Rubiarbonol B induces RIPK1-dependent necroptosis via NOX1-derived ROS production

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Abstract

The activation of receptor-interacting protein kinase 1 (RIPK1) by death-inducing signaling complex (DISC) formation is essential for triggering the necroptotic mode of cell death under apoptosis-deficient conditions. Thus, targeting the induction of necroptosis by modulating RIPK1 activity could be an effective strategy to bypass apoptosis resistance in certain types of cancer. In this study, we screened a series of arborinane triterpenoids purified from *Rubia philippinesis*, and identified rubiarbonol B (Ru-B) as a potent DISC-mediated caspase-8 activator. Ru-B elicited both the apoptotic and necroptotic modes of cell death, depending on the cellular expression of RIPK3. In RIPK3-expressing human colorectal cancer (CRC) cells, and the pharmacological or genetic inhibition of caspase-8 was sufficient to cause a switch in cell death mode from apoptosis to necroptosis following Ru-B treatment by necrosome formation which accompanied by the upregulation of RIPK1 phosphorylation. Conversely, Ru-B-induced cell death was almost completely abrogated by RIPK1 deficiency. The enhanced RIPK1 phosphorylation and necroptosis triggered by Ru-B treatment occurred independently of tumor necrosis factor receptor signaling, and was mediated by the production of reactive oxygen species via NADPH oxidase 1 in CRC cells. Thus, we propose Ru-B as a novel anticancer agent that enhances the cytotoxic potential of RIPK1, prompting the further development of Ru-B as a necroptosis-targeting compound in apoptosis-resistant CRC.

Introduction

The death domain kinase, receptor-interacting protein kinase 1 (RIPK1), is an essential mediator of the activation of programmed cell death (PCD) via apoptosis and necroptosis, and exerts its effects by integrating signaling complexes following the ligation of cell surface receptors, such as tumor necrosis factor receptor 1 (TNFR1) and Toll-like receptor 3 (Humphries 2015; Meylan et al. 2004; Silke 2011; Witt and Vucic 2017).

Upon ligation with TNFR1, RIPK1 can transduce the signal to either cell survival or PCD, depending on the engagement of activated adaptor proteins and/or the cellular context. Recently, it has been proposed that RIPK1 posttranslational modifications in spatially distinct TNFR1 complexes (complex-I and -II) play an important role in determining cell fate (Kang et al., 2019; Ting and Bertrand 2016). The conjugation of non-degradable poly-ubiquitin chains to RIPK1 bound within complex-I maintains the survival function of RIPK1 by acting as a scaffold for the recruitment of pro-survival kinases, such as IκB kinase, which are essential for the activation of nuclear factor-κB (NF-κB) (Dynek et al., 2010; Fritsch et al., 2014; Wertz, 2014). Conversely, RIPK1 lacking poly-ubiquitination detaches from complex-I and associates with caspase-8- and FADD-containing complex, namely complex-II (also termed as a death-inducing signaling complex; DISC) that causes apoptotic cell death in a RIPK1-dependent manner (Brenner et al., 2004; Micheau and Tschopp, 2003). Notably, genetic or pharmacological inhibition of caspase-8 activity greatly increases the cytotoxic potential of RIPK1 via Ser166-autophosphorylation, which promotes the switch to RIPK1-dependent necroptosis by inducing the recruitment of RIPK3 and mixed lineage kinase-domain-like (MLKL) (Kaiser et al., 2011; Li et al., 2012; Newton, 2015).

In various human cancers, genetic alterations occur that play an important role in the evasion of apoptosis, a hallmark of cancer that represents a major mechanism of cellular resistance to current cancer treatments including radiation and chemotherapeutic drugs (Croce and Reed, 2016; Hanahan and Weinberg, 2000). Caspase-8 is often inactivated by somatic mutations or epigenetic methylation in multiple types of human cancer, including colorectal cancer (CRC) (Hopkins-Donaldson et al., 2003; Kim et al., 2003; Teitz et al., 2000). In caspase-8-deficient CRC, the use of Smac mimetics can reduce cellular inhibition of apoptosis protein(cIAP)-mediated RIPK1 ubiquitination, which overcomes apoptosis resistance by inducing RIPK1-dependent necroptosis (He et al., 2017). Furthermore, DNA damaging compounds such as etoposide and doxorubicin induce apoptosis or necroptosis (depending on the cellular context) without the involvement of TNFR1 ligation via the Ripoptosome, a cytosolic complex containing RIPK1, FADD and caspase-8 (Bertrand and Vandenabeele, 2011; Koo et al., 2015; Tenev et al., 2011). Thus, the discovery of a substance capable of inducing the necroptotic mode of cell death via the Ripoptosome could lead to an effective chemotherapeutic strategy for eradicating apoptosis-resistant cancer cells.

Triterpenoids comprise the largest group of plant natural products and possess a diverse range of pharmacological activities (Gill et al., 2016). Pentacyclic triterpenoids in particular exhibit promising antitumor activity, regulating multiple cellular pathways related to apoptosis, the cell cycle and angiogenesis (Markov et al., 2017; Patlolla and Rao, 2012). Arborinane-type triterpenoids constitute a rare group of pentacyclic triterpenoids. Recently, arborinane-type triterpenoids such as rubiarbonol G and myrotheols A have attracted attention from chemists and pharmacologists due to their potential to induce apoptosis and cell cycle arrest in various cancer cell types (Basnet et al., 2019; Zeng et al., 2018). However, the activity of arborinane-type triterpenoids toward necroptotic inducers and Ripoptosome formation is largely unknown. The genus *Rubia* is a rich source of arborinane-type triterpenoids. In a previous phytochemical study, we isolated a series of arborinane-type triterpenoids from *Rubia philippinesis* (*R. philippinesis*) (Quan et al., 2016). In the present study, we show that a novel arborinane triterpenoid isolated from *R. philippinesis*, rubiarbonol B (Ru-B), elicited apoptotic and necroptotic cell death via Ripoptosome formation in RIPK3-expressing CRC cells. When apoptosis was blocked, Ru-B triggered a shift from apoptotic to RIPK1-dependent necroptotic cell death. The RIPK1-dependent cell death was mediated by NADPH oxidase 1 (NOX1)-derived reactive oxygen species (ROS) generation, which led to TNFR1-independent RIPK1 phosphorylation. Our findings provide insight into the interplay between necroptotic cell death and ROS-mediated RIPK1 phosphorylation that underlies the cytotoxic potential of Ru-B, and offer a potential therapeutic strategy for the treatment of refractory CRC, which is resistant to proapoptotic stimuli.

Materials And Methods

Extraction of rubiarbonol B

Arborinane-type triterpenoids, rubiarbonol B (Ru-B) was isolated from our previous chemical investigation on *R. phillippinensis* (Quan et al., 2016). Rubiarbonol B: white amorphous powder; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 5.50 (1H, brs, H-11), 4.50 (1H, t, J = 7.7 Hz, H-19), 4.05 (1H, m, H-7), 3.48 (1H, t, J = 7.5 Hz, H-3), 1.28 (3H, s, H_3-26), 1.25 (3H, s, H_3-23), 1.22 (3H, s, H_3-25), 1.16 (3H, s, H_3-27), 1.11 (3H, s, H_3-24), 0.90 (6H, s, overlapped, H_3-28 and H_3-29), 0.85 (3H, d, J = 4.8 Hz, H_3-30); ^{13}C NMR (pyridine-d_5, 75 MHz) δ 148.0 (C-9), 117.4 (C-11), 78.4 (C-3), 72.6 (C-7), 70.7 (C-19), 59.6 (C-18), 58.2 (C-21), 49.8 (C-8), 49.4 (C-5), 44.2 (C-17), 42.3 (C-20), 40.3 (C-14), 40.2 (C-10), 39.8 (C-4), 38.7 (C-13), 37.7 (C-12), 37.5 (C-16), 37.4 (C-1), 34.3 (C-6), 32.4 (C-15), 31.1 (C-22), 29.1 (C-2), 29.1 (C-23), 23.6 (C-30), 22.6 (C-29), 22.4 (C-25), 17.5 (C-26), 17.3 (C-27), 16.8 (C-24), 16.3 (C-28).

Structure determination of rubiarbonol B

Ru-B was determined on the basis of NMR and MS spectroscopic data analysis. The ^{13}C NMR spectrum of Ru-B exhibited typical 30 carbon signals corresponding to an arborinane-type triterpenoid. The ^{1}H NMR spectrum of Ru-B displayed characteristic resonances for one olefin (δ 5.50), three oxygenated methines (δ 4.50, 4.05, 3.48), six tertiary methyls (δ 1.28, 1.25, 1.22, 1.16, 1.11, 0.90), and two secondary methyls (δ 0.90, 0.85). Comparison of the 1D NMR spectra with those reported in the reference enabled identification as rubiarbonol B (Quan et al., 2016).

Antibodies and reagents

All commercial antibodies and chemicals were purchased from the following resources: anti-caspase-8 (#9746), anti-caspase-3 (#9662), anti-Bid (#2002), anti-Bcl-2 (#2872), anti-Bcl-xL (#2762), anti-RIPK3 (#13526, for WB and IP), anti-phospho-RIPK3-S^{227} (#93654), anti-phospho-MLKL-S^{358} (#37333), anti-phospho-RIPK1-S^{166} (#65746), anti-phospho-IKKα/β-S$^{176/180}$ (#2697), anti- IκBα (#9242), and anti-phospho-p65-S^{536} (#3033) antibodies were from Cell Signaling Technology (Beverly, MA, USA); anti-FADD (sc-5559), anti-Mox1 (sc-518023), anti-TRADD (sc-46653), and anti-TNFR1 (sc-8436) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Actin antibody (A2066), anti-Flag antibody (F7425), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Cycloheximide (CHX), Dihydroethidium (DHE), MitoTEMPO, Apocynin, Diphenyleneiodonium (DPI), Butylated hydroxyanisole (BHA), Necrostatin-1 (Nec-1), and Necrosulfonamide (NSA) were from Sigma-Aldrich (St. Louis, MO, USA); MitoTracker Green and MitoSox Red were from Invitrogen (Franklin, MA, USA); anti-PARP (556362) and anti-RIPK1 (556395) antibodies were from BD Biosciences (San Diego, CA, USA); anti-MLKL (ab184718) antibody was from Abcam (Cambridge, MA, USA); recombinant TNF-α (410-MT) and anti-caspase-8 (AF-705, for IP), anti-TNFR1 (AF-425, for IP) antibodies were from R&D system (Minneapolis, MN, USA); recombinant human TRAIL ligand (310-04) was from PeproTech (Rocky Hill, NJ, USA); SM-164, benzyloxy-carbonyl-Asp-(OMe)-Glu-(OMe)-Val-Asp-(OMe)-fluoromethyl ketone (z-DEVD-fmk), benzyloxy-carbonyl-Ile-Glu-(OMe)-Tyr-Asp-(OMe)-fluoromethyl ketone (z-IETD-fmk), arbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk), and GSK-872 were from MedChemExpress (Monmouth Junction, NJ, USA).

Cell culture, plasmids and transfection

HCT116 cells, HeLa cells, MCF7 cells, DLD1 cells, Caco2 cells, SW620 cells, Jurkat cell and HT-29 were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum, 2 mmol/L glutamine and 100 U/mL penicillin/ streptomycin in 5% CO2 atmosphere at 37°C. The point-mutant RIPK1 (C257S, C268S, and C586S) were created using a Quickchange™ Site-Directed Mutagenesis kit (Stratagene). For the knockdown of NOX1 and NOX2, siRNA specific for human NOX-1 (sc-43939) and NOX-2 (sc-106233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transient transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Franklin, MA, USA), following the manufacturer's protocol.

CRISPR/Cas-9 mediated KO cells generation

For the depletion of RIPK1, caspase-8 and FADD in HT-29 cells, oligos were synthesized and inserted into the px330-puro vector through a standard protocol to generate gRNA with hCas9 protein. gRNA sequences were designed using the open-access software provided at http://chopchop.cbu.uib.no/. gRNA sequences were as follows: RIPK1-CTCGGGCGCCATGYAGTAGA; caspase-8- CACCGAACGAGATATAT CCCGGATG; FADD-ACACGCTCTGTCAGGTTGCG. The targeting plasmid was transfected into HT-29 cells using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Franklin, MA, USA). After 24h, cells were exposed with 3μg/ml puromycin for two days, and clones propagated from single cells were picked out. The depletion of target genes was confirmed by both immunoblotting and genomic DNA sequencing.

Caspase-8 activation assay

HCT116 cells were plated in 96-well plates and treated with a series of constituents (10 μM) derived from *R. phillippinensis* for 24 h. Caspase-8 activity was measured using a Caspase-Glo 8 assay kit (Promega, USA) that utilizes luminogenic caspase-8 substrates, following the manufacturer's instructions. The luminescence intensity of each sample was measured in a plate-reading luminometer (Infinite 200pro, Tecan, Switzerland).

Determination of cell death

After treatment as described in the figure legends, a cell viability assay was conducted utilizing Cell Titer-glo Luminescent Cell Viability Assay kit (Promega, USA), which measures cell viability based on ATP levels present in live cells. Luminescent measurements were taken on a microplate leader (Infinite 200pro, Tecan, Switzerland). Representative images were also taken by an inverted microscope (EVOS M5000, Thermo Fisher Scientific, USA). For the measurement of early/late apoptotic or necrotic cell death, cells were stained with 10 μM fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI), in a Ca^{2+}-enriched binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and analyzed by two-color flow cytometry. The fluorescence of cells was analyzed by NovoCyte Flow Cytometer (ACEA Biosciences, USA).

RT-PCR analysis

After total RNA was extracted using an easy-BLUE RNA extraction kit (iNTRON Biotechnology, Korea), the ologo(dT)-primed cDNA was synthesized using a RT-PCR kit (Promega, USA). PCR amplication was carried out using the primer pairs specific to each human genes (NOX-1, 5' - GCCTGTGCCCGAGCGTCTGC – 3' and 5' - ACCAATGCCGTGAATCCCTAAGC – 3' ; NOX-2, 5' - GGAGTTTCAAGATGCGTGGAAA CTA – 3' and 5' - GCCAGACTCAGAGTTGGAGATGCT – 3' ; NOX-3, 5' - CCATGGGACGG GTCGGATTGT – 3' and 5' - GGGGGCAGAGGTAAGGGTGAAGG – 3' ; NOX-4, 5' - GTCAT AAGTCATCCCTCAGA – 3' and 5' - TCAGCTGAAAGACTCTTTAT – 3' ; NOX-5, 5' - ATCA AGCGGCCCCCTTTTTTCAC – 3' and 5' - CTCATTGTCACACTCCTCGACAGC – 3' ; GAPDH, 5' -GACCCCTTCTTGACCTC-3' and 5' - GCCATCCACAGTCTTCTG-3').

Immunoblot analysis and immunoprecipitation

After treatment as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg/mL leupeptin). Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by enhanced chemiluminescence (Thermo Fisher, USA). For immunoprecipitation assay, the lysates were precipitated with the relevant antibodies and protein A- or G-sepharose beads overnight at 4°C. The beads were washed three times with M2 buffer, and the bound proteins were resolved in 10% SDS-PAGE for immunoblot analysis. Densitometry analysis was performed on scanned immunoblot images using an image J software.

Determination of ROS production

Production of intracellular ROS was measured using a fluorescent dye dihydroethidium (DHE) in HT-29 cells. After treatment, as described in the figure legends, cells were incubated with 10 μM DHE in phosphate-buffered saline (PBS) solution containing 10% FBS for 30 min. The stained cells were analyzed with flow cytometry (NovoCyte Flow Cytometer, USA), and the mean fluorescence intensity (MFI) was calculated after correction for autofluorescence is presented. For the quantification of the mitochondrial-derived superoxide (O_2^-) production, cells were incubated with a mitochondria-target probe, MitoSOX Red (5 μM) with a MitoTracker green (200 nM) for 10 min, and the images were captured using a fluorescent microscope (EVOS M5000, Thermo Fisher Scientific, USA).

Statistical analysis

Data are expressed as the mean ± SE from at least three separate experiments performed triplicate. The difference between groups was analyzed using the Student's t-test, and $P < 0.05$ is considered statistically significant. Statistical analyses were carried out using SPSS software (ver. 24; SPSS Inc., Chicago, IL, USA)

Results

Ru-B induces caspase-8-mediated apoptosis by associating with DISC

To identify novel small molecules with cytotoxic potential that act on caspase-8, we first screened a set of arborinane-type triterpenoids purified from *R. phillippinensis* by conducting a protease activity assay using a luminogenic substrate specific for caspase-8. In initial an investigation, Ru-B was found to be a potent caspase-8 activator, exhibiting modest cytotoxicity in HCT116 human CRC cells. (Table 1, Fig. 1A). To determine whether the cytotoxic potential of Ru-B could be attributed to caspase-8 activation, we compared the effects of the irreversible caspase-8 and caspase-3 inhibitors z-IETD-fluoromethyl ketone (z-IETD) and z-DEVD-fluoromethyl ketone (z-DEVD), respectively, against Ru-B-induced cell death. Pretreatment with z-IETD effectively abrogated cell death in response to Ru-B treatment in multiple types of human cancer cells, including HCT116, HeLa and MCF7 cells, while z-DEVD had a marginally preventive effect against cell death (Fig. 1B, 1C). To examine the mode of cell death triggered by Ru-B, cells treated with Ru-B were subjected to annexin V and propidium iodide (PI) staining, followed by flow cytometry. Ru-B treatment resulted in significant increases in both the early and late phases of apoptosis (52.8% and 9.14%, respectively), while very few cells were stained exclusively with PI (2.51%) (Fig. 1D). Consistently, such an increased population of cell satined with Annexin V following Ru-B treatment was significantly reduced by pretreatment with z-IETD, but not with z-DEVD. To further investigate the signaling pathway underlying Ru-B-induced apoptosis, we analyzed the sequence of activation processes in the caspase cascades. In a kinetic analysis, treatment of Ru-B resulted in sequential activation of caspase-8 and the resultant cleavage of RIPK1, Bid, caspase-3 and PARP, which was completely inhibited by pretreatment with z-IETD (Fig. 1E). Caspase-8 is a key initiator of death receptor (DR)-mediated apoptosis upon DR ligation by associating with RIPK1 and FADD to form the DISC. Catalytic activation of caspase-8 triggers the cleavage of DISC-associated RIPK1, resulting in destabilization of the DISC (Tummer and Green, 2017). Accordingly, an immunoprecipitation assay using an anti-caspase-8 antibody showed that RIPK1, FADD and caspase-8 were recruited to the isolated DISC following Ru-B treatment in the presence of z-IETD (Fig. 1F). Taken together, these results suggest that Ru-B from *R. phillippinensis* elicits caspase-8-mediated apoptosis via DISC formation.

Ru-B triggers a shift from apoptosis to necroptosis in RIPK3-expressing cancer cells

Given that caspase-8 inhibits RIPK3-MLKL-mediated necroptosis (Shalini et al., 2015), we hypothesized that RIPK3 expression in cancer cells may be play a role in determining the cell fate (apoptosis or necroptosis) in response to Ru-B treatment. To investigate this, we compared the effects of z-IETD against Ru-B-induced cell death in pairs of CRC cells either lacking or harboring RIPK3 expression. In line with previous findings, z-IETD pretreatment drastically suppressed Ru-B-induced cell death in HCT116, DLD1 and Caco2 cells, all of which lack RIPK3 expression (Fig. 2A). Conversely, in the RIPK3-expression cell lines HT-29 and SW620, Ru-B-induced cell death was significantly enhanced by the pretreatment with

z-IETD. Furthermore, Ru-B-induced cell death was rescued by the RIPK1-specific inhibitor necrostatin-1 (Nec-1); this was more prominent in HT-29 cells treated with z-IETD (Fig. 2B). Importantly, Pretreatment of HT-29 cells with z-IETD resulted in a remarkable increase in biochemical markers of necroptosis, such as the phosphorylation of RIPK1, RIPK3 and MLKL, in response to Ru-B treatment (Fig. 2C), suggesting that Ru-B-induced apoptosis switched to necroptosis when caspase-8 was chemically inhibited. Consistent with this notion, cell death induced by Ru-B and z-IETD treatment was almost completely prevented by pretreatment with Nec-1, or with the RIPK3- and MLKL-specific inhibitors GSK872 and necrosulfonamide, respectively (Fig. 2D). These results indicate that the inhibition of caspase-8-mediated apoptosis by Ru-B sensitizes RIPK3-expressing cells to RIPK1/3- and/or MLKL-dependent necroptosis.

To further assess the functional relationship between apoptosis and necroptosis following Ru-B treatment, we used CRISPR-Cas9 to knock out caspase-8 or FADD in HT-29 cells and examined the modes of cell death. As expected, caspase-8 signaling cascades were activated without triggering necroptosis-related events after Ru-B treatment in wild-type (WT) HT-29 cells (Fig. 2E, left panel). By contrast, RIPK1, RIPK3 and MLKL were markedly phosphorylated in both caspase-8- and FADD-deficient HT-29 cells upon Ru-B treatment (Fig. 2E, middle and right panels). To gain further insight into the molecular mechanisms underlying Ru-B-induced RIPK1/3 and MLKL phosphorylation, we examined whether Ru-B induces the necrosome formation under the condition of pharmacological or genetic blockade of DISC-mediated apoptosis. As expected, RIPK1, RIPK3 and MLKL were associated with caspase-8 in HT-29 cells after treatment with Ru-B in the presence of z-IETD (Fig. 2F). In parallel, we observed the Ru-B-induced association of necrosome components including RIPK1, RIPK3 and MLKL in caspase-8 deficient HT-29 cells (Fig. 2G). Our results suggest that, in physiological conditions under which both apoptotic and necroptotic cell death are preserved, cells preferentially undergo apoptotic cell death instead of necroptosis in response to Ru-B treatment, and cell fate can revert to necroptosis via necrosome formation in apoptosis-limiting conditions.

RIPK1 phosphorylation plays an essential role in Ru-B-induced necroptosis

RIPK1 and its phosphorylation play an essential role in inducing necroptosis by forming an RIPK3-containing necrosome under apoptosis-deficient conditions (Newton 2015). To directly determine whether Ru-B-induced PCD is achieved by targeting RIPK1, we examined the cytotoxic efficacy of Ru-B and Ru-B/z-IETD treatment in WT and RIPK1-knockout (KO) HT-29 cells. As establishing that RIPK1 is not involved in the TNF/cycloheximide (CHX)-induced cell death pathway (Kang et al., 2020; Lin et al., 2004), the cytotoxic effects of TNF/CHX treatment were comparable between RIPK1-KO and WT HT-29 cells (Fig. 3A-3C). By contrast, Ru-B- and TNF/SM-164 (SM)-induced cell death, as well as caspase-8 cascade activation, were almost completely abolished in RIPK1-KO HT-29 cells (Fig. 3B-3D). Furthermore, necroptotic cell death accompanied by RIPK1, RIPK3 and MLKL phosphorylation was completely abolished in RIPK1-KO HT-29 cells treated with Ru-B or TNF/SM in the presence of z-IETD. Similar effects were found in RIPK1-deficient Jurkat T cells (Fig. 3E). These results suggest that RIPK1 plays an essential role in Ru-B-induced apoptotic and necroptotic cell death.

Because the phosphorylation of RIPK1 at serine residue 166 (Ser^{166}) triggers RIPK1 kinase activity to trigger the downstream cell death signaling (Kang et al., 2019), we next investigated whether Ru-B could induce RIPK1 phosphorylation. We found that in response to Ru-B, RIPK1 was phosphorylated in WT HT-29 cells, peaking at 1 h after Ru-B treatment (Fig. 4A, left panel). Important to note, Ru-B-induced RIPK1 phosphorylation was markedly prolonged and enhanced by z-IETD pretreatment, which was subsequently accompanied with the enhanced phosphorylation of RIPK3 and MLKL (Fig. 4A, right panel). These results suggest that persistent RIPK1 phosphorylation promotes RIPK3/MLKL-mediated necroptosis when the apoptotic pathway is blocked. Consistent with this notion, RIPK1 phosphorylation was persistent in caspase-8-KO and FADD-KO HT-29 cell following treatment with Ru-B, while RIPK1 phosphorylation was transient in WT HT-29 cells (Fig. 4B). Moreover, Ru-B-induced RIPK1 and RIPK3 phosphorylation was almost completely inhibited by Nec-1 (Fig. 4C). Subsequent immunoprecipitation assays revealed that Ru-B treatment led to the recruitment of RIPK1 and MLKL into the isolated RIPK3 in caspase-8-KO HT-29 cells, and this necrosome formation was abrogated by Nec-1 (Fig. 4D). These results indicate that the increased RIPK1 phosphorylation triggered by Ru-B treatment likely occurs upstream of RIPK3 and actively drives necroptosis via necrosome formation under apoptosis-deficient conditions.

NOX1-derived ROS production induced by Ru-B is required for RIPK1 phosphorylation and RIPK1-dependent cell death

Previous *in vitro* and *in vivo* experimental studies reported that ROS derived from superoxide (O_2^-) are involved in RIPK1-dependent necroptosis (Goossens et al., 1995; Kim et al., 2007; Roca and Ramakrishnan, 2013). Furthermore, ROS function as a positive feedback loop to enhance necrosome formation via RIPK1 autophosphorylation at Ser^{161} (Schenk and Fluda, 2015; Zang et al., 2017). Therefore, we investigated whether intracellular O_2^- levels increased following Ru-B treatment using dihydroethidium, an oxidative fluorescent dye. Ru-B and Ru-B/z-IETD treatment caused a dramatic increase in O_2^- levels within 30 min in HT-29 cells, which peaked at 1 h after treatment (Fig. 5A). This increase was attenuated when the cells were pretreated with either butylated hydroxyanisole (BHA), a general ROS scavenger, or the NOX inhibitors diphenyleneiodonium and apocynin; however, intracellular O_2^- levels were not reduced following treatment with the mitochondria-targeting antioxidant Mito-TEMPO (Fig. 5B). These results suggest that Ru-B induces non-mitochondrial ROS production, potentially via NOX. To exclude the possibility that the ROS production triggered by Ru-B treatment was mitochondrial, we used a mitochondria-targeting hydroethidine analog, MitoSOX Red, to monitor mitochondrial O_2^- production. Treatment of HT-29 cells with carbonyl cyanide chlorophenylhydrazone, a mitochondrial uncoupler, dramatically enhanced the MitoSOX Red oxidation signal, being consistent with the well-established mitochondrial uncoupling effect (Fig. 5C, bottom panel). By contrast, Ru-B treatment did not induce MitoSOX Red oxidation (Fig. 5C, middle panel), indicating that Ru-B-induced ROS production

occurs independently of the mitochondria. To determine whether increased ROS production plays a role in Ru-B or Ru-B/z-IETD-induced cell death, we pretreated HT-29 cells with various antioxidants. Pretreatment with BHA or the NOX inhibitors, but not with Mito-TEMPO, significantly prevented cell death in response to Ru-B and Ru-B/z-IETD treatment; this was correlated with their ROS quenching efficiencies (Fig. 5D). Moreover, the sequential phosphorylation of RIPK1, RIPK3 and MLKL upon Ru-B/z-IETD treatment was markedly attenuated in the presence of apocynin (Fig. 5E). These results indicate that ROS generated by NOX enzymes play an important role in RIPK1 phosphorylation, which subsequently leads to RIPK1-dependent apoptosis and necroptosis in response to Ru-B and Ru-B/z-IETD, respectively.

Of the known NOX enzymes, NOX1 is expressed in several types of non-phagocytic cells, while NOX2/gp91 is found primarily in phagocytic cells (Geiszt et al., 2003; Suh et al., 1999). Thus, we investigated whether the expression of various NOX isoforms was responsible for Ru-B-induced ROS production. As shown in Fig. 5F, two major NOX isoforms, NOX1 and NOX2, were constitutively expressed in multiple types of colon cancer cells. Notably, the mRNA levels of NOX1 were high compared to those of the other four NOX isoforms (Fig. 5F). Knockdown of NOX1 led to a significant decrease the ROS production following Ru-B treatment (Fig. 5G), suggesting that NOX1 is the major NOX responsible for Ru-B-induced ROS production in colon cancer cells. Furthermore, knockdown of NOX1, but not NOX2, caused a marked attenuation of cell death against to both apoptotic (Ru-B) and necroptotic (Ru-B/z-IETD) triggers, respectively (Fig. 5H), which was accompanied by decreased cleavage of caspase-8 cascade and reduced phosphorylation of RIPK1 and RIPK3 (Fig. 5I). These data suggest that NOX1-derived ROS production plays an essential role in RIPK1-mediated cell death in CRC cells.

Three cysteine residues on RIPK1 play a crucial role in triggering necroptosis by regulating the ROS-mediated RIPK1 phosphorylation induced by Ru-B

Three cysteine residues (C257, C268 and C586) in RIPK1 sense ROS signals and thus play a crucial role in RIPK1 autophosphorylation and necroptosis, by forming oxidized disulfide bonds and causing RIPK1 to aggregate (Zhang et al., 2017). Thus, it is interesting to test whether these cysteine residues are involved in RIPK1 phosphorylation and necroptosis mediated by Ru-B/z-IETD. To address this issue, we reconstituted wild-type (WT) or three cysteine mutants (3CS) of RIPK1 in RIPK1-KO HT-29 cells. Consistent with a previous report (Zhang et al., 2017), no significant differences in the recruitment of ubiquitinated-RIPK1 and TRADD into TNFR1 were detected between RIPK1-KO HT-29 cells reconstituted with either WT or 3CS RIPK1 (Fig. 6A). Consistently, we also observed that treatment of cells with TNF led to no obvious difference in TNF-induced NF-κB activation between these cells, as evidenced by either the phosphorylation of IKK and p65 or the degradation of IκBα (Fig. 6B), confirming that the modification of these cysteine residues on RIPK1 is unlikely involved in upstream NF-κB activation signaling upon TNFR1 ligation. By contrast, following Ru-B/z-IETD treatment, RIPK1 phosphorylation (Fig. 6C), as well as the association of the RIPK1, RIPK3 and MLKL with caspase-8 (Fig. 6D), was dramatically reduced in RIPK1-KO HT-29 cells expressing 3CS-RIPK1 compared to those expressing WT-RIPK1. This suggests that the

modification of the RIPK1 cysteine residues serves a critical role in Ru-B-mediated RIPK1 phosphorylation and necrosome formation. To further determine whether the cysteine residues of RIPK1 are directly involved in Ru-B-mediated necroptosis, we compared the cytotoxicity in RIPK1-KO cells reconstituted with WT-RIPK1 and 3CS-RIPK1. As expected, no differences were observed between RIPK1-KO HT-29 cells expressing WT or 3CS-RIPK1 following TNF-related apoptosis-inducing ligand (TRAIL) treatment. (Fig. 6E). By contrast, necroptotic cell death triggered by either Ru-B/z-IETD or TNF/SM/z-IETD was significantly lower in RIPK1-KO cells reconstituted with 3CS-RIPK1 compared with those expressing WT-RIPK1, as evidenced by cell viability, cell morphology and the phosphorylation of RIPK1, RIPK3 and MLKL (Fig. 6E-6G). Taken together, these results suggest that the 3 cysteine residues on RIPK1 are indeed essential for RIPK-1-dependent necroptosis, and function by amplifying the ROS-mediated phosphorylation of RIPK1.

Discussion

Given the pivotal role of RIPK1 in triggering necroptosis, small molecules capable of activating RIPK1 kinase activity and RIPK1-dependent PCD in human cancer cells present an alternative means of eradicating cancer cells, by inducing the necroptotic mode of cell death in apoptosis-resistant cancer cells. As part of our search for PCD-inducing bioactive compounds at the DISC level, the novel arborinane triterpenoid Ru-B, which was isolated from *R. philippinesis*, was identified as a potent inducer of dual RIPK1-dependent modes of apoptosis and necroptosis in RIPK3-expressing CRC cells. In this study, we found that Ru-B markedly enhances necroptosis by upregulating RIPK1 phosphorylation under apoptosis-limiting conditions, which is mediated by NOX1-derived ROS production. Thus, we propose that Ru-B is a novel RIPK1 activator that could provide an efficient strategy to induce CRC cell necroptosis to overcome apoptosis-resistant.

Caspase-8 activation is triggered by RIPK1-associated DISC formation following DR ligation, and functions as an initiator caspase that induces the extrinsic apoptotic signaling pathway (Tummer and Green, 2017). However, the anti-cancer properties of several bioactive compounds, including pentacyclic triterpenoids, are closely associated with the DISC-independent activation of executor caspases (*e.g.* caspase-3) by inducing mitochondrial dysfunction (Fluda, 2010; Fluda and Kroemer, 2009; Markov et al., 2017). However, in this study, we found that Ru-B induces caspase-8 activation and DISC formation without affecting the mitochondrial pathway in multiple types of CRC cells (Fig. 1). In addition, pretreatment with the caspase-8 inhibitor z-IETD protected CRC cells lacking RIPK3 expression against Ru-B-induced apoptosis (Fig. 1). Moreover, we provide evidence that Ru-B is capable of not only accelerating cell death, but also shifting the balance of cell death to necroptosis after the genetic or pharmacological inhibition of caspase-8 in RIPK3-expressing CRC cells (Fig. 2). Therefore, we propose that Ru-B-induced caspase-8 activation at the DISC level is the major determinant of cell death type (apoptotic or necroptotic). In this sense, the mechanism driving caspase-8 activation and DISC formation in response to Ru-B treatment is a question that remains yet largely unresolved. It has been reported that,

in human cancer epithelial cells, including CRC cells, certain pentacyclic triterpenoids can activate caspase-8 by upregulating DRs such as DR5 and Fas cell surface DR (FAS) (Byun et al., 2018; Mou et al.., 2011; Sung et al., 2014). However, we observed that the mRNA and protein expression levels of DR5 and FAS were not significantly affected by Ru-B treatment (data not shown). Thus, Ru-B-induced caspase-8 activation via DISC formation is unlikely associated with DR pathway.

ROS actively participate in the execution of necroptosis, which is induced by a variety of stimuli including TNF (Vanden Berghe et al., 2010; Vanlangenakker et al., 2011; Zhang et al., 2009), FAS ligand (Chen et al., 2009) and plant-derived natural products (Sun et al., 2016; Zhao et al., 2021). However, the signaling pathways governing the crosstalk between ROS and RIPK1 activation are still under debate. For example, RIPK1 plays an essential role in TNF-induced ROS generation, which is required for the initiation of necroptosis (Kim et al., 2007; Lin et al., 2004); this suggests that the ROS production driving necroptosis occurs downstream of RIPK1. On the other hand, ROS promotes RIPK1 phosphorylation at Ser161 via its three cysteine sites, which leads to RIPK1 oligomerization and promotes RIPK1/RIPK3 interaction (Zhang et al., 2017); this suggests that ROS functions as a positive feedback loop during necrosome formation upstream of RIPK1. In this study, we found that ROS accumulated after Ru-B treatment, presumably via NOX1 (Fig. 5), and both Ru-B-induced apoptosis and necroptosis were abrogated in RIPK1-deficient HT29 cells (Fig. 3). In this regard, it was of interest to determine the role of ROS in the cytotoxic potential of RIPK1 during Ru-B-induced cell death. An important finding from this study is that Ru-B-induced RIPK1 phosphorylation was markedly prolonged and enhanced under necroptotic conditions, such as caspase-8 inhibition and FADD deficiency (Fig. 4). This indicated that the upregulation of RIPK1 phosphorylation functions as a positive effector that enables RIPK3 phosphorylation, thus facilitating necrosome formation. We also found that the ROS scavenging activity of BHA and two NOX inhibitors, diphenyleneiodonium and apocynin, correlated well with the inhibition of RIPK1 phosphorylation and Ru-B/z-IETD-induced necroptosis (Fig. 5). Thus, we propose that ROS production by NOX1 likely functions upstream of Ru-B-induced RIPK1 phosphorylation, switching the cell death mode from apoptosis to necroptosis in RIPK3-expressing CRC cells.

Nevertheless, the findings from this study also raise several questions that should be addressed. Although the results including ours showed that extra-mitochondrial ROS production by NOX1 is responsible for necroptosis by TNF and some anti-cancer compounds (Kim et al., 2011; Kim et al., 2007), it has been also reported that mitochondrial involvement in this process (Schenk and Fluda, 2015; Zhang et al., 2017). Although our knowledge regarding mitochondrial structure in specific cancer types is limited, it has been established that malignant transformation disturbs redox homeostasis in cancer cells (Gorrini et al., 2013). Thus, this discrepancy may depend on the cell type and/or cellular level of molecular context such as the NOX family members. Further research is needed to elucidate the dynamic interactions between Ru-B and NOX1, as well as the mechanism by which Ru-B targets NOX1 to induce ROS generation. Further *in vivo* studies examining the anti-cancer efficacy of Ru-B in mice lacking caspase-8 or FADD could lead to the development of Ru-B as a cancer chemotherapeutic to overcome apoptosis resistance.

Declarations

Declarations

Ethics approval

Not applicable. There were no human or animal experiments in this study.

Consent to participate

Not applicable

Consent for publication

All the listed authors agree to submit the manuscript for publication in Cell Biology and Toxicology

Conflict of interest

The authors declare no competing interests

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Author contributions

HSB participated in the design of the study, carried out bench experiments and analyzed data. KAP, EJ, K-C S, CSJ helped carrying out bench experiments related to this study. JHH, HR, HYL helped drafting the manuscript by providing critical intellectual input. KTQ, IP, MN carried out the experiments for the purification of Ru-B and NMR spectra data analysis. MN, GMH designed this study and wrote the manuscript with comments from the coauthors, and all authors collaborated on the work.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

Ru-B induces caspase-8-mediated apoptosis via associating with DISC. (A) Chemical structure of rubiarbonol B (Ru-B) isolated from *R. Philippinesis.* (B) HCT116, HeLa, and MCF7 cells were pretreated with z-IETD-fmk (20 mM) and z-DEVD-fmk (10 mM) for 30 minutes, followed by Ru-B (10 mM) for indicated times. Cell death was quantified by using Cell Titer-glo Luminescent cell viability assay as described in Materials and Methods. The data represent as mean ± S.E. of three experiments carried out in triplicate. *$P < 0.05$, compared with Ru-B treated group. (C-E) HCT116 cells were pretreated with z-IETD-fmk and z-DEVD-fmk, followed by Ru-B for 24 h or indicated times. (C) After 24 h, cells were visualized using an inverted phase-contrast microscope. (D) Cells were stained with FITC-labeled annexin V and PI and analyzed by flow cytometry as described in Materials and Methods. (E) Whole cell extracts were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). (F) HCT116 cells were pretreated with z-IETD-fmk, followed by Ru-B for the indicated times. Cell extracts from each sample were subjected to immunoprecipitation (IP) with anti-caspase-8 antibody. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. A total of 1% of the cell extract volume from each sample was used as input control. Densitometry analysis of the bands from the relevant proteins was performed (right). *$P < 0.05$, compared with Ru-B treated group.

Figure 2

Ru-B triggers the necroptotic mode of cell death in RIPK3-expressing cancer cells. (A) The expression of RIPK3 in various types of human colon cancer cell lines was analyzed by immunoblotting with and the indicated antibodies (left). Cells were treated with Ru-B (10 mM) in the absence or presence of z-IETD-fmk

(20 mM), and cell death was quantified as in Fig. 1B (right). The data represent as mean ± S.E. *$P < 0.05$, compared with Ru-B treated group. (B, C) HT-29 cells were untreated or pretreated with Nec-1 (50 μM) for 30 minutes and then treated with Ru-B or in combination with z-IETD-fmk for the indicated times and 24 h, respectively. (B) Cell death was quantified as in A. The data represent as mean ± S.E. *$P < 0.05$, compared with Ru-B treated group. $^{\#}P < 0.05$, compared with Ru-B/z-IETD-fmk treated group. (C) Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). (D) HT-29 cells were treated with Ru-B or in combination with z-IETD-fmk for 24 h in the absence or presence of necroptosis inhibitors Nec-1 (50 μM), GSK-872 (3 μM), and NSA (2 μM). Cell death was quantified as in A. The data represent as mean ± S.E. *$P < 0.05$, compared with Ru-B treated group. $^{\#}P < 0.05$, compared with Ru-B/z-IETD-fmk treated group. (E) WT and indicated caspase-8 and FADD genes KO HT-29 cells were treated with Ru-B for the indicated times. Whole cell lysates were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). (F, G) WT and caspase-8 KO HT-29 cells were treated with Ru-B or in combination with z-IETD-fmk for the indicated times. Cell extracts from each sample were subjected to IP with anti-caspase-8 (F) and anti-RIPK3 (G) antibodies, respectively. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. A total of 1% of the cell extract volume from each sample was used as input control. Densitometry analysis of the bands from the relevant proteins was performed (right). *$P < 0.05$, compared with Ru-B/z-IETD-fmk treated group. $^{\#}P < 0.05$, compared with Ru-B treated group.

Figure 3

RIPK1 phosphorylation plays an essential role in Ru-B-induced necroptosis. (A) HT-29 cells deficient of RIPK1 gene were analyzed by immunoblotting with indicated antibodies. (B-D) WT and RIPK1 KO HT-29 cells were treated with Ru-B, z-IETD-fmk, TNF (15 ng/ mL), SM-164 (100 nM) or cycloheximide (CHX, 10 μg/mL) for 24 h, as indicated. (B) Cell death was quantified as in Fig. 1B. The data represent as mean ± S.E. of three experiments carried out in triplicate. *$P < 0.05$, compared with WT HT-29 cells. (C) Cells were visualized using an inverted phase-contrast microscope. (D) Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). (E) WT and RIPK1 KO Jurkat cells were treated with the indicated compounds, and cell death was quantified, as in B. *$P < 0.05$, compared with WT Jurkat cells.

Figure 4

RuB-induced RIPK1 phosphorylation at Ser166 facilitates necrosome formation. (A) HT-29 cells were pretreated with z-IETD-fmk for 30 minutes, followed by Ru-B for the indicated times. Whole cell extracts

were subjected to immunoblotting with the indicated antibodies (top), and densitometry analysis of the bands from the relevant proteins was performed (bottom). *P < 0.05, compared with Ru-B treated group. (B, C) WT and indicated caspase-8 and FADD genes KO HT-29 cells were treated with Ru-B for the indicated times (B), and indicated combination of Ru-B, z-IETD-fmk and Nec-1 for 12 h (C). Whole cell lysates were subjected to immunoblotting with the indicated antibodies (top), and densitometry analysis of the bands from the relevant proteins was performed (bottom). *P < 0.05, compared with Ru-B treated WT HT-29 cells. (D) Caspase-8-KO HT-29 cells were pretreated with Nec-1 (50 μM) for 30 minutes, followed by Ru-B for the indicated times, and whole cell extracts from each sample were subjected to IP with anti-RIPK3 antibody. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. A total of 1 % of the cell extract volume from each sample was used as input control. Densitometry analysis of the bands from the relevant proteins was performed (bottom). *P < 0.05, compared with Ru-B treated group.

Figure 5

NOX1-derived ROS production by Ru-B contributes to triggering the phosphorylation of RIPK1 and necroptosis. (A) HT-29 cells were untreated or pretreated with z-IETD-fmk for 30 minutes and then treated with Ru-B for the indicated periods of time.

Cells were then incubated with 10 μM DHE for 10 min, and analyzed for superoxide (O_2^-) production by flow cytometry. The data represent as mean ± S.E. of three independent experiments (left). Representative fluorescent images of cells are shown (right). (B) HT-29 cells were pretreated with the indicated compounds (100 mM BHA; 10 μM DPI; 10 μM Apocynin; 10 μM Mito-TEMPO) and then treated Ru-B for 1 hour and the superoxide production was analyzed in A. The data represent as mean ± S.E. of three independent experiments. *p < 0.05, compared with Ru-B treated group. (C) HT-29 cells were treated with Ru-B or CCCP (10 μM) for 1 h. Mitochondrial O_2^- production was determined using MitoSOX Red, as described in Materials and Methods. (D) HT-29 cells were pretreated with the indicated compounds for 30 min, and then treated with Ru-B in the absence or presence of z-IETD-fmk. Cell death was quantified, as in Fig. 1B. The data represent as mean ± S.E. *p < 0.05, compared with Ru-B treated group. $^{\#}p$ 0.05, compared with the Ru-B/z-IETD-treated group. (E) HT-29 cells were pretreated with 20 μM apocynin, followed by Ru-B/z-IETD for the indicated times. Whole cell lysates were performed immunoblotting with the indicated antibodies (top), and densitometry analysis of the bands from the relevant proteins was performed (bottom). *P < 0.05, compared with Ru-B/z-IETD-fmk treated group. (F) Total RNA was prepared from the indicated cell lines, and RT-PCR was performed with the primers specific to human NOX isoforms. After PCR amplification, the products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide staining. (G-I) HT-29 cells were transfected with wither a nonspecific control siRNA or siRNA specific for NOX1 and NOX2 for 48 hours. (G) Cells were treated Ru-B for 1 h, and the superoxide production was analyzed in A. (H) Cells were treated Ru-B or Ru-B/z-IETD for 24 h, cell death was quantified as in D. (I) Cells were treated Ru-B or Ru-B/z-IETD for the indicated times. Whole-cell

lysates were performed immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (bottom). The data represent as mean ± S.E. *p < 0.05, compared with Ru-B treated HT-29 cells transfected with control siRNA. $^{\#}p$0.05, compared with the Ru-B/z-IETD-treated HT-29 cells transfected with control siRNA.

Figure 6

RIPK1 phosphorylation and necroptosis by Ru-B/z-IETD-fmk is impaired in cysteine mutant of RIPK1 expressing cells. (A-D) RIPK1 KO HT-29 cells reconstituted with the indicated RIPK1 constructs were treated with TNF or Ru-B/z-IETD-fmk for the indicated times. (A, D) Whole cell extracts from each sample were subjected to IP with anti-TNFR1 and anti-caspase-8 antibodies, respectively. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. A total of 1 % of the cell extract volume from each sample was used as input control. Densitometry analysis of the bands from the relevant proteins was performed (bottom). (B, C) Whole-cell lysates were performed immunoblotting with the indicated antibodies (top), and densitometry analysis of the bands from the relevant proteins was performed (bottom). *p < 0.05, compared with Ru-B/z-IETD-treated RIPK1-KO HT-29 cells expressing WT-RIPK. (E-G) RIPK1 KO HT-29 cells reconstituted with the indicated RIPK1 constructs were treated with TRAIL (100 ng/ml) and the indicated combination of compounds (10 μM Ru-B; 20 μM z-IETD-fmk, 15 ng/ml TNF; 100 nM SM-164) for 24 h. (E) Cell death was quantified as in Fig. 1B. The data represent as mean ± S.E. *p < 0.05, compared with RIPK1 KO HT-29 cells expressing WT-RIPK1. (F) Cells were visualized using an inverted phase-contrast microscope. (G) Whole cell lysates from each sample were subjected to immunoblotting with the indicated antibodies (left), and densitometry analysis of the bands from the relevant proteins was performed (right). *p < 0.05, compared with Ru-B/z-IETD-treated RIPK1-KO HT-29 cells expressing WT-RIPK. $^{\#}p$0.05, compared with the TNF/SM/z-IETD-treated RIPK1-KO HT-29 cells expressing WT-RIPK.

Supplementary Files

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- Table1.docx