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Ferroptosis Inducer RSL3 Inhibits Cell Death by Camptothecin-Induced Apoptosis

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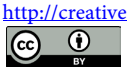

Abstract

Ferroptosis, an iron-dependent type of cell death, is being considered for new clinical treatments of malignant tumors that are difficult to treat with apoptosis inducers. Although several reports have attempted to increase the sensitivity of cells to cell death by combining ferroptosis and apoptosis inducers using a single treatment, detailed elucidation of the respective mechanisms of ferroptosis and apoptosis during cell death remains unclear. Here, we evaluated combined treatment effectiveness using the apoptosis-sensitive rat insulinoma INS-1 cell lines. DNA laddering, an indicator of camptothecin (CPT)-induced apoptosis, was abolished by adding RSL3 and ML-162, but not erastin. We found that when the cells were treated with the apoptosis inducer CPT or the ferroptosis inducer RSL3, respectively, the degree of cytotoxicity observed increased dose-dependently. However, a combined CPT and RSL3 treatment did not show a synergistic decrease in cell viability. Camptothecin did not significantly affect increases in intracellular lipid peroxidation and reactive oxygen species or increases in mitochondrial and cytoplasmic free iron levels that were induced by treatment with RSL3 alone. Moreover, deferoxamine and *α*-tocopherol were found to inhibit RSL3-induced cytotoxicity but did not protect against CPT or CPT and RSL3-induced cytotoxicity. Finally, the exogenous addition of *tert*-butyl hydroperoxide inhibited DNA ladder formation that is induced by CPT, while the addition of hydrogen peroxide or ferrous ammonium sulfate had no effect. Taken together, these results suggest that lipid peroxides generated during ferroptosis may suppress cell death induced by apoptotic mechanisms.

Keywords

Apoptosis, Cell Viability, DNA Laddering, Ferroptosis, Iron, Peroxide

1. Introduction

The proliferation, differentiation, development, and maintenance of living organisms require a variety of growth mechanisms as well as cell death mechanisms. Genetically programed apoptosis is an energy-dependent cell death mechanism that can be induced by various conditions, including anticancer drugs and oxidative stress, and its biochemical and morphological characteristics have been elucidated in various studies [1]-[3]. When apoptosis is over- or under-expressed, proper cell and tissue maintenance is not possible, which can result in impaired biological function. There are various somatic cell death pathways, including apoptosis, necrosis, and ferroptosis, and the appropriate execution of each pathway plays an important role in the fine modulation of biological functions and the maintenance of physiological functions [1]. In recent years, interactions between these pathways have been actively studied [4], but the mechanisms underlying their maintenance in a tightly regulated balance remain poorly understood.

Elucidating the pathology of cancers in which cell number control is uncontrollable and developing treatments for them are urgent issues to be solved, and the drug therapy is intended to fundamentally activate the cell death network and induce apoptosis in cancer cells [5] [6]. However, apoptotic cell death cannot easily be induced in cancer cells with abnormalities in their apoptosis signaling mechanisms; this can lead to tumor maintenance, the acquisition of cell death resistance, and cancer recurrence [7]. In addition to inducing apoptotic death in cancer cells, other efforts have focused on developing drug therapies that complement defective apoptotic pathways [8]-[10]. One uniquely regulated nonapoptotic cell death pathway, ferroptosis, can be expressed to induce cell death in apoptosis-resistant cells to facilitate effective cancer cell therapies [11]. Ferroptosis is experimentally induced by the inhibition of glutamate-cystine antiporter system xc (xCT) to reduce intracellular GSH and by the accumulation of lipid peroxides by the inhibition of the selenoprotein glutathione peroxidase 4 (GPX4) and is accompanied by increased intracellular free ferrous iron levels and generation of cell membrane phospholipid hydroperoxides (LOOH) [12]-[17]. However, it has been reported that the expression is affected by various factors such as the type and localization of lipids that undergo peroxidation and epigenetic modifications.

In one recent study, Vinik *et al.*, showed that two-drug combinations that simultaneously targeted bromodomain-containing protein 4 and the proteasome induced a continuous phenotypic gradient between ferroptosis and apoptosis in several breast cancer subtypes, thereby connecting these two cell death processes [16]. Overall, these results confirm that crosstalk between apoptosis and ferroptosis exists in pathways triggering cell death. In addition, Dixon *et al.*, and Bersuker *et al.*, both found that the proapoptotic protein ferroptosis suppressor protein 1 (FSP1) is an *in vivo* resistance factor capable of inhibiting ferroptosis by suppressing lipid peroxidation [13] [18]. The expression of FSP1 was positively correlated with ferroptosis resistance in various cancer cell lines, suggesting that inhibition of the ferroptosis pathway is essential for successful apoptosis. Taken together, these findings strongly

suggest that apoptosis and ferroptosis may interact to coordinately control the extent of cell death in biological systems, and there may be a stage at which the progression of ferroptosis impedes the progression of apoptosis. In this study, we report an investigation of the effect of the ferroptosis process on apoptotic cell death and demonstrate the inhibitory effects of ferroptosis inducers in the apoptosis-sensitive pancreatic β-cell line INS-1. We believe that the results reported here will contribute to the elucidation of multiple networks involved in the induction of cell death.

2. Material and Methods

Materials: We used the following chemicals: camptothecin (CPT), ferrous ammonium sulfate, α-Tocopherol and 70% *tert*-butyl hydroperoxide solution (*t*-BHP), all of which were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Liperfluo, Lipid Peroxidation Probe-BDP 581/591 C11 and Mito-Ferro Green were obtained from Dojindo (Kumamoto, Japan). DNA marker 1, XL-DNA Ladder 100, was obtained from Pharma Foods International Co., Ltd (Tokushima, Japan) and DNA marker 2, Gene Ladder Fast 1, were obtained from Nippon GENE (Tokyo, Japan). Finally, deferoxamine mesylate (DFO) was sourced from Novartis Pharma (Switzerland), Ferro Far Red was purchased from Goryo chemical (Hokkaido, Japan), (1S, 3R)-RSL3 and ML-162 were obtained from Funakoshi (Tokyo, Japan), erastin was obtained from MedChemExpress (Monmouth Junction NJ, USA) and a Cell Titer 96 Non-Radioactive Cell Proliferation Assay (MTT assay kit) was purchased from Promega Corporation (Madison WI, USA). All other chemicals were of analytical grade and were purchased from commercial suppliers.

Cell culture and treatment: The rat insulinoma INS-1 cell line was kindly gifted by Dr. Wollheim (University of Geneva, Switzerland). Cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) containing heat-inactivated fetal bovine serum, L-glutamine, sodium pyruvate, and 2-mercaptoethanol (2ME); this medium was produced as previously described [19]. To eliminate the influence of 2ME, a reducing substance required for the synthesis and secretion of insulin, cells were washed twice with culture medium without 2ME via centrifugation before being used in further experiments. Next, to prepare MTT assay and flow cytometer samples, cells were seeded in 96-wells plate at a density of 1×10^5 cells/100 μL. For DNA ladder observation, cells were seeded at a density of 3×10^6 cells/3 mL. After overnight preincubation at 37˚C in an atmosphere of 5% CO_2/95% air, cells were then added to 10 μM CPT and/or 10 μM RSL3 to reach final concentrations and then incubated for 24 h in a CO_2 incubator under sterile conditions.

Cytotoxicity assays: Next, we determined cell viability as the indexed capability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (*i.e.*, an MTT assay). All procedures were performed using a previously published protocol with minor modification [20]. Briefly, after rinsing the cells with PBS twice, they were incubated in fresh culture medium without 2ME (50 μL) in the presence of an MTT dye solution (7.5 μL) for 2 h. After this time had elapsed, cells were

then immersed in a solubilization/stop solution (50 μL) for an additional 2 h. Finally, we recorded solution absorbance at 570 nm (*i.e.*, the wavelength indicating the formation of formazan) using a microplate reader (Bio-Rad, Tokyo, Japan).

To determine the degree of apoptotic cell death, cells were seeded in 6-well plates at a density of 3×10^6 cells in 3 ml of culture medium without 2ME. The formation of the DNA ladder (*i.e.*, the DNA fragmentation pattern) in INS-1 cells was assessed via 1.5% agarose gel electrophoresis using a previously described protocol with minor modification [2]. Finally, electrophoresis patterns were visualized by 0.5 μg/ml ethidium bromide staining for 15 min and gels were then photographed using an epi-fluorescence detector (Ez-capture MG, ATTO CORPORATION, Tokyo, Japan).

Flow cytometry: Detection of intracellular lipid peroxidation, the presence of highly reactive oxygen species (hROS), and the amount of free ferrous iron in the mitochondria or cytoplasm were performed using Liperfluo [21], a lipid peroxidation probe [22], and Mito-Ferro Green [23] or Ferro Far Red [24], respectively. All procedures were performed according to the manufacturer's recommendations with minor modifications. The fluorescent intensity of the products was then observed using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA). Briefly, medium containing cells (1×10^5/well) treated using the above conditions was aspirated and washed twice with 200 μL of HBSS. Next, a Liperfluo probe (1 μM), a lipid peroxidation probe (stock solution × 1000), and either Mito-Ferro Green (5 μM) or Ferro Far Red (5 μM) were added to a 96-wells plate containing cells. The Plates were then incubated for 0.5 h in the dark. Cells were washed twice with PBS to remove excess probe. To suspend, cells were incubated with trypsin-EDTA (0.25%) for 5 min at room temperature in the dark, after which cells from two technical replicate wells (*i.e.*, those experiencing the same conditions) were placed in one tube (1.5 mL). Cells were then washed twice with 1 mL HBSS by centrifugation at 500 × g for 5 min, and the resulting cell pellet was resuspended in 500 μL PBS before being analyzed by flow cytometry. Finally, the fluorescent wavelengths of Liperfluo, the lipid peroxidation probe BDP 581/591 C11, and Mito-Ferro Green were all 525 nm when excited with the blue laser (488 nm) using channel FL-1, in addition, the fluorescence wavelength of Ferro Far Red was 660 nm when excited with a red laser (638 nm) using channel FL-6.

Statistical analysis: All data are represented as mean ± S.D. from at least three independent experiments. Students' t-tests were used to evaluate the statistical significance of differences among unpaired comparisons. Finally, an ANOVA followed by a Dunnett's test was used to determine pairwise differences among more than two. In all cases, a p value of <0.05 was considered to indicate a statistically significant difference among group means.

3. Results

First, we examined the effects of ferroptosis inducers on a representative apoptosis inducer, CPT, to examine the interplay between apoptosis and ferroptosis during INS-1 cell death. As shown in **Figure 1(A)**, treatment of cultured cells with CPT

resulted in the formation of DNA ladders, a typical indicator of apoptotic cell death. The ferroptosis inducer erastin, which inhibits the cystine transporter (xCT), did not affect this DNA ladder formation, whereas the GPx4 inhibitors RSL3 and ML-162 inhibited DNA ladder formation. No DNA ladders were observed with either erastin, RSL3, or ML-162 treatment alone. As shown in Figure 1(B), we found that no ladder formation was observed in response to CPT treatment, regardless of whether RSL3 was added 1 h before, simultaneously with, or 3 h after the addition of CPT. Figure 1(C) shows the cytotoxic effects of various concentrations of CPT in the presence or absence of RSL3. In general, CPT reduced cell viability in a concentration-dependent manner in the absence of RSL3, whereas RSL3 (10 mM) alone reduced cell viability by approximately 30%. In contrast, the addition of RSL3 to any concentration of CPT did not enhance the

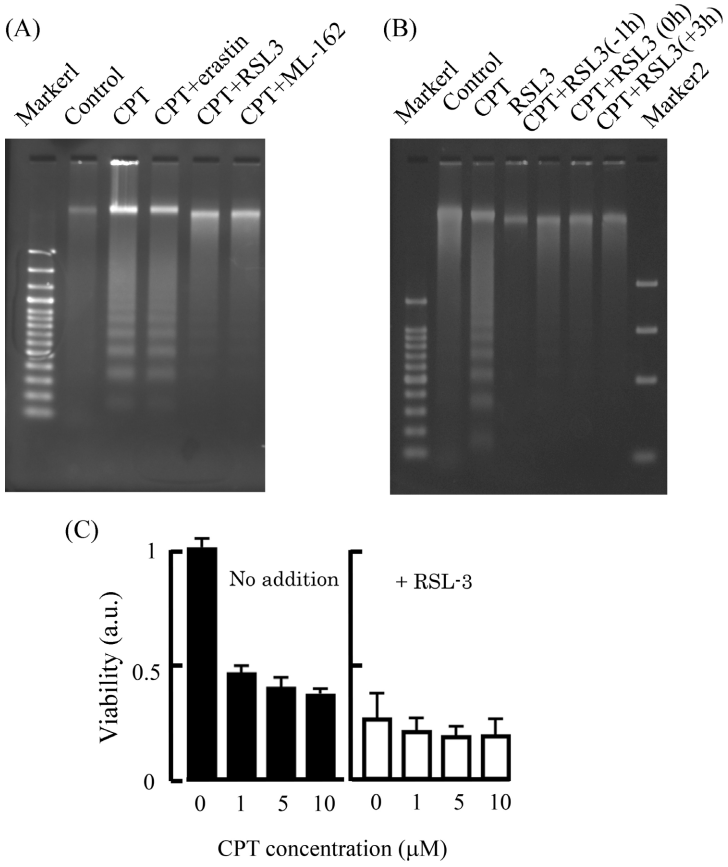

Figure 1. The effect of ferroptosis-inducer on CPT-induced cytotoxicity (A) INS-1 cells were incubated for 24 h with the apoptosis inducer 10 mM CPT and/or the ferroptosis inducers 10 mM erastin, 10 mM RSL3 or 10 mM ML-162. (B) RSL3 was added to cells 1 hour before, simultaneously with, and 3 hours after the addition of CPT, followed by incubation in the presence of CPT for 24 hours. Representative results are shown. At least three experiments were performed for confirmation. (C) Cell viability measured by MTT assay. Results show the mean ± S.D. *$p < 0.05$ compared to cells without CPT treatment. All experiments were performed with at least three technical replicates. All conditions are described in Materials and Methods.

cytotoxic effect. Furthermore, we observed that sublethal doses of CPT and RSL3 combined did not reduce cell viability (data not shown). Taken together, these results indicate that apoptosis induction by CPT treatment was inhibited by the presence of ferroptosis inducers and that combined treatment of CPT and RSL3 did not induce additive or synergistic toxic effects.

Ferroptosis is a type of cell death in which increased free iron, suppression of antioxidant function, and increased lipid peroxidation coexist. As shown in **Figure 2(A)**, a significant increase in mitochondrial free iron was observed in cells incubated with RSL3 in the presence or absence of CPT for 1 - 3 h, but not in the presence of CPT alone. We also observed a similar increase in cytoplasmic free iron by treatment with RSL3 alone (**Figure 2(B)**), whereas the presence of CPT had no effect on iron release. Taken together, these results suggest that ferroptosis inducers increase intracellular free iron without being affected by the effects of apoptosis inducers. We also found that RSL3 treatment significantly increased hROS levels within 3 - 6 h of incubation, including the levels of intracellular hydroxyl radicals, lipid radicals, and lipid peroxyl radicals (**Figure 2(C)**). Moreover, intracellular lipid hydroperoxide levels were significantly increased after 3 h of incubation and increased up to 6 h thereafter (**Figure 2(D)**). Cells treated with RSL3 alone or with camptothecin and RSL3 showed a significant and correlated increase in hROS and lipid hydroperoxide levels. However, we also found that CPT had no effect on RSL3-induced increases in free iron, lipid peroxide levels, and/or hROS. These results therefore suggest that CPT does not affect RSL3-induced ferroptosis.

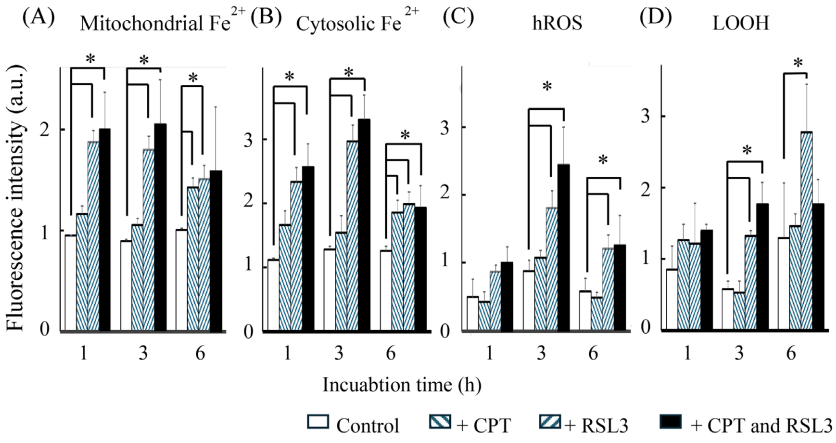

Figure 2. Time course of markers associated with ferroptosis in cells treated with CPT and/or RSL3 Here, INS-1 cells were incubated with 10 μM CPT and/or 10 μM RSL3 for 1, 3, or 6 h. The indicators of ferroptosis included free ferrous iron in the mitochondria and cytoplasm, as well as intracellular ROS and lipid hydroperoxides. All results are shown as mean ± S.D. of 3 - 9 technical replicates. *$p < 0.05$ compared to control cells. All conditions are described in the Materials and Methods.

Next, we investigated the effects of ferroptosis inhibitors, iron chelator and antioxidant, on the cytotoxicity of CPT and/or RSL3. We found that the addition of

the iron chelators DFO (Figure 3(A)) and *α*-tocopherol (Figure 3(B)) to RSL3-treated cells significantly prevented the loss of cell viability but had no effect on the cytotoxicity of cells treated with CPT alone. These results suggest that free iron and peroxides are both involved in RSL3-induced ferroptotic cell death but not in CPT-induced apoptotic cell death. In addition, neither DFO nor *α*-tocopherol showed a significant protective effect against the loss of viability observed in cells cotreated with CPT and RSL3. Thus, these results indicate that cell death was induced even when ferroptosis was inhibited in co-treated cells.

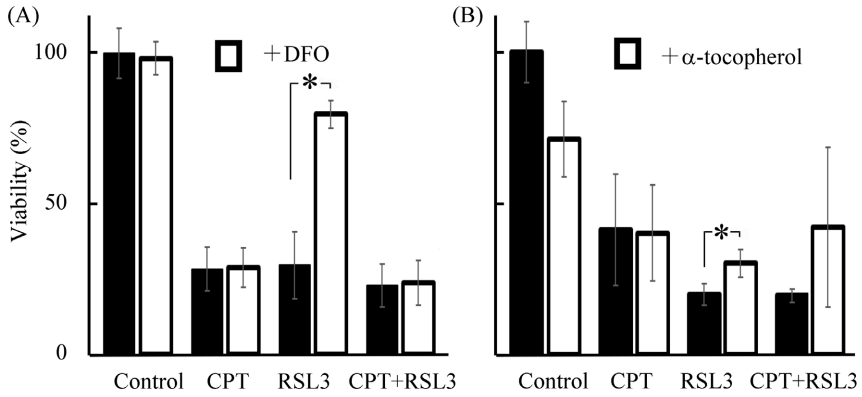

Figure 3. The effects of DFO and a-tocopherol on CPT- and/or RSL3-induced cytotoxicity INS-1 cells were incubated with 10 μM CPT and/or 10 μM RSL3 for 24 h in the presence of 0.1 mM deferoxamine (A) or 0.1 mM *α*-tocopherol (B). Cell viability was then determined by MTT assay. Results show mean ± S.D. All results indicate 3 - 6 technical replicates. All conditions are described in the Materials and Methods. * $p < 0.05$.

Since the induction of apoptosis was inhibited by ferroptosis activation, we investigated the effects of free iron and peroxides, *t*-BHP and H_2O_2, on DNA ladder formation. We found that the addition of 100 μM ferrous ammonium sulfate, which induced a fluorescent intensity equivalent to that observed in samples in which mitochondrial and cytoplasmic free iron increased following RSL3 treatment, did not significantly affect the DNA ladder formation that is induced by CPT (Figure 4(A)). Moreover, although the fluorescent intensity of the original DNA band decreased in a dose-dependent manner with the concentration of ferrous ammonium sulfate added at low concentrations, DNA ladder formation induced by CPT was still clearly observed. However, DNA strand breaks were not observed when 500 μM ferrous ammonium sulfate was added alone. Interestingly, *t*-BHP had no effect on CPT-induced DNA ladder formation at concentrations below 10 μM, but clearly inhibited ladder formation at a concentration of 50 μM. No DNA ladder formation was observed in cells treated with 50 μM *t*-BHP alone (Figure 4(B)). Moreover, the addition of various concentrations of H_2O_2 to CPT-treated cells did not inhibit DNA ladder formation, and the fluorescent intensity of the original DNA strand band decreased with increasing H_2O_2 concentration. In addition, the treatment with 50 μM H_2O_2 alone induced DNA ladder formation, unlike the pattern observed for *t*-BHP (Figure 4(C)). Taken together, these results

revealed that *t*-BHP inhibited CPT-induced DNA ladder formation, whereas free iron and H_2O_2 had no effect.

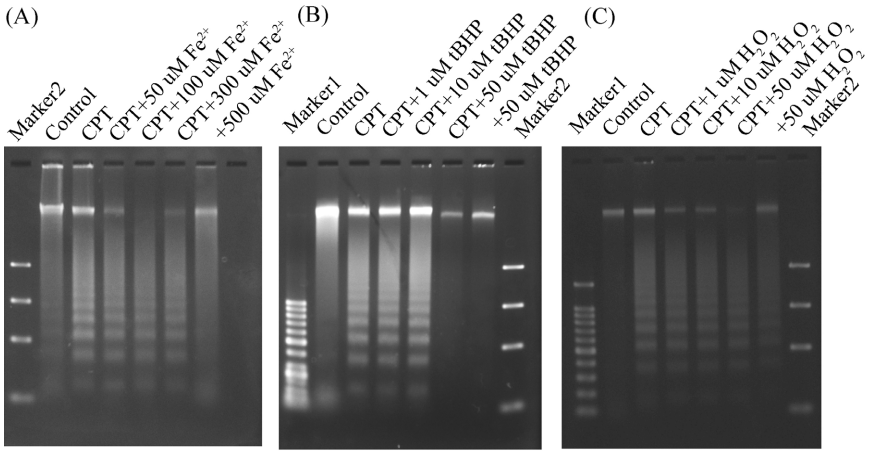

Figure 4. The effects of free ferrous iron, *t*-BHP and H_2O_2 on CPT-induced DNA ladder formation INS-1 cells were incubated with 10 μM CPT and various concentrations of ferrous ammonium sulfate (A), *t*-BHP (B) or H_2O_2 (C) for 24 h. Experiments were performed at least three times and representative results were shown. All conditions are described in the Materials and Methods.

4. Discussion

With respect to the relationship between ferroptosis and apoptosis, many studies have been conducted to examine how drugs can complement ferroptosis-inducing activity in apoptosis-resistant cells to further induce cell death [2] [25]. However, to date, the mechanistic details regarding how ferroptosis reagents affect apoptosis-sensitive cells remain poorly understood. In this study, we showed that treatment of the pancreatic β cell line INS-1 with CPT induces DNA laddering, a characteristic of apoptosis, but this laddering is not detected when treated with the ferroptosis inducer RSL3, regardless of the presence or absence of CPT. In the apoptosis mechanism, in which various factors are activated hierarchically, DNA laddering is a downstream phenomenon that directly leads to cell death [26].

Ferroptosis inhibitor protein 1 is an enzyme that reduces extramitochondrial CoQ10 in cells and is known to inhibit ferroptosis [13]. This enzyme has previously been reported as mitochondrial apoptosis-inducing factor 2 and was previously thought to be involved in the induction of apoptosis [18] [27]. Overall, these findings imply that the inhibition of ferroptosis is necessary for apoptosis induction. Moreover, in this study we specifically demonstrate that the expression of machinery linked to ferroptosis induction inhibits the completion of the apoptosis machinery in apoptosis-sensitive cells.

Next, we asked whether synergistic effects were apparent. Lee et al. demonstrated that combined treatment with the ferroptosis factor erastin and the apoptosis factor TRAIL promoted the collapse of mitochondrial membrane potential and activated the caspase system [28]. We found that although RSL3 treatment

induced a significant cytotoxic effect on INS-1 cells, the cytotoxic effect was not significantly enhanced by combining the RSL3 treatment with CPT, thereby indicating that CPT and RSL3 did not have additive or synergistic cytotoxic effects on this cell line. Moreover, although there are no absolute indicators of ferroptosis, we note that RLS3 induced an increase in cytoplasmic and mitochondrial free iron concentrations as well as in the generation of intracellular reactive oxygen species (ROS) and lipid peroxides. Under conditions of cotreatment in which indicators of apoptosis disappeared, we observed clear indicators of ferroptosis, with no attenuation. Thus, these results suggest that ferroptosis, rather than apoptosis, was carried out in cotreated cells.

Next, the iron chelator DFO and the antioxidant *α*-tocopherol both inhibited ferroptosis and significantly prevented cytotoxicity caused by RLS3 treatment alone, thereby indicating that RSL3-induced cell death was mediated by ferroptosis. However, we also observed obvious cytotoxicity in cells cotreated with RSL3 and CPT in the presence of *α*-tocopherol or DFO, which suggests that mechanisms other than ferroptosis are involved in cell death. Moreover, although not shown in the data, DNA ladder formation, which was not observed in cotreated cells, was found to be restored following the addition of DFO. In addition, since it is known that FSP1 is involved in the induction of apoptosis by inhibiting ferroptosis [13] [18] [21] [27], it may be that ferroptosis proceeds preferentially under conditions where both apoptosis and ferroptosis are induced, but also that the cellular machinery required for apoptosis can be restored by inhibiting ferroptosis under the same conditions.

Previous research has shown that ROS, free iron and lipid peroxides generated by chain reaction are all involved in the occurrence of ferroptosis [29]. In this study, we showed that apoptosis induced by CPT was inhibited by the exogenous addition of the organic peroxide *t*-BHP. However, we also found that H_2O_2 had no inhibitory effect on apoptosis by CPT and induced DNA ladder formation when added alone. Furthermore, exogenously added free iron did not affect DNA ladder formation. Overall, these results suggest that organic peroxides generated by chain lipid peroxidation, rather than free iron or H_2O_2, are involved in the inhibition of DNA ladder formation.

The GPX4 inhibitors RSL3 and ML-162 inhibited DNA ladder formation, whereas the xCT inhibitor erastin did not. Although the detailed mechanism responsible for this effect is unclear, since changes in intracellular GSH content and peroxide generation have been shown to be involved in gene expression [30] [31], it is possible that certain peroxides capable of inducing or completing ferroptosis expression may also control the expression of genes involved in apoptosis. It will be important to examine the effects of other ferroptosis inducers, such as FIN56 or glutamate, to determine whether the observed inhibition of apoptosis is a general phenomenon resulting from the activation of ferroptosis function. During the process of ferroptosis, a myriad of lipid peroxides is generated in various cellular locations, including the plasma membrane, mitochondria, and endoplasmic reticulum

[13] [29]. Further investigations are required to identify the type and site of lipid peroxidation that induces ferroptotic cell death and inhibits the apoptotic machinery. Furthermore, we note that there are multiple activation steps involved in triggering cell death via apoptosis. Camptothecin causes inhibition of topoisomerase I activity, leading to apoptotic death via cell cycle arrest, loss of mitochondrial membrane potential, release of cytochrome c, sequential activation of caspases, and activation of caspase-dependent DNase [32] [33]. Further experiments should focus on clarifying the mechanism by which ferroptosis factors inhibit apoptosis.

In this study, we showed that ferroptosis impedes the execution of apoptosis. Moreover, chemical-mediated crosstalk on the genetic level may regulate the optimization and operational coordination of apoptosis and ferroptosis and is therefore likely essential for maintaining critical life functions. We believe that this work contributes to an improved elucidation of how cell death mechanisms are regulated, including how pathogenesis can result in cancer and how such cancers may be effectively treated.

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The manuscript has been carefully reviewed by an experienced editor Publication Support Services whose first language is English and who specializes in editing papers written by scientists whose native language is not English. After using this service, the author reviewed and edited the content.

Conflicts of Interest

The authors declare no conflicts of interest regarding the contents of this article.

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Abbreviations

CPT, camptothecin; DFO, deferoxamine; FSP1, ferroptosis suppressor protein 1; GPX4, glutathione peroxidase 4; hROS, high reactive oxygen species; H2O2, hydrogen peroxide; LOOH, lipid hydroperoxides; 2ME, 2-mercaptoethanol; ROS, reactive oxygen species; t-BHP, t-butyl hydroperoxide; xCT, glutamate-cystine antiporter system xc.