A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy

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Abstract

Killing cancer cells through the induction of apoptosis is one of the main mechanisms of chemotherapy. However, numerous cancer cells have primary or acquired apoptosis resistance, resulting in chemoresistance. In this study, using a novel chalcone derivative chalcone-24 (Chal-24), we identified a novel anticancer mechanism through autophagy-mediated necroptosis (RIP1- and RIP3-dependent necrosis). Chal-24 potently killed different cancer cells with induction of necrotic cellular morphology while causing no detectable caspase activation. Blocking the necroptosis pathway with either necrostatin-1 or by knockdown of RIP1 and RIP3 effectively blocked the cytotoxicity of Chal-24, suggesting that Chal-24-induced cell death is associated with necroptosis. Chal-24 robustly activated JNK and ERK and blockage of which effectively suppressed Chal-24-induced cytotoxicity. In addition, Chal-24 strongly induced autophagy that is dependent on JNK-mediated phosphorylation of Bcl-2 and Bcl-xL and dissociation of Bcl-2 or Bcl-xL from Beclin1. Importantly, suppression of autophagy, with either pharmacological inhibitors or siRNAs targeting the essential autophagy components ATG7 and Beclin1, effectively attenuated Chal-24-induced cell death. Furthermore, we found that autophagy activation resulted in...
in c-IAP1 and c-IAP2 degradation and formation of the Ripoptosome that contributes to necroptosis. These results thus establish a novel mechanism for killing cancer cells that involves autophagy-mediated necroptosis, which may be employed for overcoming chemoresistance.

Keywords
autophagy; necroptosis; RIP1; RIP3; c-IAP; apoptosis

Introduction

Chemotherapy is used as a primary or adjuvant therapy for treating cancer patients. While different cellular actions such as to induce cytostasis and to suppress angiogenesis are involved in the anticancer activities of chemotherapeutics, the main mechanism to directly kill cancer cells is to induce cytotoxicity. However, as evading programmed cell death is one of the hallmarks of cancer, chemoresistance, whether primary or acquired, is the main obstacle that causes therapy failure. It is believed that chemotherapeutics kill cancer cells mainly through activation of apoptosis, and apoptosis resistance substantially contributes to chemoresistance. However, although extensive efforts to elucidate the mechanism and to overcome apoptosis resistance have been devoted to anticancer research, limited improvement of chemotherapy has been achieved, suggesting other cell death pathways may also be activated for inducing cytotoxicity in cancer cells.

Recent studies have suggested that necroptosis, RIP1- and RIP3-dependent necrosis, can be activated in certain cell types by chemotherapeutics. It was found that necroptosis is activated when apoptosis pathways are blocked in certain circumstances. However, necroptosis may be predominant even the apoptosis pathways are competent. Thus, necroptosis can be either a backup or an alternative cell death mode for killing cancer cells by chemotherapeutics. Many stimuli induce necroptosis, of which TNFα-induced necroptosis is extensively studied. TNFα activates TNFR1 signals to form complex II consisting of RIP1, FADD and caspase-8. If caspase-8 is activated, RIP1 will be cleaved to ensue activation of downstream caspases and apoptosis. Under conditions where caspase-8 activation or RIP1 ubiquitination is suppressed, RIP1 recruits RIP3 to form a complex called the necrosome where RIP3 is activated through phosphorylation by RIP1. Activated RIP3 is released and binds the pseudo kinase MLKL, and then migrates to the mitochondria to activate the phosphatase PAGM5, resulting in ROS production and necroptotic cell death. Therefore, suppressing c-IAP1, the E3 ubiquitin ligase of RIP1, by SMAC mimetics or activating the RIP1 deubiquitylating enzyme CYLD together with suppressing caspase-8 with z-VAD triggers necroptosis in TNFα-exposed cells. Interestingly, certain anticancer therapeutics such as etoposide are able to suppress c-IAP1 expression, thereby to induce formation of a complex called the Ripoptosome consisting of RIP1, FADD, RIP3 and caspase-8, resulting in necroptosis. Therefore, activating necroptosis could be employed for anticancer therapy.

Autophagy, a catabolic process for degradation and recycling of long-lived proteins and organelles, can lead to either cell survival or death. Autophagy is initiated by formation...
of a double-membrane vesicle called the autophagosome, which is fused to the lysosome to form the autolysosome where sequestered cellular components are digested by lysosomal enzymes. The autophagy process is tightly regulated at different stages by autophagy factors such as ATG7, ATG5 and Beclin-1. The antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL bind Beclin-1 to inhibit autophagy, and dissociation of these Bcl-2 family proteins from Beclin-1 promotes autophagy. Consistent with its contradictory roles in cell death control, the effects of autophagy in cancer cells’ response to chemotherapy are also complex: either pro- or anti-death. While the term of autophagic cell death is still a matter of dispute, it is known that autophagy can promote apoptosis. Whether therapeutic-induced autophagy regulates necroptosis is not well studied.

In this study, we report a novel anticancer pathway for killing cancer cells that involves autophagy-mediated necroptosis triggered by the novel chalcone derivative chalcone-24 (Chal-24) (Fig. S1). Chal-24 (named as 11a in Ref 29) was shown to potently inhibit xenografted tumor growth without observed signs of toxicity to animals, thus could be a potential anticancer agent. We found that Chal-24 activates autophagy that is dependent on JNK-mediated Bcl-2 and Bcl-xL phosphorylation, which triggers c-IAP1 and c-IAP2 degradation and Ripoptosome formation, thereby inducing necroptosis in cancer cells. This novel cancer cell killing mechanism could be exploited for overcoming chemoresistance.

Results

Chal-24 induced non-apoptotic death in cancer cells

A potential anticancer activity of Chal-24 was seen in a xenografted tumor model in nude mice. To investigate the mechanism by which the anticancer activity of Chal-24 is achieved, we confirmed by LDH release assay that Chal-24 induces cytotoxicity in cancer cells derived from different human tumors. The results show that Chal-24 induced cell death in both lung cancer (A549 and H1299) and bladder cancer (UM-UC-3) cell lines (Fig 1A and data not shown). Consistent with the in vivo results showing that Chal-24 has little toxicity in mice, immortalized human bronchial cells were remarkably resistant to Chal-24-induced cytotoxicity (Fig. S2). While apoptosis is one of the main mechanisms involved in chemotherapy-induced cytotoxicity, a series of experiments assessing apoptosis were conducted. Unexpectedly, results from all the experiments did not support apoptosis as the main cytotoxicity pathway induced by Chal-24. These included: no detectable activation of caspase-8 or caspase-3, and cleavage of PARP1 (Fig 1B); suppressing apoptosis with the pan-caspase inhibitor z-VAD had minimal suppression on Chal-24-induced cell death while it almost completely suppressed TRAIL-induced cytotoxicity (Fig 1C); and the dead cells showed necrotic morphological features (cell membrane rupture and relative normal nuclear morphology) (Fig 1D). These results strongly suggest that Chal-24 kills cancer cells mainly through induction of non-apoptotic cell death.

Activation of JNK and ERK is required for Chal-24-induced cell death

To explore the mechanism by which Chal-24 induces cancer cell death, we examined the activation of MAP kinases that are involved in cell survival and death regulation. Chal-24 potently activated JNK, ERK and p38, which was detected as early as 30 min (Fig 2A).
Pharmacological inhibitors were employed to investigate the role of each MAPK in Chal-24-induced cell death. While each inhibitor potently suppressed its respective kinase (Fig. 2B), suppressing JNK and ERK but not p38 effectively inhibited Chal-24-induced cell death (Fig. 2C). These results suggest that Chal-24-induced cytotoxicity involves JNK and ERK.

**Chal-24 induces autophagy in cancer cells**

Because MAPKs such as JNK are involved in autophagy induction, and autophagy regulates cell death, we investigated if Chal-24-induced cytotoxicity involves autophagy. The conversion of LC3-I to LC3-II, a hallmark of autophagy, was readily detected in A549 and UM-UC3 cells after Chal-24 treatment (Figs. 3A and 3B). The expression level of p62, which is degraded in the autophagosome during autophagy, was gradually reduced in Chal-24-treated cells (Figs. 3A and 3B). To confirm that the changes in LC3 and p62 were resulted from autophagy induction, a turnover assay for LC3 and p62 to detect autophagy flux was conducted. The lysosome inhibitor chloroquine (CQ) was used for suppressing lysosomal protein degradation so as to determine if Chal-24-induced LC3-II increase is due to enhanced LC3-II production or reduced LC3-II clearance. In both cell lines, while either Chal-24 or chloroquine alone induced a moderate increase of LC3-II, the co-treatment with both Chal-24 and CQ further elevated LC3-II level (Figs. 3C and 3D). Consistently, the reduction of p62 induced by Chal-24 was effectively attenuated by CQ (Figs. 3C and 3D). These data strongly substantiate that Chal-24 induces autophagy in cancer cells. In addition, with transfection of a GFP-LC3 plasmid, we examined if Chal-24 induces LC3 puncta formation, another hallmark of autophagy, in A549 cells. The results show that Chal-24 strongly increased both the number of cells with increased LC3 puncta and LC3 puncta number within the cell (Figs. 3E and 3F). Altogether, these experiments provide strong evidence suggesting that Chal-24 induces autophagy in human cancer cells.

**Autophagy is required for Chal-24-induced cell death**

We next investigated the role of autophagy in Chal-24-induced cytotoxicity with different autophagy inhibitors: wortmannin (WTM), 3-methyladenine (3MA) and CQ. These inhibitors block autophagy at different steps along the autophagic pathway. Although little cytotoxicity caused by the inhibitors alone was detected, a significant suppression of Chal-24-induced cell death was observed (Fig. 4A). The inhibitors were confirmed to block Chal-24-induced autophagy, which was shown by reduction of Chal-24-induced LC3-II accumulation, p62 degradation by WTM and 3MA (Fig. 4B). As expected, the inhibition of lysosomal degradation by CQ increased LC3-II and p62 expression (Fig. 4B). These observations were further supported by knockdown of ATG7 or Beclin1 expression, which was confirmed by Western blot, effectively suppressing Chal-24-induced cytotoxicity (Fig. 4C, 4D). Inhibition of autophagy by WTM, CQ and 3MA, or siRNAs against ATG7 or Beclin1 protected cells against Chal-24-induced death and ensured long-term cell survival, which was detected by clonogenic growth assay (Figs. 4E, S3), excluding the possibility that Chal-24 delays cell death or otherwise changes the kinetics of the LDH release. Taken together, these results suggest that autophagy is required for Chal-24-induced cancer cell death.
**JNK mediates autophagy through phosphorylation of Bcl-2 and Bcl-xL and reduction of the Bcl-2/Beclin-1 and Bcl-xL/Beclin-1 complex**

We then investigated the signaling pathways that are involved in Chal-24-induced autophagy with use of specific inhibitors against JNK, ERK and p38. Similar to WTM, the JNK inhibitor remarkably reduced Chal-24-induced LC3-II levels, while the ERK and p38 inhibitor had little effect (Fig. 5A). The JNK inhibitor also inhibited Chal-24-induced autophagic flux (Fig. 5B). Further, JNK suppression effectively inhibited Chal-24-induced GFP-LC3 puncta formation (Data not shown). It has been reported that JNK phosphorylates the prosurvival Bcl-2 family proteins, which leads to disruption of the complex of these proteins with Beclin-1 to promote autophagy during starvation\(^\text{30}\). Therefore, we examined if Chal-24 induces JNK-mediated phosphorylation of prosurvival Bcl-2 family proteins. Indeed, we detected a clear mobility shift of Bcl-2 and Bcl-xL, which started as early as 2 hr post Chal-24 exposure and gradually increased as time elapsed (Fig 5C, 5D). The shift of Bcl-2 and Bcl-xL is highly likely due to phosphorylation because it was completely eliminated by treatment with lambda protein phosphatase (Fig. 5E). The band shifts of Bcl-2 and Bcl-xL were suppressed by the JNK inhibitor, suggesting that JNK is involved in phosphorylation of Bcl-2 and Bcl-xL. (Fig. 5E). In addition, the effect of Chal-24-induced JNK activation on the complex consisting of Bcl-2/Beclin-1 or Bcl-xL/Beclin-1 was examined by co-immunoprecipitation. The results showed that Beclin-1 constitutively binds to Bcl-xL and Bcl-2 in untreated cells. However, Chal-24 treatment caused reduction of Bcl-2/Beclin-1 or Bcl-xL/Beclin-1 complex (Fig. 5F). Interestingly, the reduction of the Bcl-2/Beclin-1 or Beclin-1/Bcl-xL interaction was attenuated when JNK was blocked, which was associated with suppression of phosphorylation of Bcl-xL and Bcl-2 (Fig. 5F). These results suggest that Chal-24-induced JNK activates autophagy through phosphorylation of Bcl-xL and Bcl-2 to disrupt the Bcl-2/Beclin-1 or Bcl-xL/Beclin-1 complex.

**Chal-24 induces degradation of IAPs depending on autophagy and ERK**

Because the IAP family proteins are important cell survival factors that are involved in non-apoptotic cell death\(^\text{12}\), we further investigated the effect of Chal-24 on expression of the IAPs. Chal-24 treatment caused reduction in expression levels of c-IAP1 and c-IAP2 in both A549 and UM-UC-3 cells, which was detected starting at 16h post Chal-24 exposure (Fig. 6A, Fig. S4). In contrast, Chal-24 induced little change in XIAP expression (Fig 6A, Fig. S4). Because ERK and JNK are involved in Chal-24-induced cytotoxicity (Fig. 2C), we examined the role of these MAPKs in Chal-24-induced c-IAP1 and c-IAP2 suppression. Due to the inconsistent result of the JNK inhibitor that is likely due to its nonspecific effect, we decided to use RNA interference for blocking JNK or ERK. Blocking either ERK by targeting ERK1 and ERK2, or JNK by targeting JNK1 and JNK2 effectively attenuated c-IAP1 and c-IAP2 reduction (Fig. 6B), suggesting these pathways are involved in Chal-24-induced suppression of the c-IAP proteins. Interestingly, the autophagy inhibitors 3MA, WTM and CQ also suppressed c-IAP1 and c-IAP2 reduction (Fig. 6C). To further determine the role of autophagy in Chal-24-induced c-IAP1 and c-IAP2 reduction, knockdown of ATG7 and Beclin-1 was conducted, which suppressed Chal-24-induced c-IAP1 and c-IAP2 reduction (Fig. 6D). The knockdown of ATG7 and Beclin-1 was confirmed by Western blot (Figs. 4C, 4D). These results suggest that autophagy is involved in Chal-24-induced c-IAP1 and c-IAP2 suppression. Because ERK is unlikely to be involved in autophagy activation.
(Fig. 5A), we examined how ERK works in c-IAP degradation. No detectable ubiquitination or sumoylation of c-IAP1 was induced by Chal-24 (Fig. S5). However, using 8% PAGE gels that have a much better resolution of the molecular range for c-IAP1, a clear band shift of c-IAP1 was detected from 4 h to 12 h, which was dramatically suppressed by the ERK inhibitor U0126 and completely removed by lambda-phosphatase, suggesting that Chal-24 caused ERK-dependent phosphorylation of c-IAP1 (Fig. 6E). These results suggest that ERK mediates a signaling pathway for c-IAP1 phosphorylation, which may trigger c-IAP1 for autolysosomal degradation. Although more effort is needed to clearly elucidate the defined mechanism, our data suggest that JNK-mediated autophagy and ERK cooperatively mediates Chal-24-induced c-IAP suppression.

Chal-24 induces Ripoptosome formation depending on autophagy

It was reported that loss of IAPs triggers Ripoptosome formation that leads to either apoptosis or necroptosis\textsuperscript{10,11}. We then investigated if Chal-24 induces formation of the Ripoptosome by co-immunoprecipitation with an anti-RIP1 antibody. Indeed, Chal-24 induced ripoptosome formation after 24h treatment (Fig. 6F). To investigate the role of autophagy in Ripoptosome formation, CQ was applied to block autophagy during Chal-24 treatment. The results show that suppressing autophagy effectively suppressed the association of FADD and caspase-8 with RIP1 (Fig. 6G), substantiating that induction of autophagy is required for Chal-24-induced Ripoptosome formation.

Chal-24 induces necroptosis in different cell lines

While Chal-24-induced cytotoxicity was mainly non-apoptotic and the Ripoptosome mediates necroptosis, we then examined whether Chal-24 kills cancer cells through necroptosis. The release of cyclophilin A, a necrotic cell death marker\textsuperscript{31}, was readily detected in media from Chal-24-treated cells (Fig. 7A). Chal-24-induced cytotoxicity was effectively blocked with the RIP1 inhibitor necrostatin-1 (Fig. 7B). Furthermore, suppression of RIP1 and RIP3, the two key proteins in the necroptosis pathway, by RNAi effectively alleviated Chal-24-induced cytotoxicity in both A549 and UM-UC-3 cells (Fig. 7C). Together with the finding that autophagy is required for Ripoptosome formation that is involved in necroptosis, these results strongly suggest that Chal-24 induces cytotoxicity through autophagy-mediated necroptosis.

Discussion

This study provides evidence that an autophagy-mediated necroptosis pathway can be activated in cancer cells (Fig. 7D): Chal-24 induced non-apoptotic cell death that was blocked by suppression of the key necroptosis pathway components RIP1 and RIP3; Chal-24 strongly induced autophagy through JNK-mediated phosphorylation of Bcl-2 and Bcl-xL and dissociation of the Bcl-2/Beclin-1 or Bcl-xL/Beclin1 complex; Suppression of autophagy effectively inhibited Chal-24-induced formation of the Ripoptosome and necroptotic cell death. This novel cancer cell killing mechanism that involves autophagy-mediated necroptosis could be targeted for overcoming cancer chemoresistance.

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The concept that autophagic cell death is one of the main cell death pathways has been challenged because independent autophagic cell death may rarely exist. In most cases, autophagy-associated death is accompanied with apoptosis or necrosis. Due to the complex role of autophagy in cell death regulation, modulation of autophagy for cancer therapy has been assessed with caution. While autophagy is often seen to protect cancer cells against chemotherapy, suppressing autophagy has been tested for sensitizing anticancer therapy. However, under some situations, autophagy may potentiate cell death. Utilization of autophagy-mediated cell death may be a new avenue for improving chemotherapy.

Indeed, bufalin was shown to induce autophagy-mediated nonapoptotic death in human colon cancer cells through JNK activation. A combination of the small molecule obatoclax and dexamethasone induced autophagy-mediated necroptosis in glucocorticoid-resistant acute lymphoblastic leukemia cells. In this study, Chal-24 independently induced autophagy-mediated necroptosis in cancer cells. To our knowledge, this is the first report showing a naturally occurring derivative compound that individually activates autophagy-mediated necroptosis to effectively kill cancer cells.

MAPKs are important cellular signaling kinases that are involved in cell death regulation. Despite that p38 and ERK are shown to directly contribute to autophagy, blocking these two MAPKs had little effect on Chal-24-induced autophagy. Thus, although Chal-24 activates all the three MAPKs, only JNK is involved in autophagy induction, suggesting that the involvement of each MAPK in autophagy may be cell type- and/or stimulation-dependent. We further demonstrate that JNK activates autophagy through phosphorylation of Bcl-2 and Bcl-xL that disrupts the Bcl-2/Beclin-1 and Bcl-xL/Beclin-1 complex, consistent with the reported mechanism of JNK in starvation-induced autophagy. The sharing of the JNK/Bcl-2-Bcl-xL cascade by Chal-24 and starvation for autophagy induction suggests this is a common autophagy-regulating pathway. Indeed, our recent report clearly showed that JNK-mediated regulation of Bcl-xL is required for cytoprotective autophagy induced by TRAIL. Therefore, targeting this pathway may be utilized for modulating autophagy-mediated cell survival or death. Further, we found that Chal-24 does not inhibit mTORC1 activity, suggesting that the mTORC1 pathway is not involved in Chal-24-induced autophagy (Fig. S6). It is noteworthy that in a recent report, Chal-24 was shown to reduce viable A549 cell number via activation of ERK or JNK, which is inconsistent with our finding. The reason of the discrepancy is currently unknown but is likely due to different concentrations of Chal-24 were used. Indeed, Chal-24 at 0.3 μM, which was used in the report by Warmka and colleagues, mainly suppresses cell proliferation but merely induces cell death (Fig. S7), while at higher concentrations (4–32 μM) it mainly induces cytotoxicity (Fig. 1). Thus, ERK and JNK may have distinct roles in distinct cellular mechanisms of Chal-24. Nevertheless, despite Chal-24 functions differently at different concentrations is of great interest for future study, our results clearly demonstrate that Chal-24 kills cancer cells through a novel autophagy-mediated necroptosis pathway that involves ERK and JNK.

We further determined that autophagy mediates necroptosis in Chal-24’s cytotoxicity. While the detailed mechanism of this action of autophagy needs further investigation, it likely involves destruction of c-IAPs. It is well known that c-IAPs put necroptosis in check during TNFR1 signaling through mediating RIP1 ubiquitination. Recent studies also found that degradation of c-IAPs triggers Ripoptosome formation and necroptosis induced by...
anticancer therapeutics or TRL3 activation\textsuperscript{10–12}. Thus, c-IAPs are located in a crucial position for necroptosis induction by different stimuli. Consistent with these reports, we clearly show that autophagy, together with ERK, strongly reduced c-IAPs expression, which is associated with necroptosis induction. Although how autophagy downregulates c-IAPs expression deserves further investigation, our results clearly suggest that a pathway involving autophagy-mediated downregulation of c-IAPs is activated for Chal-24-induced cytotoxicity.

While our results clearly show that both JNK and ERK are required for autophagy-mediated c-IAP degradation, the mechanism is not fully understood. While JNK promotes autophagy activation through releasing Beclin-1 from the inhibition by Bcl2 and Bcl-xL, how ERK works in c-IAP degradation is currently unclear. We found that Chal-24 causes ERK-dependent phosphorylation of c-IAP1 at late time points that occurred behind the timing of ERK activation. Thus, it is unlikely that ERK is the direct c-IAP1 kinase. Instead, ERK may mediate a currently unidentified pathway for c-IAP1 phosphorylation that is needed for targeting c-IAPs to the autophagosome for autophagic degradation. Further studies are needed to answer this important question.

As opposed to apoptosis, necroptosis results in release of cellular components that may stimulate the inflammatory response. Although there is a concern whether necroptosis will induce excess and systematic inflammation that may have adverse effect on patients, moderate local inflammation at the tumor site may be beneficial because it may elicit anticancer immunity \textsuperscript{43}. Indeed, Chal-24 treatment effectively suppressed tumor growth while having little adverse effect on mice in a xenografted tumor therapy model\textsuperscript{29}, suggesting that induction of necroptosis in tumors is likely safe for patients, which needs careful assessment in the future.

Taken together, our results establish a novel mechanism for killing cancer cells that involves autophagy-mediated necroptosis (Fig. 7D), which may be employed for overcoming chemoresistance. Further research is warranted for determining the effectiveness and possible side effects of targeting this pathway for anticancer chemotherapy.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Antibodies against Bcl-2 (sc-7328), Beclin1 (sc-48341), GAPDH (sc-32233) and RIP3 (sc-47368) were from Santa Cruz Biotechnology. Anti-phospho-JNK (44-682G), Anti-phospho-ERK (AHO0061) were from Invitrogen. Anti-caspase-8(551242), caspase-3 (559565), c-IAP-1 (AF8181), c-IAP-2 (552782), FADD (556402), JNK1 (554286), p62 (610832) and RIP1 (610458) were from BD Biosciences. Anti-poly(ADP-ribose) polymerase (PARP, ALX-210-302) was from Enzo. Anti-β-actin (A1978) and Anti-LC3B (L7543) was purchased from Sigma-Aldrich. Antibodies for Bcl-xL (#2762), phospho-p38 (#9211) and XIAP (#2042) were from Cell Signaling. Antibodies against ATG7 (PA5-17216) were from Thermo Scientific (Barrington, IL). Cyclophilin A (H00005478-P01) was from Abnova. The JNK inhibitor SP600125(129-56-6), p38 inhibitor SB203580 (152121-47-6), Wortmannin (19545-26-7) were from Calbiochem. Chloroquine (CQ,
50-63-5) was from Sigma-Aldrich. 3-Methyladenine (3MA, sc-205596) and Necrotatin-1(4311-88-0) was from Santa Cruz Biotechnology. Pan-caspase inhibitor z-VAD(AXL-260-039) was from Enzo. The ERK inhibitor U0126 (#9903) was from Cell Signaling. Short-interfering RNAs for selected genes (ATG7 siRNA, M020112 and RIP1 siRNA, M-00445-02-005) and the nontargeting siRNA (Silencer® negative control #1 siRNA) were obtained from Dharmacon. siRNAs targeting RIP3 (sc-61483), Beclin1 (sc-29797), ERK1 (sc-29307), ERK2 (sc-35335), JNK1 (sc-29380) and JNK2 (sc-39101) were from Santa Cruz Biotechnology.

**Cell Culture**

A549 and UM-UC-3 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

**Cytotoxicity Assay**

A cytotoxicity assay based on the release of lactate dehydrogenase (LDH) was conducted using a cytotoxicity detection kit (Promega, Madison, WI). Cells were seeded in 48-well plates at 70% to 80% confluence. After overnight culture, cells were treated as indicated in each figure legend. LDH release was determined as described previously. For long-term survival assay, cells were treated with Chal-24 (3 μM) for 3 days. The medium was refreshed every 3 days, and the cells were cultured for 8 days before fixing with methanol and staining with methylene blue.

**Western Blot and Immunoprecipitation**

Cell lysates were prepared by suspending cells in M2 buffer (20 mmol/L Tris-HCl [pH 7.6], 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, and 1 μg/mL leupeptin). Equal amounts of protein from each cell lysate were resolved by 12%, or 15% SDS-PAGE and analyzed by Western blot. The proteins were visualized by enhanced chemiluminescence according manufacturer’s instructions (EMD Millipore, Billerica, MA). Each experiment was repeated at least thrice and representative results are shown. For immunoprecipitation, cells were cultured in 60-mm dishes, treated as indicated in figure legends, and lysed in M2 buffer. Immunoprecipitation was performed as described previously. Briefly, 20 μl of protein A agarose beads (50%) were coupled to 1 μg Beclin 1 antibody in PBS for 2 h at room temperature. Then, 1 mg cell lysates were added and incubated with the beads by rotating overnight at 4 °C. The beads were washed seven times with M2 buffer. The immunoprecipitants were eluted off the beads using electrophoresis sample buffer. The samples were boiled for 5 min and loaded on 12% SDS-PAGE gel. BCL-XL, Bcl-2, Beclin-1 RIP1, FADD and caspase-8 were detected by Western blot.

**Knockdown of ATG7 and Beclin1 by RNAi**

Short-interfering RNAs for ATG7, Beclin1 and the nontargeting siRNA (Silencer® negative control #1 siRNA) were obtained from Thermo Scientific (Barrington, IL). A549 and UM-
UC-3 cells were seeded in a 12-well plate and 48-well plate the day before transfection at 50% confluency. INTERFERin™ transfection reagent (Polyplus-transfection, New York, NY) was used to transfect cells with siRNA. Twenty-four hours after transfection Chal-24 (8 μM for A549 and 4 μM for Um-UC-3) was added to the culture for 30 h and LDH release was determined to examine Chal-24-induced cytotoxicity, and knockdown confirmed by Western blot.

**Fluorescence microscopy**

A549 cells stably tranfected with GFP-LC3 were grown on glass coverslips, treated with Chal-24 (8 μM) for 2 h, then examined under a fluorescence microscope. Images shown are representative of three independent experiments. The percentage of punctate cells and average of puncta per positive cells were calculated.

**Statistics**

All data are expressed as means ± SD. Statistical significance was examined by one-way ANOVA. In all analyses, p<0.05 was considered statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Chal-24 induces non-apoptotic cell death

(A) The cells were treated with the indicated concentrations of Chal-24 for 30 h. Cell death was measured by LDH release assay. Data shown are the mean ± SD. (B) Cells were treated with Chal-24 (8 μM for A549, 4 μM for UM-UC-3) for the indicated time points. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. The cells treated with TRAIL (200 ng/mL for A549, 60 ng/mL for UM-UC-3) for 8 h were used as a control of apoptotic cell death. (C) A549 were pretreated with the z-VAD (5 μM) for 30 min and then treated with Chal-24 (8 μM) or TRAIL (200 ng/mL) for an additional 30 h. Cell death was measured by LDH release assay. Data shown are the mean ± SD. (D) A549 treated with Chal-24 (8 μM) or TRAIL (200 ng/mL) for 24 h and stained with acridine orange and ethidium bromide. White arrows indicate necrotic cells. Red arrows indicate apoptosis cells.
Fig 2. JNK and ERK activation is required for Chal-24-induced cell death

(A) A549 cells were treated with Chal-24 (8 μM) for indicated times. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. (B) A549 were pretreated with the indicated inhibitors for 30 min and then treated with Chal-24 (8 μM) for 30 min. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. (C) A549 cells were pretreated with the indicated inhibitors, SP600125 (10 μM), U0126 (10 μM), SB203580 (5 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 30 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD. * p<0.01.

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**Fig 3. Chal-24 induces autophagy in cancer cells**

**(A–B)** The cells were treated with Chal-24 (8 μM for A549, 4 μM for UM-UC-3) for indicated times. The indicated proteins were detected by Western blot. β-actin was detected as an input control. **(C–D)** The cells were pretreated with chloroquine (CQ, 20 μM) for 30 min and then treated with Chal-24 (8 μM for A549, 4 μM for UM-UC-3) for an additional 2 h (for LC3) or 8h (for p62). The indicated proteins were detected by Western blot. GAPDH was detected as an input control. **(E)** A549 stably transfected with GFP-LC3 were treated with Chal-24 (8 μM) for 2h or remained untreated. Photographs were taken under a fluorescence microscope. **(F)** Quantification of cell numbers with GFP puncta (left) and number of puncta per positive cell. Data shown are the mean ± SD. * p<0.01.
Fig 4. Chal-24 induces cell death depending on Autophagy  
(A) A549 cells were pretreated with 3MA (10 mM), CQ (20 μM), or Wortmannin (WTM, 1 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 30 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD. * p<0.01. (B) A549 cells were pretreated with different inhibitors (CQ, 20 μM; WTM, 1 μM; 3MA, 10 mM) for 30 min and then treated with Chal-24 (8 μM) for an additional 2h. The indicated proteins were detected by Western blot. β-actin was detected as an input control. (C, D) The cells were transfected with the indicated siRNAs (10nM) for 30 h and then treated with Chal-24 (8 μM) for an additional 30 h. Cytotoxicity was detected by LDH release assay. Data shown
are the mean ± SD. * p<0.01. Inserts, knockdown of each protein was confirmed by Western blot. β-actin was detected as an input control. (E). A549 cells were transfected with the indicated siRNAs (10 nM) for 30 h and then treated with Chal-24 (3 μM) for 3 days followed by culture in fresh medium that was refreshed every 3 days. The cells were fixed with methanol and staining with methylene blue after culturing for 8 days. Representative mages of whole wells are shown.
Fig 5. Chal-24-induced autophagy involves JNK-mediated phosphorylation of Bcl-2 and Bcl-xL.

(A, B) A549 were pretreated with the indicated inhibitors, SP600125 (10 μM), U0126 (10 μM), SB203580 (5 μM), WTM (1 μM) or CQ (20 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 2 h. The indicated proteins were detected by Western blot. β-actin or GAPDH was detected as an input control. (C, D) The cells were treated with Chal-24 (8 μM for A549, 4 μM for UM-UC-3) for indicated times. The indicated proteins were detected by Western blot. β-actin or GAPDH was detected as an input control. (E) A549 cells were pretreated with SP600125 (10 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 2 h. For Lambda protein phosphatase (Lambda PP) treatment, the cell extracts were incubated with Lambda PP (400 u/μl) at 30 °C for 1h. The indicated proteins were detected by Western blot. β-actin or GAPDH was detected as an input control. (F) A549 were pretreated with SP600125 (10 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 2 h. The indicated proteins were detected by Western blot after co-immunoprecipitation with an antibody for Beclin-1.
Fig 6. Chal-24 induces degradation of c-IAPs depending on autophagy and ERK
(A) A549 cells were treated with Chal-24 (8 μM) for indicated times. The indicated proteins were detected by Western blot. β-actin or GAPDH was detected as an input control. (B) A549 cells were transfected with combination of ERK1 and ERK2 siRNAs or JNK1 and JNK2 siRNAs (10 nM each) for 24 h and then treated with Chal-24 (8 μM) for an additional 30 h. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. (C) A549 cells were pretreated with 3MA (10 mM), CQ (20 μM), or Wortmannin (WTM, 1 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 30 h. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. (D) A549 were transfected with the indicated siRNAs (10nM) for 24 h and then treated with Chal-24 (8 μM) for an additional 30 h. GAPDH was detected as an input control. (E) A549 cells were transfected with the indicated siRNAs (10nM) for 24 h and then treated with Chal-24 (8 μM) for an additional 30 h. GAPDH was detected as an input control.
input control. (E) A549 cells were pretreated with U0126 (10 μM) for 30 min and then treated with Chal-24 (8 μM) for indicated times. Lambda phosphatase treatment was as described in 5E. The indicated proteins were detected by Western blot. GAPDH was detected as an input control. (F) A549 were treated with Chal-24 (8 μM) for the indicated times. The indicated proteins were detected by Western blot after co-Immunoprecipitation with an antibody for RIP1. (G) A549 were pretreated with CQ (20 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 30 h. The indicated proteins were detected by Western blot after co-immunoprecipitation with an antibody for RIP-1. GADPH was detected as a control for specificity of co-Immunoprecipitation.
Fig 7. Chal-24 induces necroptosis in cancer cells

(A) A549 cells were treated with Chal-24 (8 μM) for indicated times. The indicated proteins in culture media were detected by Western blot. Comassie blue staining for the main protein component, BSA, in medium was used as an input control. (B) A549 cells were pretreated with the Necrostatin-1 (20 μM) for 30 min and then treated with Chal-24 (8 μM) for an additional 30 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD. * p<0.01. (C) A549 and UM-UC-3 cells were transfected with the indicated siRNAs (10 nM) for 30 h and then treated with Chal-24 (8 μM for A549, 4 μM for UM-UC-3) for an additional 30 h. Cytotoxicity was detected by LDH release assay. Data shown are the mean ± SD. * p<0.01. Knockdown of each protein was confirmed by Western blot. β-actin was detected as an input control. (D) A model of Chal-24-induced cell death.