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Original Research Article

Zinc protoporphyrin IX improves the sensitivity of colorectal cancer cells to paclitaxel by inactivating AKT/mTOR pathway via HO-1

Quanfu Li[1], Tian Xia[1], Guowei Yang[2], Houlai Yang[1], Juan Zhang[3]*
[1]Department of General Surgery, Wuhan third Hospital (Tongren Hospital Affiliated with Wuhan University), Wuhan, Hubei 430000, [2]Department of Traditional Chinese Medicine, The First Hospital Affiliated with Lanzhou University, [3]Department of Proctology, The Hospital Affiliated with Gansu University of Traditional Chinese Medicine, Lanzhou, Gansu 730000, China

For correspondence:* ***Email:* *zhangjuan_0531@163.com;* ***Tel:*** *+86-18709429225*

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Abstract

Purpose: *To examine the effect of zinc protoporphyrin IX (ZnPPIX) on the sensitivity of colorectal cancer (CRC) cells to paclitaxel.*
Methods: *Paclitaxel-resistant CRC cells were established by incubating CRC cells with increasing concentrations of paclitaxel. Colorectal cancer cells were incubated with ZnPPIX for 1 h before treatment with paclitaxel. Cell viability, proliferation, and apoptosis were determined by MTT, colony formation, and flow cytometry assays, respectively. The effect of ZnPPIX on DNA damage response in paclitaxel-resistant cells was assessed using western blot analysis of γH2AX, p-DNA-PK, and Rad51.*
Results: *Paclitaxel suppressed proliferation of SW480 cells at an IC_{50} (half-maximal inhibitory concentration) of 0.23 μM, while that of proliferation of paclitaxel-resistant SW480 cells was also suppressed by paclitaxel at an IC_{50} of 2.58 μM ($p < 0.05$). Incubation with ZnPPIX attenuated the paclitaxel-induced decrease in viability and proliferation of SW480 cells. Paclitaxel-induced apoptosis of SW480 cells was enhanced upon incubation with ZnPPIX ($p < 0.05$). Similarly, protein expression of γH2AX was enhanced while protein expressions of p-DNA-PK and Rad51 reduced in paclitaxel-induced SW480 cells. On incubation with ZnPPIX, protein expression of heme oxygenase-1 (HO-1) and AKT and mTOR phosphorylation decreased in SsW480 cells ($p < 0.05$).*
Conclusion: *ZnPPIX enhances paclitaxel-induced chemosensitivity of CRC by suppression of HO-1-mediated AKT/mTOR activation, and could potentially be used to facilitate the management of CRC.*

Keywords: *Zinc protoporphyrin IX, Paclitaxel, Chemosensitivity, Colorectal cancer, AKT/mTOR*

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common tumors worldwide [1]. Paclitaxel is a common chemotherapeutic agent widely used in clinical treatment of a variety of human tumors. Paclitaxel inhibits metastasis of CRC [2]. However, it has been shown that CRC cells are resistant to paclitaxel and that this resistance occurs via activation of the mitogen-activated protein/extracellular signal-regulated kinase pathway (MEK)/ERK [3]. Strategies that

increased the chemosensitivity of CRC conferred cytotoxicity of paclitaxel to CRC [4].

Zinc protoporphyrin IX (ZnPPIX) is a derivative of protoporphyrin IX (PPIX), a precursor of heme, that shows antitumor activity in solid tumors [5]. Zinc protoporphyrin IX inhibits heme oxygenase-1 (HO-1) leading to suppression of breast, ovarian, and prostate cancers and melanoma [6]. Zinc protoporphyrin IX blocks activity of HO-1 (heme oxygenase-1) to reduce release of vascular endothelial growth factor, and represses angiogenesis in CRC [7]. Zinc protoporphyrin IX has been shown to enhance chemosensitivity of hepatoma cells to cisplatin [8]. The effect of ZnPPIX on paclitaxel-resistant CRC cells was investigated in this study.

Heme oxygenase-1 cleaves heme to generate antioxidants, thus promoting tumor growth and conferring chemoresistance of cancer cells [9]. Overexpression of HO-1 enhanced chemoresistance of hepatoma cells to cisplatin, while inhibition of HO-1 augmented cisplatin-induced cytotoxicity [8]. Therefore, inhibition of HO-1 may enhance the cytotoxic effects of various chemotherapeutics and could be used in combination with chemotherapeutics to kill cancer cells. Heme oxygenase-1 was upregulated in CRC tissues and this upregulation-associated with CRC cell chemosensitivity [10]. Suppression of HO-1 with ZnPPIX promoted pirarubicin-induced cytotoxicity of CRC cells [10].

However, the mechanism of ZnPPIX-mediated paclitaxel resistance in CRC cells has not yet been reported. In this study, paclitaxel-resistant CRC cells were established, and the effects of ZnPPIX on their proliferation and apoptosis were investigated.

EXPERIMENTAL

Cell Culture

The SW480 human colon cancer cells (American Type Culture Collection; Manassas, VA, USA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Wisent Corporation, Wisent, Canada) and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO, USA) in a 37°C incubator.

Paclitaxel-resistant CRC cell model

SW480 cells were treated with 0.1 μM paclitaxel (Sigma-Aldrich) for 24 h, and then the culture medium was replaced with fresh medium containing 0.1 μM paclitaxel and incubated for another 24 h. This was repeated three times before the paclitaxel concentration was elevated gradually to 0.2, 0.5, 1.0, and 2.0 μM. Cells that grew stably in 2.0 μM paclitaxel were regarded as paclitaxel-resistant CRC cells.

Determination of half maximal inhibitory concentration (IC_{50})

Paclitaxel-resistant SW480 and SW480 cells were seeded into 96-well plates and incubated with 0, 0.25, 0.5, 1, 2.5, 5, 10, and 20 μM paclitaxel for 24 h. MTT solution (2 mg/mL; Beyotime, Beijing, China) was added to each well, and the plates were incubated for another 4 h. Then, the medium was removed and DMSO was added to the wells. The absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). The inhibition percent for each well = (1-A_{490} of paclitaxel group)/A_{490} of control group) × 100 %.

Cell viability and proliferation studies

Paclitaxel-resistant SW480 cells were incubated with 0.5 μM ZnPPIX (Sigma-Aldrich) in 96-well plates for 1 hour, 1 μM paclitaxel was then added to each well, and the plates were incubated for 24 h. A total of 10 μL MTT solution (Beyotime) was added to each well, and the plates were incubated for another 4 h. Then, DMSO was added to the wells, and the absorbance at 490 nm was measured with a microplate reader (Bio-Rad). For the cell proliferation assay, paclitaxel-resistant SW480 cells were incubated with 0.5 μM ZnPPIX (Sigma-Aldrich) in 6-well plates for 1 h,1 μM paclitaxel was added to each well, and the plates were incubated for 14 d. Cells were fixed in paraformaldehyde and stained with crystal violet before observing under light microscopy (Olympus, Tokyo, Japan).

Flow cytometry

Paclitaxel-resistant SW480 cells (1×10^6 cells/mL) treated with ZnPPIX and paclitaxel were harvested and resuspended in 100 μL binding buffer (Annexin V Fitc Apoptosis Detection Kit, KeyGen BioTECH, Nanjing, China). The cells were then incubated with 5 μL Annexin V-APC and 5 μL propidium iodide in the dark before flow cytometric analysis using the Beckman FC500 MCL flow cytometer (Beckman Coulter, Brea, CA, USA).

Western blot

Paclitaxel-resistant SW480 cells treated with ZnPPIX and paclitaxel were lysed in RIPA buffer (Ding Guo Chang Sheng Biotech, Beijing,

China). The supernatants were collected following centrifugation at 12000 × *g* for 1 h. Protein concentrations of the supernatants were determined using bicinchoninic acid protein assay kit (Sigma Aldrich). Protein samples (30 µg) were separated using SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked with 5 % non-fat milk and probed with anti-Bcl-2, anti-Bax, and anti-cleaved caspase 3 (1:2000; Abcam, Cambridge, MA, USA); with anti-γH2AX, anti-p-DNA-PK, and anti-Rad51 (1:2500; Abcam); with anti-HO-1 (1:3000; Abcam); with anti-AKT, anti-p-AKT, anti-mTOR, and anti-p-mTOR (1:3500; Abcam); and with anti-β-actin (1:4000; Abcam) at 4 °C overnight. Then, the membranes were washed, and incubated with horseradish peroxidase-conjugated immunoglobulin G (1:5000; Abcam). Enhanced chemiluminescence (KeyGen) was used for the assessment.

Statistical analysis

Results from at least three independent experiments are presented as mean ±standard deviation. Statistical analyses of different groups were performed with one-way analysis of variance or a Student's t test in SPSS19.0. $P < 0.05$ were considered significant.

RESULTS

ZnPPIX enhanced the chemosensitivity of paclitaxel-resistant SW480 cells

The IC_{50} of SW480 was 0.23 µM, while the IC_{50} of paclitaxel-resistant SW480 was 2.58 µM (Figure 1 A), indicating that paclitaxel-resistant SW480 was indeed resistant to paclitaxel. Incubation with paclitaxel or ZnPPIX decreased the viability of paclitaxel-resistant SW480 cells, and incubation with paclitaxel and ZnPPIX further decreased the viability of paclitaxel-resistant SW480 cells (Figure 1B). In addition, proliferation of paclitaxel-resistant SW480 cells was suppressed by paclitaxel or ZnPPIX (Figure 1 C and D). Zinc protoporphyrin IX combined with paclitaxel further decreased proliferation of paclitaxel-resistant SW480 cells (Figure 1 C and D), indicating that ZnPPIX enhanced the cytostatic effect of paclitaxel on paclitaxel-resistant CRC cells.

ZnPPIX enhanced paclitaxel-induced apoptosis of paclitaxel-resistant SW480 cells

Flow cytometry analysis showed that incubation with ZnPPIX or paclitaxel alone increased apoptosis of paclitaxel-resistant SW480 cells when compared with untreated cells (Figure 2A) and that incubation with ZnPPIX + paclitaxel induced more cell apoptosis than ZnPPIX or paclitaxel alone (Figure 2 A). Treatment with ZnPPIX + paclitaxel enhanced the paclitaxel-induced decrease in Bcl-2 expression and enhanced the paclitaxel-induced increase in Bax and cleaved caspase 3 expression (Figure 2 B), suggesting that ZnPPIX increased the cytotoxic effect of paclitaxel on paclitaxel-resistant CRC cells.

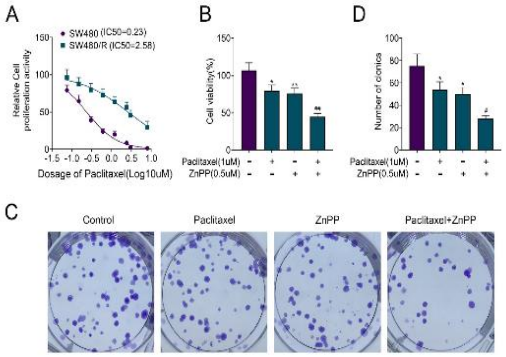

Figure 1: ZnPPIX enhanced chemosensitivity of paclitaxel-resistant SW480 cells. (A) The MTT assay was used to determine the IC_{50} of paclitaxel against SW480 cells and paclitaxel-resistant SW480 cells. (B) The effect of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on the viability of paclitaxel-resistant SW480 cells determined using the MTT assay. (C) The effect of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on cell proliferation of paclitaxel-resistant SW480 cells determined by colony formation. (D) The effect of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on the number of paclitaxel-resistant SW480 colonies. *, # $p < 0.05$, **, ## $p < 0.01$

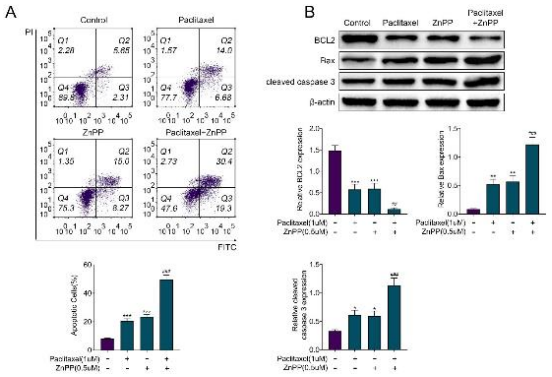

Figure 2: ZnPPIX enhanced paclitaxel-induced apoptosis of paclitaxel-resistant SW480 cells. (A) The effect of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on apoptosis of paclitaxel-resistant SW480 cells. (B) The effect of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on protein expression of Bcl-2, Bax, and cleaved caspase 3 in paclitaxel-resistant SW480 cells. * $p < 0.05$, **, ## $p < 0.01$. ***, ### v $p < 0.001$

ZnPPIX enhanced paclitaxel-induced DNA damage of paclitaxel-resistant SW480 cells

Protein expression of the DNA damage marker γ-H2AX and of the DNA repair-related proteins p-DNA-PK and Rad51 were higher in paclitaxel-resistant SW480 cells on incubation with ZnPPIX or paclitaxel alone (Figure 3). Treatment with ZnPPIX + paclitaxel attenuated the paclitaxel-induced increases p-DNA-PK and Rad51 expression but it enhanced the paclitaxel-induced increases γ-H2AX expression (Figure 3), suggesting that ZnPPIX disrupted DNA damage repair in paclitaxel-resistant SW480 cells post-incubation with paclitaxel.

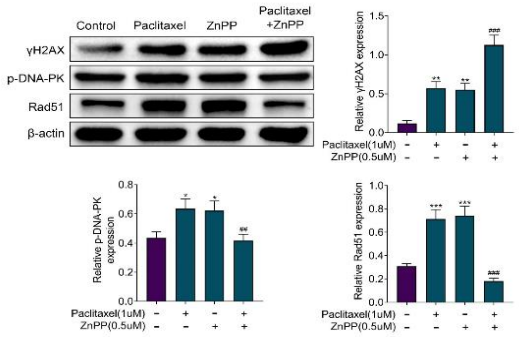

Figure 3: ZnPPIX enhanced paclitaxel-induced DNA damage of paclitaxel-resistant SW480 cells. The effects of paclitaxel, ZnPPIX, and ZnPPIX + paclitaxel on protein expression of γ-H2AX, p-DNA-PK, and Rad51 in paclitaxel-resistant SW480 cells. * $p < 0.05$, **, ## $p < 0.01$. ***, ### v $p < 0.001$

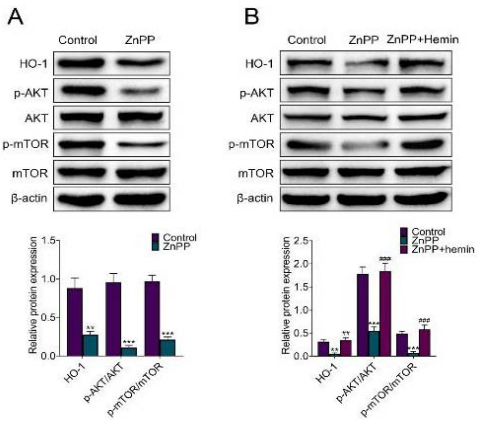

Figure 4: ZnPPIX suppressed HO-1-mediated AKT/mTOR activation in paclitaxel-resistant SW480 cells. (A) The effect of ZnPPIX on protein expression of HO-1, AKT, p-AKT, mTOR, and p-mTOR in paclitaxel-resistant SW480 cells. (B) The effects of ZnPPIX and hemin on protein expression of HO-1, AKT, p-AKT, mTOR, and p-mTOR in paclitaxel-resistant SW480 cells. **, ## $p < 0.01$. ***, ### v $p < 0.001$

ZnPPIX suppressed HO-1-mediated AKT/mTOR activation in paclitaxel-resistant SW480 cells

Protein expressions of HO-1 and phosphorylated AKT and mTOR were lower in paclitaxel-resistant SW480 cells post-incubation with ZnPPIX (Figure 4 A). Moreover, incubation with 1 μM hemin attenuated the ZnPPIX-induced decrease protein expression and p-AKT and p-mTOR (Figure 4 B), indicating that ZnPPIX suppressed HO-1-mediated AKT/mTOR activation in paclitaxel-resistant SW480 cells.

DISCUSSION

Drug resistance is the major reason for the limited clinical outcomes of chemotherapies and targeted therapies for CRC [11]. The CRC exhibits relatively low sensitivity to chemotherapeutic agent, paclitaxel, due to drug resistance [12]. Enhancement of the chemosensitivity of CRC cells is a promising approach for the treatment of CRC [13]. Zinc protoporphyrin IX, an anti-angiogenesis factor in CRC [7], enhances the chemosensitivity of CRC cells to pirarubicin [10]. The effect and mechanism of ZnPPIX on paclitaxel-resistant CRC cells were investigated in this study.

A previous study showed that paclitaxel suppressed the proliferation of SW480 cells, with IC_{50} values of 0.26 μM for SW480 cells and 2.94 μM for paclitaxel-resistant SW480 cells [14], thereby indicating resistance of SW480 cells to paclitaxel. In this study, paclitaxel-resistant SW480 cell model was established, and the results showed that the IC_{50} of paclitaxel-resistant SW480 was 2.58 μM, while the IC_{50} of SW480 was 0.23 μM. Incubation with ZnPPIX aggravated paclitaxel-induced decrease of cell viability in paclitaxel-resistant SW480, and promoted the cell apoptosis.

It was shown that apoptosis contributed to the cytotoxic effect of paclitaxel against CRC cells. It was also found that paclitaxel-induced apoptosis was suppressed in paclitaxel-resistant CRC cells [3]. This study showed that combination of ZnPPIX and paclitaxel led to a higher percent of apoptosis of paclitaxel-resistant SW480 cells than ZnPPIX or paclitaxel alone, demonstrating that that ZnPPIX enhanced the cytotoxic effect of paclitaxel on paclitaxel-resistant CRC cells. The cytotoxicity of ZnPPIX on SW480 cells was shown to involve increased oxidative stress [5].

In chemoresistant CRC cells, DNA damage repair mechanisms are altered, thereby exhibiting resistance to chemotherapeutic agents

[15]. Deoxyribonucleic acid damage repair was enhanced in 5-fluorouracil-resistant CRC cells. However, the DNA damage repair system was suppressed in paclitaxel-resistant breast cancer cells. In this study, incubation with both ZnPPIX attenuated paclitaxel-induced increase of p-DNA-PK and Rad51, while aggravated paclitaxel-induced increase of γ-H2AX, suggesting that ZnPPIX disrupted the DNA damage repair system in paclitaxel-resistant CRC cells, thereby increasing the cytotoxic effect of paclitaxel.

Zinc protoporphyrin IX was shown to inhibit HO-1, and enhanced sensitivity of CRC cells to pirarubicin [10]. Protein expression of HO-1 in paclitaxel-resistant SW480 cells was reduced upon incubation with ZnPPIX. HO-1 inhibited tumor apoptosis and to increase the survival rate and drug resistance of breast cancers through activation of the AKT/mTOR pathway [16]. Moreover, activation of the PI3K/AKT/mTOR pathway contributed to paclitaxel-induced resistance of cancer cells, and inhibition of PI3K/AKT/mTOR-attenuated chemotherapy resistance [17]. In this study, treatment with ZnPPIX reduced p-AKT and p-mTOR in paclitaxel-resistant SW480 cells, suggesting that ZnPPIX suppressed activation of the AKT/mTOR pathway in paclitaxel-resistant CRC cells. Moreover, hemin, an inducer of HO-1, attenuated the ZnPPIX-induced decreased AKT and mTOR phosphorylation in paclitaxel-resistant SW480 cells. These data suggest that ZnPPIX improved the sensitivity of CRC cells to paclitaxel by inactivation of the AKT/mTOR pathway through inhibition of HO-1.

CONCLUSION

ZnPPIX lowers paclitaxel-induced resistance of CRC cells by suppressing cell proliferation, promoting cell apoptosis, and disrupting DNA damage repair. The HO-1-induced activation of AKT/mTOR is suppressed by ZnPPIX in paclitaxel-resistant CRC cells. The combined strategy of using ZnPPIX and paclitaxel has promise for the treatment of CRC.

DECLARATIONS

Conflict of Interest

No conflict of interest 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. Quanfu Li and Tian Xia designed the study and supervised the data collection. Guowei Yang analyzed and interpreted the data. Houlai Yang and Juan Zhang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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