Leucovorin enhances curcumin-induced inhibition of cell viability and ferroptosis in colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality. Curcumin can inhibit the viability and induce ferroptosis in CRC cells, while leucovorin is known to enhance the cytotoxic effects of fluorouracil in the same cells. Therefore, the present study aimed to investigate whether leucovorin could potentiate curcumin-induced inhibition of cell viability and induce ferroptosis in CRC cells. The CRC cell lines, Caco-2 and HCT116, were treated with 50 μM curcumin, 2 μM leucovorin, a combination of curcumin and leucovorin, or leucovorin pre-treatment followed by co-treatment with curcumin and leucovorin. Cell viability and ferroptosis were assessed after 24 h of treatment. The results indicated that curcumin suppressed the viability of both Caco-2 and HCT116 cells. Furthermore, it also reduced glutathione, mitochondrial membrane potential and the protein expression levels of solute carrier family 7 member 11, while increasing the levels of malondialdehyde, reactive oxygen species, ferrous iron and the expression levels of acyl-CoA synthetase long chain family member 4, thus indicating that curcumin could induce ferroptosis in these cells. Cell treatment with curcumin and leucovorin further suppressed cell viability compared with curcumin alone in both cell lines. Furthermore, this combination, compared with curcumin alone, more notably promoted ferroptosis in HCT116 cells compared with that in Caco-2 cells. However, pre-treatment with leucovorin followed by co-treatment with curcumin and leucovorin had no further effect on cell viability or ferroptosis compared with the synchronous treatment of Caco-2 and HCT116 cells with curcumin and leucovorin. In conclusion, leucovorin could potentiate curcumin-induced inhibition of CRC cell viability and ferroptosis. However, additional leucovorin pre-treatment did not display further benefits compared with curcumin and leucovorin co-treatment. The findings of the present study suggested that curcumin and leucovorin may serve as a potential regimen for the treatment of CRC.

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality, accounting for 9.3% of all cancer-related mortality worldwide (1). Patients with CRC are commonly diagnosed at an advanced stage of the disease due to the subtle nature of early-stage CRC (2). Although targeted therapies and immunotherapies have been applied to treat patients with advanced CRC, chemotherapy remains the cornerstone of standard treatment regimens (3,4). However, a notable proportion of patients with advanced CRC gradually develop resistance to chemotherapy (5-7). Therefore, it is necessary to identify novel therapeutic targets to improve the management of CRC. In recent years, studies have explored novel treatment modalities for CRC, such as glucagon-like peptide 1 receptor agonists and the combination of autocrine motility factor peptide and glycyrrhetinic acid (8,9). Potential molecular targets of CRC have also been identified, such as heat shock transcriptional factor 4, E3 ubiquitin ligase-related genes and ribosomal protein L22-like 1 (10-12).

Curcumin, a compound derived from turmeric (*Curcuma longa*), exhibits an extensive range of biological activities, such as anti-inflammatory, anti-diabetic and antitumor effects (13). Emerging evidence has suggested that curcumin can serve as a promising agent for the treatment of CRC. For example, Dal *et al* (14) demonstrated that curcumin could inhibit the viability of CRC cells via activating nucleotide-binding oligomerization domain-like receptor protein 3-mediated pyroptosis. Furthermore, Liu *et al* (15) demonstrated that curcumin increased reactive oxidative species (ROS) in CRC cells, thus further activating the Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2/microRNA-34 axis and suppressing the metastasis of CRC cells.

Ferroptosis is a type of programmed cell death characterized by glutathione deprivation, accumulation of ferrous iron

(Fe^{2+}) and enhanced oxidative stress (16,17). Inducing ferroptosis has been considered as a potential strategy for cancer treatment. For example, allicin, a garlic-derived organosulfide, exhibits the potential to treat nasopharyngeal cancer due to the ability of promoting ferroptosis (18). Progesterone is able to induce ferroptosis, thus promoting the efficacy of niraparib in ovarian cancer (19). Furthermore, it has been extensively reported that curcumin can induce ferroptosis in CRC (20-22).

Leucovorin is commonly incorporated into chemotherapeutic regimens for CRC due to its effect on sensitizing tumor cells to fluorouracil (23,24). Considering this effect of leucovorin, and that curcumin inhibits cell viability and induces ferroptosis in CRC cells, we made a preliminary hypothesis that leucovorin could also sensitize curcumin-mediated ferroptosis in CRC cells. In the present study, CRC cells were treated with the combination of curcumin and leucovorin to investigate the effect of this combination on cell survival and ferroptosis.

Materials and methods

Cell culture. Caco-2 cells (Tongwei Co., Ltd.) and HCT116 cells (iCell Gene Therapeutics, Inc.) were cultured in DMEM (cat. no. 4511; Wuhan Servicebio Technology Co., Ltd.) and RPMI1640 medium (cat. no. G4531; Wuhan Servicebio Technology Co., Ltd.) respectively, supplemented with 10% FBS (cat. no. 164210; Procell Life Science & Technology Co., Ltd.). All cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Curcumin and leucovorin incubation. Initially, Caco-2 and HCT116 cells were cultured with various concentrations of curcumin (0, 5, 10, 20, 50 and 100 μM; cat. no. HY-N0005; MedChemExpress) and leucovorin (0, 1, 2, 4, 8 and 16 μM; cat. no. HY-17556; MedChemExpress) for 24 h. Caco-2 and HCT116 cells were also incubated with combinations of 1, 2, 4, 8 and 16 μM leucovorin, with 50 and 100 μM curcumin in combination for 24 h. Experimental groups established were as follows: Leu, 2 μM leucovorin; Cur, 50 μM curcumin; Leu + Cur, 2 and 50 μM leucovorin and curcumin, respectively, in combination (24 h incubation). Furthermore, Caco-2 and HCT116 cells were pre-treated with 2 μM leucovorin for 24 h, followed by co-treatment with 2 μM leucovorin and 50 μM curcumin (pre-Leu + Leu + Cur group) for an additional 24 h. Caco-2 and HCT116 cells cultured under normal conditions without any treatment served as the control group. The duration of treatment was in accordance with previous studies (25-27).

Ferrostatin-1 (Fer-1) incubation. The Caco-2 and HCT116 cells in Control, Leu, Cur, Leu + Cur and pre-Leu + Leu + Cur groups were incubated with 10 μM Fer-1 (cat. no. HY-100579, MedChemExpress) at 37˚C for 24 h (28). Subsequently, cell viability and Fe^{2+} were analyzed.

Cell viability. Cell viability was evaluated utilizing a Cell Counting Kit-8 assay (CCK-8; Wuhan Servicebio Technology Co., Ltd.). Briefly, Caco-2 and HCT116 cells were cultured in a 96-well plate and treated with curcumin and leucovorin as aforementioned. Following incubation, the cell medium (DMEM for Caco-2, and RPMI-160 for HCT-116 cells) supplemented with CCK-8 reagent was added in each well and cells were cultured in an incubator at 37˚C for 2 h. The optical density (OD) at a wavelength of 450 nm was measured using a microplate reader (Nanjing Huadong Electronics Group Co., Ltd.). The relative cell viability was calculated based on the following formula: Cell viability rate=(OD of experimental groups/OD of the corresponding control group) x100%.

Glutathione (GSH) analysis. Cells were collected at 24 h after treatment with curcumin and leucovorin as aforementioned. Subsequently, following collection, the cells were lysed using repeated freeze-thaw cycles. The cell lysates were centrifuged at 100 x g at 4˚C for 10 min to obtain the cell supernatant. GSH content in cell supernatant was assessed utilizing the corresponding GSH Assay Kit (cat. no. S0053; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The OD was measured using a microplate reader (Nanjing Huadong Electronics Group Co., Ltd.).

Malondialdehyde (MDA) and Fe^{2+}assessment. Briefly, following treatment with the indicated compounds for 24 h, cells were lysed with RIPA Buffer (Wuhan Servicebio Technology Co., Ltd.) for 30 min. The protein concentration in cell supernatant was quantified using a BCA Kit (Wuhan Servicebio Technology Co., Ltd.). MDA and Fe^{2+} levels were assessed using the Lipid Peroxidation MDA Assay Kit (cat. no. S0131S; Beyotime Institute of Biotechnology) and Ferrous Ion Content Assay Kit (cat. no. BC5415; Beijing Solarbio Science & Technology Co., Ltd.), respectively, according to the manufacturer's protocol.

Reactive oxygen species staining. Following incubation for 24 h, ROS levels in CRC cells were calculated utilizing a ROS Assay Kit (cat. no. S0033S; Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Prior to analysis, the working probe was freshly prepared. Cells were then incubated with the working probe at 37˚C for 10 min. Images were captured using an inverted fluorescence microscope (Motic Incorporation, Ltd.).

Mitochondrial membrane potential (MMP) detection. To assess MMP, CRC cells were incubated with MMP Assay Kit with JC-1 (cat. no. C2006; Beyotime Institute of Biotechnology) for 24 h. The JC-1 working solution was prepared according to the manufacturer's instructions. The cells were incubated with the JC-1 working solution for 20 min at 37˚C. After the working solution was removed, images of the stained cells were captured using an inverted fluorescence microscope (Motic Incorporation, Ltd.).

Western blot analysis. Following incubation with curcumin and leucovorin as aforementioned for 24 h at 37˚C, cells were lysed using a RIPA buffer (Wuhan Servicebio Technology Co., Ltd.). To ensure complete lysis, cells in RIPA buffer were scrapped and incubated on ice for 30 min. Following centrifugation at 12,000 x g for 5 min at 4˚C, the cell supernatant was obtained. The protein concentration was measured utilizing a BCA kit (Wuhan Servicebio Technology Co., Ltd.), according to the manufacturer's instructions. After mixing with loading

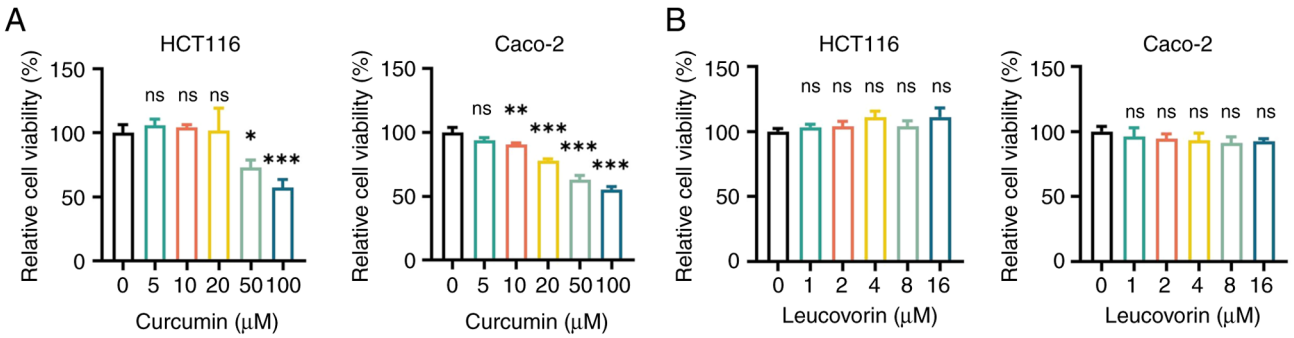

Figure 1. Dose-dependent effect of curcumin and leucovorin on cell viability. (A) Viability of Caco-2 and HCT116 cells treated with concentrations of curcumin. (B) Viability of Caco-2 and HCT116 cells treated with increasing concentrations of leucovorin. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group. ns, not significant. Error bars represent the SD.

buffer, the supernatant was denatured at 98˚C for 5 min. Subsequently, the protein samples (10 μg) were separated using a 10% Precast Gel (Willget) for 30 min, then transferred onto a nitrocellulose membrane (MilliporeSigma) for 90 min. Following by washing with Tris Buffered Saline with Tween 20 (TBST; cat. no. G2150; Wuhan Servicebio Technology Co., Ltd.), the membrane was immersed in 5% BSA (Beyotime Institute of Biotechnology) for 90 min at 37˚C. Subsequently, the membrane was incubated with primary antibodies against solute carrier family 7 member 11 (SLC7A11; 1:1,000; cat. no. GB150180; Wuhan Servicebio Technology Co., Ltd.), acyl-CoA synthetase long chain family member 4 (ACSL4; cat. no. 81196-1-RR; dilution, 1:1,000; Proteintech Group, Inc.) and GAPDH (cat. no. GB15004; 1:5,000, Wuhan Servicebio Technology Co., Ltd.) at 4˚C overnight. After the primary antibodies were discarded, the membrane was incubated with the corresponding secondary antibody (cat. no. GB23303; 1:10,000; Wuhan Servicebio Technology Co., Ltd.) for 90 min at 37˚C. Subsequently, the membrane was washed using TBST and immersed in ECL Reagent (cat. no. MA0186; Dalian Meilun Biology Technology Co., Ltd.). Lastly, the protein bands were visualized on X-ray films. The experiments were conducted in triplicate. The intensity value was measured utilizing ImageJ software (version 1.8.0; National Institutes of Health).

Target gene analysis. The target genes of curcumin and leucovorin were analyzed from the PharmMapper database (https://www.lilab-ecust.cn/pharmmapper/index.html). The common target genes of curcumin and leucovorin were displayed using Cytoscape (version 3.10.3). The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was performed using the Database for Annotation, Visualization and Integrated Discovery tool (https://david.ncifcrf.gov/). The significance of each enrichment item was identified with the cut-off FDR <0.05.

Statistical analysis. All data were expressed as the mean ± SD. The experiments were performed in triplicate. All analyses were performed by GraphPad software (version 9.0; Dotmatics). The differences among groups were compared using one-way ANOVA followed by Dunnett's or Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

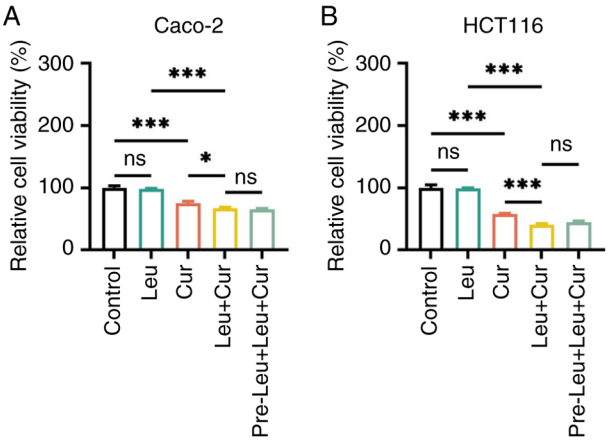

Figure 2. Effect of curcumin and leucovorin on cell viability. Comparison of cell viability among treatment groups in (A) Caco-2 and (B) HCT116 cells. *P<0.05 and ***P<0.001. Leu, leucovorin; Cur, curcumin; ns, not significant. Error bars represent the SD.

Results

Effect of curcumin and leucovorin on cell viability. The results indicated that treatment of Caco-2 and HCT116 cells with curcumin alone significantly suppressed their viability in a dose-dependent manner. Statistically significant differences were observed in Caco-2 cells treated with 10, 20, 50 and 100 μM curcumin, and in HCT116 cells treated with 50 and 100 μM (all P<0.05; Fig. 1A). By contrast, treatment with 1-16 μM leucovorin alone had no effect on the viability of Caco-2 or HCT116 cells (all P>0.05; Fig. 1B). Considering that 50 μM curcumin exhibited significant inhibitory effect in both cell lines, this concentration was selected for the subsequent experiments, which was also in accordance with a previous study (29). Furthermore, 2 μM leucovorin was chosen for further experiments, as previously described (30). Co-treatment in the Leu + Cur group resulted in significantly reduced cell viability compared with the Cur group in both Caco-2 and HCT116 cells (both P<0.05; Fig. 2A and B). Furthermore, the pre-Leu + Leu + Cur group displayed similar inhibitory effect on the viability of Caco-2 and HCT116 cells compared with the simultaneous Leu + Cur group (both P>0.05; Fig. 2A and B). The dose-dependent effects in the Leu + Cur group were further investigated with varying

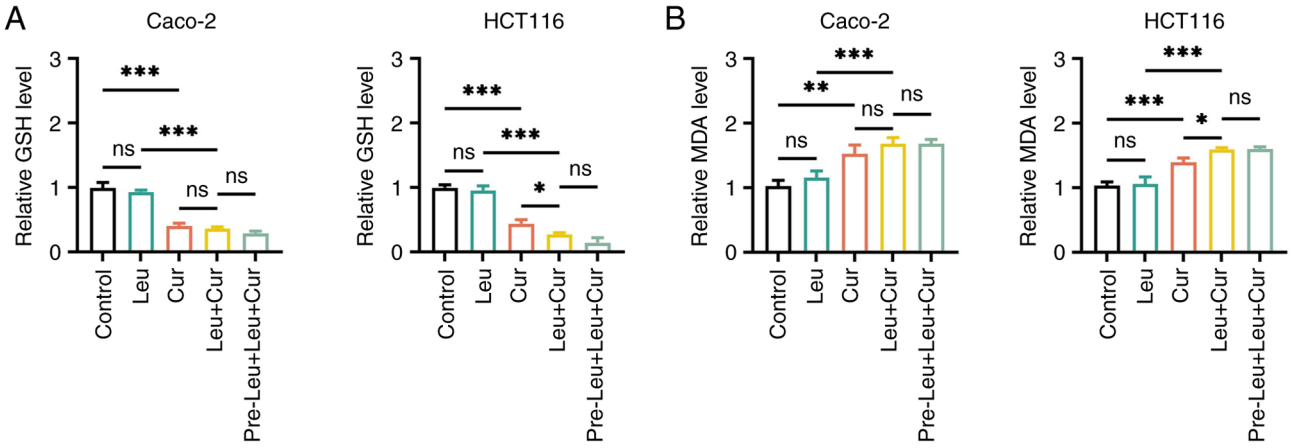

Figure 3. Effect of curcumin and leucovorin on GSH and MDA levels. Comparison of (A) GSH and (B) MDA levels among treatment groups in Caco-2 and HCT116 cells. *P<0.05, **P<0.01 and ***P<0.001. Leu, leucovorin; Cur, curcumin; GSH, glutathione; MDA, malondialdehyde; ns, not significant. Error bars represent the SD.

concentrations of curcumin and leucovorin (Figs. S1A-D and S2A-D).

Oxidative stress and Fe^{2+} levels in CRC cells treated with curcumin/leucovorin. In both Caco-2 and HCT116 cells, treatment with curcumin alone in the Cur group significantly reduced the relative GSH levels (both P<0.001; Fig. 3A) and enhanced those of relative MDA (both P<0.01; Fig. 3B) and ROS (both P<0.001; Fig. 4A and B) compared with the control group. Curcumin alone in the Cur group also elevated relative intracellular Fe^{2+} levels in both cell lines compared with the control group (both P<0.001; Fig. 4C). However, CRC cell treatment with leucovorin alone in the Leu group had no effect on GSH, MDA, ROS or Fe^{2+} levels compared with the control group (all P>0.05). In addition, co-treatment in the Leu + Cur group significantly further reduced the relative GSH levels and significantly increased those of MDA and ROS compared with Cur group in HCT116 cells (all P<0.05). However, this effect was not observed in Caco-2 cells (all P>0.05). Although in HCT116 cells, the Leu + Cur group showed notable increases in relative Fe^{2+} levels compared with that of the Cur group, a statistically significant difference was not reached (P>0.05). Consistently, no significant difference in Fe^{2+} levels was detected between the Cur and Leu + Cur groups in Caco-2 cells (P>0.05; Fig 4C). The pre-Leu + Leu + Cur cells did not demonstrate a more predominant effect on GSH, MDA, ROS and Fe^{2+} levels compared with that of the Leu + Cur group in both CRC cell lines. However, the relative GSH, ROS and Fe^{2+} levels in HCT116 cells were markedly lower in the pre-Leu + Leu + Cur group (all P>0.05). Furthermore, ferroptosis inhibitor, Fer-1, was used for verification. In both Caco-2 and HCT116 cells, the addition of Fer-1 [Fer-1 (+) cells] improved cell viability and reduced Fe^{2+} level in the Cur, Leu + Cur and pre-Leu + Leu + Cur groups compared with Fer-1 (-) cells, confirming ferroptosis (Fig. S3A and B).

MMP in curcumin/leucovorin-treated CRC cell lines. MMP, which was assessed by the ratio of aggregates/monomers, was notably decreased in the Cur group in both Caco-2 and HCT116 cells compared with the control group (both P<0.001). However, treatment with leucovorin in the Leu group had no effect on MMP in either cell line compared with the control group (both P>0.05). The combination treatment in the Leu + Cur group exhibited minor effects on MMP levels compared with Cur group in both Caco-2 and HCT116 cells (both P>0.05). Furthermore, the pre-Leu + Leu + Cur group did not show significantly altered MMP levels compared with Leu + Cur group in HCT116 cells. In Caco-2 cells in pre-Leu + Leu + Cur group a slight, but not statistically significant, decrease in MMP levels were observed compared with the Leu + Cur group (both P>0.05; Fig. 5A and B).

Expression of ferroptosis-related markers in curcumin/leucovorin-treated CRC cell lines. In both Caco-2 and HCT116 cells, treatment with curcumin alone in the Cur group upregulated ACSL4 and downregulated SLC7A11 compared with the control group (all P<0.01). However, leucovorin alone treatment in the Leu group had no significant effect on the expression levels of both proteins compared with the control group (all P>0.05). Furthermore, the Leu + Cur group showed numerically increased ACSL4 levels compared with Cur group in Caco-2 and HCT116 cells; however, a statistically significant difference was not reached (both P>0.05). The Leu + Cur group also presented significantly attenuated the expression levels of SLC7A11 compared with Cur group in HCT116 cells (P<0.05), while the reduction observed in Caco-2 cells did not reach statistical significance (P>0.05). Furthermore, the pre-Leu + Leu + Cur group displayed a similar effect on ACSL4 and SLC7A11 expression compared with the Leu + Cur group in both cell lines (all P>0.05; Fig. 6A and B).

Potential mechanisms of the interaction between curcumin and leucovorin. A total of 218 co-target genes of curcumin and leucovorin were identified from the PharmMapper database. GO analyses indicated that the co-target genes of curcumin and leucovorin were mainly enriched in biological processes of 'signal transduction' and 'peptidyl-serine phosphorylation', cellular component of 'cytosol' and 'extracellular exosome', and molecular function of 'enzyme binding' and 'nuclear

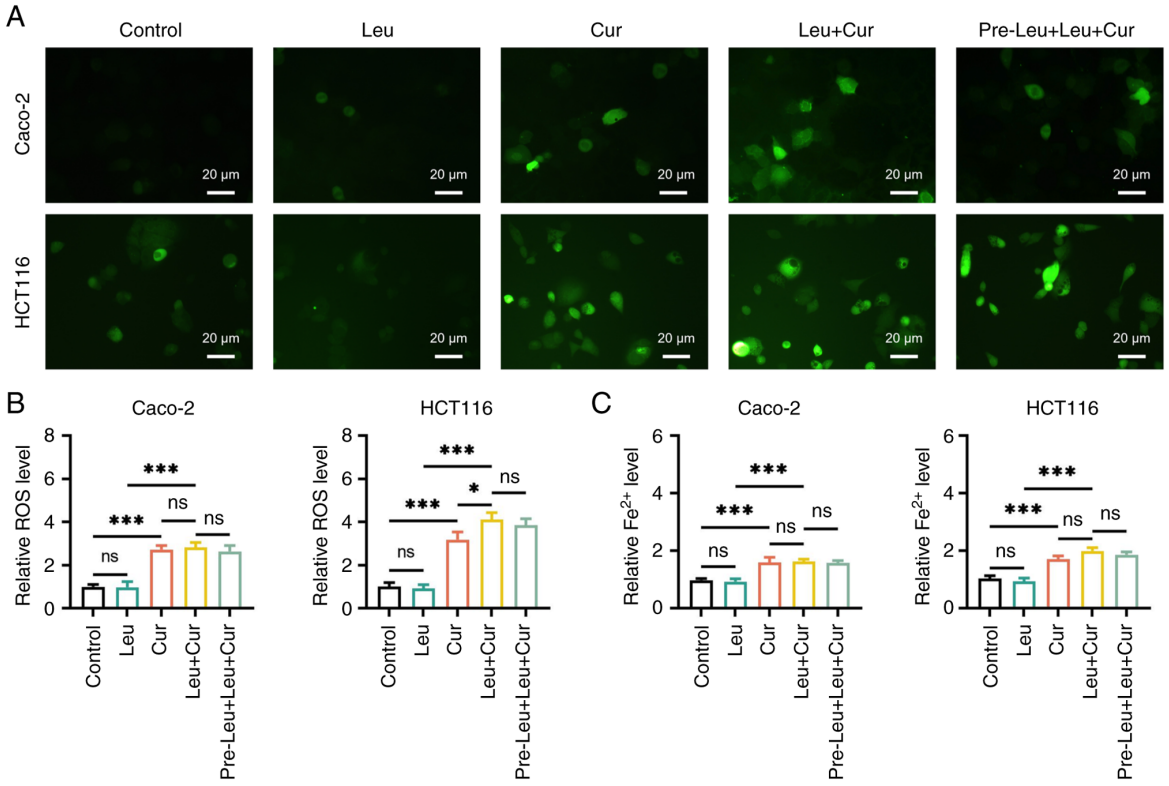

Figure 4. Effect of curcumin and leucovorin on ROS and Fe^{2+} levels. (A) Representative images illustrating ROS levels (scale bar, 20 μm). Comparison of (B) ROS and (C) Fe^{2+} levels among treatment groups in Caco-2 and HCT116 cells. *P<0.05 and ***P<0.001. Leu, leucovorin; Cur, curcumin; ns, not significant; ROS, reactive oxygen species; Fe^{2+}, ferrous iron. Error bars represent the SD.

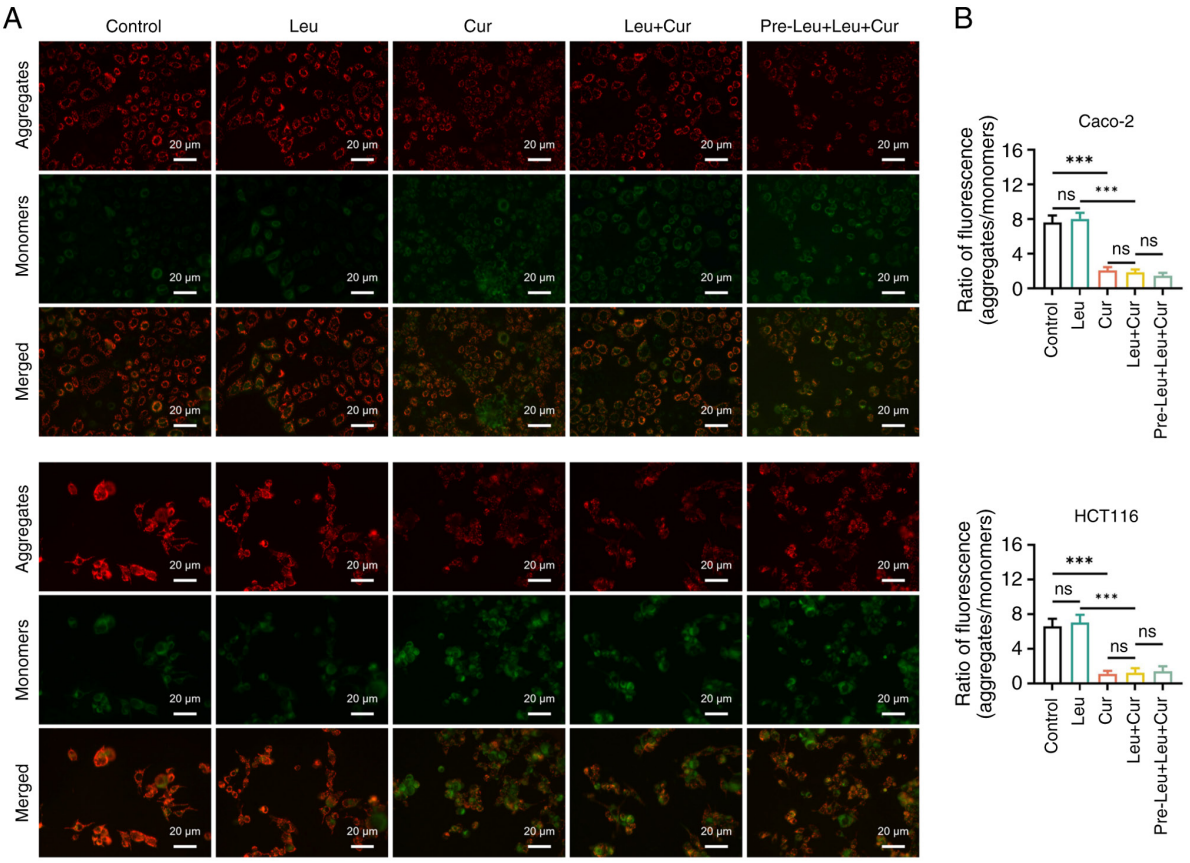

Figure 5. Effect of curcumin and leucovorin on MMP in colorectal cancer cell lines. (A) Representative images illustrating MMP levels (scale bar, 20 μm). (B) Comparison of MMP levels among different treatment groups in Caco-2 and HCT116 cells. ***P<0.001. Leu, leucovorin; Cur, curcumin; MMP, mitochondrial membrane potential; ns, not significant. Error bars represent the SD.

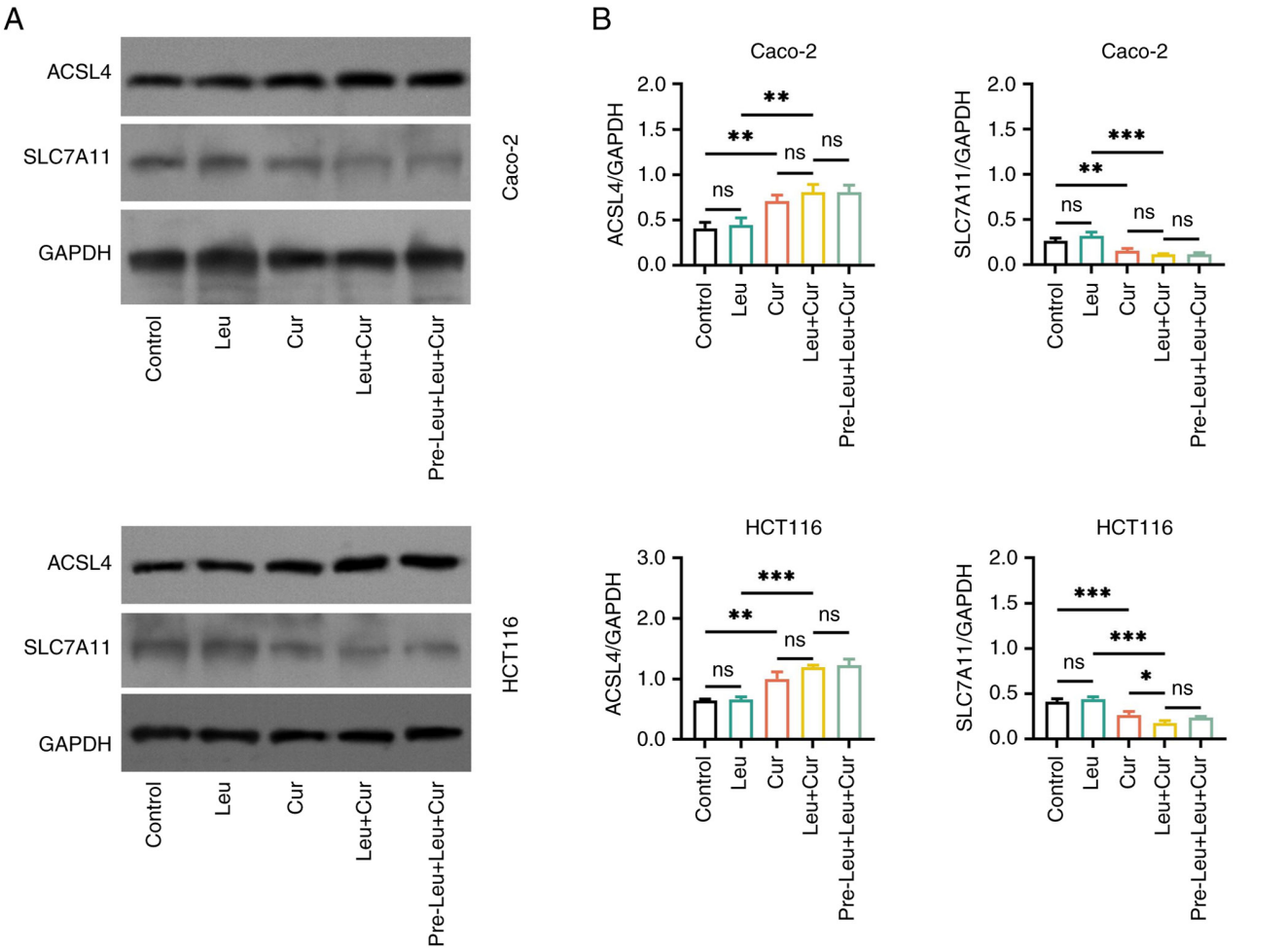

Figure 6. Effects of curcumin and leucovorin on the expression of ACSL4 and SLC7A11. (A) The protein expression levels of ACSL4 and SLC7A11 are shown. (B) Comparison of ACSL4 and SLC7A11 expression among different treatment groups in Caco-2 and HCT116 cells. *P<0.05, **P<0.01 and ***P<0.001. Leu, leucovorin; Cur, curcumin; ACSL4, acyl-CoA synthetase long chain family member 4; SLC7A11, solute carrier family 7 member 11; ns, not significant. Error bars represent the SD.

receptor activity'. KEGG analyses indicated that these genes were mainly enriched in metabolic pathways and pathways in cancer (Fig. S4A-C).

Discussion

In the present study, two well-established CRC cell lines, namely HCT116 and Caco-2, were utilized to perform the *in vitro* experiments to assess the effect of curcumin and leucovorin in CRC (31-33). The CCK-8 assay revealed that curcumin inhibited the viability of HCT116 and Caco-2 cells in a dose-dependent manner, thus supporting the anti-CRC capacity of curcumin. These findings were consistent with those reported in previous studies (20,25,34). Other studies indicated that curcumin exerted its antitumor activity in CRC by inducing ferroptosis (20-22). Therefore, the present study further verified this mechanism. The results demonstrated that treatment of HCT116 and Caco-2 cells with curcumin significantly increased oxidative stress and Fe^{2+} levels, and the expression levels of the ferroptosis-related marker ACSL4, thus supporting the notion that curcumin could induce ferroptosis in CRC cells.

Leucovorin, a clinically notable medication adjunct in CRC chemotherapy, is known to augment the efficacy of fluorouracil via inhibition of thymidylate synthase (35), thus enhancing the cytotoxicity of fluorouracil in CRC (36,37). However, whether leucovorin can affect the antitumor effect of curcumin in CRC has not been previously investigated. In the present study, the results demonstrated that leucovorin alone had no effect on CRC cell viability. However, the combination of curcumin and leucovorin more effectively suppressed the viability of CRC cells compared with curcumin alone. These findings suggested that leucovorin could potentiate the antitumor effect of curcumin on CRC. Furthermore, the present study revealed that leucovorin had a modest effect on promoting curcumin-induced ferroptosis. However, this effect was less pronounced compared with that observed on cell viability. It was hypothesized that this discrepancy could be due to the fact that apart from ferroptosis, curcumin could inhibit the viability of CRC cells via additional mechanisms, including apoptosis and pyroptosis (14,38). Notably, the results also demonstrated that the effect of leucovorin on enhancing curcumin-induced inhibition of cell viability and ferroptosis was more evident in HCT116 cells compared with Caco-2 cells. Furthermore, curcumin exerted a stronger effect on inhibiting the viability of HCT116 cells compared with Caco-2 cells. This finding could be attributed to intrinsic differences between the two

cell lines used. For instance, HCT116 cells exhibit microsatellite instability and mutations in TGF-β (39), a known target of curcumin in CRC (40). Meanwhile, the p53 status, differentiation and ferroptosis sensitivity also differ markedly between these two cell lines. Therefore, curcumin and curcumin combined with leucovorin displayed enhanced efficacy in HCT116 cells. However, further studies are warranted to verify this hypothesis.

Potentiating the efficacy of one therapeutic modality with pre-treatment of another one is a common strategy used in studies on treatments for cancer. For example, pre-treatment with inhibitors of EGFR, c-Jun N-terminal kinase or protein kinase C improved the efficacy of hypericin photodynamic therapy in cancer cells (41). Another previous study reported that the cytotoxicity of cisplatin in resistant ovarian cancer cells could be promoted by pre-treatment of quercetin (42). Furthermore, it has been reported that pre-treatment of colon cancer cells with leucovorin enhances the antitumor capacity of fluorouracil (43). Therefore, the present study further explored whether pre-treatment with leucovorin could further potentiate the effect of curcumin on CRC. However, pre-treatment of CRC cells with leucovorin followed by curcumin + leucovorin treatment had no effect on cell viability and ferroptosis compared with co-treatment with curcumin + leucovorin. These findings suggested that the capacity of leucovorin in potentiating curcumin in CRC could not be further improved by pre-treatment. A potential explanation for these findings is that the co-treatment strategy with leucovorin already fully promoted the effects of curcumin. However, this hypothesis requires verification.

The present study demonstrated that leucovorin could augment the antitumor efficacy of curcumin in CRC, thus suggesting that their combination could serve as a potential therapeutic regimen for patients with CRC. The detailed molecular mechanisms of their interaction were not explored in the present study. Nevertheless, the present study identified 218 co-target genes of leucovorin and curcumin, which were mainly enriched in 'signal transduction', 'peptidyl-serine phosphorylation', 'enzyme binding' and 'nuclear receptor activity', as well as 'metabolic pathways' and 'pathways in cancer'. Based on these findings, further studies could explore the detailed mechanisms by which leucovorin enhanced the effects of curcumin in CRC. Furthermore, the present study findings suggested that leucovorin promoted curcumin-induced ferroptosis in CRC. Therefore, an assumption could be made that leucovorin might affect ferroptosis-related mechanisms in CRC, such as redox metabolism, iron metabolism and ferroptosis regulators, thus promoting the effect of curcumin in CRC. Nevertheless, this assumption should be further verified. However, *in vivo* studies are warranted to verify the findings of the present study. In addition, further clinical research is necessary before this regimen can be considered for clinical application; the findings of the present study should be verified in other cell lines of CRC. Additionally, the lack of enhanced effect with leucovorin pre-treatment should be interpreted with caution, as timing and pharmacodynamics may differ between experimental models and clinical settings.

In conclusion, the present study indicated that leucovorin could enhance curcumin-induced suppression of cell viability and ferroptosis in CRC cells, demonstrating the potential of curcumin combined with leucovorin as a therapeutic regimen for treatment of CRC in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HX contributed to the study conception and design. Material preparation, data collection and analysis were performed by RM and QX. Data interpretation was performed by XW. The first draft of the manuscript was written by XW and all authors commented on previous versions of the manuscript. HX and RM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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