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RESEARCH

Mitochondrial disruption resulting from Cepharanthine-mediated TOM inhibition triggers ferroptosis in colorectal cancer cells

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Abstract

Background Chemotherapy for colorectal cancer (CRC) urgently needs low-toxicity and highly effective phytomedicine. Cepharanthine (Cep) shown to have multiple anti-tumor effects, including colorectal cancer, whose pivotal mechanisms are not fully understood. Herein, the present work aims to reveal the impact of Cep on the mitochondrial and anti-injury functions of CRC cells.

Methods The TOM70/20 expression was screened by bioinformatic databases. SW480 cells were utilized as the colorectal cancer cell model. The expression of TOM70/20 and the downstream molecules were measured by western blots (WB). The ferroptosis was analyzed using Transmission electron microscopy (TEM), C11-BODIPY, PGSK, and DCFH-DA probes, wherein the detection was performed by flow cytometry and laser confocal microscopy. The anti-cancer efficacy was conducted by CCK-8 and Annexin-V/PI assay. The rescue experiments were carried out using Fer-1 and TOM70 plasmid transfection.

Results Bioinformatic data identified TOM20 and TOM70 were highly expressed in colorectal cancer, which could be down-regulated by Cep. Further findings disclosed that Cep treatment destroyed the mitochondria and inactivated the NRF2 signaling pathway, an essential pathway for resistance to ferroptosis, thereby promoting reactive oxygen species (ROS) generation in CRC cells. As a result, prominent ferroptosis could be observed in CRC cells in response to Cep, which thereby led to the reduced cell viability of cancer cells. On the contrary, recovery of TOM70 dampened the Cep-elicited mitochondria damage, ferroptosis, and anti-cancer efficacy.

Conclusion In summary, Cep-mediated TOM inhibition inactivates the NRF2 signaling pathway, thereby triggering ferroptosis and achieving an anti-colorectal cancer effect. The current study provides an innovative chemotherapeutic approach for colorectal cancer with phytomedicine.

Keywords TOM70 · NRF2 · Ferroptosis · Colorectal cancer (CRC) · Mitochondrial function · Cepharanthine (Cep)

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Abbreviations

Cep	Cepharanthine
CRC	Colorectal cancer
COAD	Colon adenocarcinoma
READ	Rectum adenocarcinoma
WB	Western blotting
PI	PropidiumIodide
MFI	Mean fluorescence intensity
TOM70	Translocase of outer membrane 70 kDa subunit
TOM20	Translocase of outer membrane 20 kDa subunit
ROS	Reactive oxygen species
TIMER	Tumor IMmune Estimation Resource
GEPIA	Gene Expression Profiling Interactive Analysis
TEM	Transmission electron microscopy

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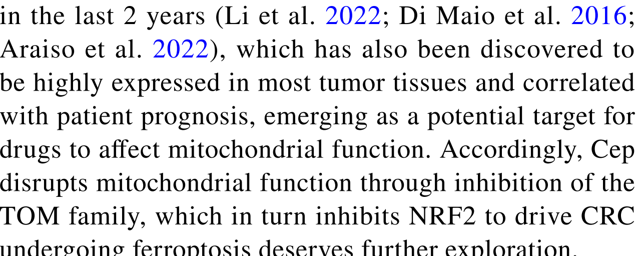
Introduction

Oxidative stress is the fundamental function of cells, which has important regulatory roles in cell death, proliferation, and even differentiation and motility (Jaganjac et al. 2022; Denu and Hematti 2021; Lin et al. 2023). Oxidative stress serves as an important mediator in triggering tumor chemotherapy, photodynamic therapy (PDT) & immunotherapy (Huang et al. 2021; Garg et al. 2011; Wan et al. 2023), plus an important endogenous cause of malignancy cell damage (Hayes et al. 2020). Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive system, which is showing an increasing incidence trend year by year all over the world (Siegel et al. 2023; Brody 2015). Patients with progressive CRC are more likely to be resistant to conventional treatment modalities, and experience recurrence and metastasis, leading to poor prognosis (Shinji et al. 2022). The induction of oxidative stress mentioned above is an essential pathway for the modulation of the function of CRC cells. Numerous reports have now identified that oxidative stress has a major regulatory effect on the pathogenesis, progression, and metastasis of CRC (Liu et al. 2022, 2023b; Lv et al. 2023; Dong et al. 2022). In this regard, the pattern of the novel cell death model, ferroptosis, in CRC cells in case of excessive hyperactivity of reactive oxygen species (ROS), and caused by an imbalance in the antioxidant system would be significant for the inhibition of CRC growth.

Cepharanthine (Cep) is an effective anti-tumor chemotherapeutic agent, which our team and some preliminaries have found its inhibitory effect on the growth of some malignant tumors (e.g., lung cancer) as well (Lu et al. 2023; Shen et al. 2022; Liu et al. 2023a), where it could damage DNA by inducing the generation of ROS (Yang et al. 2023), yet there was no systematic mechanistic study in CRC. In our concurrent study, Cep was revealed to suppress the antioxidant system in several cancer cells, mainly dominated by NF-E2-related factor 2 (NRF2), which has been reported to be activated by ROS or tissue damage (Sánchez-Ortega et al. 2021; Chen 2022). Generally, NRF2 is highly expressed in cancer cells, which may cause organelle or DNA damage in malignant cells if NRF2 is not functioning properly, which in turn results in various types of death (ferroptosis or pyroptosis) (Anandhan et al. 2023; Chen et al. 2023; Huang et al. 2023).

As reported in the literature, the body's antioxidant system is regulated by mitochondrial function (Balaban et al. 2005). Thus, mitochondrial dysfunction may contribute to the inhibition of NRF2. The TOM protein family represents an important molecule capable of regulating mitochondrial homeostasis that has been discovered 
in the last 2 years (Li et al. 2022; Di Maio et al. 2016; Araiso et al. 2022), which has also been discovered to be highly expressed in most tumor tissues and correlated with patient prognosis, emerging as a potential target for drugs to affect mitochondrial function. Accordingly, Cep disrupts mitochondrial function through inhibition of the TOM family, which in turn inhibits NRF2 to drive CRC undergoing ferroptosis deserves further exploration.

The present study initially screened CRC cell lines with high expression of TOM20 and TOM70, before verifying the inhibitory effect of Cep on TOM and the consequent disruption of mitochondria. Then the modulation of NRF2 signaling by mitochondrial disruption in CRC cells was investigated with its subsequent ferroptosis and anti-CRC efficacy was validated. Lastly, recovery experiments were carried out to demonstrate the important role of TOM proteins in Cep-induced mitochondrial disruption and anti-tumor efficacy. This study constitutes an innovative discovery by revealing the role of Cep in the regulation of the TOM molecules, which was found to intervene in the intracellular oxidative stress of CRC and trigger ferroptosis.

Materials and methods

CRC cells culture and Cep treatment

The SW480, LOVO, HCT116, and SW620 cell lines were cultured as the CRC cell models. Alternatively, the NCM460 cell line was utilized as the normal intestinal epithelial cell model, which was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences in China. All kinds of cells were nurtured in Dulbecco's Modified Eagle Medium added with 10% fetal bovine serum (QmSuero/Tsingmu Biotechnology, Wuhan), which were incubated In a constant temperature and humidity environment (5% CO_2/95% air, 37 °C.). The Cep harnessed in the present work was purchased from Macklin (481–49-2, Macklin, Shanghai, China, 98% purity).

Screening for TOM molecules expression

The TOM70 and TOM20 expression in pan-cancer and CRC tissues, including colorectal adenocarcinoma (COAD) and rectum adenocarcinoma (READ) was analyzed using the differential gene expression analysis in Tumor, Normal, and Metastatic tissues (TNMplot) website (https://tnmplot.com/analysis/) and Gene Expression Profiling Interactive Analysis (http://gepia2.cancer-pku.cn/#index) databases. Then the gene expression was presented. Furthermore, the

TOM70 and TOM20 expression in CRC cells and normal intestinal epithelial cells was detected using western blots. In the following experiments, the SW480 cells, which have higher TOM70 and TOM20 expression, were utilized as the major CRC cell model.

Cep treatment for CRC cells

The concentrations and processing time of Cep for treating CRC cells were screened by CCK-8 experiments. Briefly, the cells seeded in 96-well plates were treated with Cep (different concentrations) for 24 h and then incubated with CCK-8. The absorbance at 450 nm was assayed with a fluorescence spectrophotometer. The effective Cep concentrations were 5 μM and higher concentrations. Hence, in the following research, the cells were treated with Cep (5, 10, 20 μM) for 24 h.

Mitochondria damage assay

The expression of TOM70 and TOM20 in CRC cells was detected by western blots, which was further described. The variation of membrane potential was measured by JC-1 probe labeling. The JC-1 polymers (red fluorescence) and monomers (green fluorescence) in CRC cells were detected using flow cytometry and laser confocal microscopy. The generation of superoxide within cells was stained with mito-sox and then observed by laser confocal microscopy.

NRF2 signaling and redox function analysis

For detection of the NRF2 expression, the proteins extracted from CRC cells were used to carry out the western blots assay. The contents of catalase (CAT), superoxide dismutase (SOD) were detected using corresponding kits (S0051 and S0101S, Beyotime). The GPX4 expression of measured by western blots. The cells labeled with DCFH-DA probe were harnessed to detect the ROS generation with flow cytometry. Additionally, the intracellular superoxide accumulation was observed with laser confocal microscopy (FV3000RS, Olympus, Japan, Hubei University of Medicine) in cells stained with mito-sox probe.

Ferroptosis detection

For the detection of CRC cells' ferroptosis, the expression of biomarkers (TfR-1 and ACSL4) was analyzed using western blots. In addition, the cells labeled with the C11-BODIPY probe were harvested for flow cytometry. The decreased PE fluorescence and increased FITC fluorescence suggested the LPO generation in the membrane according to the principle. Furthermore, the fixed cells were dehydrated and prepared as ultrathin slices before observation with TEM. The iron ions in the cells were detected with the PGSK probe, which could be quenched by the elevated iron ions. The PGSK fluorescence (FITC) of CRC cells was measured by flow cytometry. The colorectal cancer cells were fixed, wherein the morphology of mitochondria was observed using transmission electron microscopy (TEM).

Cell viability assay of CRC cells

To assay the cell viability of CRC cells, the CCK-8 kits were utilized to incubate the treated cells before detection using the absorbance in 450 nm. Alternatively, the cells were seeded in the six-well plate for culturing 7 days. The cell cloning formation was stained with crystalline purple for measurement. Additionally, The expression of proliferation and apoptosis proteins (PCNA, Bax, etc.) was assayed by western blots. Finally, the cell death of harvested CRC cells, which were labeled by Annexin-V/PI, was measured by flow cytometry.

Overexpression of TOM70

The CRC cells were seeded in 24 or 6-well plates and treated with TOM70 plasmid and Lipofectamine 8000 (2:1 at volume) for 10 h. Then the conditioned medium including plasmid and lipofectamine was replaced by a fresh medium. The successful overexpression of TOM70 was validated by the elevated expression of TOM70 measured by qRT-PCR and western blots. The TOM70 overexpression cells were then treated with Cep. The ferroptosis and cell viability were assayed.

Flow cytometry

The harvested CRC cells were washed and then stained with probes described above (JC-1, PGSK, C11-BODIPY, etc.). The fluorescence-labeled cells were collected by flow cytometry. At least 1×10^4 cells per sample were acquired for every collection. Each channel was adjusted to the appropriate voltage before collecting the cells. Geometric means (GM) were used to quantify the mean fluorescent intensity (MFI). Additionally, the excitation and emission wavelengths in the FITC channel were 488 and 525 nm, respectively, and those in the PE channel were 561 and 585 nm, respectively.

Western blots

The proteins extracted from CRC were treated with RIPA and protease inhibitors for 60 min, which were further centrifuged to obtain the supernatant. The concentration of proteins was assayed using a bicinchoninic acid (BCA) assay kit before western blots. Equal amounts of protein (10–30 µg) were fractionated by SDS-PAGE electrophoresis. The proteins within the SDS-PAGE were transferred to the PVDF membranes, which were blocked with 3–5% BSA and then incubated with primary antibodies against Bax (GTX109683, GeneTex, Taiwan, China), Caspase-3 (GTX110543, GeneTex, Irvine, California, USA), PCNA (bs-2006R, Bioss, Beijing, China), Ki67 (bs-23103R, Bioss, Beijing, China), TOM70 (14,528–1-AP, Proteintech, Wuhan, China), TOM20 (11,802–1-AP, Proteintech, Wuhan, China), GPX4 (#52,455, Cell Signaling, Danvers, Massachusetts, USA), ACSL4 (YT8070, Immunoway, Newark, DE, USA), TfR-1 (YT5374, Immunoway, Newark, DE, USA), NRF2 (16,396–1-AP, Proteintech, Wuhan, China), and GAPDH (PMK053C, BioPM, Wuhan, China) at 4 °C overnight. The primary-incubated membranes were then treated with horseradish peroxidase-conjugated secondary antibody. Finally, the protein bands were treated with an ECL kit (PMK003, BioPM, Wuhan, China) and exposed to a Bio-Imaging system (170–8265, Bio-Rad).

Statistical analysis

The generated data in the present work were exhibited using the mean ± standard deviation (SD). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ were considered statistically significant.

Results

Cep could inhibit TOM proteins and thereby destroy the function of mitochondria

As the TOM molecules of mitochondrial outer membrane proteins are closely associated with tumor development and pharmacological treatment (Li et al. 2022; Di Maio et al. 2016; Araiso et al. 2022), we then proceeded to explore whether TOM molecules are highly expressed in tumors, including those within CRC. As expected, both TOM70 and TOM20 expression was higher in almost most tumor tissue compared with the normal tissues, with no exception in COAD and READ (Fig. 1A–D). Correspondingly, the expression of TOM70 and TOM20 was examined in one normal intestinal epithelial cell line (NCM460) and 4 malignant colorectal cancer cell lines (SW480, LOVO, HCT116, SW620). Agreed with the results of bioinformatics, the TOM molecules showed obvious high abundance in malignant CRC cells (Fig. 1E–G). Both SW480 and SW620 cells were identified for subsequent experiments based on the results of TOM70 and TOM20 expression. To explore whether Cep has a regulatory effect on the TOM molecules in CRC cells, the TOM70 and TOM20 expression of Cep-treated SW480 was measured. The reduced expression of TOM molecules in SW480 and SW620 cells demonstrated that Cep could inhibit TOM proteins (Figs. 2A–C, S1). Not surprisingly, the CRC cells' mitochondrial functions were destroyed in response to Cep, as confirmed by the decreased membrane potential detected by flow cytometry and confocal microscopy (Fig. 2D–F). In a nutshell, evidence has emerged suggesting an essential role of Cep in regulating mitochondrial functions through inhibiting TOM molecules.

Cep suppressed the NRF2 signaling pathway of anti-ferroptosis

The mitochondrial impairment caused by Cep may further disrupt the cellular antioxidant system, of which NRF2 is the most important antioxidant system in cancer cells (Sajadimajd and Khazaei 2018). We therefore followed with a network pharmacology assay of the interaction target of Cep and CRC first. In accordance with our speculation, Cep treatment down-regulated the NRF2 in SW480 and SW620 cells as presented in Fig. 3A, B. Based on the suppression of the anti-oxidative ability of colorectal cancer, the representative productions were measured. As presented, intracellular anti-oxidative GPX4 (Figs. 3A, C and S2), CAT (Fig. 3D), and SOD (Fig. 3E) were inhibited in the presence of Cep.

We therefore further investigate the superoxide (Fig. 3F) and ROS accumulation (Fig. 3G, H) and, two kinds of classical oxidative damage production, in colorectal cancer, which was up-regulated in response to the Cep. These results were strong proof that Cep treatment significantly regulated the redox system. Altogether, the present experiments revealed that Cep was a kind of powerful inhibitor for blocking NRF2, which further led to the depression of reduction and antioxidant systems in CRC cells.

Cep induced ferroptosis and thereby exhibited anti-*cancer* efficacy

To provide further confirmation of whether inhibition of NRF2, which was induced by Cep, can activate ferroptosis of CRC, the iron content, lipid peroxidation (LPO), and critical

Fig. 1 The expression of TOM70 and TOM20 was elevated in colorectal cancer. **A–D** The TOM70 and TOM20 expression was presented in tumor tissues and normal tissues, especially COAD and READ, analyzed using the TNMplot and GEPIA databases. **E–G** The expression of TOM70 and TOM20 in the normal intestinal epithelial cell line (NCM460) and four malignant colorectal cancer cell line (SW480, LOVO, HCT116, SW620) was detected by western blots. The mean gray of bands was quantitative measured. Values were means ± SD (n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

molecules of ferroptosis were detected in CRC cells. Firstly, as displayed in Fig. 4A, C–E, the biomarkers of ferroptosis (TfR-1, ACSL4, and COX-2) were driven in the presence of Cep. In agreement with these results, Cep-treated colorectal cancer cells displayed accumulated intracellular iron ions, as evidenced by the enhanced FeRhoNOX-1 fluorescence and decreased PGSK fluorescence, which indicated the increased iron ions (Fig. 4B and Fig. S3). Most importantly, TEM observation revealed Cep treatment promoted the atrophy of the mitochondria in the colorectal cancer cells (Fig. 4F, G). What's more, another evidence suggestive of ferroptosis was the significantly elevated LPO in SW480 and SW620 cells treated by the Cep (Figs. 4H, I and S4). Thus, the above data strongly spoke that Cep induced vigorous ferroptosis of colorectal cancer cells.

Building on this foundation, our efforts further investigated the efficacy of Cep-mediated anti-CRC efficacy. Firstly, the viability assay experiment exhibited that the Cep incubation decreased the viability of SW480 and SW620 cells as detected by the CCK-8 experiment (Figs. 5A and S5), which was also corroborated by the depressed proliferation of colorectal cancer cells validated by reduced cell

Fig. 2 Cep treatment depressed TOM20 and TOM70, destroying the function of mitochondria. **A–C** The expression of TOM20 and TOM70 in Cep-treated CRC cells was measured using western blots. The mean gray of bands was quantitatively analyzed. **D–F** The mitochondrial membrane potential was labeled by JC-1 probe and measured by flow cytometry (**D**, **E**) and laser confocal microscopy (**F**). Geometric means were used to quantify the MFI. Values were means ± SD (n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

migration and colony formation (Fig. 5B, G and D, H). More impressively, the expression of the death-associated molecule (Bax) elevated, and the proliferation-associated molecule (PCNA) down-regulated in the presence of Cep (Fig. 5C, E, F). Furthermore, increased Annexin-V positive colorectal cancer cells with the treatment of Cep indicated the anti-cancer efficacy of Cep (Fig. 5I, J).

Fig. 3 In vitro experiments showed that Cep inhibited NRF2 signaling pathway. **A–C** The expression of NRF2 and GPX4 in CRC cells was detected using western blots, whose mean gray was calculated. **D-E:** The intracellular catalase (CAT) and superoxide dismutase (SOD) were assayed by reagent kit. **F** The superoxide within mitochondria was detected by mito-sox prove and observed with laser confocal microscopy. **G**, **H** The ROS generation of CRC cells stained with DCFH-DA probe was measured by flow cytometry. MFI: mean geometric fluorescence intensity. Values were means ± SD (n = 3, *** p < 0.001, **** p < 0.0001)

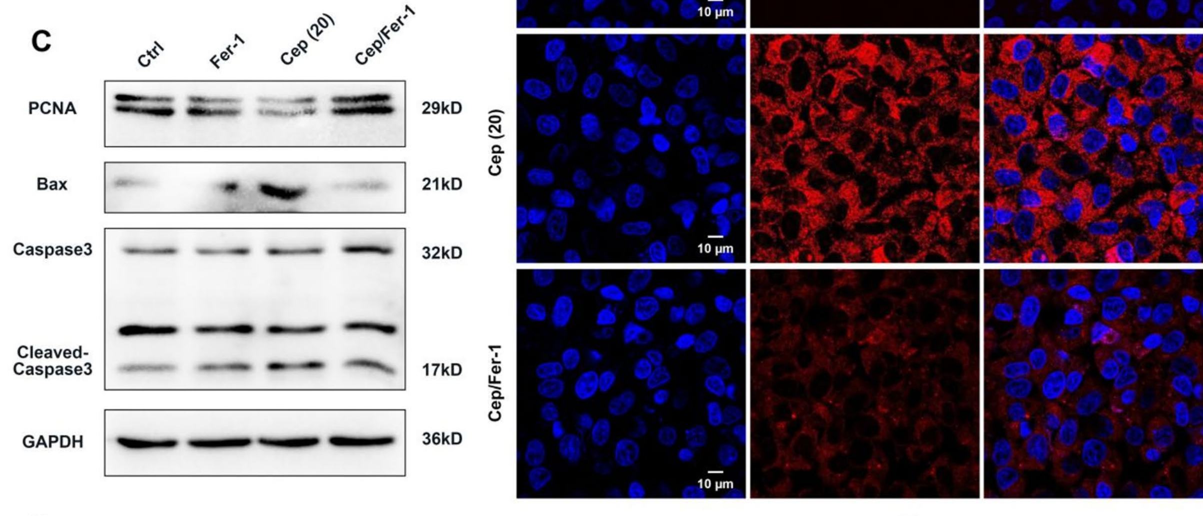
Since Cep can also destroy tumor cells through multiple mechanisms (Shahriyar et al. 2018). Hence, to deeply explore whether the Cep-induced anti-CRC efficacy is derived from ferroptosis, logical validations using ferroptosis inhibitors were carried out. As expected, Fer-1, a kind of ferroptosis inhibitor, which could effectively dampen the LPO generation (Fig. S6A-B), TfR-1 up-regulation, and GPX4 inhibition (Fig. 6A) induced by Cep, suggesting the effectiveness of Fer-1. On this basis, the oxidative stress triggered by Cep was mitigated, as characterized by the reduced mito-sox fluorescence (Fig. 6B), ROS (Fig. S6C, D), and JC-1 monomers (Fig. S6E). Notably, Fer-1 treatment dampened the suppression of PCNA (Fig. 6C), up-regulation of Bax and Cleaved-caspase-3 (Fig. 6C), slowed cell migration (Fig. 6D-E), increased apoptosis rate (Fig. 6F-G), and attenuated colony formation (Fig. S7) induced by Cep, confirming Cep-mediated ferroptosis was the critical effect for the anti-colorectal cancer therapy.

Rescue of TOM70 weakened the Cep-driven ferroptosis and anti-*cancer* effect

To confirm the essential function of TOM70 in Cep-mediated ferroptosis and anti-cancer efficacy, TOM70 was over-expressed in CRC cells for in-depth explorations. The

Fig. 4 Cep triggered ferroptosis in colorectal cancer cells. A, C–E The expression of ferroptosis's biomarkers (TfR-1, ACSL4 and COX-2) was identified using western blots. The mean gray of bands was measured. **B** The iron ions within colorectal cancer cells labeled with FeRhoNox-1 probe were observed by laser confocal microscopy. **F**, **G** The morphology of mitochondria was observed with TEM, wherein atrophic mitochondria indicated the ferroptosis. **H**, **I** The LPO production within CRC cells was detected with C11-BODIPY probe. The FITC fluorescence, which indicated the LPO generation, was calculated through flow cytometry. Values were means ± SD (n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Fig. 5 Cep possessed vigorous anti-colorectal cancer efficacy. **A** CCK-8 experiment was carried out to assay the cell viability of CRC cells. **B**, **G** The migration of CRC cells was verified using scratch test. **D**, **H** The anti-cancer efficacy was also detected with clone formation experiment. **C, E, F:** The cell death-associated proteins (Bax), proliferation-associated protein (PCNA) were detected by western blots, which were further quantitatively measured. **I**, **J** The cell death was analyzed by Annexin-V/PI staining, which was further detected by flow cytometry. MFI: mean geometric fluorescence intensity. Values were means ± SD (n = 3, *** p < 0.001, **** p < 0.0001)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Cep (20)
Cep/Fer-1
10 μm
C
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Fer-1
Cep (20)
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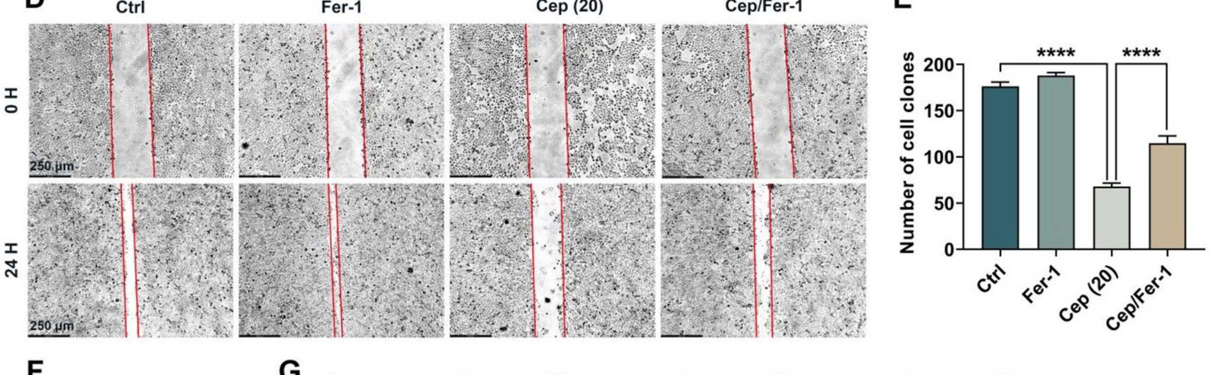
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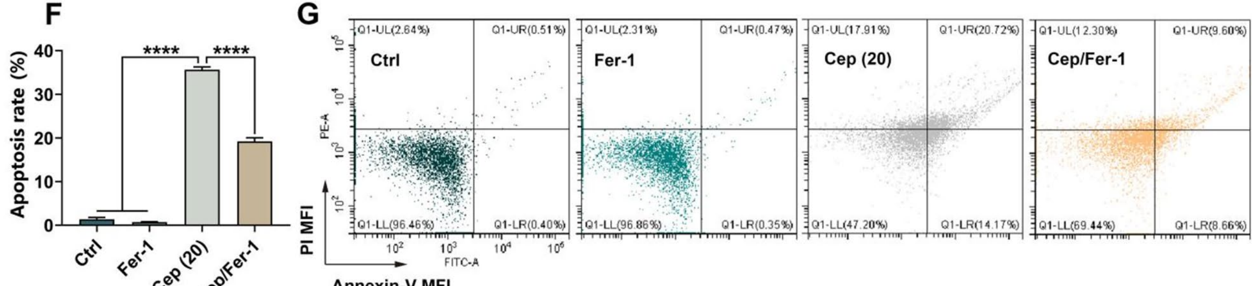
F
Apoptosis rate (%)
****
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Ctrl
Fer-1
Cep (20)
Cep/Fer-1
G
Ctrl
Q1-UL(2.64%)
Q1-UR(0.51%)
Q1-LL(96.46%)
Q1-LR(0.40%)
Fer-1
Q1-UL(2.31%)
Q1-UR(0.47%)
Q1-LL(96.86%)
Q1-LR(0.35%)
Cep (20)
Q1-UL(17.91%)
Q1-UR(20.72%)
Q1-LL(47.20%)
Q1-LR(14.17%)
Cep/Fer-1
Q1-UL(12.30%)
Q1-UR(9.60%)
Q1-LL(69.44%)
Q1-LR(8.66%)
PE-A
FITC-A
PI MFI
Annexin-V MFI

◂**Fig. 6** Inhibition of ferroptosis dampened anti-colorectal cancer efficacy induced by Cep. The CRC cells were co-treated by ferroptosis inhibitor (Fer-1) and Cep. **A** The biomarkers of ferroptosis (TfR-1 and GPX4) were analyzed by western blots. **B** The superoxide within CRC cells was detected by mito-sox fluorescence, which was observed using laser confocal microscopy. **C**: The expression of Bax, Caspase-3, and PCNA was measured by western blots. **D**, **E** The migration of CRC cells was measured by scratch test. The wound closure was calculated. **F**, **G**Annexin-V/PI staining and flow cytometry were utilized to assay the cell death. *MFI* mean geometric fluorescence intensity. Values were means ± SD (n = 3, *** p < 0.001, **** p < 0.0001)

successful over-expression of TOM70 (OE-TOM70) in SW480 CRC cells was verified by up-regulation of TOM70 in OE-TOM70 group compared with control CRC cells (Fig. 7A). The results of rescue experiments confirmed that over-expression of TOM70 attenuated Cep-induced ferroptosis, as evidenced by the decreased TfR-1 expression, ACSL4 expression, and increased GPX4 expression in Cep-treated OE-TOM70 CRC cells compared with Cep-treated control CRC cells (Fig. 7A). In addition, OE-TOM70 also dampened the iron ions in Cep-incubated CRC cells as presented in Fig. 7B. The above data suggested that the recovery of TOM70 dampened Cep-triggered ferroptosis. Furthermore, TOM70 replenishment attenuated the anti-cancer efficacy of Cep, as evidenced by the accelerated cell migration in the presence of OE-TOM70 and Cep treatment in comparison to Cep monotreatment (Fig. 7C-D). More data evidence corroborated this, wherein diminished apoptosis in the OE-TOM70 and Cep group could be observed (Fig. 7E-F). In conclusion, the above proof demonstrated that recovery of TOM70 weakened the anti-colorectal cancer effect induced by Cep. Taken together, TOM70 was the critical molecule for Cep-induced ferroptosis and anticancer efficacy in colorectal cancer cells.

Discussion and conclusion

This cutting-edge study presents that Cep inhibits TOM molecules and destroys the mitochondria, thereby abolishing the anti-oxidative function of NRF2, resulting in ferroptosis in colorectal cancer cells (Fig. 8).

Mitochondrial function generally is strongly relevant to cellular homeostasis, for instance, in neurons where it has an important role (Pekkurnaz and Wang 2022). Nevertheless, tumor cells tend to be more robust than normal cells, which may also be attributed to their abnormally vigorous mitochondria. Many explorations have also been done by scientists to investigate the differences between the mitochondria of tumor cells and normal cells, resulting in the discovery that a variety of proteins in the outer membrane of the mitochondria maintain mitochondrial stability (Li et al. 2022; Fu et al. 2020). Rather, it happens that TOM70 and TOM20 of the TOM family are aberrantly overexpressed in many tumor species, with no exception in CRC (Fig. 1). The present study coincided with the findings that Cep inhibited TOM series proteins, which in turn disrupted mitochondrial homeostasis, thus both unveiling and elucidating the pivotal reason for the regulatory action of Cep on mitochondria.

There have been several reports on how mitochondria sustain cellular homeostasis. A number of investigations have suggested that mitochondria can remove intracellular ROS and prevent cellular aging (Guo et al. 2023; Janikiewicz et al. 2018). So the question is, how do mitochondria clear ROS? Recent studies have found that mitochondria can activate the cellular antioxidant system, which is represented by NRF2 signaling, with this phenomenon being more prominent in tumor cells. Tumor cells tend to be highly resistant to damage, which is dependent on the function of this antioxidant molecule. NRF2 and some other antioxidant molecules showed high expression in most of the tumor tissues and thus had a strong survival capacity (Härkönen et al. 2023). During chemotherapy of tumors, often severe resistance is caused by long-term use of chemotherapeutic agents that may further up-regulate antioxidant molecules. On the contrary, Cep can inhibit the function of NRF2 by destroying mitochondria when killing tumors and directly weaken the antioxidant capacity of malignant cells, making it very beneficial for chemotherapy of tumors. One of our previous reports revealed that Cep synergized photodynamic therapy and rescued its resistance, which may also have some relevance to this mechanism (Yang et al. 2023). There must be other novel discoveries in the future of Cep in combination with other drugs in the treatment of malignant tumors.

In addition, since ferroptosis has been reported to greatly increase cellular immunogenicity (Han et al. 2023), which implies that Cep may change the immune properties of the cancer tissues, thereby allowing more immunocytes to infiltrate locally into the tumor immune microenvironment and reversing immunosuppressed immunocytes to an immune-activated phenotype. Therefore, investigating the regulation of the tumor immune microenvironment by Cep in the future will be a very promising topic.

In summary, Cep-mediated TOM inhibition suppresses the anti-oxidative stress signaling pathway, thereby triggering ferroptosis and achieving an anti-colorectal cancer effect (Fig. 8). The current study provides an innovative chemotherapeutic approach for colorectal cancer with phytomedicine.

Fig. 7 Recovery of TOM70 reduced the ferroptosis and anti-cancer efficacy induced by Cep. The SW480 CRC cells were transfected with TOM70 plasmid before incubating with Cep. **A** The expression of TOM70, TfR-1, ACSL4, GPX4, and Bax was detected with western blots. **B:** The iron ions within CRC cells were determined by FeRhoNOX-1 probe. **C**, **D** The migration of CRC cells was measured by scratch test. The wound closure was calculated. **E**, **F** Annexin-V/PI staining and flow cytometry were utilized to assay the cell death. MFI: mean geometric fluorescence intensity. OE-TOM70: Overexpression of TOM70. Values were means ± SD (n = 3, *** p < 0.001, **** p < 0.0001)

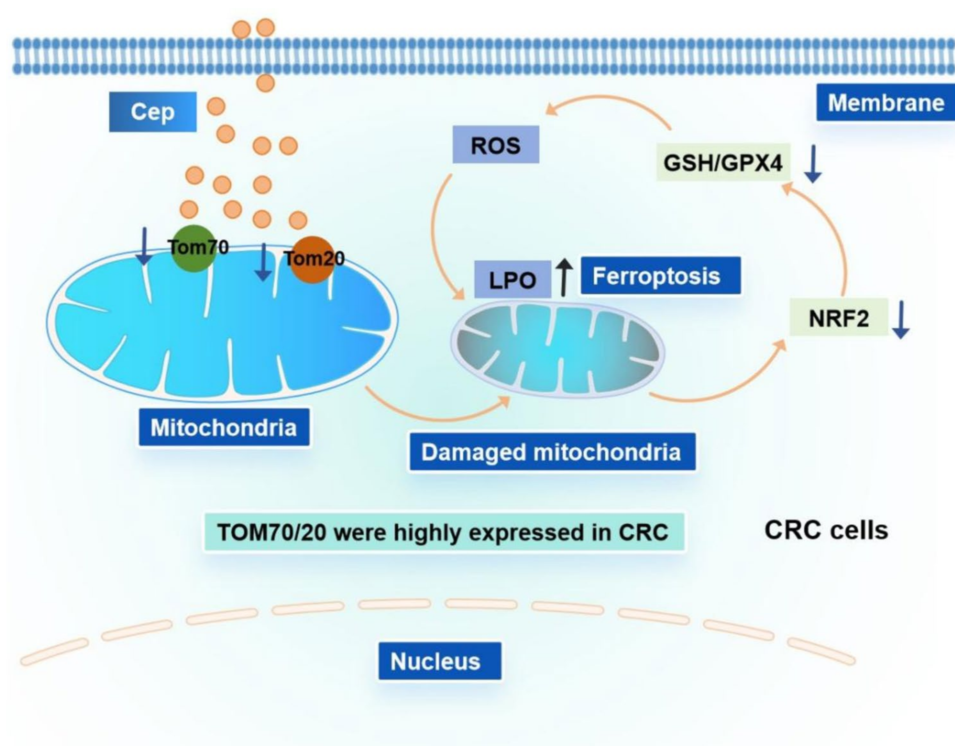

Fig. 8 The working diagram of Cep in the treatment of colorectal cancer cells

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Author contributions Liu-Gen Li, Di Zhang, and Qi Huang carried out the in vitro experiments. Min Yan, Nan-Nan Chen, Rong-Cheng Xiao, and Yan Yang made data analysis. Ning Han, Abdul Moiz Qureshi, and Hui Liu conducted the methodology. Yuan-Jian Hui provided the funding for research. Yuan-Jian Hui and Jun Hu conceptualized the work. Yuan-Jian Hui and Fan Leng wrote the manuscript. Yuan-Jian Hui and Fan Leng reviewed the original manuscript.

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Availability of data and material No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication All the authors agree for the publication.

Specific statement The present study was carried out in compliance with the journal guidelines.

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