 (10μM) decreased the effects of LCL161-induced cell death (Fig. 3B). These observations indicate that LCL161-induced cell death was RIP1-dependent.

2.6. LCL161 and z-VAD-fmk cooperate to activate RIP3-induced necroptosis

The addition of LCLl61 combined with caspase inhibitor lead to a significant increase in protein expression levels of RIP3, MLKL and Phospho-MLKL (Figs. 3A & B). Western blot analysis demonstrated that LCL161 and z-VAD-fmk synergistically triggered RIP1 to RIP3 necroptosis activation, with these necroptosis-related proteins downregulated in the presence of Necrostatin-1($P<0.01$). Taken together, these results demonstrate that upregulation of necroptosis-associated proteins is the principal mechanism underlying the sensitization of MCF-7/DDP cells to RIP1/RIP3/MLKL-induced necroptosis by LCL161 after caspase inhibition.

2.7. LCL161 and z-VAD-fmk cooperate to induce necroptosis as observed by changes in fluorescence

Laser scanning confocal microscopy demonstrated that the expression of RIP1 and RIP3 proteins in cells treated with LCL161 combined with z-VAD-fmk increased compared with the blank group (Fig. 4) (both $P<0.01$). The nuclei of cells in the blank and z-VAD-fmk groups were light blue, intact with uniform chromatin. Compared with the blank group, the nuclei of cells in the LCL161 group were densely stained with apparent nuclear fragmentation. When combined with caspase inhibition, the nuclei of cells treated with LC161 were fragmented and condensed, indicating necroptosis.

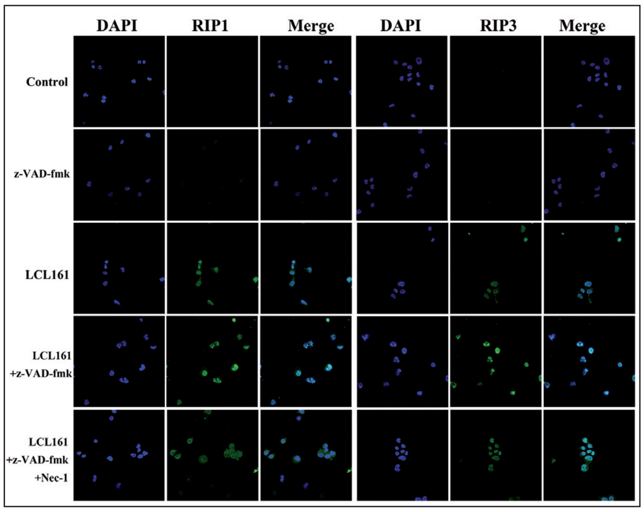

Fig. 4: LCL161 and z-VAD-fmk cooperate to activate RIP1/RIP3 induced necroptosis. Groups comprised: control; 10μM z-VAD-fmk; 2 μM LCL161; 10μM z-VAD-fmk + 2 μM LCL161; 10 μM z-VAD-fmk + 2 μM LCL161 + 10 μM Nec-1 for 24 hours. MCF-7/DDP cells treated in different groups were incubated with primary antibodies at 4°C overnight. Localization of target proteins was assessed by immunofluorescence staining. Nuclei of treated cells were stained with DAPI. Scale bar is 50 μm.

2.8. Knockdown of RIP3 using siRNA protected MCF-7/DDP cells against CL161-induced necroptosis after caspase inhibition

To clarify the effect of RIP3 protein on the sensitivity of LCL161-induced necroptosis in MCF-7/DDP cells, and further explore the mechanism of action of this drug, we explored the effect of RIP3 knockdown in the necroptosis signaling pathway in MCF-7/DDP cells (Figs. 3E & F). A CCK-8 assay and flow cytometry were then used to evaluate cell viability and apoptosis. The results demonstrated that silencing RIP3 prevented the induction of cell death and apoptosis by LCL161 combined with z-VAD-fmk. The protective effects of Nec-1 weakened after knockdown (Figs. 3G & H). These results indicate that down-regulation of RIP3 protein expression attenuates necroptosis induced by LCL161 in MCF-7/DDP cells in conditions of caspase inhibition.

3. Discussion

In the process of tumor treatment, cancer cells can gradually develop drug resistance characterized by blocking the apoptotic pathway, which is a principal explanation for multidrug resistance of cancer (Yang et al. 2016). Tumor multidrug-resistance is the lead cause of chemotherapy failure and also an important factor in cancer recurrence and metastasis (Tang et al. 2016). Some cancer cells exhibit intrinsic resistance to chemotherapeutic drugs, often due to high endogenous expression of transporters such as MDR (Steding et al. 2016). In order to combat the problems of congenital and acquired drug-resistance, we studied caspase-mediated apoptosis in the death pathway of cancer cells and the mechanisms of action of the SMAC analog LCL161.

In this study, we used the MCF-7/DDP cell line as a research object and used different concentrations of LCL161 to treat the cells. CCK-8 assay results demonstrated that LCL161 inhibited proliferation of the MCF-7/DDP cells, with increasing concentrations leading to increased cell death rate (Fig. 1B). By detection of cell viability using CCK-8, colony cloning and flow cytometry, we found that LCL161 alone had limited inhibitory effect on the cells (Figs. 2A & B), but after the inhibition of caspase, it significantly induced the death of MCF-7/DDP cells. In some experiments we pre-treated the cells with the necroptosis inhibitor Nec-1 prior to the addition of LCL161 and z-VAD-fmk, interestingly finding that the combined induction of necroptosis was inhibited.

Tumor suppressor protein family IAPs plays a major role in the regulation of tumors, and IAP is highly expressed in many tumor tissues. Its level is closely related to prognosis (Finlay et al. 2017). SMAC inhibits tumors by degrading IAPs. The drug investigated in this study, LCL161, is one of a number of small molecule mimics developed in recent years and is expected to form the basis of a new therapy for tumors (Dynek et al. 2017). SMAC analogs bind and downregulate multiple IAPs and induce apoptosis by activating caspase (Silke et al. 2016). Previous studies have demonstrated that SMAC mimics can activate caspase dependent apoptotic pathways by degrading IAPs. The mechanism of action of LCL161 is to bind XIAP, cIAP1 and cIAP2. By binding cIAP1, LCL161 induces ubiquitination, thus allowing degradation by proteases, the activation of caspase followed by TNF-α-dependent apoptosis (Beug et al. 2016). The clinical efficacy of LCL161 has not been clearly reported. Based on the close relationship between SMAC analogs and tumors, most current research is devoted to the joint study of SMAC analogs and chemotherapeutic drugs, including paclitaxel, gemcitabine and everolimus (Fulda et al. 2016). Unlike other studies, our initial aim was to induce different death patterns with LCL161 in drug-resistant cells, such as necroptosis, under the induction of caspase inhibitors. Therefore, this new apoptotic pathway is an effective means to solve cancer drug resistance.

Necroptosis is a non-caspases-dependent programmed death pattern discovered just recently (Galluzzi et al. 2017). In the case where caspases are inhibited, death receptors bind to ligands and regulate the expression of a relevant protein to trigger necroptosis (Conrad et al. 2016). Necroptosis is defined as the process of programmed cell death characterized by morphological features similar to necrosis: swelling of cell volume and intracellular organelles, cytoplasmic granulation, destruction of envelope integrity, ultimately leading to the leakage of the intracellular content (Shan et al. 2018). When caspase-inhibited cells are stimulated, they cause necroptosis instead of apoptosis. For example, TNF induces programmed necrosis of z-VAD-fmk-treated mouse fibroblasts but also causes caspase-8 deficiency in Jurkat leukemia cells. Therefore, programmed necrosis may be the manner in which cells die when apoptosis is not initiated correctly. Different cells undergo apoptosis or necroptosis depending on their environment and degree of activation (Kopalli et al. 2016). Necroptosis can be regulated by activation and high expression of a series of factors, of which the RIP1, RIP3 and caspase families are the most effective (Chen et al. 2016). The most important factor in the induction of necroptosis is the inhibition of caspase-8 kinase and the formation of complex IIb by RIP1 and RIP3, the downstream signal subsequently transmitted by the necrosome to finally perform the necroptosis program (Fuchs et al. 2016). MLKL is a functional RIP3 substrate that binds to RIP3 through its kinase-like domain but lacks its own kinase activity. RIP3 phosphorylates MLKL at T357 and S358. Phosphorylated MLKL forms oligomers that bind to phosphatidylinositol lipids and cardiolipin. This allows MLKL to be transferred from the cytosol to the plasma and intracellular membranes where it directly disrupts membrane integration, leading to necrotic death. The target of Nec-1 is the kinase site of RIP1. Nec-1 prevents the synthesis of complex IIb by blocking the phosphorylation of RIP1 and RIP3, and in this way blocks the necroptotic signaling pathway (Su et al. 2016).

In this study, we examined the differential expression of necroptosis-related proteins in MCF-7/DDP cells after treatment with LCL161 and the pan-caspase inhibitor z-VAD-fmk by Western blot analysis and immunofluorescence. RIP1, RIP3, MLKL and Phospho-MLKL were activated after caspase was inhibited (Figs. 3B & C). Previous studies have investigated whether caspase family protein activity is inhibited and if the interaction between RIP1 and RIP3 causes terminal apoptosis or necroptosis (Hanson et al. 2016). RIP1 and RIP3 interact through the RIP homotypic interaction motif to form RIP1/RIP3 complexes that mediate classical necroptosis, which can be inhibited by Nec-1. The RIP protein family has been widely recognized as a regulatory switch for cell death or survival, and it has been confirmed that RIP3 protein is a switch for the necroptosis pathway (He et al. 2016). In addition, knockout of RIP3 using siRNA demonstrated that LCL161-induced necrotic apoptosis requires RIP3 after caspase inhibition. In the present study, when cells were pretreated with Nec-1, the rate of apoptosis decreased, as did the expression of the corresponding apoptotic protein, indicating that necroptosis induced by LCL161 after caspase inhibition was blocked. After stimulation of the cells by stress factors, TNFα binds to the ligand, and after Fas-death domain-associated protein (FADD) is recruited, RIP1 binds to caspase-8 to form complex I after which caspase-8 is activated, RIP1 protein is cleaved and the cells begin to apoptose. When caspase-8 activity is specifically blocked by z-VAD-fmk, RIP1 and RIP3 proteins combine, with FADD and caspase-8 recruited to form complex II. The activity of this metabolism-related enzyme is enhanced, with the cell tending to become necrotic. Compared with apoptosis, apoptotic bodies are not formed and chromatin does not agglomerate during necroptosis. Apoptosis and necroptosis are mutually inhibiting processes. If the necroptosis pathway is inhibited, the rate of apoptosis will increase, and if so, cell death may become necroptosis. These results demonstrate the important role of RIP family proteins in the induction of necroptosis of MCF-7/DDP cells by LCL161 in combination with caspase inhibitors.

In summary, LCL161 is a second mitochondria-derived cysteine protease agonist, and when treatment is combined with a pan-caspase inhibitor, it triggers MCF-7/DDP cells to undergo necroptosis by regulating the expression of RIP1, RIP3, MLKL and Phospho-MLKL proteins. In this way, LCL161 exerts an anti-tumor effect. These data suggest that the combination of LCL161 and the caspase inhibitor z-VAD-fmk may be a promising chemotherapy regimen for addressing breast cancer chemotherapy resistance. Unfortunately, because of the limitations of time, an in-depth *in vivo* antitumor study of the efficacy of LCL161 with z-VAD-fmk has not been conducted, but this is likely to be the focus of subsequent work.

4. Experimental

4.1. Reagents and chemicals

LCL161, Nec-1 and z-VAD-fmk were purchased from MCE Company (USA). RPMI 1640 medium was obtained from HyClone (USA). Fetal bovine serum (FBS) was obtained from Pan Company (Germany). The following antibodies were used in the study: anti-RIP, anti-RIP3, anti-MLK, anti-Phospo-MLKL anti-HMGB1, anti-PGAM-5 and CypA (Abcam, Cambridge, MA, USA). Anti-GAPDH antibody, HRP-labeled goat anti-rabbit Ig, CCK-8 and Annexin V-FITC/PI staining kits were purchased from Biosharp (Hefei, China).

4.2. Cell culture and treatment

The human breast cancer cell line MCF-7/DDP was purchased from Jiangsu Kaiji Biotechnology Co., Ltd. (Jiangsu, China). The cells were maintained in RPMI-1640 medium containing 10 % FBS, 100 U/mL penicillin, 250 ng/ml cisplatin and 100 µg/mL streptomycin. Cells were cultured in an atmosphere containing 5 % CO_2 at 37 °C. Culture medium was completely refreshed every other day.

4.3. CCK-8 kit detects cell viability

Cells were plated at a density of 6 × 103 cells/well in 96-well plates and cultured for 24, 48 and 72 h then treated with different concentrations of LCL161 (0 to 16 µM). A combination of z-VAD-fmk (10 µM) and/or Nec-1 (10 µM) was utilized to treat the cells 1 h prior to the addition of LCL161. At each time point, 10 µL CCK-8 was added to the appropriate wells and the absorbance at 450 nm measured after a further 1 h incubation at 37 °C, using a microplate reader.

4.4. Colony formation assay

Logarithmic growth phase cells were uniformly seeded in 6-well plates at a density of 7000 cells per well. The medium was subsequently exchanged with fresh medium containing LCL161 and z-VAD-fmk or Nec-1. The plates were incubated for 7 days, after which they were washed 3 times with PBS then placed in air to dry. Cells were fixed using 4 % paraformaldehyde at 4 °C for 20 min, which was then discarded and the cells air dried. Colonies were stained with 2 % crystal violet for 10 min, washed with double-distilled water and finally dried at room temperature prior to imaging by light microscopy.

4.5. Flow cytometry

MCF-7/DDP cells were seeded in 6-well plates and treated with LCL161, z-VAD-fmk or Nec-1. After incubation at 37 °C for 24 h the cells were harvested by trypsin digestion then washed with PBS. Five hundred µL of binding buffer were added to each cell suspension which was then stained with 10 µL propidium iodide (PI) and 10 µL Annexin V-FITC in the dark for 15 min and then red and green fluorescence detected using an Accuri C6 flow cytometer.

4.6. Detection of mitochondrial membrane potential

Cells were seeded into the wells of a 6-well plate (2 × 105 cells/well). After the cells were attached to the plates, different treatments were added to the medium. The cells were then collected after 5 h of incubation. A luminometric-based ATP assay kit was used to detect intracellular ATP levels for the different treatments, according to the manufacturer's instructions. Absorbance was measured using a microplate reader and ATP levels calculated as a percentage of the blank group.

4.7. Immunostaining assay

Cells in exponential growth were cultured in 12-well plates (1.2 × 105 cells/well) and then treated with different groups for 24 h. The cells were fixed with 4 % paraformaldehyde for 20 min, permeabilized for 10 min in 0.5 % Triton X-100, then incubated for 2 h in blocking buffer (5 % BSA in PBS). The cells were then incubated with the primary antibody rabbit anti–RIP1/RIP3 (1:1000) at 4 °C overnight. The cells were washed with PBS then incubated with goat anti-rabbit IgG. Cell nuclei were stained for 1 h using DAPI (1:500) then visualized by fluorescence microscopy.

4.8. Western blot analysis

Proteins were extracted from the culture plates of cells that had received different treatments using ice-cold RIPA buffer and separated on a 10 % SDS-PAGE gel then transferred to a PVDF membrane. This membrane was incubated with primary antibodies at 4 °C overnight, including rabbit anti-RIP1, anti-RIP3, anti-MLKL and anti- Phospho-MLKL (1:1000). After washing in Tris-buffered saline, the membranes were incubated with secondary antibodies at room temperature for 2h (1:10000). Quantification of protein bands was achieved using ImageJ software. GAPDH served as the loading control.

4.9. Statistical analysis

Data are expressed as means±SEM of three separate experiments. Statistical significance was determined using a two-tailed Student's t-test or a 1-way ANOVA. SPSS v.22.0 software was used for data analysis. A P value < 0.05 was considered statistically significant.

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Conflicts of interest: The authors declare no conflicts of interest.

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