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ML385, an Nrf2 Inhibitor, Synergically Enhanced Celastrol Triggered Endoplasmic Reticulum Stress in Lung Cancer Cells

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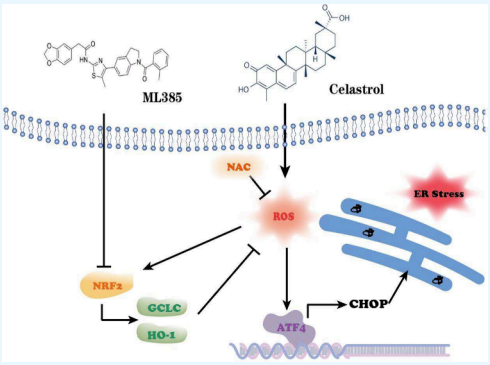
ABSTRACT: Lung cancer is one of the leading causes of death. Celastrol is a natural product that has shown anticancer activity but has not yet been applied in clinical settings due to its narrow therapeutic window. In this study, we discovered that celastrol stimulates an abnormal rise in the reactive oxygen species (ROS) level in lung cancer cells and that the ROS scavenger N-acetylcysteine (NAC) could counteract the cell death caused by celastrol. At the same time, celastrol upregulated the expression of cytoprotective transcription factor Nrf2 and its downstream proteins, which are effective in preventing the oxidative damage caused by ROS accumulation. Notably, we found that the overexpression of Nrf2 enhances the tolerance of lung cancer cells to celastrol and that lung cancer cells H460 with a Keap1 mutation are insensitive to celastrol. This indicates that the increase in Nrf2 contributes to the survival of lung cancer cells. Thus, we brought in an Nrf2 inhibitor ML385 to suppress the activation of Nrf2. We found that when ML385 and celastrol were added together the survival rates of lung cancer cells decreased more and the detected ROS level became much higher compared to treatment with celastrol alone. We also discovered that ML385 suppressed the expression of HO-1 and GCLC, which amplified celastrol-induced ATF4/CHOP-dependent endoplasmic reticulum stress (ER stress). Above all, our study found that ML385 enhanced celastrol-induced increases in ROS and ER stress, leading to lung cancer cell death. This research provides a potential strategy for the preclinical investigation of celastrol.



1. INTRODUCTION

Lung cancer is considered a high-risk cancer due to its high incidence and mortality rates. Based on the statistics calculated by the American Cancer Society and the National Center for Health Statistics, although the incidence of lung cancer has declined steadily since 2006, it still causes a significant number of deaths each year, much more than other cancers.[1] Patients with phase I or II non-small cell lung cancer (NSCLC) are typically recommended for surgical resection, while those with phase III or IV NSCLC are usually advised to undergo chemotherapy or radiotherapy.[2] Although chemotherapy has enormous potential, drug resistance is still an intractable issue in clinical application.[3] Thus, research and development on combination therapeutic methods using available drugs are especially important.

Celastrol is a pentacyclic triterpenoid extracted from the root bark of *Tripterygium wilfordii*, a plant in the family *Celastraceae R. Br.*[4] Recently, a mass of research has found that celastrol possesses a strong therapeutic effect in rheumatoid arthritis (RA).[5−7] Yang and his group found that celastrol activates autophagy by blocking the PI3K/AKT/mTOR pathway, thereby ameliorating RA.[8] Moreover, the antitumor activity of celastrol has been confirmed in various cell lines and *in vivo*. In lung cancer cells, Liu found that celastrol and low concentrations of erastin activated the ROS signaling pathway, leading to the death of NSCLC cells.[9] *In vivo*, Zhao discovered that celastrol demonstrated significant antitumor activity via the STAT3 pathway.[10]

Reactive oxygen species (ROS) are short-lived molecules, including O_2^-, H_2O_2, and •OH, that induce oxidative stress.[11] The influence of ROS on regulating physiological activities *in vivo* is double-edged.[12] This double-edged influence is reflected in the role of ROS as signaling molecules that support cellular functions, while their abnormal accumulation may lead to cancer and programmed cell death.[13,14] Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor which has a tight relationship with oxidative stress.[15] There are 7 Nrf2-ECH homologous (Neh) structural domains in Nrf2, and they perform diverse functions, respectively.[16] The Keap1-Nrf2 pathway is a crucial cellular defense mechanism that regulates oxidative stress by encoding antioxidants, enzymes, and components involved in glutathione regeneration.[17] It has

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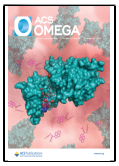

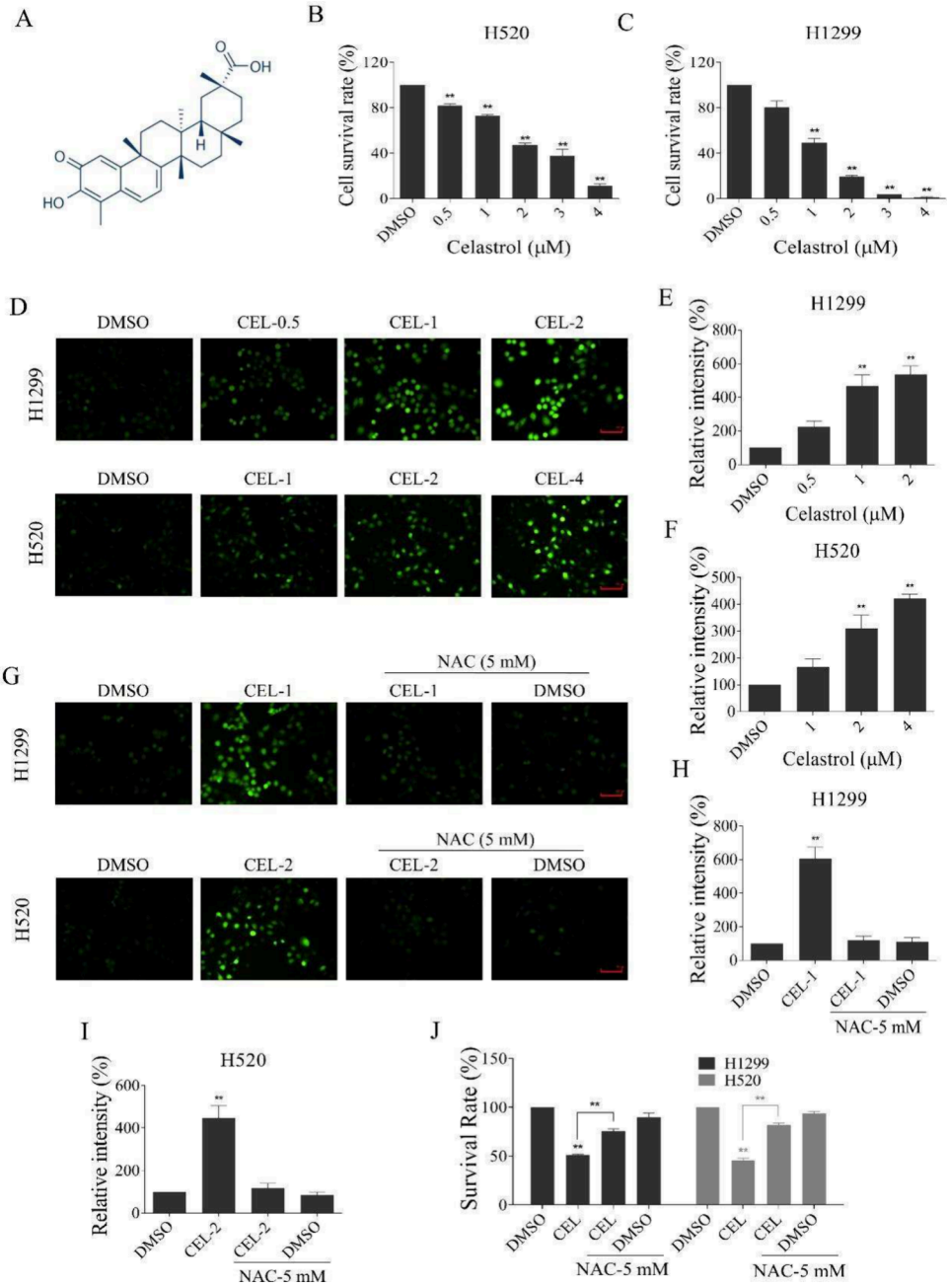

Figure 1. Structure of celastrol (A). H1299 and H520 cells were treated with celastrol at gradient concentrations ranging from 0.5 μM to 4 μM for 24 h. The survival rates were assessed following treatment with celastrol (B and C). H1299 cells were treated with celastrol at concentrations ranging from 0.5 μM to 2 μM, and H520 cells were from 1 μM to 4 μM for 1 h. The ROS level was measured using the DCFH-DA probe (D−F). The ROS level was detected after H1299 and H520 cells were treated with NAC for 1 h, followed by celastrol for an additional 1 h (G−I). Survival rates were calculated after treatment with NAC for 1 h, followed by celastrol for 24 h (J).

been reported that BDH2 promotes the interaction between Keap1 and Nrf2, increasing the ubiquitination of Nrf2, which in turn raises ROS levels and inhibits the phosphorylation of Akt at Ser473 and mTOR at Ser2448.[18]

In this study, we discovered that celastrol-induced cell proliferative inhibition was related to oxidative stress. At the same time, celastrol can upregulate the expression of Nrf2 and its downstream proteins. We applied the Nrf2 inhibitor ML385 with celastrol to further reveal the function of Nrf2. We found that the application of ML385 enhanced celastrol-induced ATF4/CHOP-dependent ER stress, thereby further augmenting its antilung cancer activity. In summary, our research provides new insights for the further application of celastrol.

2. MATERIALS AND METHODS

2.1. Reagents. Celastrol (>98% pure) was purchased from Desite Company (Chengdu, China). ML385 (>98% pure), Z-VAD-FMK, and necrostatin-1 were purchased from Targetmol (Boston, USA). *N*-Acetyl-L-cysteine (NAC) and ciclopirox were purchased from Aladdin Industrial Corporation (Shanghai, China). ATF4 (diluted 1:2000, Cat. #11815S) was purchased from Cell Signaling Technology (Danvers, USA). CHOP/DDIT3 (diluted 1:2000, Cat. #15204-1-AP), Nrf2

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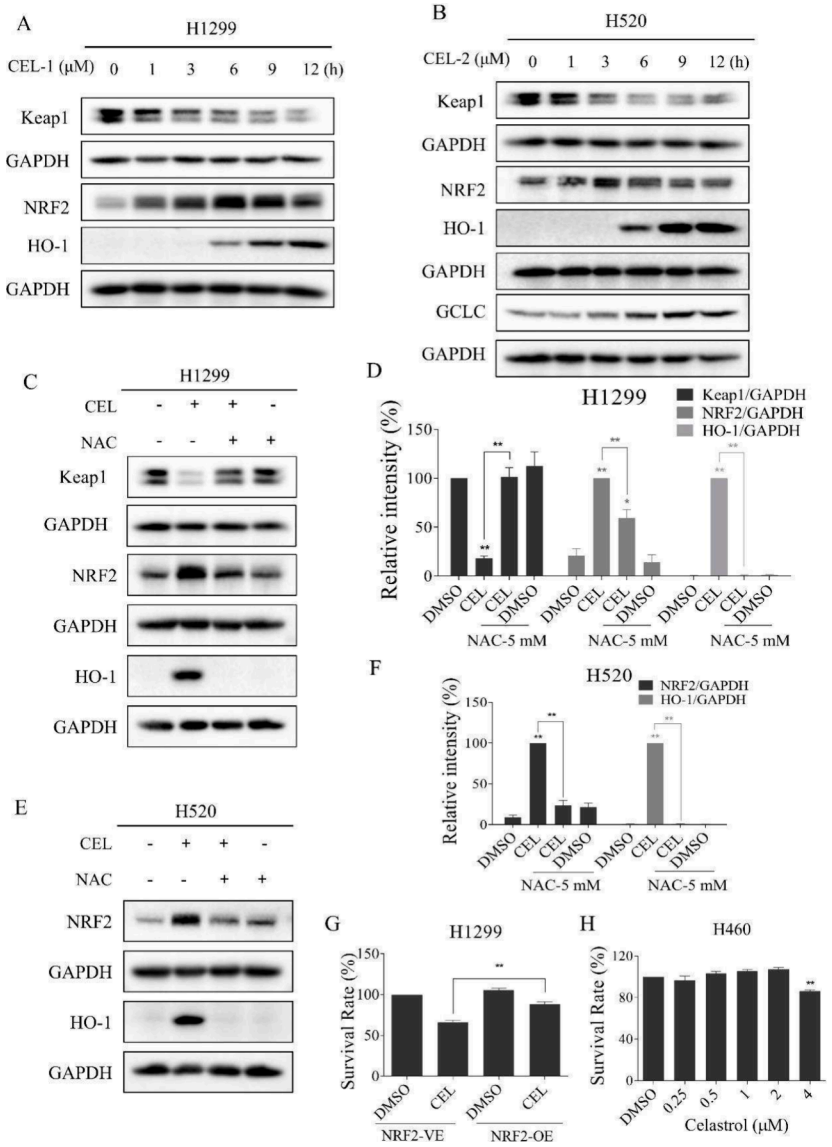

Figure 2. H1299 cells were treated with celastrol at a concentration of 1 μM for durations ranging from 0 to 12 h, after which samples were collected for Western blot analysis (A). H520 cells were treated with celastrol at a concentration of 2 μM for durations ranging from 0 to 12 h, and protein levels were measured by Western blot (B). H1299 and H520 cells were treated with NAC for 1 h, followed by celastrol treatment to assess the expression levels of Nrf2, HO-1, and Keap1 using Western blot analysis (C−F). Nrf2-overexpressing H1299 cells and vehicle control cells were treated with celastrol for 24 h. The survival rates were assessed following this treatment (G). H460 cells were cultured with celastrol at gradient concentrations ranging from 0.25 μM to 4 μM for 24 h, after which the survival rate was calculated (H).

(diluted 1:2000, Cat. #16396-1-AP), and GAPDH (diluted 1:10000, Cat. #10494-1-AP) were purchased from Proteintech (Wuhan, China). HO-1 (diluted 1:200, Cat. #sc-136960) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell Culture. H520, H1299, H460, and BEAS-2B cell lines were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). H520, H1299, and H460 cell lines are all cultured in 1640 basic medium with 10% FBS in a humidified incubator with 5% CO_2 at 37 °C. The BEAS-2B cell line is cultured in DMEM basic culture medium with 10% FBS. After growing to a specific density, cell lines are digested with trypsin enzyme solution, which contains 0.25% EDTA. Cell suspensions are collected into centrifuge tubes and centrifuged at 1200 rpm for 3 min. 2 mL of culture medium was added to resuspend centrifuged cells after supernatants were discarded carefully. Part of the cell suspensions are added into the culture plate with 5−6 mL of culture medium, and the rest of them are scheduled to complete other experiments.

2.3. Cell Viability Assay. Equal amounts of cells are seeded into a 6-well plate and cultured in a 5% CO_2 humidified incubator at 37 °C for 24 h. The next day, after replacing nutrient-depleted medium with new medium, cells are treated with different sorts and concentrations of drugs for specific times. Cell viability is assessed, and CI values are calculated by CompuSyn software.[19]

2.4. Measurement of ROS. Cells grow on the round slide in the 6-well plates. After drug treatment, we add fluorescence probe 2,7-dichlorofluorescin-diacetate (DCFH-DA) which is diluted to 1:3000 with nonfetal bovine serum 1640 medium into wells for 30 min. Then, the slides were observed under an inverted fluorescence microscope, with all of the operations conducted entirely in the dark.

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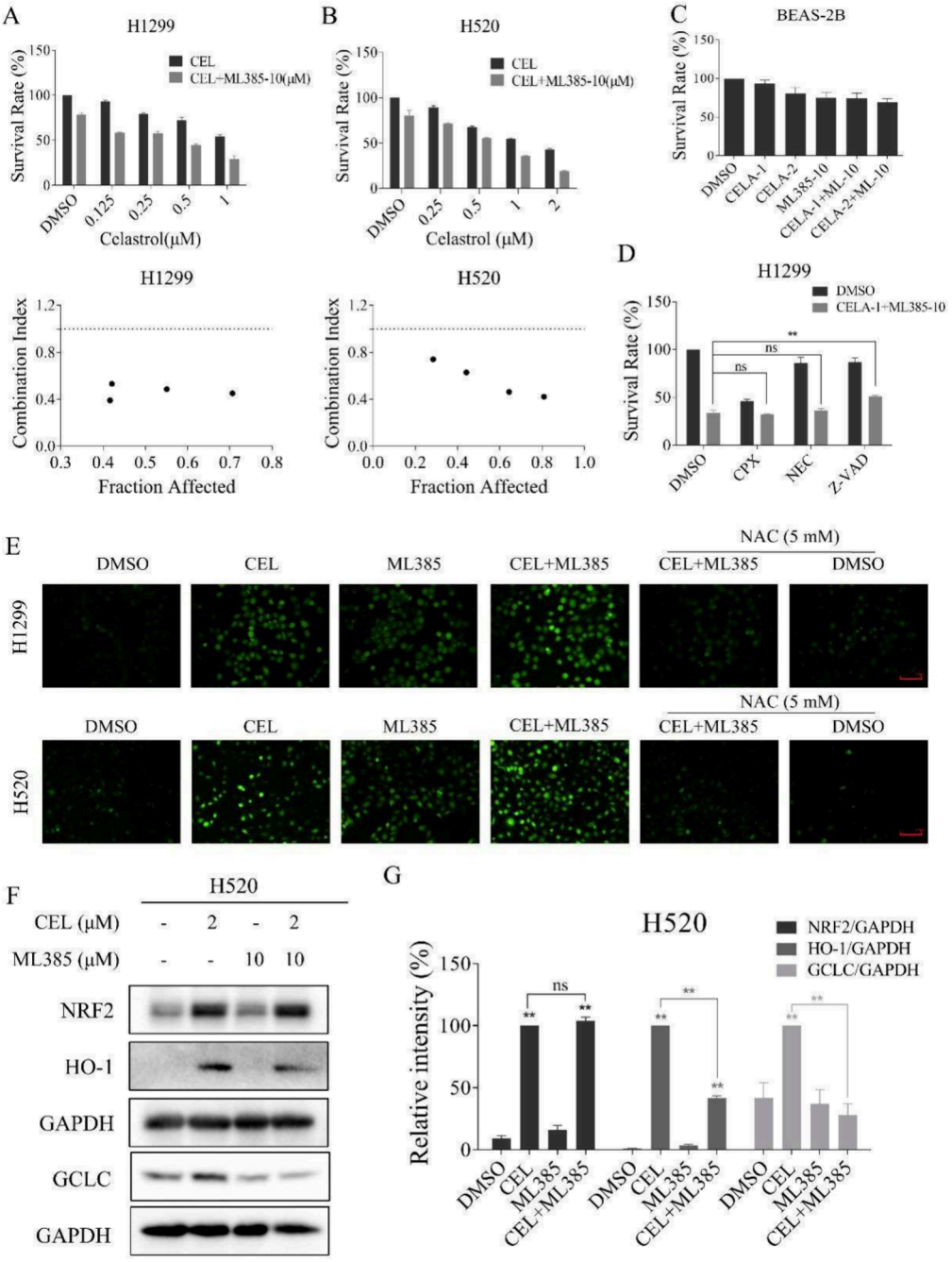

Figure 3. H1299 cells were treated with celastrol at concentrations ranging from 0.125 μM to 1 μM and also with celastrol at the same concentrations plus ML385 at 10 μM, for 24 h. The CI value was calculated by CompuSyn (A and B). The BEAS-2B cells were treated with celastrol and ML385 at different concentrations, after which their survival rate was measured (C). H1299 cells were treated with celastrol and ML385 for 24 h after being treated with Z-VAD-FMK, necrostatin-1, or ciclopirox for 1 h, respectively. The survival rate was then measured (D). The ROS levels were examined after the cells were treated with NAC for 1 h, followed by treatment with celastrol and ML385, both individually and together, for 1 h (E). Western blot was applied to measure the protein levels of Nrf2, HO-1, and GCLC after treatment with celastrol and ML385 (F and G).

2.5. Western Blot. Cells grow in the 6-well plates and are treated with drugs of various concentrations according to the experimental schedule. Cells are lysed with 70−80 μL of lysis buffer according to the quantity of cells in each well for nearly 10 min on the ice. Cell lysates are then collected into 1.5 mL centrifuge tubes, respectively, in an ice bath. Cell lysates are centrifuged at 4 °C and 12000 rpm for 10 min. The protein concentration is quantified by the measured absorbance of the solution mixed with 20 μL of PBS, 2 μL of cell lysates, and 200 μL of Brilliant Blue G with a microplate reader. Next, pure water, cell lysates, and loading buffer are added successively into 0.6 mL tubes and heated in boiling water at 100 °C for 10 min. These protein samples are separated with SDS-PAGE at 80 V for 1 h according to their molecular weight and then transferred onto a poly(vinylidene fluoride) membrane under the condition of 300 mA for 90 min. After being blocked with 5% nonfat milk for 90 min, specific antibodies were used to combine onto the membranes for more than 6 h, followed by

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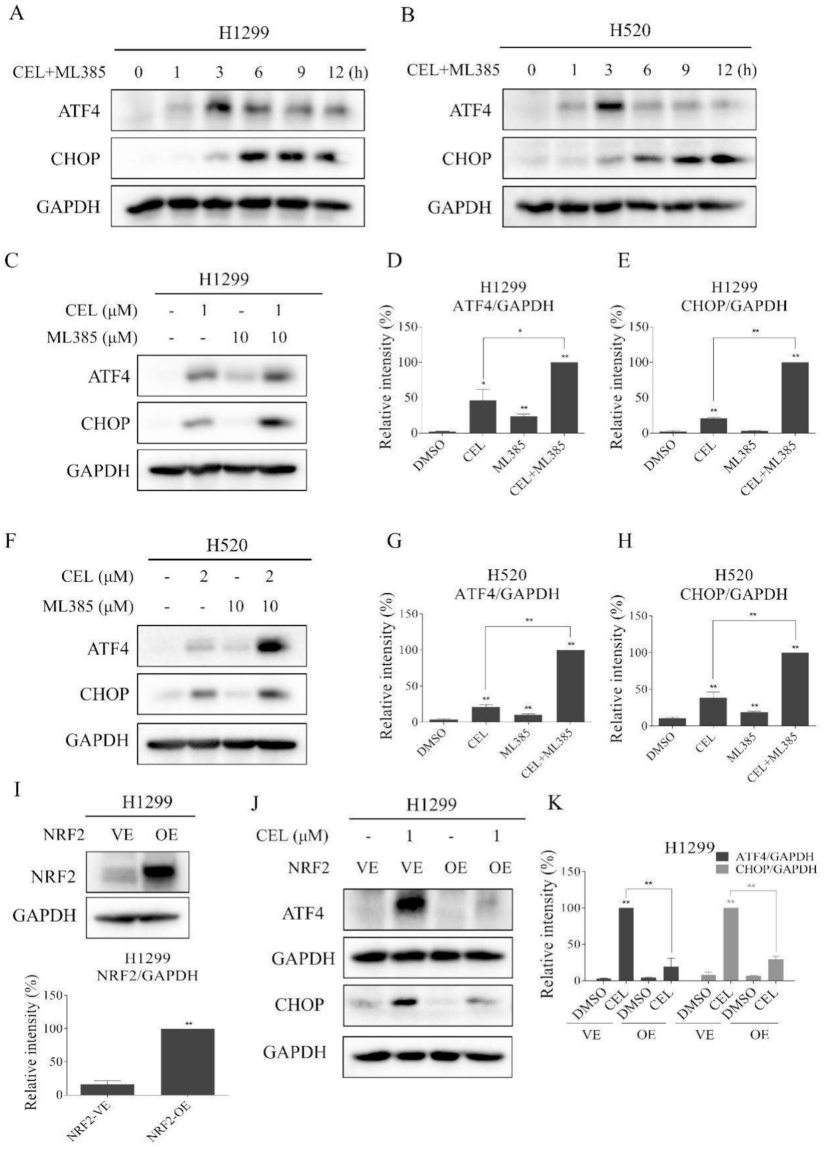

Figure 4. H1299 cells were treated with celastrol at 1 μM and ML385 at 10 μM at the same time for durations ranging from 0 to 12 h, after which the samples were collected for Western blot analysis (A). H520 was treated with celastrol at 2 μM and ML385 at 10 μM for the same duration, and then the samples were collected for Western blot analysis (B). Celastrol and ML385 were added to the cells either separately or together, and the samples were then used for Western blot analysis (C–H). Nrf2-overexpressing H1299 cells and vehicle cells (I) were treated with celastrol, and the levels of ATF4 and CHOP were then detected by Western blot assay (J–K).

the secondary antibody for 1 h. Then, protein bands can be detected by an ECL solution.

2.6. Generation of a CHOP Knockdown or Nrf2 Overexpressing Cell Line. Recombinant lentiviruses of CHOP/DDIT3 and Nrf2/NFE2L2 were obtained from GeneChem (Shanghai, China). Cells were planted into a 12-well plate and cultured for 24 h. According to the manual, cells were infected with the lentivirus for 14 h. Next, the lentivirus-containing culture medium was replaced with complete medium and cultured for more than 24 h. Cells were then expanded to 10 cm culture dishes and selected with puromycin.

2.7. Statistical Analysis. Data were analyzed by using GraphPad prism 6.0 software (CA, USA). Statistical differences were calculated using Student's t test or One-way ANOVA. Statistical significance: * $p < 0.05$, ** $p < 0.01$.

3. RESULTS

3.1. Celastrol Inhibits the Proliferation of the Tumor Cell and Activates Intracellular ROS Level. We first assessed the cellular activities of celastrol and found that the cell survival rates were close to those at their semilethal concentrations, when the concentration of celastrol was 2 μM in H520 cells and 1 μM in H1299 cells (Figure 1B and C). Celastrol has been reported to markedly increase ROS level in cancer cells, subsequently leading to cell death.[20] To ascertain whether celastrol elevates ROS level in nonsmall cell lung cancer cell lines, we utilized the DCFH-DA probe to measure ROS level in H1299 and H520 cells across a range of celastrol concentrations. We found that ROS accumulation increased with the rising concentrations of celastrol (Figure 1D, E, and F). N-Acetylcysteine (NAC) is a widely used ROS scavenger that acts as a GSH precursor.[21] Fluorescence detection showed that NAC could reverse the ROS accumulation caused by

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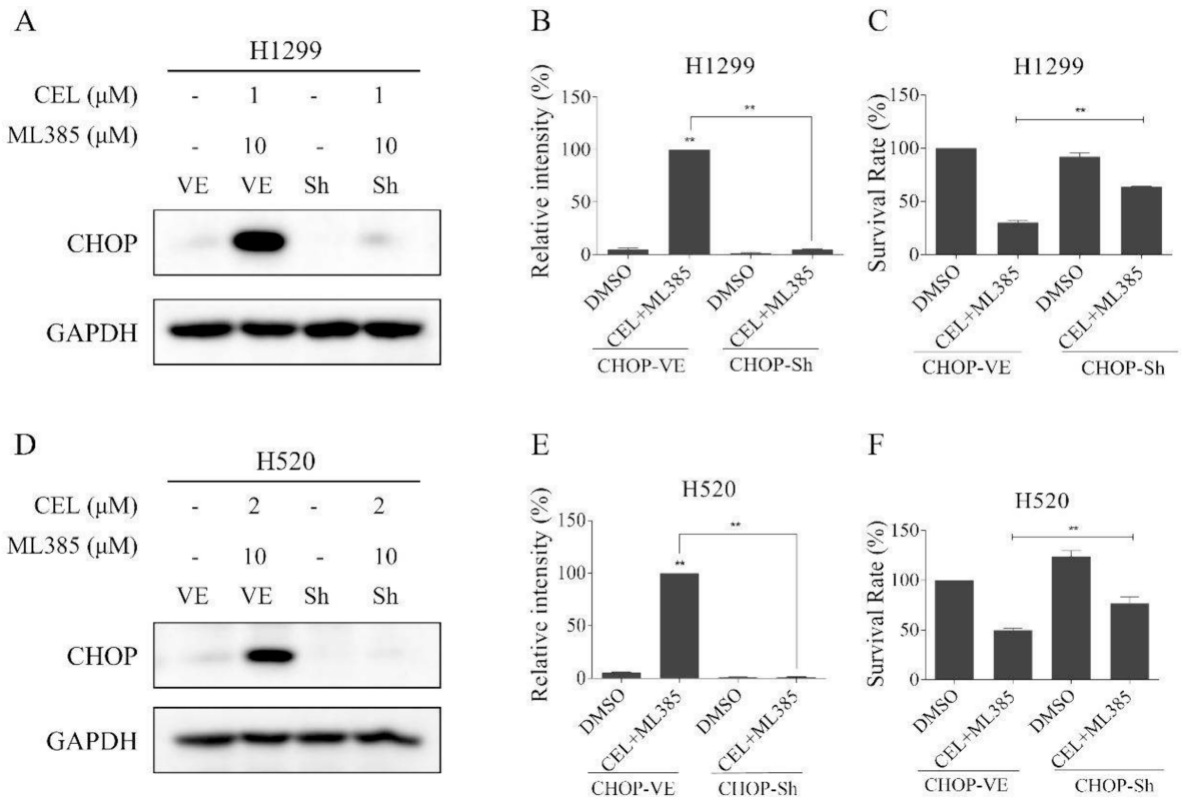

Figure 5. Celastrol (1 μM) and ML385 (10 μM) were added to H1299 CHOP-knockdown cells and vehicle cells, after which samples were collected for Western blot analysis (A,B). Celastrol (2 μM) and ML385 (10 μM) were added to H520 CHOP-knockdown cells and vehicle cells, and samples were subsequently collected for Western blot analysis (D,E). CHOP-knockdown cells and vehicle cells were seeded into 6-well plates and treated with celastrol and ML385 for 24 h, after which the survival rate was calculated (C and F).

celastrol (Figure 1G, H, and I). In addition, NAC was also able to counteract celastrol's ability to inhibit cell proliferation (Figure 1J).

3.2. Celastrol Promotes the Expression of Nrf2 and Its Downstream Proteins. Nrf2, which is considered a cytoprotective transcription factor that regulates the gene expression of many cytoprotective enzymes, is related to the balance between ROS aggregation and dissipation. Among these enzymes are HO-1 and GCLC.[22] It can be observed that the expressions of Nrf2 in H1299 and H520 cells were upregulated by celastrol. Following the increase in Nrf2 expression, the levels of HO-1 and GCLC were also upregulated (Figure 2A and B). It is worth noting that Keap1 expression significantly decreased after celastrol treatment, and this decrease, along with the upregulation of Nrf2 and HO-1, could be reversed by NAC (Figure 2C−F). This suggests that celastrol-induced ROS can reduce Keap1 levels, thereby activating Nrf2 and its downstream proteins. Additionally, we constructed an Nrf2-overexpressing H1299 cell line to assess the role of Nrf2 following celastrol treatment. The results showed that Nrf2 overexpression reduced the cells' sensitivity to celastrol (Figure 2G). Furthermore, H460 cells with a Keap1 mutation exhibited resistance to celastrol (Figure 2H). Overall, these findings suggest that the upregulation of Nrf2 decreases the efficacy of celastrol.

3.3. ML385, an Inhibitor of Nrf2, Functions with Celastrol Synergistically. ML385 has been reported to interact with Nrf2 directly and specifically.[23] The cell viability assay demonstrated that when the concentration of ML385 was at 10 μM with celastrol at 1 μM in H1299 cells and with celastrol at 2 μM in H520 cells ML385 obviously strengthened the effect of celastrol (Figure 3A and B). Additionally, we found that, compared to tumor cells H1299 and H520, the combination of celastrol and ML385 had a smaller effect on the proliferation of normal BEAS-2B cells, indicating that this combination exhibits good selectivity (Figure 3C). Moreover, we applied Z-VAD-FMK, necrostatin-1, and ciclopirox to tentatively investigate the type of cell death occurring after the combination treatment. The results indicated that only Z-VAD-FMK obviously reversed the effect of celastrol in combination with ML385, preliminarily suggesting that this combination may promote cell apoptosis; however, further confirmation is needed (Figure 3D). The ROS level after treatment with celastrol and ML385 was much higher than that observed with celastrol or ML385 alone, and this phenomenon could also be reversed by NAC (Figure 3E). The outcomes of the Western blot assay exhibited that the level of Nrf2 did not change significantly, but its downstream effectors, HO-1 and GCLC, exhibited a noticeable decrease after celastrol treatment (Figure 3F and G).

3.4. Celastrol and ML385 Jointly Stimulate Endoplasmic Reticulum Stress (ER Stress). The out-of-balance of ROS production and consumption triggers numerous physiological activities, such as ER stress.[24] After treatment with celastrol and ML385 together, ATF4 and CHOP which occupy a vital position in ER stress increased over time (Figure 4A and B). ATF4 peaked at 3 h, while CHOP peaked at 9−12 h. Furthermore, when celastrol and ML385 were added simultaneously, ATF4 and CHOP exhibited a much greater increase compared with when they were added individually

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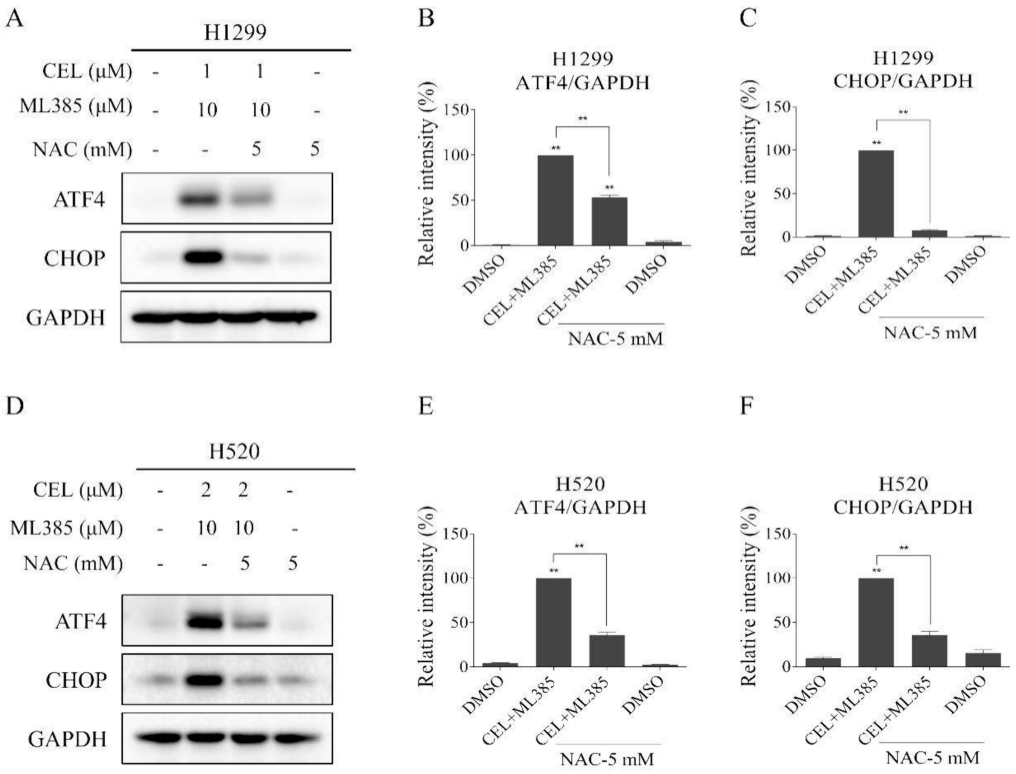

Figure 6. Celastrol and ML385 were added to the H1299 cells either separately or together after the cells were treated with NAC for 1 h, and then samples were collected for Western blot analysis (A−C). Celastrol and ML385 were added to the H520 cells either separately or together after the cells were treated with NAC for 1 h, and then samples were collected for Western blot analysis (D−F).

(Figure 4C−H). It is worth noting that a high expression of Nrf2 can reverse the celastrol-induced expression of ATF4 and CHOP, indicating that the increase in Nrf2 is a compensatory response of the cells to celastrol-induced ER stress (Figure 4I−K).

To identify the importance of CHOP in the ER stress process, we structured CHOP knockdown cell lines of H1299 and H520 and then treated them with celastrol and ML385. The expression of CHOP elevated in the vehicle cell lines but remained almost unchanged in the CHOP knockdown cell lines (Figure 5A,B and D−E). Importantly, the ability of celastrol in combination with ML385 to inhibit cell proliferation was also reversed (Figure 5C and F).

3.5. Celastrol in Combination with ML385 Triggers ROS-Mediated ER Stress. According to all of the results above, we used NAC to function with celastrol plus ML385. It showed that NAC reversed celastrol and ML385 triggered the rise of ATF4 and CHOP in H1299 and H520 cell lines (Figure 6A−F). This result indicates that the activation of ER stress is caused by the ROS accumulation induced by this combination.

4. DISCUSSION

Lung cancer is one kind of malignant cancer that is interlinked with other pulmonary diseases, making clinical therapy more difficult.[24] Celastrol is emerging as a promising drug due to its powerful abilities in combating tumors, reducing inflammation, and fighting cardiovascular diseases.[4,25] Celastrol is well-known as an antioxidant,[26] but it also has various abilities, such as triggering histone acetylation[27] and inducing autophagy, apoptosis, and ferroptosis.[8,28] However, there are also many studies on its induction of ROS elevation.[28−31] From our assay, the application of celastrol inhibited the growth of H1299 and H520 cells by promoting the amount of ROS in the cells. These results indicate that its ability to induce ROS varies with the cell type. Notably, although we have demonstrated that upregulation of ROS mediates the antitumor effect of celastrol, further investigation is needed to determine the specific types and sources of ROS in lung cancer.

It has been reported that Nrf2 plays a crucial role in safeguarding cells from oxidative stress.[13] The antioxidant ability of Nrf2 is efficient not only in metabolism and inflammation but also in cancer cells.[11] In our study, we proved that celastrol obviously activates the expression of Nrf2. It also increased the expression of HO-1 and GCLC which meant that under the stimulation of celastrol Nrf2 and its downstream proteins exert a cytoprotective effect in lung cancer cells. It is worth noting that the addition of celastrol significantly downregulated the expression of Keap1, and this decrease, along with the upregulation of Nrf2 and HO-1, can be reversed by NAC. These results suggest that the accumulation of ROS leads to the degradation of Keap1, resulting in an increase in Nrf2 and its downstream proteins. The cellular protective function of Nrf2 has been verified extensively.[32] The block of the Nrf2 pathway may promote cellular oxidative stress.[32] In our study, we used ML385 to inhibit the activity of Nrf2. We found that with the addition of ML385 the rise of celastrol-triggered ROS level grew higher. At the same time, the expression of HO-1 and GCLC was downregulated.

The endoplasmic reticulum is an essential organelle where proteins are handled, modified, and folded.[33] However, external factors and cell-intrinsic events can lead to protein misfolding, resulting in endoplasmic reticulum stress.[34] It has been reported that overproduced ROS can provoke ER

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stress.[35] In our study, we observed an abnormal increase in ROS following stimulation with celastrol. Simultaneously, ATF4 and CHOP were markedly upregulated. These results proved that celastrol-induced ROS accumulation provoked ER stress in lung cancer cells. Importantly, application of the Nrf2 inhibitor ML385 led to elevated levels of ATF4 and CHOP induced by celastrol. Furthermore, overexpression of Nrf2 can prevent this increase in ATF4 and CHOP. These findings indicate that Nrf2 plays a protective role against ROS-induced ER stress caused by celastrol.

In summary, celastrol upregulated the ROS level to trigger the cytoprotective function of Nrf2. The Nrf2 inhibitor ML385 which functions with celastrol would promote more ROS. With the activity of Nrf2 inhibited, celastrol-induced oxidative stress and ATF4/CHOP-dependent ER stress are enhanced. This study provides a possible strategy for further application of celastrol.

■ ASSOCIATED CONTENT

Data Availability Statement

The data presented in this study are included in the article material.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Siegel, R. L.; Giaquinto, A. N.; Jemal, A. Cancer statistics, 2024. *CA Cancer J. Clin* **2024**, *74*, 12−49.

(2) Li, Y. T.; Yan, B. S.; He, S. M. Advances and challenges in the treatment of lung cancer. *Biomed. Pharmacother.* **2023**, *169*, 115891.

(3) Guo, H. Y.; Zhang, J.; Qin, C.; Yan, H.; Liu, T.; Hu, H. Y.; Tang, S. J.; Tang, S. J.; Zhou, H. N. Biomarker-Targeted Therapies in Non-Small Cell Lung Cancer: Current Status and Perspectives. *Cells* **2022**, *11*, 3200.

(4) Wang, C.; Dai, S.; Zhao, X. T.; Zhang, Y. F.; Gong, L. H.; Fu, K.; Ma, C.; Peng, C.; Li, Y. X. Celastrol as an emerging anticancer agent: Current status, challenges and therapeutic strategies. *Biomed. Pharmacother.* **2023**, *163*, 114882.

(5) Deng, C. F.; Zhang, Q.; He, P. H.; Zhou, B.; He, K.; Sun, X.; Lei, G. H.; Gong, T.; Zhang, Z. R. Targeted apoptosis of macrophages and osteoclasts in arthritic joints is effective against advanced inflammatory arthritis. *Nat. Commun.* **2021**, DOI: 10.1038/s41467-021-22454-z.

(6) Hua, P.; Liang, R. F.; Yang, S. L. X.; Tu, Y. B.; Chen, M. W. Microneedle-assisted dual delivery of PUMA gene and celastrol for synergistic therapy of rheumatoid arthritis through restoring synovial homeostasis. *Bioactive Materials* **2024**, *36*, 83−95.

(7) Wong, V. K. W.; Qiu, C. L.; Xu, S. W.; Law, B. Y. K.; Zeng, W.; Wang, H.; Michelangeli, F.; Dias, I.; Qu, Y. Q.; Chan, T. W.; Han, Y.; Zhang, N.; Mok, S. W. F.; Chen, X.; Yu, L.; Pan, H. D.; Hamdoun, S.; Efferth, T.; Yu, W. J.; Zhang, W.; Li, Z.; Xie, Y. S.; Luo, R. Q.; Jiang, Q.; Liu, L. Ca^{2+} signalling plays a role in celastrol-mediated suppression of synovial fibroblasts of rheumatoid arthritis patients and experimental arthritis in rats. *Br. J. Pharmacol.* **2019**, *176*, 2922−2944.

(8) Yang, J. J.; Liu, J. Y.; Li, J.; Jing, M.; Zhang, L. M.; Sun, M. M.; Wang, Q. Y.; Sun, H. L.; Hou, G. G.; Wang, C. H.; Xin, W. Y. Celastrol inhibits rheumatoid arthritis by inducing autophagy via inhibition of the PI3K/AKT/mTOR signaling pathway. *Int. Immunopharmacol.* **2022**, *112*, 109241.

(9) Liu, M.; Fan, Y. M.; Li, D. Y.; Han, B. H.; Meng, Y. X.; Chen, F.; Liu, T. C.; Song, Z. Y.; Han, Y.; Huang, L. Y.; Chang, Y. Z.; Cao, P. X.; Nakai, A.; Tan, K. Ferroptosis inducer erastin sensitizes NSCLC cells to celastrol through activation of the ROS-mitochondrial fission-mitophagy axis. *Mol. Oncol.* **2021**, *15*, 2084−2105.

(10) Zhao, Z. C.; Wang, Y. M.; Gong, Y. Y.; Wang, X.; Zhang, L. Y.; Zhao, H. Y.; Li, J. F.; Zhu, J. D.; Huang, X. Y.; Zhao, C. G.; Yang, L. H.; Wang, L. X. Celastrol elicits antitumor effects by inhibiting the STAT3 pathway through ROS accumulation in non-small cell lung cancer. *J. Transl. Med.* **2022**, *20*, 16.

(11) Srinivas, U. S.; Tan, B. W. Q.; Vellayappan, B. A.; Jeyasekharan, A. D. ROS and the DNA damage response in cancer. *Redox Biol.* **2019**, *25*, 101084.

(12) Wang, Y. W.; Qi, H.; Liu, Y.; Duan, C.; Liu, X. L.; Xia, T.; Chen, D.; Piao, H. L.; Liu, H. X. The double-edged roles of ROS in cancer prevention and therapy. *Theranostics* **2021**, *11*, 4839−4857.

(13) Cheung, E. C.; Vousden, K. H. The role of ROS in tumour development and progression. *Nat. Rev. Cancer* **2022**, *22*, 280−297.

(14) Perillo, B.; Di Donato, M.; Pezone, A.; Di Zazzo, E.; Giovannelli, P.; Galasso, G.; Castoria, G.; Migliaccio, A. ROS in cancer therapy: the bright side of the moon. *Exp. Mol. Med.* **2020**, *52*, 192−203.

(15) He, F.; Ru, X. L.; Wen, T. NRF2, a Transcription Factor for Stress Response and Beyond. *Int. J. Mol. Sci.* **2020**, *21*, 4777.
(16) He, F.; Antonucci, L.; Karin, M. NRF2 as a regulator of cell metabolism and inflammation in cancer. *Carcinogenesis* **2020**, *41*, 405−416.
(17) Loboda, A.; Damulewicz, M.; Pyza, E.; Jozkowicz, A.; Dulak, J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell. Mol. Life Sci.* **2016**, *73*, 3221−3247.
(18) Liu, J. Z.; Hu, Y. L.; Feng, Y.; Jiang, Y.; Guo, Y. B.; Liu, Y. F.; Chen, X.; Yang, J. L.; Chen, Y. Y.; Mao, Q. S.; Xue, W. J. BDH2 triggers ROS-induced cell death and autophagy by promoting Nrf2 ubiquitination in gastric cancer. *J. Exp. Clin. Cancer Res.* **2020**, *39*, 18.
(19) Chou, T. C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. *Cancer Res.* **2010**, *70*, 440−446.
(20) Luo, P.; Liu, D. D.; Zhang, Q.; Yang, F.; Wong, Y. K.; Xia, F.; Zhang, J. Z.; Chen, J. Y.; Tian, Y.; Yang, C. B.; Dai, L. Y.; Shen, H. M.; Wang, J. G. Celastrol induces ferroptosis in activated HSCs to ameliorate hepatic fibrosis via targeting peroxiredoxins and HO-1. *Acta Pharm. Sin. B* **2022**, *12*, 2300−2314.
(21) Raghu, G.; Berk, M.; Campochiaro, P. A.; Jaeschke, H.; Marenzi, G.; Richeldi, L.; Wen, F. Q.; Nicoletti, F.; Calverley, P. M. A. The Multifaceted Therapeutic Role of N-Acetylcysteine (NAC) in Disor-ders Characterized by Oxidative Stress. *Curr. Neuropharmacol.* **2021**, *19*, 1202−1224.
(22) Kanzaki, H.; Shinohara, F.; Kajiya, M.; Kodama, T. The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling (vol 288, pg 23009−23020, 2013). *J. Biol. Chem.* **2014**, *289*, 5536−5536.
(23) Singh, A.; Venkannagari, S.; Oh, K. H.; Zhang, Y. Q.; Rohde, J. M.; Liu, L.; Nimmagadda, S.; Sudini, K.; Brimacombe, K. R.; Gajghate, S.; Ma, J. F.; Wang, A.; Xu, X.; Shahane, S. A.; Xia, M. G.; Woo, J. Y.; Mensah, G. A.; Wang, Z. B.; Ferrer, M.; Gabrielson, E.; Li, Z. Y.; Rastinejad, F.; Shen, M.; Boxer, M. B.; Biswal, S. Small Molecule Inhibitor of NRF2 Selectively Intervenes Therapeutic Resistance in KEAP1-Deficient NSCLC Tumors. *ACS Chem. Biol.* **2016**, *11*, 3214−3225.
(24) Qi, C.; Sun, S. W.; Xiong, X. Z. From COPD to Lung Cancer: Mechanisms Linking, Diagnosis, Treatment, and Prognosis. *Int. J. Chronic Obstr. Pulm. Dis.* **2022**, *17*, 2603−2621.
(25) Li, Z. X.; Zhang, J. Y.; Duan, X. L.; Zhao, G. A.; Zhang, M. Celastrol: A Promising Agent Fighting against Cardiovascular Diseases. *Antioxidants* **2022**, *11*, 1597.
(26) Xu, H.; Zhao, H. F.; Ding, C. Y.; Jiang, D. F.; Zhao, Z. J.; Li, Y.; Ding, X. Y.; Gao, J.; Zhou, H.; Luo, C.; Chen, G. Q.; Zhang, A.; Xu, Y.; Zhang, H. Celastrol suppresses colorectal cancer via covalent targeting peroxiredoxin 1. *Signal Transduct. Target. Ther.* **2023**, *8*, 16.
(27) Chen, G. E.; Zhu, X. Y.; Li, J. Q.; Zhang, Y.; Wang, X. X.; Zhang, R.; Qin, X. C.; Chen, X.; Wang, J. Y.; Liao, W. L.; Wu, Z. J.; Lu, L.; Wu, W. Y.; Yu, H. J.; Ma, L. J. Celastrol inhibits lung cancer growth by triggering histone acetylation and acting synergically with HDAC inhibitors. *Pharmacol. Res.* **2022**, *185*, 106487.
(28) Liu, X. H.; Zhao, P. Y.; Wang, X. J.; Wang, L.; Zhu, Y. J.; Song, Y. D.; Gao, W. Celastrol mediates autophagy and apoptosis via the ROS/JNK and Akt/mTOR signaling pathways in glioma cells (vol 38, 184, 2019). *J. Exp. Clin. Cancer Res.* **2019**, *38*, 2.
(29) Chen, X.; Zhao, Y.; Luo, W.; Chen, S. A.; Lin, F.; Zhang, X.; Fan, S. J.; Shen, X.; Wang, Y.; Liang, G. Celastrol induces ROS-mediated apoptosis via directly targeting peroxiredoxin-2 in gastric cancer cells. *Theranostics* **2020**, *10*, 10290−10308.
(30) Niu, W. N.; Wang, J. G.; Wang, Q. Y.; Shen, J. J. Celastrol Loaded Nanoparticles With ROS-Response and ROS-Inducer for the Treatment of Ovarian Cancer. *Front. Chem.* **2020**, *8*, 9.
(31) Li, H. Y.; Zhang, J.; Sun, L. L.; Li, B. H.; Gao, H. L.; Xie, T.; Zhang, N.; Ye, Z. M. Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells: an in vitro and in vivo study. *Cell Death Dis.* **2015**, *6*, e1604.
(32) DeBlasi, J. M.; DeNicola, G. M. Dissecting the Crosstalk between NRF2 Signaling and Metabolic Processes in Cancer. *Cancers* **2020**, *12*, 3023.
(33) Chen, X.; Cubillos-Ruiz, J. R. Endoplasmic reticulum stress signals in the tumour and its microenvironment. *Nat. Rev. Cancer* **2021**, *21*, 71−88.
(34) Ajoolabady, A.; Kaplowitz, N.; Lebeaupin, C.; Kroemer, G.; Kaufman, R. J.; Malhi, H.; Ren, J. Endoplasmic reticulum stress in liver diseases. *Hepatology* **2022**, *77*, 619−639.
(35) Bobrovnikova-Marjon, E.; Grigoriadou, C.; Pytel, D.; Zhang, F.; Ye, J.; Koumenis, C.; Cavener, D.; Diehl, J. A. PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. *Oncogene* **2010**, *29*, 3881−3895.

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