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RESEARCH ARTICLE

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Piperlongumine increases the sensitivity of bladder cancer to cisplatin by mitochondrial ROS

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Abstract

Background: The development of cisplatin resistance often results in cisplatin inefficacy in advanced or recurrent bladder cancer. However, effective treatment strategies for cisplatin resistance have not been well established.

Methods: Gene expression was measured by qRT-PCR and Western blotting. CCK-8 assay was performed to detect cell survival. The number of apoptotic cells was determined using the Annexin V-PI double-staining assay. The level of reactive oxygen species (ROS) was measured using 2',7'-dichlorodihydrofluorescein diacetate fluorescent dye, and the ATP level was detected using an ATP measurement kit.

Results: The expression of receptor-interacting protein kinase 1 (RIPK1), a key regulator of necroptosis, gradually decreased during cisplatin resistance. We first used piperlongumine (PL) in combination with cisplatin to act on cisplatin-resistant BC cells and found that PL-induced activation of RIPK1 increased the sensitivity of T24 resistant cells to cisplatin treatment. Furthermore, we revealed that PL killed T24 cisplatin-resistant cells by triggering necroptosis, because cell death could be rescued by the mixed lineage kinase domain-like (MLKL) protein inhibitor necrotic sulfonamide or MLKL siRNA, but could not be suppressed by the apoptosis inhibitor z-VAD. We further explored the specific mechanism and found that PL activated RIPK1 to induce necroptosis in cisplatin-resistant cells by stimulating mitochondrial fission to produce excessive ROS.

Conclusions: Our results demonstrated the role of RIPK1 in cisplatin-resistant cells and the sensitization effect of the natural drug PL on bladder cancer. These may provide a new treatment strategy for overcoming cisplatin resistance in bladder cancer.

KEYWORDS
cisplatin resistance, piperlongumine, reactive oxygen species, receptor-interacting protein kinase 1

1 | INTRODUCTION

Bladder cancer is the most common malignancy of the urinary tract. In recent years, the incidence of bladder cancer has shown a gradual upward trend in China.[1] Transurethral resection is the main treatment for early bladder cancer; however, this standard approach is limited to advanced invasive urothelial carcinoma. Currently, cisplatin is the main chemotherapy drug for advanced bladder cancer as a single

agent or key component in the treatment of metastatic bladder cancer, and it can also be used in neoadjuvant therapy combined with radical cystectomy.[2,3] Unfortunately, many patients in practice are considered to be "cisplatin-ineligible." These patients initially benefit from cisplatin treatment but ultimately develop resistance in the final stage, leading to progressive disease and therapy failure.[4] Therefore, it is necessary and urgent to explore additional approaches to effectively inhibit the progression of advanced bladder cancer.

Necroptosis is a form of programmed cell death that is strictly regulated by the activation of receptor-interacting protein kinases (RIPKs).[5] RIPK1 belongs to the RIPK family, including the seven members RIPK1–RIPK7, and controls necroptosis through its kinase function.[6,7] Activated RIPK1 combines with and phosphorylates RIPK3 to form a complex called the necrosome, which then transmits the phosphorylation signal to the downstream mixed lineage kinase domain-like protein (MLKL) to localize to the cell membrane, causing cell membrane rupture and finally leading to cell death.[8,9] Recently, necroptosis has been considered to play a key role in the regulation of cancer biology, including oncogenesis, metastasis, immunity, and cancer subtypes.[10,11] Therefore, pivotal regulators of the necroptotic signaling pathway, such as RIPK1, are considered promising therapeutic targets.

Piperlongumine (PL) is an alkaloid natural product derived from pepper plants, and it has a well-characterized structure ($C_{17}H_{19}NO_5$).[12] PL has traditionally been used to treat gastrointestinal and respiratory diseases.[13] In recent years, a growing number of studies have reported that PL has antitumor activity against several types of tumors, such as hepatocellular carcinoma,[14] breast cancer,[15] gastric cancer,[16] and bladder cancer.[17] Although Liu et al. have reported that PL can suppress bladder cancer invasion by inhibiting epithelial–mesenchymal transition and F-actin reorganization, the role of PL in "cisplatin-ineligible" bladder cancer remains obscure.

In our study, we first explored the important role of RIPK1 in cisplatin resistance in bladder cancer and discovered that PL could increase the sensitivity of bladder cancer to cisplatin by mitochondrial reactive oxygen species (ROS)-induced necroptosis. These results may provide a new treatment strategy for overcoming cisplatin resistance in bladder cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture and drug treatment

All bladder cancer cell lines, including J82, T24, RT4, and TCCSUP, were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. These cells were maintained at 37°C and 5% CO_2 in a humid environment and cultured in a suitable medium containing 10% fetal bovine serum. Cells were collected in mid-log phase for related experiments. As previously described [4], a progressively increasing concentration gradient was used to induce cisplatin resistance in bladder cancer cell lines. Then, 10 μM piperlongumine was used to treat the cisplatin-resistant cells.

2.2 | Tissue samples

Human bladder cancer tissues were obtained from 27 patients who underwent cisplatin-based chemotherapy at The Affiliated People's Hospital of Ningbo University. All patients provided written informed consent prior to treatment and surgery. The patients were divided into two groups based on their therapy outcomes: 14 in the sensitive group and 13 in the resistant group, of which 12 cases of normal adjacent tissues were taken from the two groups. Patients who relapsed after cisplatin treatment were considered cisplatin-resistant. The study protocol was approved by the Ethics Committee of The Affiliated People's Hospital of Ningbo University.

2.3 | Quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). Reverse transcription was performed using a PrimeScript RT Master Mix kit (Takara). The cDNAs were amplified by qRT-PCR using SYBR Green PCR Master Mix (Roche,) on a LightCycler480 system, and relative abundance was normalized to the expression of the endogenous control, GAPDH. The PCR primers used are listed in Table 1.

2.4 | Western blot analysis

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to molecular weight, transferred to a polyvinylidene fluoride membrane (Bio-Rad), and blocked with TBS-T containing 5% non-fat dry milk. Anti-RIPK1 (1:1000, Cell Signaling Technology,), anti-p-RIPK1 (1:1000 CST), anti-RIPK3 (1:1000; Abcam,), anti-p-RIPK3 (1:1000; Abcam), anti-MLKL (1:1000; Abcam), anti-p-MLKL (1:1000; Abcam), anti-p-DRP1(1:1000; Abcam), and anti-GAPDH (1:1000; Abcam) antibodies were diluted with TBS-T containing 3% non-fat dry milk and incubated overnight at 4°C. After washing in TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam,) for 1 h. It was washed again in TBS-T and developed using ECL-Plus (GE Healthcare, Life Sciences,).

2.5 | Cell survival assay

The cells were seeded in a 96-well plate at 1×10^5 cells/well and cultured for 24 h. Cisplatin was then added at different concentrations to continue the culture for 24 h. A total of 10 μl of CCK-8 (Dojindo, Japan) solution was added to each well and incubated at 37°C for 3 h. An iMark™ microplate reader (Bio-Rad,) was used to measure the absorbance at 450 nm.

2.6 | ATP measurement

ATP levels were measured using an ATP measurement kit (Beyotime Biotech,), according to the manufacturer's instructions. Briefly,

TABLE 1 Oligonucleotide sequence of primer set used to amplify in each cDNA

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
RIPK13	GACACGGAGACTAGGTGGCA	CTCTGTCTAGCGGAGGAAGC
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
mtDNA	CCTATCACCC TTGCCATCAT	GAGGCTGTTGCTTGTGTGAC
Nuclear DNA	ATGGAAAGCCTGCCATCATG	TCCTTGTTGTTCAGCATCAC

approximately 1×10^6 cells per well were seeded into a 6-well plate. After treatment with or without targeted drugs for a specified period of time, the corresponding cells were collected and washed. The harvested cells were dissolved in ATP extract and then centrifuged at 12,000 × *g* at 4°C for 5 min. The supernatant was used as the sample to be detected and kept in an ice bath for later use. Samples were mixed with ATP reaction buffer, and a photometer (SpectraMax ID3, Molecular Devices) was used to assay the RLU values. The values of the different groups were standardized according to their own protein content.

2.7 | Apoptosis assays

2.7.1 | Annexin V-PI double-staining assay

The integrity of the cell membrane was evaluated using an annexin V-fluorescein (FITC)/propidium iodide (PI) double-staining assay kit (China Unitech Biotechnology Co., Ltd.). Briefly, the cells were collected and resuspended in 500 μl of 1× binding buffer. After adding 10 μl of PI and 5 μl of Annexin V-FITC to the cell suspension, the samples were incubated in the dark at 25°C for 15 min. The cells were then detected on a FACScan flow cytometer (Becton Dickinson,), and the results were analyzed using FlowJo software (BD, USA).

2.7.2 | Caspase-3/7 activity assays

According to the manufacturer's manual (G8090, Promega), the caspase-3/7 activity was analyzed using the Caspase-Glo 3/7 assay system.

2.8 | Plasmid and siRNA transfection

The T24 cisplatin-resistant cell line was selected for the knockdown of MLKL mRNA. Specific and non-specific scrambled siRNAs were purchased from GenePharma. The T24 resistant cell line was also chosen to overexpress RIPK1. The pGV657-3Flag-RIPK1 plasmid and control plasmids were purchased from GeneChem. Cell transfection was performed using Lipofectamine™ 3000 reagent (Invitrogen, Thermo Fisher Scientific,), following the manufacturer's instructions.

2.9 | Measuring ROS

The cells were seeded in a 6-well plate at 3×10^5 cells/well and cultured overnight. The cells were then treated with or without 10 μM N-acetylcysteine (NAC) for 6 h. Then, the cells were incubated with 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 30 min to assess the ROS-mediated oxidation of DCFH-DA to the fluorescent compound DCF. Images of green fluorescence of DCF in the cells were acquired using a Nikon Ti-U fluorescence microscope. Next, the stained cells were harvested and resuspended in 1 ml PBS. Fluorescence intensity was analyzed using a FACScan flow cytometer (Becton-Dickinson,) at an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

2.10 | Mitochondrial DNA quantitation

The Kaneka Easy DNA Extraction Kit (KN-T110005, Kaneka) was used to extract total DNA from the cells following the manufacturer's instructions. The specific primers used to quantify mitochondrial DNA and nuclear DNA are listed in Table 1. The comparative change-in-cycle method (ΔΔCT) was used to quantify the relative change in transcript copy number.

2.11 | Statistical analysis

An unpaired t test was used to analyze the differences between the two groups. All experiments were performed at least three times and expressed as mean ±standard error (SEM). Statistical significance was set at $p < 0.05$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3 | RESULTS

3.1 | RIPK1 is an important tumor suppressor and related to the cisplatin resistance in bladder cancer tissues

To detect the level of RIPK1 in bladder cancer, we first analyzed the TCGA dataset and found that RIPK1 was downregulated in bladder cancer samples compared with that in normal tissues (Figure 1A). Furthermore, we found that RIPK1 expression was associated with tumor metastasis (Figure 1B). We collected our own clinical samples, including 12 normal tissues, 14 cancer tissues from patients who were sensitive to cisplatin, and 13 cancer tissues from patients who were resistant to cisplatin, and used qRT-PCR to detect the expression of RIPK1 in these samples. The results showed that RIPK1

had higher levels in normal tissues than in cancer tissues, which was similar to the TCGA dataset analysis (Figure 1C). Interestingly, we found that the RIPK1 expression in resistant patients was significantly lower than that in sensitive patients. In addition, we standardized RIPK1 activity with its total protein expression level and found that the relative activity of RIPK1 in patients with cisplatin resistance was also obviously decreased (Figure 1D). These findings indicate that RIPK1 may play an important role in tumor progression and that its expression may be closely related to cisplatin resistance in bladder cancer.

3.2 | RIPK1 expression was downregulated with the development of cisplatin resistance in bladder cancer cells

To further explore the relationship between RIPK1 expression and cisplatin resistance, we detected the expression of RIPK1 in different bladder cancer cell lines by Western blotting and tested their sensitivity to cisplatin using the CCK-8 assay. The results showed that the cell lines with high RIPK1 expression were significantly more sensitive to cisplatin than the cell lines with low RIPK1 expression (Figure 2A,B). Next, we constructed two bladder cancer cell lines resistant to cisplatin therapy. Compared with their parental cells, J82 and T24 resistant cells were obviously insensitive to cisplatin treatment (Figure 2C,D). The IC50 values of cisplatin in J82 and T24 resistant cells were also significantly higher than those in parental cells (Figure 2C,D). We further detected the expression of RIPK1 in J82 and T24 parental and resistant cells and found that the level of RIPK1 was conspicuously reduced compared with that in the parental cells (Figure 2E). Altogether, our data indicate that RIPK1 expression was downregulated with the development of cisplatin resistance in vitro.

3.3 | Apoptosis was inhibited in cisplatin-resistant bladder cancer cells

We used flow cytometry to test the apoptosis levels of J82 and T24 parental and resistant cells, as apoptosis is an important mode of cisplatin-induced cell death. The data showed that cisplatin treatment barely induced apoptosis in the J82 and T24 resistant cells (Figure 3A,B). Consistently, caspase-3/7 activation was also inhibited in resistant cells (Figure 3C). Therefore, our results suggest that apoptosis is inhibited in cisplatin-resistant bladder cancer cells.

3.4 | PL increased the sensitivity of resistant cells to cisplatin by activating RIPK1-induced non-apoptotic cell death

As PL is an effective anti-cancer agent that selectively kills cancer cells [12], we further studied whether PL could improve the sensitivity of cisplatin-resistant cells. We discovered that PL treatment of T24 resistant cells significantly increased the expression of RIPK1 and activated RIPK1 phosphorylation (Figure 4A), suggesting that PL is an activator of RIPK1. In contrast to cisplatin treatment alone, PL activation of RIPK1 alone triggered increased cell death in T24 resistant cells (Figure 4B). Furthermore, PL in combination with cisplatin caused a higher percentage of cell death in T24 resistant cells (Figure 4B), indicating that RIPK1 activation by PL could amplify the killing effect of cisplatin. Unexpectedly, PL alone or in combination with cisplatin did not significantly enhance apoptosis in T24 resistant cells (Figure 4C). However, PL treatment caused a decrease in ATP level in T24 resistant cells (Figure 4D), suggesting that PL induced another way of cell death rather than apoptosis. To further explore the effect of RIPK1, we overexpressed RIPK1 by transfecting it with the plasmid and found that RIPK1

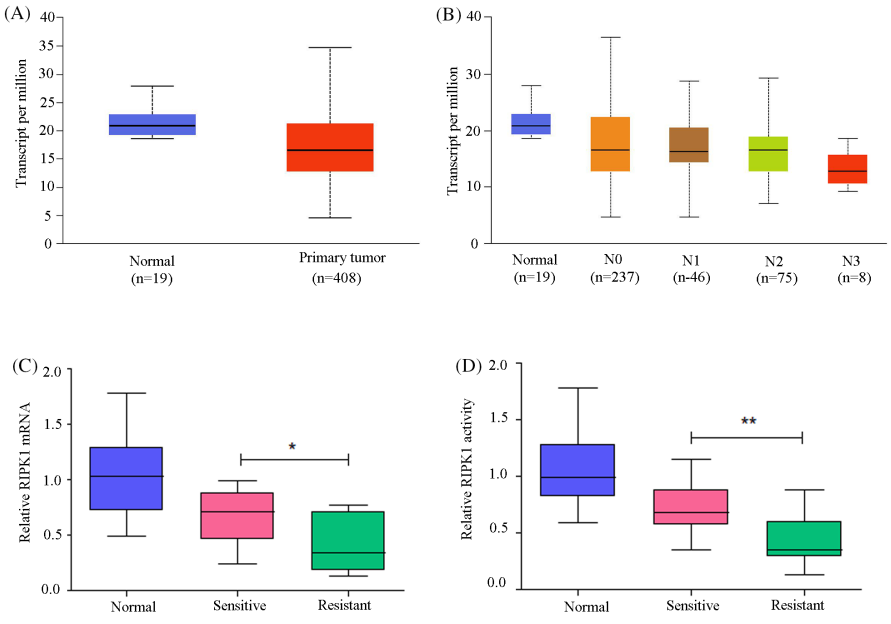
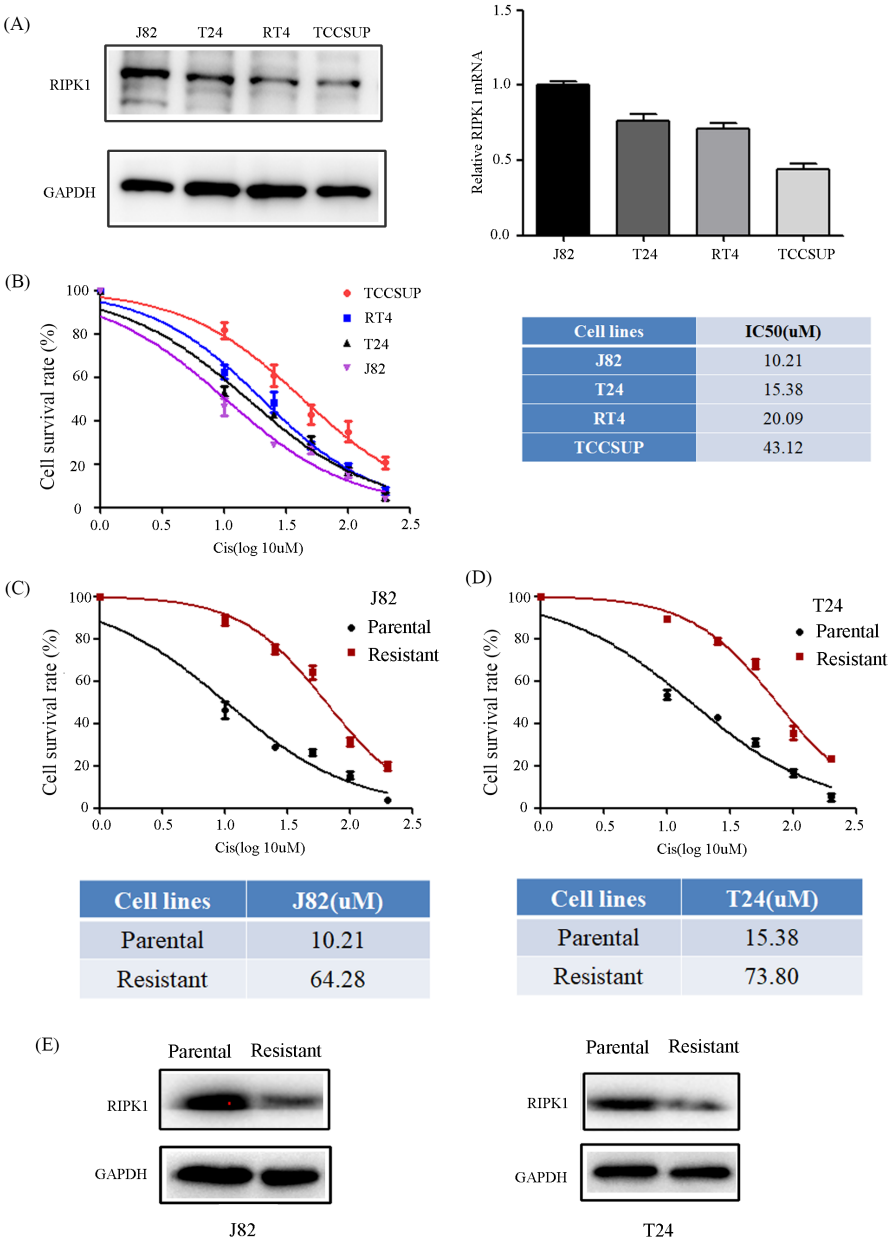


FIGURE 1 RIPK1 expression was downregulated in patients with cisplatin-resistant bladder cancer. (A). Expression of RIPK1 in bladder cancer based on sample types in the TCGA datasets. (B). Expression of RIPK1 in bladder cancer based on nodal metastasis status in the TCGA datasets. (C). The mRNA expression of RIPK1 in tumor tissue samples from normal ($N = 12$), sensitive ($N = 14$), and cisplatin-resistant ($N = 13$) bladder cancer patients. (D). The RIPK1 activity (phosphorylated form) in tumor tissue samples from normal ($N = 12$), sensitive ($N = 14$), and cisplatin-resistant ($N = 13$) bladder cancer patients, which is standard to its own protein expression level



Cell lines	IC50(uM)
J82	10.21
T24	15.38
RT4	20.09
TCCSUP	43.12

Cell lines	J82(uM)
Parental	10.21
Resistant	64.28

Cell lines	T24(uM)
Parental	15.38
Resistant	73.80

FIGURE 2 RIPK1 expression was downregulated with the development of cisplatin resistance in bladder cancer cells. (A). Western blotting and qRT-PCR were performed to test the level of RIPK1 in bladder cancer cells (J82, T24, RT4, and TCCSUP). (B). J82, T24, RT4, and TCCSUP cells were treated with cisplatin for 24 h according to the specific concentration gradient and then analyzed with CCK-8 assays. The survival curve was shown on the left, and the IC50 values were shown on the right. (C, D). J82 and T24 parental and resistant cells were treated with cisplatin for 24 h according to the specific concentration gradient and then analyzed with CCK-8 assays. The survival curve was shown above, and the IC50 values were shown below. (E). Western blotting was performed to detect the expression of RIPK1 in J82 and T24 parental and resistant cells

overexpression increased the sensitivity of cisplatin-resistant cells (Figure 4E,F). Altogether, our results suggest that PL increased the sensitivity of cisplatin-resistant cells by activating RIPK1-induced non-apoptotic cell death.

3.5 | PL-induced necroptosis in bladder cancer resistant cells

Since PL alone or in combination with cisplatin could indeed cause the death of T24 resistant cells, the level of apoptosis did not increase significantly; therefore, we further explored its underlying mechanism. In previous experiments, we found that PL could activate PIPK1, which is the key regulator of necroptosis; thus, we hypothesized that PL could trigger necroptosis in T24 resistant cells. As expected, treatment with PL alone or in combination with cisplatin did not change the total protein expression levels of RIP3 and MLKL in T24 resistant cells, but their phosphorylation levels were significantly enhanced (Figure 5A). In addition, pretreatment with an MLKL inhibitor (NSA) inhibited the cell death caused by PL treatment or its combination with cisplatin (Figure 5B). However, pretreatment with the apoptosis inhibitor z-VAD did not achieve similar effects (Figure 5C).

Furthermore, siRNA knockdown of MLKL had an effect similar to that of NSA and inhibited cell death and MLKL phosphorylation that were induced by PL alone or in combination with cisplatin (Figure 5D,E). Therefore, our results suggest that PL could eliminate T24 resistant cells by inducing necroptosis.

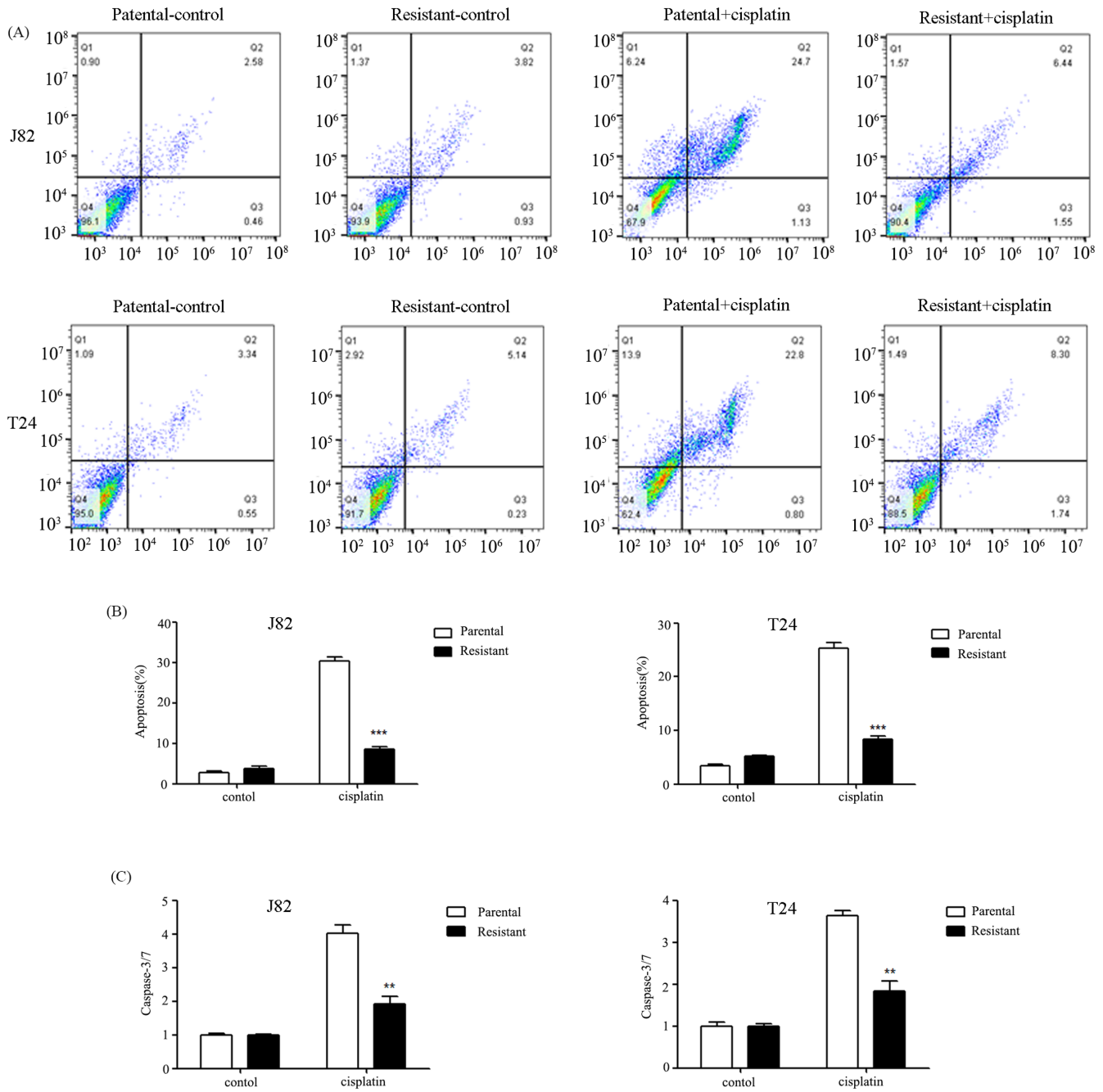


FIGURE 3 Apoptosis was inhibited in cisplatin-resistant bladder cancer cells. (A). J82 and T24 parental and resistant cells were treated with 20 μM cisplatin for 24 h. Representative density plots of flow cytometric analysis on the fraction of apoptosis cells were detected with Annexin V/PI. The histogram represents the mean values of three independent experiments shown in B. (C). After treating the J82 and T24 parental cells with 20 μM cisplatin and resistance for 24 h, the caspase-3/7 activity in the cells was detected

3.6 | Excessive ROS production by mitochondria contributed to necroptosis in cisplatin-resistant cells induced by PL

We investigated why PL-induced necroptosis in the drug-resistant cells. It has been revealed that PL can induce ROS production and that ROS is closely associated with the anti-cancer effects of PL.[13,18] ROS can activate RIPK1 by modifying three crucial cysteine residues to induce necroptosis.[19,20] Therefore, we used the DCFH-DA fluorescent dye to detect ROS levels in the different drug treatment groups. As shown in Figure 6A, the level of ROS increased in the group treated with PL alone and the group treated with PL in combination with cisplatin. Because mitochondria are the major source of ROS production,[21] we next assayed their function in the group treated with PL alone and in the group treat with PL in combination with cisplatin. Surprisingly, we found a significant increase in the copy number of mitochondrial DNA (Figure 6B). This suggests that the mitochondria undergo fission

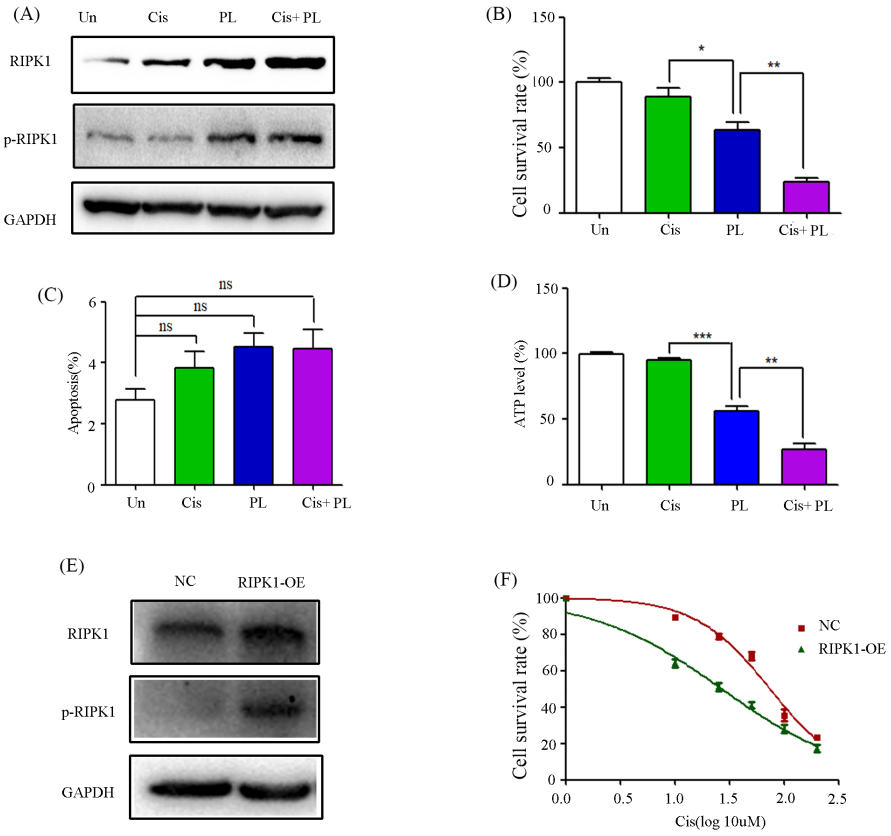


FIGURE 4 PL increased the sensitivity of resistant cells to cisplatin by activating the RIPK1-induced non-apoptotic cell death. A total of 5 μM PL and 20 μM cisplatin alone or their combination were used to treat T24 resistant cells for 24 h. (A). The expression of RIPK1 in T24 resistant cells was assessed by Western blotting. (B). The cell viability was detected by CCK-8 assay. (C). The apoptosis level was determined by Annexin V/PI staining. (D). The ATP level was tested using an ATP measurement kit. (E). The expression of RIPK1 in T24 resistant cells transfected with RIPK1 plasmid was analyzed by Western blotting. (F). T24 resistant cells were transfected with RIPK1 plasmid and then treated with cisplatin for 24 h. Cell viability was detected using the CCK-8 assay

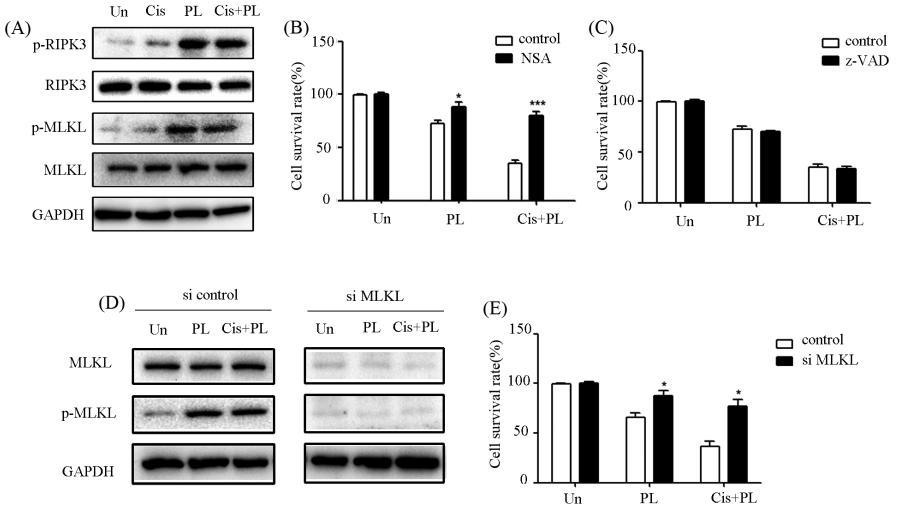


FIGURE 5 PL-induced necroptosis in T24 resistant bladder cancer cells. (A). The 5 μM PL and 20 μM cisplatin (Cis) alone or their combination (Cis+PL) were used to treat T24 resistant cells for 24 h. The expression of p-RIP3, RIP3, p-MLKL, and MLKL were analyzed by Western blotting. (B). The 5 μM MLKL inhibitor NSA was used to pretreat T24 resistant cells for 3 h, and then, 5 μM PL or its combination with 20 μM cisplatin were used to treat the cells for another 24 h. The cell viability was detected by CCK-8 assay. (C). After pretreating with 10 μM caspases inhibitor z-VAD for 3 h, the T24 resistant cells were treated with 5 μM PL or its combination with 20 μM cisplatin for another 24 h. The cell viability was detected by CCK-8 assay. (D, E). After transfecting with MLKL siRNA, the T24 resistant cells were treated with 5 μM PL or its combination with 20 μM cisplatin for another 24 h. D. The expressions of MLKL and p-MLKL were analyzed by Western blotting. E. The cell viability was analyzed by CCK-8 assay

under the action of PL to produce more ROS. Indeed, treatment with PL or its combination with cisplatin increased the phosphorylation level of dynamin-related protein 1 (DRP1), the key protein of mitochondrial fission, suggesting that mitochondria are involved in excessive ROS production (Figure 6C).

To further address the role of ROS, we treated the combination group with the antioxidant NAC to clear ROS (Figure 6A). The expression of p-RIPK1 in the combination group and the survival of drug-resistant cells were reexamined. NAC restored the high expression of p-RIPK1 and enhanced the survival of drug-resistant cells

(Figure 6D,E), suggesting that ROS contributed to PL-induced necroptosis in cisplatin-resistant cells. In summary, PL induces necroptosis in cisplatin-resistant cells by stimulating mitochondrial fission to produce excessive ROS.

4 | DISCUSSION

The occurrence and development of cisplatin resistance in bladder cancer is a clinical problem that urgently needs to be solved. To overcome cisplatin resistance and improve its efficacy, an increasing number of studies have investigated treatment strategies that can be combined with natural phytochemicals such as baicalein[22] eugenol,[23] fucoxanthin,[24] and emodin.[25] In our study, we found that PL, which is the major biologically active alkaloid in long peppers, can also increase the sensitivity of bladder cancer cells to cisplatin by activating RIPK1 to trigger necroptosis by excessive mitochondrial ROS.

In our study, we first found that the expression of RIPK1 gradually decreased as the malignancy of bladder tumors increased (normal bladder>bladder cancer>cisplatin-resistant bladder cancer tissues). In bladder cancer cells, cells with high RIPK1 expression are more sensitive to cisplatin than those with low expression. Next, we constructed cisplatin-resistant J82 and T24 cell lines. We found that the resistant cells had a significantly lower expression of RIPK1 than the parental cells. Upregulation of RIPK1

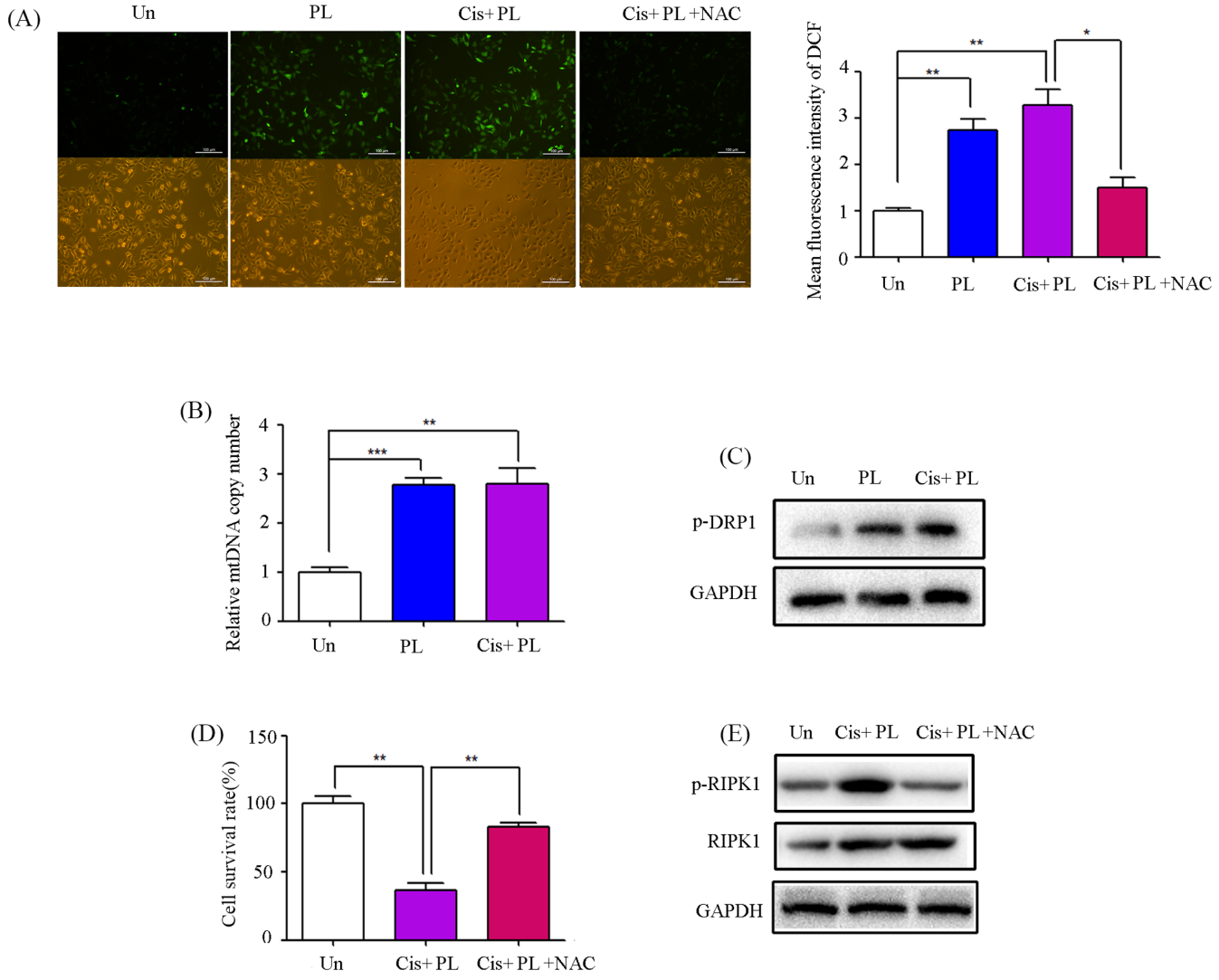


FIGURE 6 Over-excessive of ROS from mitochondria contributed to PL-induced necroptosis in cisplatin-resistant cells. (A). T24 resistant cells were pretreated with/without 10 mM NAC for 6 h; then, the cells were treated with 5 μM PL alone or in combination with 20 μM cisplatin (Cis+PL) for 24 h. Fluorescence microscopy of cells was conducted following the 10 μM DCFH-DA staining for 30 min (scale bar, 100 μm). Flow cytometry was used for the quantitative analysis of DCF. T24 resistant cells were treated with 5 μM PL alone or in combination with 20 μM cisplatin (Cis+PL) for 24 h. Mitochondrial DNA relative to the control is shown in B, as measured by qRT-PCR. The expression of p-DRP1, as analyzed by Western blotting, is shown in C. T24 resistant cells were pretreated with or without 10 mM NAC for 6 h and then treated with 5 μM PL in combination with 20 μM cisplatin (Cis+PL) for 24 h. Cell viability analyzed using the CCK-8 assay is shown in D. The expression of RIPK1 and p-RIPK1 analyzed by Western blotting is shown in E

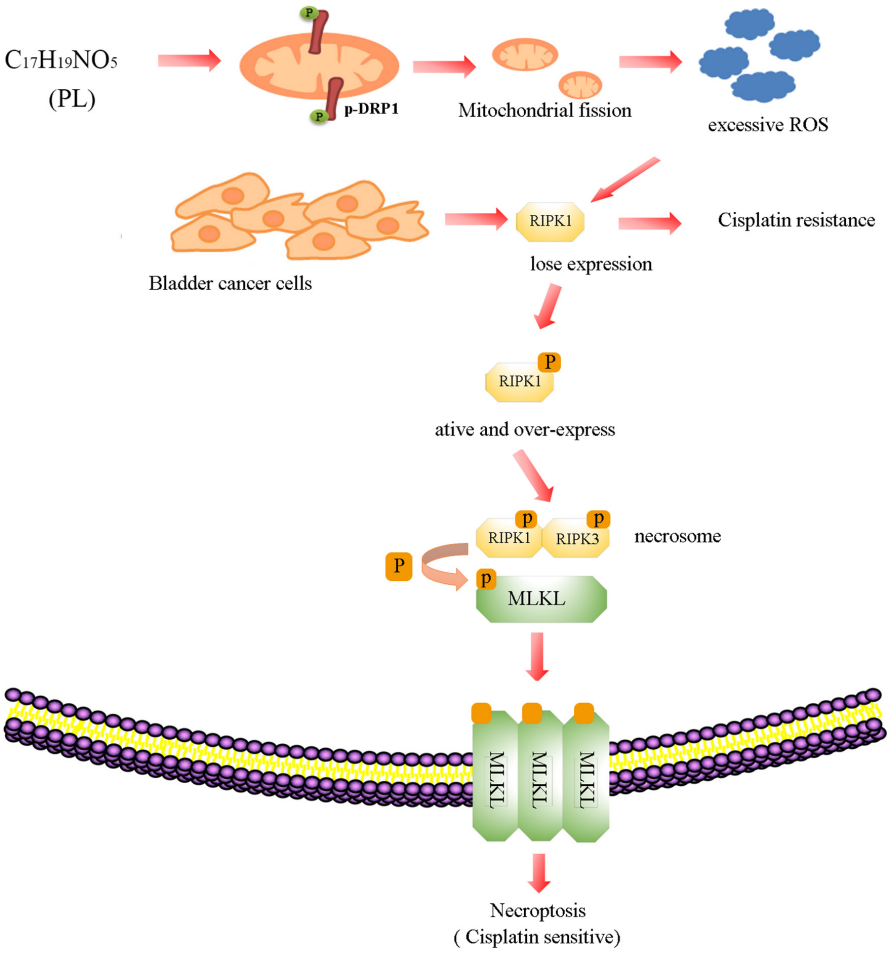


FIGURE 7 Schematic diagram of the mechanism by which PL enhanced the sensitivity of cisplatin to bladder cancer cells. In the process of cisplatin resistance in bladder cancer cells, RIPK1 expression was gradually lost. PL promotes mitochondrial fission by activating DRP-1, which results in excessive ROS production. Then, ROS induces necroptosis of resistant cells by activating the expression of RIPK1, thereby enhancing the effect of cisplatin on bladder cancer cells

re-sensitizes bladder cancer cells to cisplatin treatment. These results indicate that RIPK1 may play an important role in tumor progression and that its expression is closely related to cisplatin resistance in bladder cancer.

Cisplatin-induced apoptosis was significantly inhibited in resistant cells. However, after treatment with PL in combination with cisplatin, the survival rate of the cells was distinctly reduced. Interestingly, cell apoptosis did not increase significantly; therefore, we believe that PL can increase the sensitivity of bladder cancer cells to cisplatin by inducing cell death other than apoptosis. RIPK1 is one of the most important regulators of necroptosis because of its kinase function, which can be activated by ROS.[20] It was reported that PL could induce ROS production and that ROS is heavily involved in the anti-cancer effects of PL[13,18]; cisplatin-resistant cells in our study simply lack the expression of RIPK1. Therefore, we hypothesized that PL triggered necroptosis in resistant cells. As predicted, PL treatment enhanced the effect of cisplatin by activating the RIPK1/RIPK3/MLKL signaling pathway.

Regarding the mechanism of RIPK1 activation by PL, it has been reported that ROS can induce autophosphorylation on S161 by modifying the three key cysteine residues in RIPK1. This phosphorylation event allows for efficient recruitment of RIP3 to RIP1 to form a functional necrosome, leading to necroptosis.[20] Thus, we detected the level of ROS in the group treated with PL alone and the group treated with PL in combination with cisplatin and found that ROS was obviously increased. Using the ROS scavenger NAC could resist most of the effects of PL, which suggested that ROS was necessary for PL-induced necroptosis in cisplatin-resistant cells.

Because mitochondria are the major source of ROS production,[21] we also assayed mitochondrial function. Surprisingly, we found a significant increase in the copy number of mitochondrial DNA after PL treatment, suggesting that mitochondria undergo fission under the action of PL to produce more ROS. To prove this, we tested the mitochondrial fission protein dynamin-related protein 1(DRP1). The data showed that the phosphorylation level of DRP1 (p-DRP1) was significantly increased after treatment with PL alone or in combination with cisplatin. Previous studies have shown that PL is an effective anti-cancer agent, which increases the level of ROS by disturbing the balance of oxidative stress in cells, thereby selectively killing cancer cells.[13] However, in our study, we found that PL stimulated mitochondrial fission to produce excess ROS.

In summary, we report for the first time that RIPK1 expression is significantly reduced in the process of cisplatin resistance in bladder cancer. Upregulation of RIPK1 by PL can overcome cisplatin resistance in bladder cells and revert cell death from apoptosis to necroptosis. Furthermore, our study also revealed that the function of RIKP1 in PL-induced necroptosis was also mediated by excessive ROS from the mitochondria (Figure 7).

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

XP conceived the study and established its initial design. GC and WH performed the experiments and analyzed the data. XP prepared the study. All the authors have read and approved the final study. XP and GC confirmed the authenticity of the raw data.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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