Novel Ridaifen-B Structure Analog Induces Apoptosis and Autophagy Depending on Pyrrolidine Side Chain

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Received August 20, 2018; accepted December 6, 2018

Ridaifen (RID)-B is an analog derived from tamoxifen (TAM). TAM has an antitumor effect by acting as an antagonist to estrogen receptor (ER). However, TAM is known to also induces apoptosis in cancer cells that do not have ER. We clarified that RID-B induces cell death at a lower concentration than TAM, and causes ER-independent apoptosis and autophagy. Based on the results of previous studies, we assumed that RID-B had a unique target different from ER and examined structural activity correlation to determine what kinds of structural features are related to RID-B activity. As a result, we found there was activity even without one of phenyl groups (Ar3) in RID-B and revealed that two pyrrolidine side chains peculiar to RID-B are related to the action. Furthermore, analogs with shorter alkyl side chains induced autophagy, but analogs with certain length of alkyl side chains induced apoptosis. Also, although there is no doubt that RID-B induces apoptosis by causing mitochondrial injury, our results suggested that such injury induced mitochondria-selective autophagy. We revealed that RID-B induce mitophagy and that this mitophagy is a defense mechanism against RID-B. Our results suggest that autophagy was induced against apoptosis caused by mitochondrial dysfunction in RID-B, so the combination of autophagy inhibitor and anticancer-drug can be effective for cancer treatment.

Key words: ridaifen; tamoxifen; apoptosis; autophagy; mitophagy

INTRODUCTION

Usually, in vivo, aged cells are quickly removed and replaced with new cells to maintain homeostasis. Thus, programmed cell death kill hundreds of millions of cells to keep the individual healthy.1) Apoptosis is induced when abnormalities occurred in cells. These unnecessary cells are removed and normal cells are maintained.2) Apoptosis prevents tumor formation and growth; when apoptosis does not function properly, cancer cells form tumors. The chemical materials that induce apoptosis in cancer cells are used as anticancer drugs.

Autophagy is a cytoprotective action that decomposes its intracellular constituent to the amino acid level and reuses it initially when the cell falls into a starvation state.3) However, recently it was reported that medicines induce autophagy, and autophagy affects cancer cell proliferation. Also, it has been observed repeatedly that the autophagy mechanism developed in cancer cells, unlike that in normal cells, is a nutrient source for cancer cells and thus a cause of proliferation. The autophagy induction mechanism in cancer cells is different from that in healthy cells, suggesting that it is deeply involved in drug resistance.4) Therefore, by controlling autophagy, we may be able to improve the utility of anticancer drugs.

Estrogen is a steroid hormone that binds to the estrogen receptor (ER) of breast cancer cells and controls the proliferation of cells. Tamoxifen (TAM) is now widely accepted to have an antitumor effect through antiproliferative action by acting as estrogen and antagonist in the ER.5) However, TAM induces apoptosis in cancer cells that do not have ER.6,7) Although TAM is used for breast cancer patients, about 30% breast cancer patients acquire resistance by autophagy.8) Ridaifen (RID)-B is an analog derived from TAM screening focusing on apoptosis and autophagy independent of ER of TAM.9) In our previous study,10) RID-B fragmented DNA of Jurkat cells, activated caspases, and induced ER-independent apoptosis. Also, we revealed that RID-B-induced apoptosis was dependent on mitochondria dysfunction, since RID-B-induced apoptosis is suppressed against cells overexpressing Bcl-2. Furthermore, although RID-B induced ER-independent autophagy, autophagy was not suppressed in Bcl-2-overexpressing cells that inhibited apoptosis.11) Although RID-B induces autophagy and apoptosis in cancer cells, it is not yet clear what kind of structural features of RID-B induce autophagy and apoptosis.

In this study, we aim to clarify the relationship between autophagy, apoptosis and chemical structure using the newly synthesized structural analog of RID-B.

MATERIALS AND METHODS

Cell Lines and Cell Culture HepG2 and Hep3B cells were purchased from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Mouse embryonic fibroblast (MEF) cells generated from wild type (WT) (Atg5+/+) and Atg5−/− embryos12) were obtained from the RIKEN BRC cell bank (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (Biofill...
Australia, Victoria, Australia) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂.

**Drug Treatments** RID-B analogs were chemically synthesized in our laboratory based on the methods described by Shiina et al.13) (Fig. 1). The analogs were stocked in ethanol solution at a concentration of 20 mM.

**Determination of Cell Viability (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Assay and Sulforhodamine B (SRB) Assay)** HepG2 cell viability was assessed by a conventional MTT reduction assay. Briefly, cells were suspended at a final concentration of 1 × 10⁵ cells/mL and seeded in 96-well plates in a final volume of 100 µL. After the cells attached to the bottom of the well, a different concentrations (0–40 µM) of RID-B analogs was added to each well in triplicate. After incubation for 23 h, 10 µL of MTT (5 mg/mL) was added and each cultures was incubated at 37°C, 5% CO₂ atmosphere for 1 h. After the medium was discarded, the formazan precipitate was dissolved in 100 µL dimethyl sulfoxide (DMSO) and the absorbance at 570 nm was measured using a microplate spectrometer (Awareness Technology, Palm City, FL, U.S.A.). Percent cell viability was calculated as the ratio of absorbance of the experimental samples to that of the control samples ×100.

MEF cell viability was assessed by a conventional SRB assay. Cells were suspended at a final concentration of 1 × 10⁴ cells/well and seeded in 96-well plates. After the cells attached to the bottom of the well, a different concentration (0–20 µM) of RID-B analogs was added to each well in triplicate. After 24 h, protein synthesis was evaluated through the SRB assay according to Peter et al.15) SRB is an anionic purple dye that binds to proteins through electrostatic bonds. After solubilization, the fixed dye can be measured by spectrophotometry, and the results correlate with the total protein measurement. Absorbance at 550 nm was measured using the Awareness Technology microplate spectrometer. The percent cell growth rate was calculated as the ratio of absorbance of the experimental samples to that of the control samples ×100.

**Western Blotting** Cells were washed twice with phosphate buffered saline (PBS), and then lysed with lysis buffer [50mM N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.5, 150 mM NaCl, 10% glycerine, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 1.5 mM MgCl₂, and 1% protease inhibitor cocktail (Sigma-Aldrich)]. Total protein (20 µg) was fractionated by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for blotting.

Antibodies against anti-Caspase-3 (Cell Signaling Technologies, Danvers, MA, U.S.A.) was used at 1 : 1000 dilution, anti-LAMP1 at 1 : 1000 (Cell Signaling Technologies), anti-LC3B at 1 : 1000 (Cell Signaling Technologies) and anti-Cathepsin B at 1 : 1000 (Santa Cruz Biotechnology, TX, U.S.A.) for blotting overnight at 4°C. Anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technologies) was used as a secondary antibody at 1 : 2000 dilution. Antibody against anti-β-actin (Cell Signaling Technologies) was used for normalization at 1 : 1000 dilution. Immunoreactivity was visualized with ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, U.S.A.) using an enhanced chemiluminescent substrate for detection of HRP (Thermo Scientific, Waltham, MA, U.S.A.).

**Determination of DNA Fragmentation by Measurement of Sub-G1 Population of Cells** Apoptosis was characterized by measuring the sub-G1 pool of cells as an indication of DNA fragmentation. Treated cells were pelleted (300 × g/5 min) and the cells were suspended in 2 mL 70% ethanol (−20°C) and fixed at −20°C for 30 min. The ethanol-fixed cells were sedimented again and permeabilized with 0.1% Triton X-100.

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Fig. 1. 4-OH-TAM, RID-B, and RID-B Structural Analogs
containing 50 μg/mL RNase A for 15 min. The DNA remaining inside the permeabilized cells was stained with 5 μg/mL of propidium iodide (PI) for at least 10 min before FACS analysis. PI fluorescence was measured by FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

**Measurement of the Mitochondrial Membrane Potential** Treated cells were pelleted (300 × g/5 min) and the cells were exposed to 100 nM DiOC6 (Molecular Probes, Eugene, OR, U.S.A.) and were incubated at 37°C, 5% CO2 atmosphere for 15 min. At the end of the experiment, the cells were centrifuged at 300 × g for 5 min at 4°C. The supernatant was removed, and the pellet was washed with 500 μL of PBS. After centrifugation, the pellet was resuspended in 1 mL of PBS followed by finger tapping to effect a gentle mixing. DiOC6 fluorescence was measured by FACS Calibur (Becton Dickinson).

**Fluorescence Microscopy** Cells were cultured on 15-mm glass coverslips in 12-well dishes overnight in DMEM containing 10% FBS. After incubation for 23 h, 1 nM LysoTracker Red (Thermo Fisher Scientific) was added and the cultures were incubated at 37°C, 5% CO2 atmosphere for 1 h. Cells were washed twice with PBS, and fixed with 4% paraformaldehyde (Wako, Osaka, Japan) for 20 min, permeabilized with 0.1% Triton X-100 in PBS, blocked with 3% BSA-PBS, and then incubated with the indicated primary antibodies of rabbit anti-LC3 antibody at 1:100 (Cell Signaling Technologies) and rabbit anti-LAMPI antibody at 1:100 (Cell Signaling Technologies). After Alexa Fluor 488 conjugate goat anti-rabbit IgG (Thermo Fisher Scientific) was added, the samples were washed with 1× 300, samples were observed using an FV10i confocal microscope (Olympus, Tokyo, Japan).

**Magic Red Cathepsin-B Assay** Cells were cultured on 15-mm glass coverslips in 12-well dishes overnight in DMEM containing 10% FBS. After treatment, the cells were further loaded with Magic red cathepsin B reagents (Immunochemistry Technologies, LLC, Bloomington, MN, U.S.A.) for 15 min. Cells were washed twice with PBS, and fixed with 4% paraformaldehyde (Wako) for 20 min. Samples were observed using an FV10i confocal microscope (Olympus).

**Detection of Mitophagy** For the detection of mitophagy in cells, a novel Mitophagy Detection Kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used. Cells were treated according to the manufacturer’s recommendations. In brief, cells were washed twice with FBS-free DMEM and afterwards incubated at 37°C for 30 min with 100 μM Mtphagy Dye diluted in FBS-free DMEM. After this incubation, the cells were again washed twice with DMEM (FBS-) followed by the addition of DMEM containing 10% FBS. Subsequently, cells were detected by the fluorescence intensity of Mtphagy Dye measured by the confocal microscope at 537 nm excitation and 619 nm emission.

**Mitochondrial Reactive Oxygen Species (ROS) Measurement** Treated cells were incubated with 5 μM MitoSOX Red mitochondrial superoxide indicator (Molecular Probes) for 10 min at 37°C in the dark, according to the manufacturer’s instructions. Approximately 10000 gated events were acquired for each sample on a FACS Calibur (Becton Dickinson). Dead cells and debris were excluded based upon forward scatter and side scatter measurements.

**Statistical Analysis** The values shown in the graphs are the mean ± standard deviation (S.D.) from three independent experiments. The statistical significance of all data was determined by the t-test, with values of p < 0.05 considered statistically significant. All experiments were performed in triplicate. The fluorescence images were quantified by dividing the fluorescence area by the area of the cell measured by Image J (NIH, Bethesda, MD, U.S.A.).

**RESULTS**

**RID-B and RID-B Structural Analogs Induce Cell Death in HepG2 Cells** We reported that RID-B of TAM derivative induces apoptosis. However, it is not clear what kinds of structural features are involved in RID-B-induced apoptosis. Therefore, in order to investigate the structure-activity correlation between RID-B-induced apoptosis and the structural features of RID-B, we synthesized several RID-B structural analogs. RID-B original compound TAM is metabolized by CYP2D6 to 4-OH-TAM, so this metabolite binds to ER (Fig. 1). The 4-hydroxyl group in Ar of 4-OH-TAM hydrogen bonds to Glu353 and Arg394 of ER. Ar2 and Ar3 fit in the ER hydrophobic pocket. Therefore, we synthesized RID-B, which instead of a 4-hydroxyl group, a pyrrolidine side chain was introduced into Ar and Ar to eliminate the ability to bind to ER (Fig. 1). In this study, to further reduce the binding ability of RID-B to ER, we substituted the alkyl chain by eliminating the phenyl group of Ar of RID-B. We introduced dimethyl (SB9), diethyl (SB10), dipropyl (SB22), dibutyl (SB23), dipentyl (SB17), dihexyl (SB24), diheptyl (SB25), dioctyl (SB26), dinonyl (SB27), didecyl (SB28) and diundecyl (SB18) alkyl chain groups in order to clarify how length correlated to cell death efficiency. We also synthesized structural analog RID-S10-(B/Me) lacking one of the pyrrolidine rings of RID-SB10, to estimate the efficiency of the pyrrolidine structure without affecting ER.

We measured the survival rates of RID-B and their synthesized analogs on human hepatoma HepG2 cells using the MTT assay. The IC50 values, calculated from the graphs, are presented in Table 1. As a result, RID-SB10, RID-SB22, RID-SB23, RID-SB17, and RID-SB24, all having side chain alkyl chains of 2 to 6, had equivalent cytotoxicities to RID-B. However, it was found that the cytotoxicity of structural analogs with side chain alkyl chains shorter than the diethyl group

| Compound name | IC50 (μM) | ClogP | Alkyl chain length |
|---------------|---------|------|-------------------|
| RID-B         | 2.45 ± 0.23 | 8.19 | 2                 |
| RID-SB9       | 8.35 ± 2.41 | 6.62 | 1                 |
| RID-SB10      | 2.71 ± 1.03 | 7.68 | 2                 |
| RID-SB22      | 4.68 ± 1.98 | 8.73 | 3                 |
| RID-SB23      | 2.64 ± 0.53 | 9.79 | 4                 |
| RID-SB17      | 2.59 ± 0.30 | 10.85| 5                 |
| RID-SB24      | 3.96 ± 0.95 | 11.91| 6                 |
| RID-SB25      | 4.96 ± 0.88 | 12.97| 7                 |
| RID-SB26      | 15.11 ± 3.38 | 14.02| 8                 |
| RID-SB27      | 36.54 ± 16.99 | 15.08| 9                 |
| RID-SB28      | 32.83 ± 16.80 | 16.14| 10                |
| RID-SB18      | 16.65 ± 3.99 | 17.20| 11                |
| RID-S10-(B/Me)| 34.80 ± 9.78 | 6.90 | 2                 |

The cells were cultured for 24 h and then further incubated with different concentrations of RID-B for 24 h. Cell viability was determined by the MTT assay. The ClogP value was calculated using ChemiBioDraw Ultra 13.0.
RID-SB10 or longer than the dihexyl group (RID-SB24) attenuated toxicity. Furthermore, it was revealed that the cytotoxicity of RID-S10-(B/Me) is very weak. This is a structural analog of RID-SB10 having only one pyrrolidine structure. In order to confirm whether the cytotoxicity of this RID-B structural analog was due to apoptosis similar to the case with RID-B, the activation of caspase-3, a hallmark of apoptosis, was detected by treating 3µM compounds for 24h (Figs. 2A–D). Since RID-SB10 to RID-SB24 of the RID-B and RID-B structural analogs showed a cleaved caspase-3 band (Figs. 2A, B), which is an activated form, the RID-B structural analog also induced apoptosis to HepG2 cells, similar to RID-B. Also, cleavage of caspase-3 did not occur in RID-SB9 (dimethyl alkyl chain without Ar') and RID-S10-(B/Me) (only one pyrrolidine structure).

Furthermore, at the final stage of apoptosis, chromosomal DNA is fragmented into nucleosome units. We measured the SubG1 phase during apoptosis by PI staining as described in Materials and Methods. The SubG1 phase were detected by flow cytometric analysis with PI staining as described in Materials and Methods. *p<0.05, **p<0.01, ***p<0.001. ΔΨm was assessed by the uptake of the fluorescent dye DiOC6 as described in Materials and Methods. The data are presented as means ± S.D. of the results for three independent experiments. *p<0.05, **p<0.01, ***p<0.001.
In general, autophagy is often induced when apoptosis is provoked. Autophagy is induced when cells become nutrient starved, but some apoptosis-inducing signals such as tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and fas-associated death domain (FADD) also induce autophagy. Since RID-B induces autophagy, we used Western blotting to further investigate whether the RID-B structural analog also induces autophagy by the transition from type I to type II of the LC3 PE bond forms as autophagy progresses (Figs. 3A, B). As a

**Fig. 3. Induction of Autophagy by RID-B Analogs**

The cells were incubated with 1 µM RID-B and its analogs for 24h. (A) HepG2 lysates were separated by 15% SDS-PAGE, and the expression of LC3-I and LC3-II was detected by Western blot analysis. (B) The relative density of the cleaved LC3-II and β-actin bands were estimated and the mean ± S.D. of three independent experiments are presented as quantified data. *(p < 0.05, **p < 0.01. (C) HepG2 cells were treated with RID-B and increased the expression level of LC3-II, which was demonstrated to induce the formation of LC3 and of Lysotracker RED-positive autolysosomes. Each white bar indicated at the lower right in the figure denotes 10 µm. (D) HepG2 cells were treated with RID-B and, where indicated, also with E64d and pepstatin A (Pep A) 10 µg/mL each. HepG2 lysates were separated by 15% SDS-PAGE, and the expression levels of LC3-I, LC3-II, and p62 were detected by Western blot analysis. (E) The relative density of the LC3-II and β-actin bands were estimated and the mean ± S.D. of three independent experiments are presented as quantified data. *(p < 0.05, **p < 0.01. (Color figure can be accessed in the online version.)
result, transitions of LC3 to type II were confirmed in RID-SB10 to RID-SB24, which induced high cytotoxicity to the cells. Surprisingly, dimethyl alkyl chain analogs of RID-B, RID-SB9 also form LC3 type II, instead of low cytotoxicity (Table 1). After the autophagosome membrane forms, autophagy is completed by fusing the autophagosome with the lysosome to become an autolysosome followed by decomposition of the contents. Therefore, the localization of LC3 and lysosome revealing autolysosome formation was observed by immunofluorescent staining (Fig. 3C). Since LC3, whose expression was induced by RID-B, colocalized with lysosome, it presumed that autolysosome was formed. We decided to use RID-SB10 and RID-SB17 among the RID structural analogs to further study the effect of autophagy and apoptosis on lipid solubility. These two compounds differ greatly in ClogP values but have equivalent cytotoxicities.

However, just this result could not be proved autophagy because the sometimes lysosome inhibition is an example of an increase in LC3. To prove autophagy, it is necessary to measure the amount of LC3-II degraded in lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors.17) To further examine the autophagy induced in HepG2 cells in more detail, cells were treated with pepstatin A and E64D, which are inhibitors of aspartic protease and cysteine protease, respectively. In this “autophagy flux” assay, treatment of intact cells capable of inducing autophagy with the inhibitors increases the amount of LC3-II, whereas no increase is observed in cells impaired in the autophagic degradation.17) p62 is a major autophagic adapter in most processes of selective autophagy.20) Therefore, p62 is also used as a marker in the autophagy flux assay like LC3. When lysosome inhibitors were added, LC3 and p62 increased. LC3 and p62 degradation by autophagy was induced by RID-B and RID-SB10 (Figs. 3D, E). Surprisingly, RID-SB17 slightly improve the autophagy flux. These results suggested that RID-B and RID-SB10 induced autophagy maybe selective autophagy, but RID-SB17 was not inducing autophagy. Since autophagy is generally controlled by Atg5,21) we examined whether autophagy induced by RID-B or structural analogs depends on whether Atg5. As shown in Figs. 3F and G, the increase in LC3 expression was confirmed in Atg5+/− cells, suggesting that autophagy induced by RID-B or their analog, RID-SB10, was dependent on Atg5.

RID-B and RID-B Structural Analogs Induce Increased Formation and Activation of Lysosomes After autophagy is induced, autolysosome formation induces lysosome formation and improves digestive activity.22) Therefore, lysosome was labeled with LysoTracker Red and LAMP 1, the latter of which is a lysosomal membrane protein22) (Fig. 4A).

Compared with Control, lysosome formation was greater when RID-B was applied. To quantify the fluorescence treated with RID-B and their analogs compared to a untreated control, the obtained confocal microscopic image was quantified by a relative ratio obtained by determining the percentage of the area of fluorescence in the cell by Image J (Fig. 4B). Similar to the result with RID-B, in this case lysosome increased during RID-SB10 treatment but not during RID-SB17 treatment. Furthermore, LAMP 1, which is a lysosomal membrane protein, was measured by Western blotting (Figs. 4C, D). Among the cells to which RID-B and the structural analogs was applied, RID-B and RID-SB10 treatment augmented LAMP-1. However, RID-SB17 treatment did not alter LAMP-1 level.

Activation of lysosomes is important not only for their formation but also for improving digestive activity.22) Therefore, in order to examine digestive activity, cathepsin B activity was observed by fluorescence using Magic Red-Cathepsin B, which fluoresces in a substrate-specific manner by cathepsin B as a degradation enzyme in the lysosome and fluorescence (Fig. 4E). The red fluorescence due to Magic Red in which RID-SB10 was hydrolyzed was stronger than in the control. Thus, lysosomal digestive activity was improved. But the fluorescence of cells treated with RID-SB17 was similar level as that of the control. We quantified the amounts of fluorescence treated with RID-B and their analogs compared to the untreated control. The obtained confocal microscopic image was quantified by the relative ratio of the area of the fluorescence in the cell obtained by Image J (Fig. 4F). Furthermore, using Western blot analysis, cathepsin B level was augmented by RID-B and RID-SB10 treatment (Figs. 4G, H). Since RID-B and RID-SB10 form a lot of lysosomes (Figs. 4A, B), cathepsin B, which is a lysosomal degradation enzyme, is increased (Figs. 4G, H), and thus lysosomal decomposition was activated. However, in cells treated with RID-SB17, there was no increase in the formation of lysosome or in the level of cathepsin B.

RID-B and RID-B Structural Analogs Induce Mitophagy in HepG2 Cells RID-B induced mitochondrial-mediated apoptosis in previous studies.23) Abnormal mitochondria promote ubiquitination. It is widely accepted that this ubiquitination is a signal of abnormal mitochondrial elimination by autophagy.23,24) This mitochondria selective autophagy is called mitophagy. Next, we examined whether autophagy induced by RID-B and their structural analogs was mitophagy induced by RID-B-mediated mitochondrial dysfunction. To detect mitophagy, a fluorescent dye was used that chemically bonds to mitochondria called Mphagy Dye and emits red fluorescence under acidic conditions fused with lysosomes. RID-B and RID-SB10, which induced autophagy and improved lysosomal digestion activity, provoked that red mitophagic fluorescence and the co-localization with lysosomes could be observed, suggesting these compounds induced mitophagy (Fig. 5A). As we argued, RID-SB17, which did not improve lysosomal digestion activity, did not induce mitophagy. Since mitophagy is induced by the transition of Parkin, p53 which binds to Parkin is known as a mitophagy inhibitor.25) In order to investigate the relationship between RID-B-induced mitophagy and cell death, we examined p53 status and RID-B-induced cell death. p53 wild-type HepG2 cells and p53-deficient Hep3B cells were used. Western blot revealed that p53 wild type HepG2 and p53-null Hep3B both expressed parkin (Fig. S1). Hep3B was more resistant to RID-B than HepG2 (Fig. 5B). Inhibition of mitophagy with E64d and Pepstatin A enhanced cytotoxicity in p53-null Hep3B cells (Fig. 5C), suggesting that the cells are protected by mitophagy.

Mitophagy Rescues Cells from Cytotoxicity by RID-B Furthermore, we examined the relationship between autophagy and apoptosis by allowing autophagy-inducing structural analogs or non-induced structural analogs to MEF (Atg5+/−) or MEF (Atg5−/−) cells. Mitophagy is also known to be controlled by Atg5.26) SRB assay was used to investigate the effect on RID-B proliferation. It became clear that RID-B and RID-SB10 act more strongly on MEF (Atg5−/−), which can not induce mitophagy. In addition, RID-SB17 not induc-
Fig. 4. RID-B and RID-B Structural Analogs Induce Increased Formation and Activation of Lysosomes

The cells were incubated with 1 µM RID-B and its analogs for 24 h. (A) HepG2 cells were double stained with LysoTracker Red (Lysotracker; red) and lysosomal-associated membrane protein 1 (LAMP1; green) after RID-B treatment. Each white bar indicated at the lower right in the figure denotes 40 µm. (B) HepG2 cells were stained with LysoTracker. For each image, the area of LysoTracker staining above a uniform threshold was quantified in the cell area (n = 3 experiments with one control and one treatment in each experiment). *p < 0.05, ***p < 0.001. (C) HepG2 lysates were separated by 15% SDS-PAGE, and the levels of LAMP1 and β-actin expression were detected by Western blot analysis. (D) The relative density of the LAMP1 and β-actin bands were estimated and the mean ± S.D. of three independent experiments are presented as quantified data. ***p < 0.001. (E) After treatment with Magic Red Cathepsin B for 37°C at 15 min, individual lysosomes fluoresced red in the presence of active cathepsin B. Each white bar indicated at the lower right in the figure denotes 40 µm. (F) HepG2 cells were stained with Magic Red Cathepsin B. For each image, the area of active cathepsin B staining above a uniform threshold was quantified in the cell area (n = 3 experiments with one control and one treatment in each experiment). The data were presented as the mean ± S.D. of the results of three independent experiments. *p < 0.05. (G) HepG2 lysates were separated by 12% SDS-PAGE, and the levels of cathepsin B and β-actin expression were detected by Western blot analysis. (H) The relative density of the cathepsin B and β-actin bands were estimated and the mean ± S.D. of three independent experiments are presented as quantified data. *p < 0.05. (Color figure can be accessed in the online version.)
ing mitophagy had similar cytotoxicities to both (Atg5+/− and Atg5−/−) cells (Fig. 6A).

Several reports have suggested that ROS, an indicator of mitochondrial injury, is involved in mitophagy induction. 27,28) Therefore, when ROS derived from mitochondria was measured by Mitosox Red, it was revealed that ROS increased by the action of RID-B and RID-SB10 (Fig. 6B). Interestingly, RID-SB17, not inducing mitophagy, also augmented mitochondrial ROS.

Fig. 5. RID-B and RID-B Structural Analogs Induce Mitophagy in HepG2 Cells

The cells were incubated with 1 μM RID-B and its analogs for 24 h. (A) HepG2 cells were treated with Mtpahgy Dye, a mitophagy detection reagent, for 30 min. The cells were then incubated with 1 μM RID-B and its analogs 24 h and then with Lyso Dye, a lysosome detection reagent, for 30 min. Representative images are shown. Each white bar indicated at the lower right in the figure denotes 10 μm. (B) HepG2 and Hep3B were cultured for 24 h and then further incubated with different concentrations of RID-B for 24 h. Viability was determined by MTT assay. *p < 0.05. **p < 0.001. (C) Hep3B cells were cultured for 24 h and then further incubated with another 4 μM of RID-B and where indicated also with E64d and pepstatin A (Pep A) 10 μg/mL each for 24 h. Viability was determined by MTT assay. The data are presented as the means ± S.D. of the results of three independent experiments. ***p < 0.001. (Color figure can be accessed in the online version.)

DISCUSSION

The results of this study showed that RID-B has an alkyl side chain that affects cytotoxicity and that two pyrrolidine side chains are necessary for cytotoxicity (Table 1). On the basis of these results, it is considered that the action induced by RID-B has nothing to do with the binding with ER. RID-B cytotoxicity was low due to the difficulty of entering cells as the side chain of RID-B becomes certain length, longer than dihexyl (SB24). The lipid bilayer of the cell membrane acts as a barrier to the passage of most polar molecules because of its
increased intracellular ROS. It is possible that RID-SB17 induces apoptosis by inhibiting lysosome, thus not induce mitophagy, RID-SB17 treatment may lower the mitochondrial membrane potential and result in mitochondrial damage, inducing apoptosis without activating mitophagy. Furthermore, RID-SB9, with very short alkyl side chain, strongly induced autophagy with low cell toxicity. Since RID-SB9 increased cathepsin B activity and lysosomal formation (data not shown), it can be considered that the digestive activity of mitophagy can be controlled by changing the length of the alkyl chain of the side chain. The RID-B structural analog is thus a unique compound inducing a reverse action on lysosomal activity due to a slightly different lipophilicity.

It was reported that cationic amphiphilic drugs (CAD) accumulate in lysosomes to raise lysosomal pH. Lysosomes have various enzymes and play important roles in digestion, but it seems that the influence of CAD does not coincide with the optimum pH of the enzyme and that the enzyme activity is lowered. The literature suggests CAD strongly interacts with biological membranes. It has been clarified that not only was the electrostatic interaction between the positive charge of CAD and the negative charge of the phosphate group of the phospholipid involved, but so was the hydrophobic interaction between the hydrophobic parts. Because lysosomes have many acidic phospholipids, CAD with high lipid solubility accumulates in lysosomes and tends to form a complex with phospholipids. Therefore, it is considered that our synthesized CAD compound, RID-SB17, induces lysosome inhibition, because with high lipid solubility, it is easy to accumulate in lysosomes and tends to form a complex with phospholipids.

We can infer that lysosomal inhibition is induced when RID-SB17 causes mitochondrial membrane potential to decrease but without mitophagy. Since RID-B is less effective against cells that can induce mitophagy, mitophagy is thought suppress cytotoxicity by RID-B. From an examination of the findings that RID-SB17 does not induce mitophagy, we can expect RID-SB17 to be effective against various cancer cells at low concentrations. In the future, it is speculated that identification of the molecular target of RID-B will lead to the development of novel anticancer drugs.

Acknowledgments We thank Dr. Noboru Mizushima...
Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.

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