

## Original Research Article

# Z-ligustilide reduces cisplatin-induced nephrotoxicity via activation of NRF2/HO-1 signaling pathways

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### Abstract

**Purpose:** To investigate the effect of Z-ligustilide (Z-lig) on cisplatin-induced nephrotoxicity and examine whether NRF2 signaling mediates the underlying mechanism of action.

**Methods:** Human proximal tubular epithelial cells (HK-2) were pretreated with 20 or 100  $\mu$ M Z-lig for 2 h, followed by 10  $\mu$ M cisplatin treatment for 24 h. Cell viability was measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A commercial kit was used to determine lactate dehydrogenase (LDH) release. Apoptosis was determined by flow cytometry while Western blotting was used to evaluate protein levels. Levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-Px) were assessed by enzyme-linked immunosorbent assay (ELISA).

**Results:** Cisplatin decreased HK-2 cell viability and increased LDH release, while Z-lig increased cell viability and decreased LDH release in a dose-dependent manner ( $p < 0.05$ ). Moreover, Z-lig reduced cisplatin-induced apoptosis ( $p < 0.01$ ), and alleviated cellular oxidative stress caused by cisplatin ( $p < 0.05$ ). Furthermore, Z-lig activated NRF2/HO-1 signaling in cells treated with cisplatin ( $p < 0.05$ ).

**Conclusion:** Z-lig reduces cisplatin-induced nephrotoxicity via activation of NRF2/HO-1 signaling. Thus, Z-lig is a potential drug for the treatment of nephrotoxicity caused by cisplatin.

**Keywords:** Z-ligustilide, Cisplatin, Nephrotoxicity, Oxidative stress, Apoptosis, Nuclear factor erythroid 2-related factor 2, Heme oxygenase-1

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## INTRODUCTION

Cisplatin is a primary chemotherapy drug used to treat various cancers [1]. Nevertheless, nephrotoxicity limits the use of cisplatin [2]. Previous studies revealed that over 30% of patients present symptoms of acute renal injury following cisplatin administration [3,4]. The mechanism of cisplatin-induced nephrotoxicity is

related to many factors, such as cellular oxidative stress, mitochondrial dysfunction, and apoptosis [5]. Oxidative stress is considered to be an important factor leading to cisplatin nephrotoxicity [6]. In some cell types, Nuclear factor erythroid 2-related factor 2 (NRF2) is involved in oxidative stress [7].

Activation of the NRF2 signaling pathway may alleviate cisplatin-induced nephrotoxicity [8]. For example, melatonin alleviated cisplatin-induced nephrotoxicity through activation of the NRF2/heme oxygenase-1 (HO-1) pathway [9]. Accumulating evidence suggests that a variety of Chinese herbal extracts, including curcumin, resveratrol, and sulforaphane, serve as NRF2 activators [10-12]. Therefore, these extracts may promote resistance to cisplatin-induced nephrotoxicity by activating the Nrf2 pathway.

Z-Ligustilide (Z-lig), the main phthalide component of medicinal plants in the Apiaceae (Umbelliferae) family, exerts protective effects in diseases of the nervous system [13]. For example, Z-lig decreased cerebral infarct size and brain swelling in rats with focal ischemic injury [13].

Moreover, Wu *et al* found that Z-lig alleviated oxidative stress induced by ultraviolet B irradiation, reduced release of inflammatory factors via activation of NRF2/HO-1 signaling, and suppressed the Nuclear Factor Kappa B (NF- $\kappa$ B) pathway [14]. These results imply that Z-lig also functions as an NRF2 activator. Therefore, Z-lig may alleviate cisplatin-induced nephrotoxicity via NRF2 signaling.

Hence, the goals of the present study were to determine effects of Z-lig on cisplatin-induced nephrotoxicity, and to investigate whether NRF2 signaling mediates its underlying mechanism of action.

## EXPERIMENTAL

### Cell culture

Cells of the human proximal tubular epithelial cell line HK-2 were obtained from American Type Culture Collection, and maintained on Keratinocyte Serum Free Medium plus 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Gibco, Grand Island, NY, USA), in a 5% CO<sub>2</sub> atmosphere at 37°C. Cells were pretreated with 20 or 100  $\mu$ M Z-lig for 2 h, followed by 10  $\mu$ M cisplatin treatment for 24 h.

### (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were inoculated into 96-well plates. After 24 h of culture, cells were treated with 20  $\mu$ L of MTT for 4 h. Subsequently, the medium was removed, and 200  $\mu$ L of DMSO (Sigma, St. Louis, Mo, USA) was added to cells to solubilize the formazan product. The absorbance at 490 nm was measured by a microplate reader.

### Assessment of lactate dehydrogenase (LDH) release

LDH release from HK-2 cells after cisplatin or Z-lig treatments was determined using the CyQUANT LDH Cytotoxicity Assay (Thermo Fisher, Waltham, MA, USA), according to the protocols of the manufacturer. Absorbance at 490 nm was determined using a microplate reader.

### Flow cytometry

After cisplatin or Z-lig treatments, cells were suspended, centrifuged, resuspended in 1 $\times$  binding buffer, and stained with 5  $\mu$ L of Annexin V-FITC and 10  $\mu$ L of PI (BD, San Jose, CA, USA) for 15 min. The proportions of apoptotic cells were determined using flow cytometry (BD, San Jose, CA, USA).

### Western blotting

Cellular proteins were extracted utilizing RIPA buffer (Thermo Fisher, Waltham, MA, USA), and quantified by the BCA method. The lysates were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked by incubation with 4% non-fat milk, then probed with anti-Cleaved caspase-3 (1 : 500), anti-Cleaved caspase-9 (1:500), anti-NRF2 (1:500), anti-HO-1 (1:500), or anti-GAPDH (1:1,000) (all from Abcam, Cambridge, UK) for 12 h at 4°C. After the washing step, the membrane was incubated with HRP-labeled anti-IgG secondary antibody (Abcam, Cambridge, UK). The blots were visualized using the ECL chemiluminescence kit (Beyotime, Shanghai, China).

### Enzyme-linked immunosorbent assay (ELISA)

After treatment of cells with cisplatin or Z-lig and harvesting, levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-Px) were determined using ELISA assay kits (R & D Systems, Minneapolis, MN, USA), following the protocols of the manufacturer.

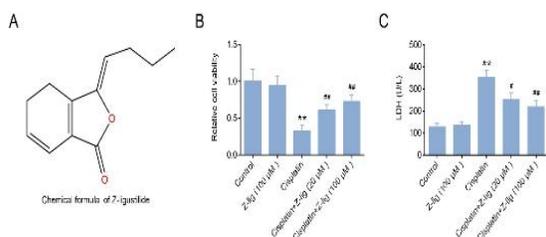
### Statistical analysis

The SPSS statistics software (Chicago, IL, USA) was used for statistical analysis of the data. Data are presented as mean  $\pm$  standard deviation (SD). The differences among multiple groups were determined by one-way ANOVA. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### Z-lig reduces cisplatin-induced HK-2 cell injury

To elucidate the effect of Z-lig on nephrotoxicity caused by cisplatin, we first assessed cell viability and LDH release by HK-2 cells that had been treated with Z-lig or cisplatin. The chemical structure of Z-lig is shown in Figure 1 A. Cisplatin significantly reduced cell viability ( $p < 0.01$ , Figure 1 B). However, 20  $\mu\text{M}$  and 100  $\mu\text{M}$  Z-lig significantly increased the viability of cells treated with cisplatin ( $p < 0.01$ , Figure 1 B). Furthermore, cisplatin treatment led to release of LDH from cells ( $p < 0.01$ , Figure 1 C). However, 20  $\mu\text{M}$  and 100  $\mu\text{M}$  Z-lig significantly reduced LDH release ( $p < 0.05$ , Figure 1 C). In addition, 100  $\mu\text{M}$  Z-lig alone did not alter cell viability or LDH leakage (Figures 1 B and C). These results indicated that Z-lig was nontoxic to the cells, and that Z-lig reduced cellular injury caused by cisplatin.

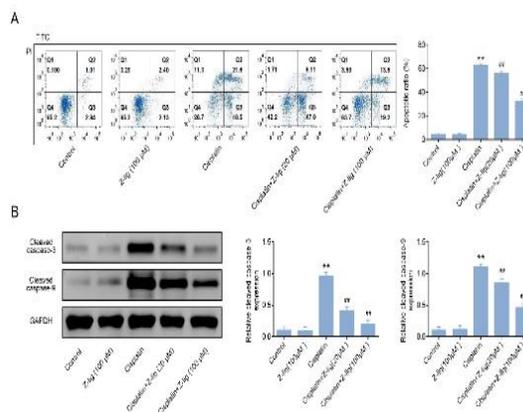


**Figure 1:** Z-lig reduces cisplatin-induced HK-2 cell injury. A. Chemical structure of Z-lig. B. Cell viability was determined by MTT assay in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h before stimulation with 10  $\mu\text{M}$  cisplatin for 24 h. C. LDH release from cells that were pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by treatment with 10  $\mu\text{M}$  cisplatin for 24 h;  $^{**}p < 0.01$  compared to control;  $^{\#}p < 0.05$  compared to cisplatin;  $^{\#\#}p < 0.01$  compared to cisplatin

### Z-lig reduces cisplatin-induced apoptosis of HK-2 cells

To further investigate the effect of Z-lig on nephrotoxicity caused by cisplatin, apoptosis of cells treated with Z-lig or cisplatin was determined. Flow cytometry revealed increased apoptosis of cells treated with cisplatin ( $p < 0.01$ , Figure 2 A). However, apoptosis of cisplatin-treated cells was significantly reduced by either 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig ( $p < 0.01$ , Figure 2). Moreover, levels of cleaved caspase-3 and cleaved caspase-9 were significantly increased in cells treated with cisplatin ( $p < 0.01$ , Figure 2 B). In contrast, 20  $\mu\text{M}$  and 100  $\mu\text{M}$  Z-lig decreased the elevation of cleaved caspase-3 and cleaved caspase-9 by cisplatin treatment ( $p < 0.01$ , Figure 2 B). Thus, Z-lig reduced

apoptosis of cells treated with cisplatin.



**Figure 2:** Z-lig suppresses cisplatin-induced apoptosis of HK-2 cells. A. Apoptosis of cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by treatment with 10  $\mu\text{M}$  cisplatin for 24 h, was measured by flow cytometry. B. Levels of cleaved caspase-3 and cleaved caspase-9 in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by stimulation with 10  $\mu\text{M}$  cisplatin for 24 h, were determined by Western blot;  $^{**}p < 0.01$  compared to control;  $^{\#\#}p < 0.01$  compared to cisplatin

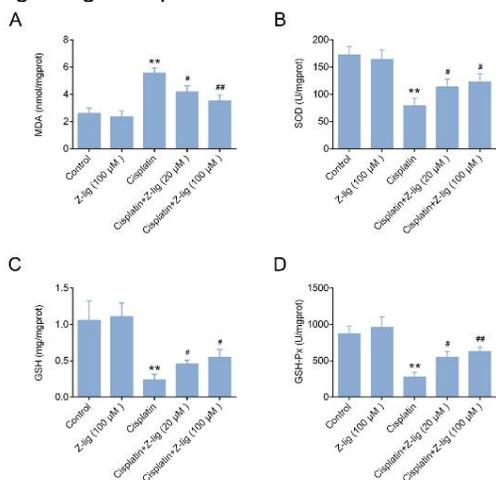
### Z-lig reduces cisplatin-induced oxidative stress in HK-2 cells

To further study the effect of Z-lig on nephrotoxicity caused by cisplatin, we determined oxidative stress in cells treated with Z-lig or cisplatin. Cells treated with cisplatin showed increased MDA ( $p < 0.01$ ), while pretreatment with either 20  $\mu\text{M}$  ( $p < 0.05$ ) or 100  $\mu\text{M}$  Z-lig ( $p < 0.01$ ) decreased this level (Figure 3 A). Cisplatin treatment decreased cellular SOD ( $p < 0.01$ ), while pretreatment with either 20  $\mu\text{M}$  ( $p < 0.05$ ) or 100  $\mu\text{M}$  Z-lig ( $p < 0.05$ ) increased SOD levels in cisplatin-treated cells (Figure 3 B). GSH was also reduced in cisplatin-treated cells ( $p < 0.01$ ), whereas 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig increased levels of GSH in cisplatin-treated cells (all  $p < 0.05$ , Figure 3 C). Moreover, cellular levels of GSH-Px were suppressed by cisplatin ( $p < 0.01$ ), and this effect was diminished by 20  $\mu\text{M}$  ( $p < 0.05$ ) or 100  $\mu\text{M}$  Z-lig ( $p < 0.01$ ) (Figure 3 D). Hence, Z-lig reduced cellular oxidative stress caused by cisplatin.

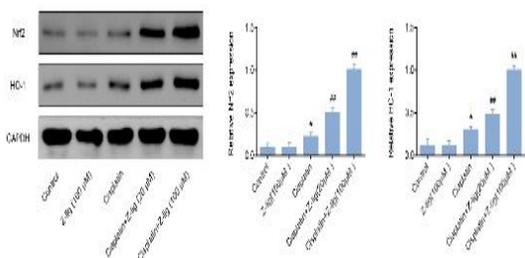
### Z-lig modulates the NRF2/HO-1 signaling pathway in cisplatin-treated HK-2 cells

To explain the mechanism of modulation of cisplatin-induced cell injury by Z-lig, we determined apoptosis, cellular oxidative stress, and changes in the activation of NRF2/HO-1 signaling in cells treated with Z-lig or cisplatin.

Levels of NRF2 and HO-1 expression were slightly increased in cells treated with cisplatin (both  $p < 0.05$ ), while 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig further elevated NRF2 and HO-1 expression in cisplatin-treated cells (all  $p < 0.05$ , Figure 4). Consequently, Z-lig activated NRF2/HO-1 signaling in cisplatin-treated cells.



**Figure 3:** Z-lig inhibits cisplatin-induced oxidative stress in HK-2 cells. A. MDA levels in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by stimulation with 10  $\mu\text{M}$  cisplatin for 24 h, were measured using ELISA. B. SOD levels in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by treatment with 10  $\mu\text{M}$  cisplatin for 24 h, were measured by ELISA. C. Levels of GSH in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by stimulation with 10  $\mu\text{M}$  cisplatin for 24 h, were measured by ELISA. D. Levels of GSH-Px in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by stimulation with 10  $\mu\text{M}$  cisplatin for 24 h, were measured using ELISA; \*\* $p < 0.01$  compared to control; # $p < 0.05$  compared to cisplatin; ## $p < 0.01$  compared to cisplatin. All experiments were performed in triplicate



**Figure 4:** Z-lig activates NRF2/HO-1 signaling in cisplatin-treated HK-2 cells. Levels of NRF2 and HO-1 protein in cells pretreated with 20  $\mu\text{M}$  or 100  $\mu\text{M}$  Z-lig for 2 h, followed by stimulation with 10  $\mu\text{M}$  cisplatin for 24 h, were determined by Western blot; \* $p < 0.01$  compared to control; \*\* $p < 0.01$  compared to control; ## $p < 0.01$  compared to cisplatin. All experiments were performed in triplicate

## DISCUSSION

Cisplatin is a primary chemotherapy drug for the treatment of various cancers. However, more than 30% of patients present acute renal injury symptoms following cisplatin treatment [1,3]. Therefore, nephrotoxicity induced by cisplatin limits its use in cancer chemotherapy. Hence, it is essential to search for effective drugs to prevent nephrotoxicity.

The present study investigated the protective effect of Z-ligustilide, a phthalide component of medicinal plants in the family Apiaceae (Umbelliferae), on cisplatin-induced nephrotoxicity. We found that Z-lig reversed both the loss of viability and increase in LDH leakage induced in HK-2 cells by cisplatin, consistent with previous studies [15,16]. For instance, research by Wu *et al* also revealed that Z-lig enhanced cell viability and reduced LDH release in neurons subjected to oxygen-glucose deprivation [15]. Therefore, Z-lig reduces cellular injury caused by cisplatin.

Because apoptosis is among the causes of cisplatin-induced neurotoxicity, we studied the effects of Z-lig on apoptosis induced in HK-2 cells by cisplatin and found that Z-lig reduced cisplatin-induced apoptosis in these cells. Anti-apoptotic effects of Z-lig were also proven in neurons treated with Abeta25-35 [17]. In a study conducted by Zhang *et al*, Z-lig reversed the decrease in neuron viability and the increase in apoptosis caused by Abeta25-35, and prevented Abeta25-35 cytotoxicity [17]. Furthermore, a study by Bunel *et al* comparing protection against cisplatin toxicity by ferulic acid, Z-lig, and E-lig revealed that Z-lig reduced cisplatin-induced apoptosis [18].

Oxidative stress has been regarded as the critical factor leading to cisplatin nephrotoxicity [6]. The effect of Z-lig in modulating oxidative stress was investigated in a previous study, in which levels of primary indicators of oxidative stress were determined, including MDA, SOD, GSH, and GSH-Px [19]. Z-Lig reversed the cisplatin-induced increase in MDA formation, the reduced GSH level, and the decreased expression of SOD and GSH-Px in that study, in accordance with other previous reports [14,20].

Z-lig also functions as an antioxidant in ischemic brain tissues, suppressing MDA content and elevating SOD, GSH, and GSH-Px [20]. In addition, Wu *et al* concluded that Z-lig attenuates oxidative stress induced by ultraviolet B [14]. Thus, these results are consistent with reduction of cisplatin-induced oxidative stress by Z-lig.

NRF2 signaling is known to be involved in oxidative stress [21,22], and activation of this signaling pathway alleviates cisplatin-induced nephrotoxicity [7,9]. To study the underlying mechanism of Z-lig protection against cisplatin nephrotoxicity, we investigated activation of NRF2 signaling in cisplatin-treated HK-2 cells and showed that Z-lig activated the NRF2/HO-1 signaling pathway, a result consistent with previous studies [23,24].

Z-lig was shown to alleviate cerebral ischemia-reperfusion injury through NRF2/HO-1 activation [23], whereas in human keratinocytes, Z-lig repressed CYP1A1 upregulation caused by benzo(a)pyrene through activation of NRF2 [24]. We conclude that Z-lig activates NRF2/HO-1 signaling in cisplatin-treated cells, and that Z-lig reduces cell injury, apoptosis, and cellular oxidative stress via NRF2/HO-1 activation.

## CONCLUSION

Z-lig attenuates cisplatin-induced nephrotoxicity via activation of NRF2/HO-1 signaling. Thus, Z-lig is a potential drug for the therapy of nephrotoxicity caused by cisplatin. However, further studies are required to strengthen this assertion.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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