Discovery a novel hybrid with resveratrol and hans ester derivatives as activators induce autophagic cell death in tumoral NCI-H460 cells through production of ROS

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Abstract

Based on the integrated fragment-based drug design, synthesis, and in vivo evaluations, a series of novel resveratrol and hans ester (**ReHa**) derivatives are discovered as autophagic death inducer. Compound **ReHa-2** is the most potent inducer with IC_{50} values as low as 9.9 mM compare with the molecule (**NIP**) in autophagic cell death. Compound **ReHa-2** lead to autophagic cells death instead of necrosis or apoptosis in human NCI-H460 cells. Mechanistic study uncovers that **ReHa-2** is capable of increasing protein LC3-II (a marker of autophagy) and reducing p62 in a time- and dose-dependent manner. Furthermore, **ReHa-2** can activate MAPKs and Akt signal pathway. In addition, we identified that **ReHa-2** triggered more ROS generation compare with **NIP** in NCI-H460 cells. Noticeably, the cytotoxicity induced by **ReHa-2** against NCI-H460 cells can be significantly revised by pretreatment of the cells with the CAT (a specific scavenger of H_2O_2) and DTT (a sulfhydrylcontaining nucleophile for quenching ROS), suggesting that the ROS (mainly including H_2O_2) induced by **ReHa-2** is responsible for its cytotoxicity against NCI-H460 cells. Our results suggest that **ReHa-2** displays potent activators by inducing autophagic cell death through production of ROS.

Highlights

- **ReHa-2** is synthesized with using simple and cheap raw materials.
- **ReHa-2** is capable of increasing protein LC3-II (a marker of autophagy) and reducing p62 in a time- and dose-dependent manner.
- **ReHa-2** can activate MAPKs and Akt signal pathway.
- **ReHa-2** induces the autophagic cell death of human NCI-H460 cells mediated by triggered ROS.

1. Introduction

Carcinoma of the lung is one of the most largest cause of morbidity and mortality in the world. The most potent defenses against lung cancer is apoptosis. Autophagy has been involved in turnover of proteins, cytoplasmic contents and elimination of damaged organelles. Concretely, the LC3-I in the cytoplasm is mated to phosphatidylethanolamine to form LC3-II and p62 is a ubiquitin-binding protein that transfers metabolites to be degraded[1–2]. However, inappropriate activation of autophagy may lead to cell death[3–4] which is different from apoptosis[5]. The autophagic cell death is also form of programmed cell death and plays an important to target tumor cells.

For the past few decades, various investigations have sought to identify new compounds which are able to induce autophagic cell death without deleterious effects on healthy cells. Among these compounds, Resveratrol (Res) is an active ingredient from our food sources, such as grapes and peanuts, which has long been used in traditional Chinese medicine. Numerous studies have demonstrated that Res can stimulate apoptosis, arrest cell cycle and suppress kinase pathways[6–9] and it can promotes cell death in many kinds of tumor cells by triggering both autophagy and apoptosis[10–11]. In addition, the previous

literature reported that hans ester derivatives showed a broad of biological activity such as antioxidant, antitumor, antiviral[12–14] and were potent anti-inflammatory agents[15]. However, the effects of hybrid with resveratrol and hans ester derivatives on cells autophagy and apoptosis have not been reported up to now.

Reactive oxygen species (ROS) were closely related with cellular redox and metabolism[16]. Excess level of ROS can cause cellular oxidative stress, such as proteins, DNA and lipids are were oxidized and damaged[17]. ROS also plays an important role in autophagic cell death[18]. Over the past few decades, many chemotherapeutic drugs can induce autophagy death. In addition, natural products with diverse chemical skeleton induce the autophagic cells death by promoting the production of ROS[19–23].

In this work, we have synthesized and selected the hybrid with resveratrol and hans ester derivatives (**ReHa**) as molecules to explore the autophagic cell death in human NCI-H460 cells (Scheme 1). Our results suggest that compared with the parent **NIP**, the *meta*-rtho trifluoromethyl-substituent hybrid with resveratrol and hans ester derivatives **ReHa-2** is capable of increasing protein LC3-II (a marker of autophagy) and reducing p62 in a time- and dose-dependent manner. Meanwhile, it can effectively promote ROS (mainly including H_2O_2), that resulted in killing of NCI-H460 cells which mainly based on ROS-mediated autophagic cell death.

2. Materials And Methods

2.1 Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco, Inc. (Solon, OH, USA). Roswell Park Memorial Institute (RPMI)-1640 was obtained from Gibco. Propidium iodide (PI), RNase, 2', 7'- dichlorofluorescein diacetate (DCFH-DA), dithiothreitol (DTT), catalase (CAT) and chloroquine (CQ) were obtained from Sigma-Aldrich ((St. Louis, MO, USA). 3-methyladenine (3-MA), Z-VAD-FMK (Z-VAD) and Necrostatin-1 (NEC) were from Selleck Chemicals. The primary antibodies against inducible LC3, p62, p-mTOR, p-p38, p-JNK, p-ERK1/2 and p-Akt were supplied from Cell Signaling Technology (Beverly, MA, USA) and antibodies against β-Actin was supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled secondary antibody was a product of TransGen Biotech (Beijing, China). All other chemicals were of analytical grade.

2.2. Synthesis of compounds ReHa analogs

The compounds **ReHa** analogs in Scheme 1 were synthesized by the Wittig-Horner reaction as described in our previous papers[24–25]. The compounds **ReHa** analogs in Scheme 1 were synthesized by the Wittig-Horner reaction between phosphonates and corresponding appropriate aldehydes (Scheme 2). The detailed synthesis process, characterization of all the **ReHa** analogs, structural determinations of the compounds which based on ^{1}H NMR, ^{13}C NMR spectra and HRMS were included in the supplementary material.

2.3 Cell Culture

NCI-H460 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and culture according to Ref. 26.

2.4 Cell Viability Assay

The cytotoxicity of **ReHa** analogs for 24 h against NCI-H460 cells was tested by the MTT assay as described previously[26].

2.5 Assay for the ROS levels by a flow cytometry

The related procedure of production of ROS were detailed in Ref. 27.

2.6 Apoptosis and Cell cycle analysis using flow cytometry

The cell cycle and apoptosis in NCI-H460 cells were further detected as described previously[27].

2.7 Western Blot Analysis

Western blot analysis was carried out as described previously[28].

2.8 Statistical Analysis

All experiments were repeated independently at least three times. Results are expressed as the mean values ± standard deviation (SD) and analyzed with SPSS 10.0 statistical software. Statistical analyses were performed by a one-way ANOVA followed by LSD test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results And Discussion

3.1 Synthesis of ReHa analogs with different substituents

ReHa analogs with different substituents were prepared by following the synthesis procedure shown in Scheme 2. In brief, we used *p*-hydroxybenzaldehyde as raw material and then it was reduced to alcohol by sodium borohydride. The reaction were went through bromination, Wittig-Horner reaction between appropriate aldehydes and corresponding phosphonates in the presence of NaH in tetrahydrofuran (for synthetic details and characterization of all the **ReHa** analogs see the supplementary material)[24–25].

3.2 Cytotoxicity of the ReHa analogs

As as shown in the Table 1, MTT assay showed that among the **ReHa** analogs tested, comparison with the **ReHa-7** (no substituent hybrid with resveratrol and hans ester), the trifluoromethyl and Cl-substituent were more high toxicity than methoxyl groups and the *meta*-rtho trifluoromethyl (**ReHa-2**), Cl (**ReHa-5**)-substituent exhibited highest cytotoxicity on NCI-H460 cells, suggesting that the electron-withdrawing group were more toxic than electron-donating group. Because the electron pulling activity of

trifluoromethyl is stronger than that of Cl-substituent, hence, **ReHa-2** is the most cytotoxic among the compounds and its IC_{50} values was 9.90 μM in NCI-H460 cells. Therefore, we choose **ReHa-2** to carry out the subsequent experiment.

Next, in order to assess the death models induced by **ReHa-2** in NCI-H460 cells, some inhibitors on the cytotoxicity were examined. They contain 3-MA (an autophagy inhibitor), Z-VAD (an apoptotic inhibitor) and NEC (a necrotic inhibitor). As shown in Fig. 1A, 3-MA partally reversed the cytotoxicity, whereas Z-VAD or NEC further no affected the cytotoxicity. The above results suggested that the cytotoxicity of **ReHa-2** depended partally on autophagy process rather than necrosis or apoptosis. In order to confirm the role of ROS in the cytotoxicity induced by **ReHa-2**, two redox modulators including CAT (a specific scavenger of H_2O_2) and DTT (a ROS scavenger) were use to conduct the experiment. Figure 1B exhibited that both CAT and DTT partally reversed the cytotoxicity induced by **ReHa-2** in NCI-H460 cells. These results suggested that the cytotoxicity of **ReHa-2** were related to ROS generation.

Table 1
Cytotoxicity of **ReHa analogs** against NCI-H460 cells[a].

ReHa	IC_{50} (μM)	Comp.	IC_{50} (μM)
1	46.1 ± 2.3	7	27.0 ± 1.8
2	9.90 ± 0.2	8	29.4 ± 0.7
3	27.6 ± 1.0	9	> 80
4	34.7 ± 3.7	10	> 80
5	14.4 ± 0.4	Nitrendipine	72 ± 4.9
6	27.4 ± 1.1		

[a]Values for IC_{50} (concentrations to cause 50% inhibition of cell viability after 48 h of treatment) are presented as means ± SD for at least three independent experiments.

3.3 ReHa-2 induces ROS accumulation in NCI-H460 cells

Considering that ROS generation is essential for the cytotoxicity of **ReHa-2** in NCI-H460 cells, we further used DCFH-CA, a widely used ROS fluorescent probe, to assay intracellular ROS levels in the cells. As shown in Fig. 2A, **ReHa-2** induced an obvious intracellular ROS accumulation with 30 μM which increased 2.1-fold relative to the control at 9 h. In contrast, the cells treatment with diverse concentrations of **NIP** for 9 h induced negligible changes (Fig. 2B). The ROS accumulation induced by **ReHa-2** is fully consistent with the design expectation.

3.4 ReHa-2 had no effect on cell cycle and apoptosis

Figure 1A showed that the cytotoxicity of **ReHa-2** depends partally on autophagy process rather than necrosis or apoptosis process. To further clarify and make sure this point, we thus examined its effect on cell cycle distribution and apoptosis by using flow cytometry. As show in Fig. 3, **ReHa-2** did not affect

both cell cycle arrest and apoptosis in NCI-H460 cells. The above results suggested that the cytotoxicity of **ReHa-2** in NCI-H460 cells depends on cell autophagic death.

3.5 ReHa-2 induces autophagic cell death in NCI-H460 cells

Since **ReHa-2** had no apparent cyclic retarded and apoptotic effect on NCI-H460 cells, we investigated the effects of **ReHa-2** on autophagy by using Western blotting. During the formation of autophagosomes, the autophagy-associated protein LC3 in cytoplasm changed from LC3-I form to LC3-II form with autophagosomal membrane specific binding. Conversion of LC3-I to LC3-II is associated with the formation of autophagosomes. During the autophagy process, protein p62 can transport substrates to be degraded to autophagosomes and then be degraded together with autophagosomes under the action of lysosomes[29]. Therefore, changes in p62 content during autophagy can reflect the completion of autophagy flow. Moreover, mTOR activity is regulated by amino acids and glucose levels in mammalian cells. The inhibition of mTOR were induced by rapamycin or starvation, which the conditions induced autophagy[30]. Figure 4 exhibited that **ReHa-2** triggered time dependently up-regulation of LC3-II as well as down-regulation of p62, p-mTOR and dose dependently up-regulation of LC3-II as well as down-regulation of p62. From these results we infer that **ReHa-2** can induce enrichment of autophagosomes by increasing the formation of autophagosomes rather than blocking its flow. Ultimately, **ReHa-2** resulted of autophagic cell death in NCI-H460 cells.

3.6 ReHa-2 induces dysfunction of autophagy degradationautophagic and further leads to cell death in NCI-H460 cells

As an inhibitor of lysosome, chloroquine (CQ) can inhibit the fusion of autophagy and lysosome. Thus, it is often used in the study of autophagy and autophagy flow[31]. We investigated the effects of CQ on LC3 expression level induced by **ReHa-2**. As shown in Fig. 5, in this case of chloroquine, the expression levels of LC3-I and LC3-II were significantly enhanced with the increased of **ReHa-2** concentration. In compare with parent molecule **NIP**, **ReHa-2** exhibited down-regulation of p-mTOR expression. These results indicated that **ReHa-2** activated autophagy by enhancing autophagy formation and then lead to autophagic death in NCI-H460 cells.

3.7 ReHa-2 activates MAPKs and Akt

MAPKs and Akt have also been confirmed to participate in the regulation of autophagy[32–33]. For example, in presence of Beclin complex, the apoptosis-related protein Bcl-2 enables the complex inhibits autophagy in vivo. Under starvation conditions, JNK can be activated and phosphorylation of Bcl-2 which weakens its binding ability with Beclin1, then it activates autophagy[34]. Figure 6 shows that **ReHa-2** induced a time-dependent phosphorylation of Akt, ERK and JNK. In brief, **ReHa-2** induced the highest protein expression of phosphorylation of Akt, JNK and ERK at 30 and 60 min respectively. These results

suggested that MAPKs and Akt have been participated in the **ReHa-2-**induced autophagic death in NCI-H460 cells.

In summary, this work describes a novel autophagic cell death inducer **ReHa-2**, which based on hybrid with resveratrol and hans ester derivatives in NCI-H460 cells. Mechanistic study focused on NCI-H460 cells reveals that this molecule compared with the parent **NIP** could more effectively increase of intracellular ROS (mainly including H_2O_2), resulting in the increasing protein LC3-II and reducing p62 in a time- and dose-dependent manner. Moreover, **ReHa-2** activated phosphorylation of Akt, JNK and ERK signal pathway (Scheme 3).

Declarations

CRediT authorship contribution statement

Yu-Ting Du performed and designed the research, supervised the whole project and wrote the manuscript which was reviewed by all the authors; Hongliang Wang performed the research; San-Hu Zhao and Xiao-Juan Zhang contributed to the data analysis. All authors have read and approved this version of the article. The authors declare that all data were generated in-house and that no paper mill was used.

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Declaration of Competing Interest

All items in the Submission Checklist have been provided and checked carefully. We declare no conflict of interest, and do not want color reproduction of figures in printed version but want color reproduction in wed version.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figures

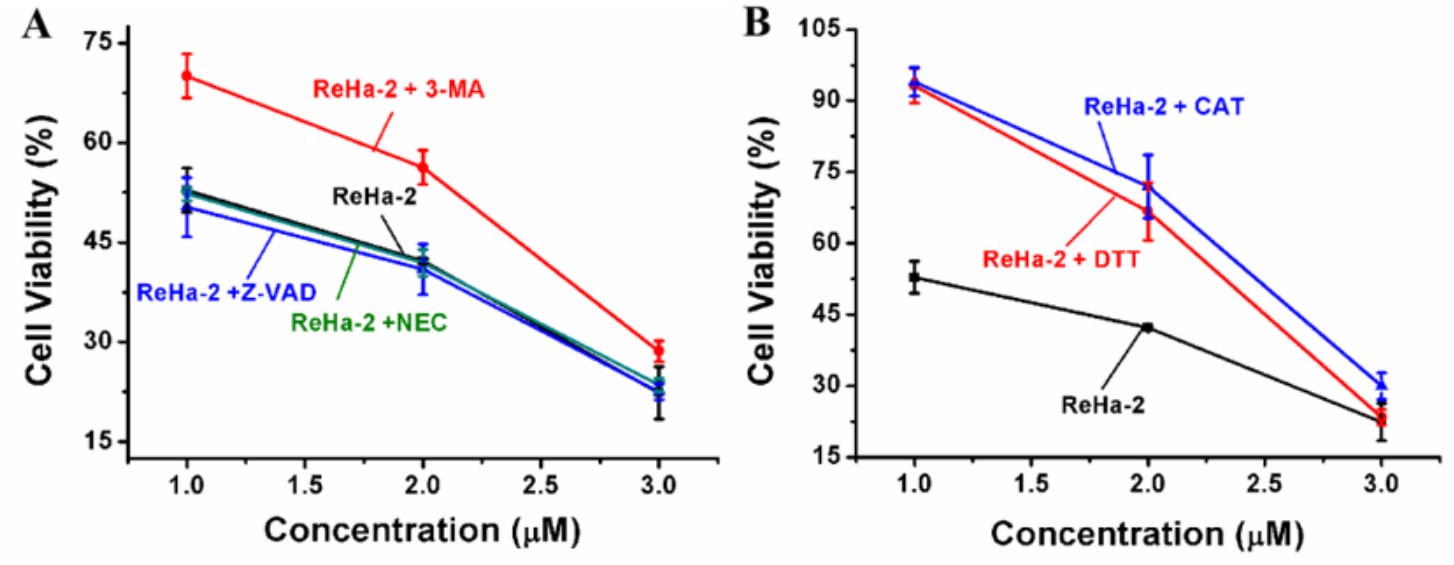

Figure 1

Effects of various cell death pattern inhibitors and redox modulators including 3-MA, Z-VAD, NEC (A) and CAT, DTT (B) on the cytotoxicity induced by **ReHa-2** against NCI-H460 cells. Data were expressed as mean ± SD from three independent experiments.

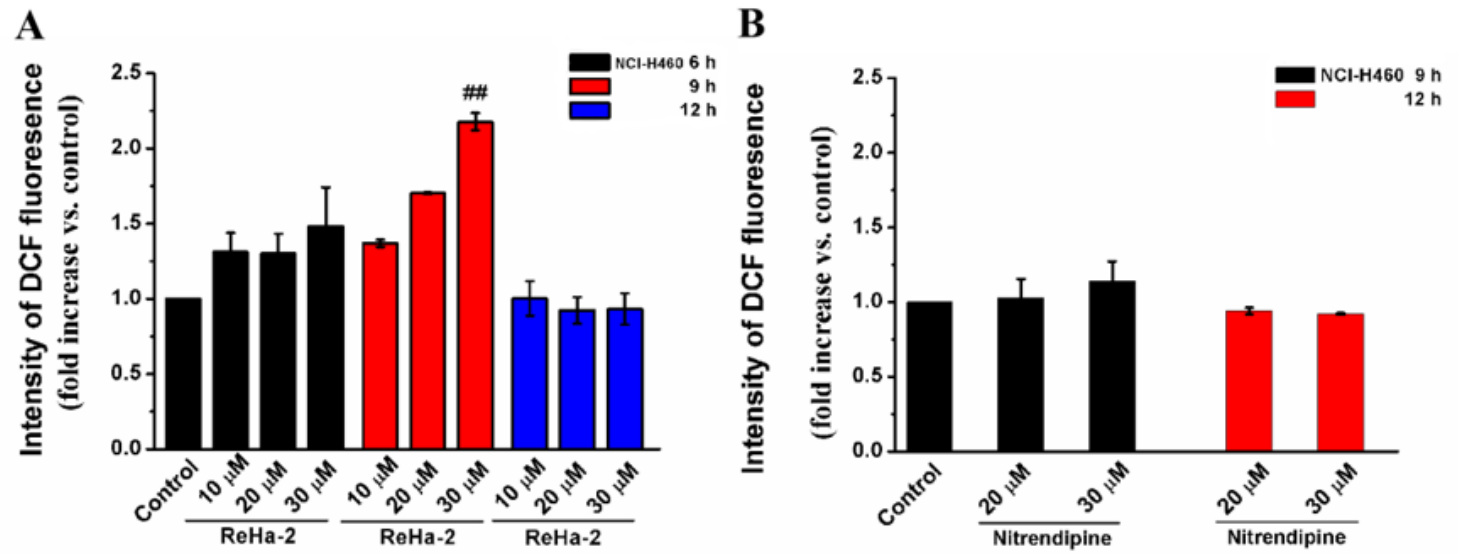

Figure 2

(A) **ReHa-2** induces the ROS accumulation at the indicated concentrations and time points in NCI-H460 cells. (B) Fold change of ROS in NCI-H460 cells treated with **NIP** (20, 30 mM) for 9, 12 h respectively. Data were expressed as mean ± SD from three independent experiments. $^{\#\#}p < 0.01$ versus the vehicle group.

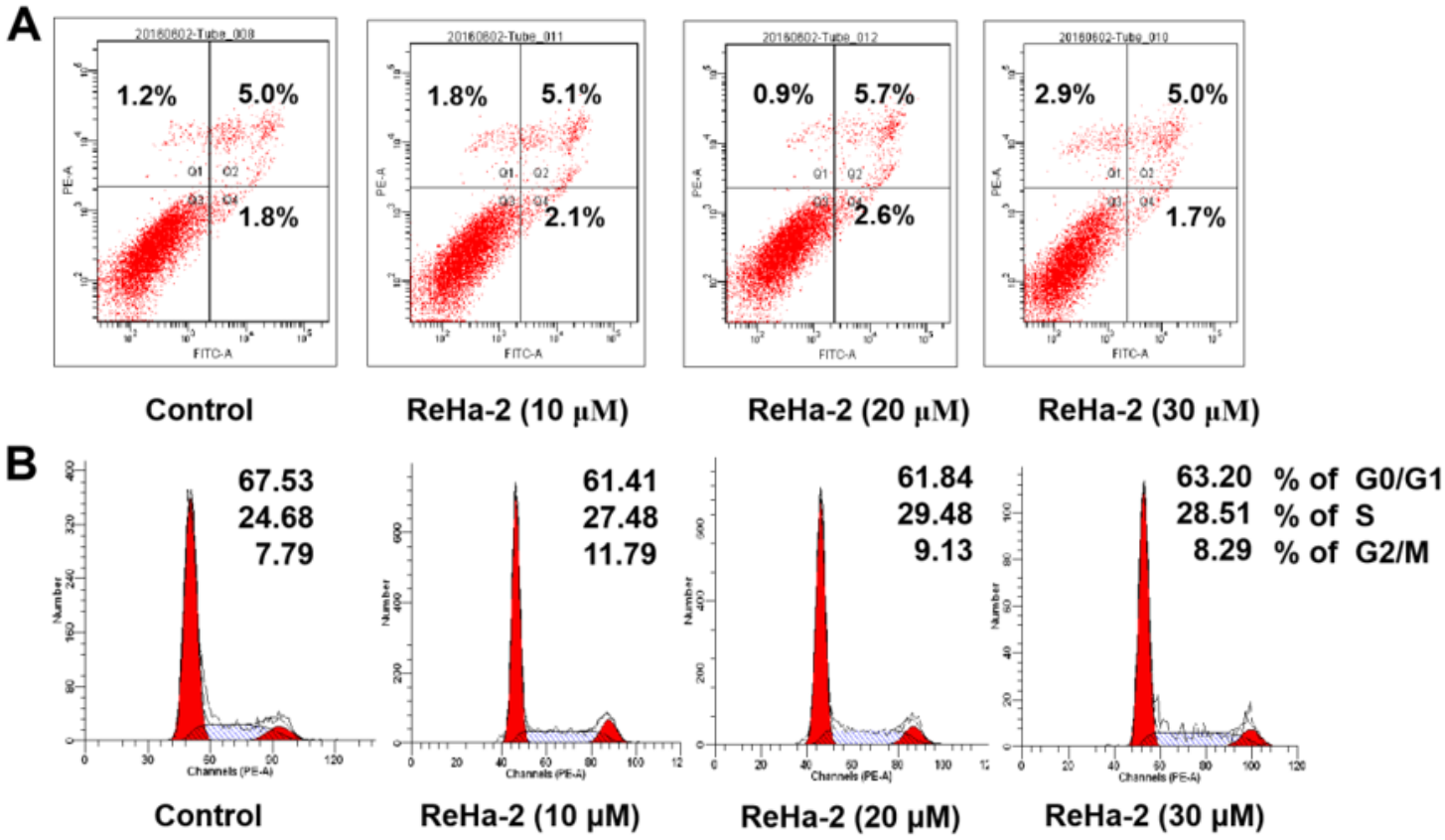

Figure 3

ReHa-2 had no effect on cell cycle and apoptosis of NCI-H460 cells. Flow cytometric analysis for apoptotic for 24 h (A) and cyclic retarded for 12 h (B) induction of NCI-H460 cells.

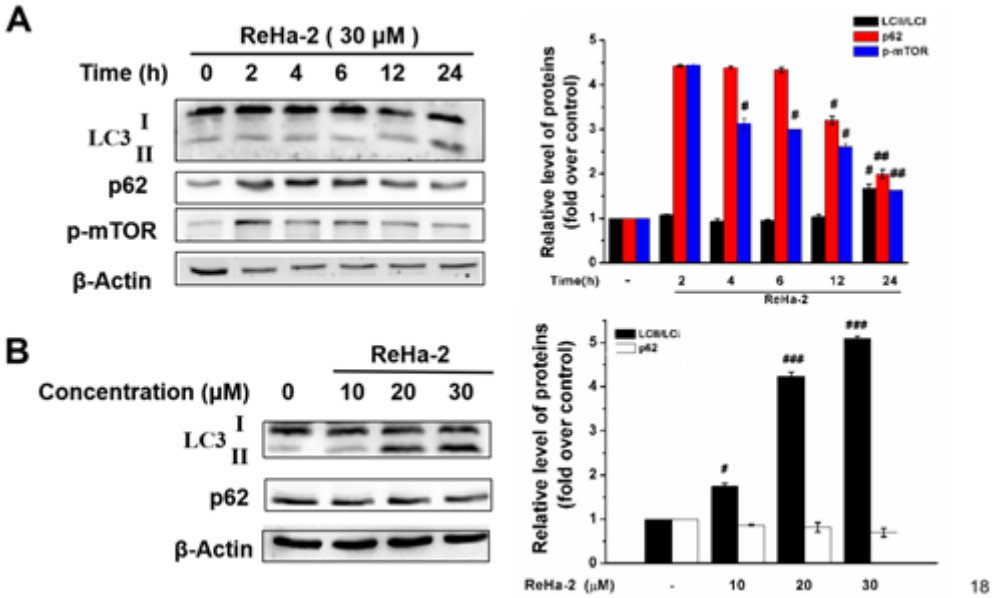

Figure 4

ReHa-2 induces autophagy in NCI-H460 cells. After treatment with 30 μM **ReHa-2** for 0-24 h (A) or the indicated concentrations of **ReHa-2** for 24 h (B) The cells were analyzed by immunoblotting with antibodies against LC3, p62, p-mTOR and β-Actin. Each experiment was performed in triplicate. $^{###}p < 0.001$, $^{##}p < 0.01$ and $^{#}p < 0.05$ versus the vehicle group.

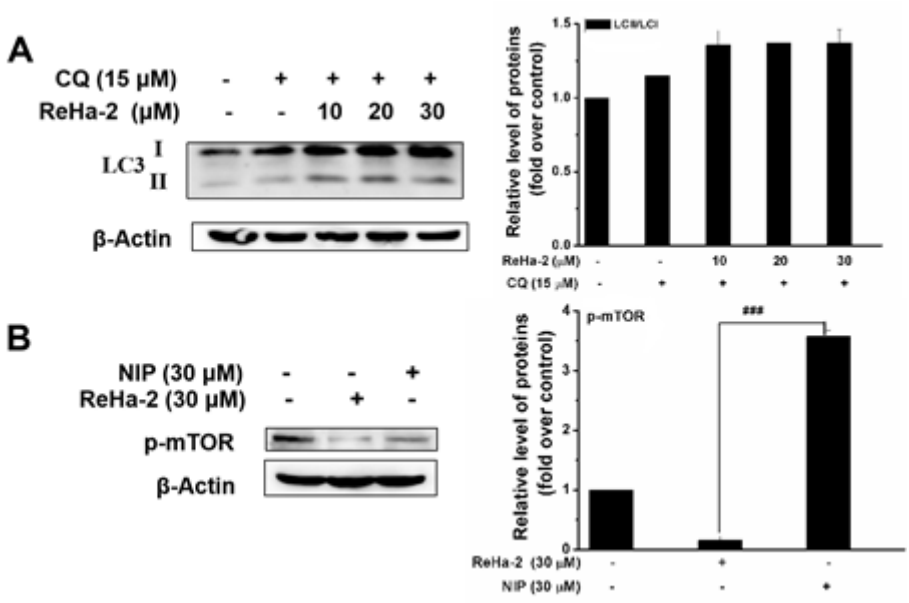

Figure 5

ReHa-2 increases autophagic flux in NCI-H460 cells. (A) The cells were treated with CQ (15 mM) for 1 h before incubation with **ReHa-2** (10, 20 and 30 mM) for 24 h. (B) The cells were treated with **ReHa-2** (30 mM) and **NIP** (30 mM) for 24 h. Each experiment was performed in triplicate. Total cellular proteins were prepared and analyzed by Western blotting. Each experiment was performed in triplicate. $^{###}p < 0.001$ versus the **ReHa-2** and **NIP**-treated group.

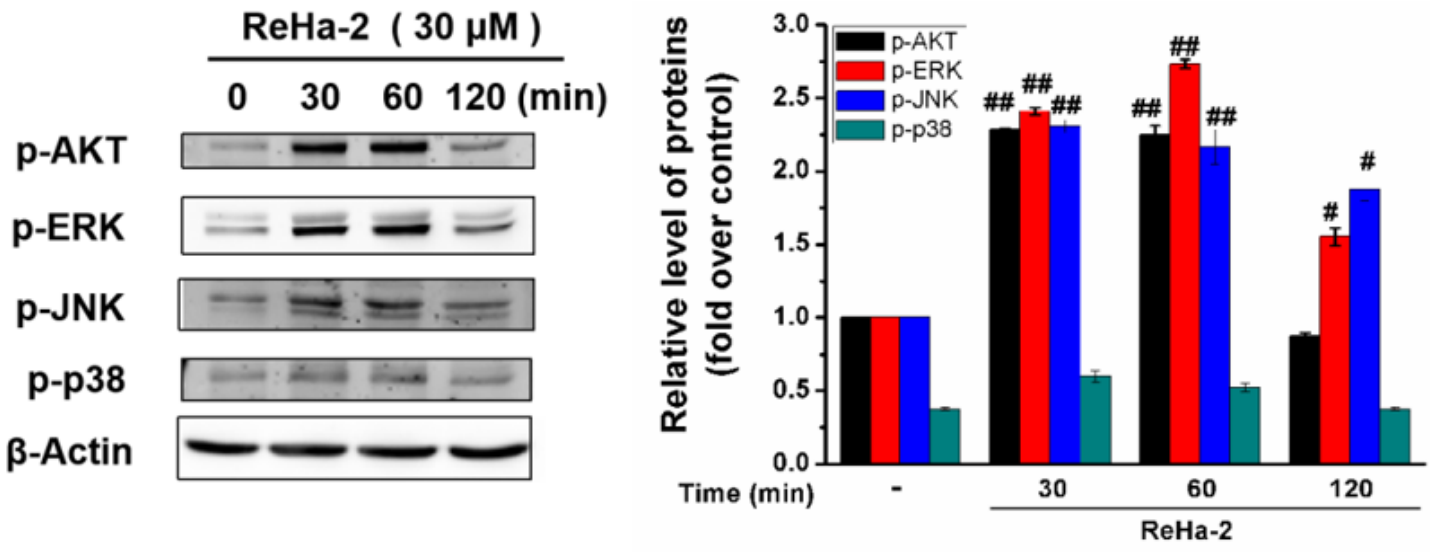

Figure 6

ReHa-2 activates phosphorylation of Akt, ERK and JNK in NCI-H460 cells. The cells were treated with indicated concentrations (30 mM) of **ReHa-2** for 24 h. Total cellular proteins were prepared and analyzed by Western blotting. Each experiment was performed in triplicate. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ versus the vehicle group.

Supplementary Files

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