

Loperamide reverses 5-FU resistance in colorectal cancer by activating autophagy

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Abstract. 5-Fluorouracil (5-FU), a cornerstone chemotherapeutic agent used for colorectal cancer therapy, has long been established as a first-line treatment. However, clinical evidence has suggested that a substantial proportion of patients develop resistance to 5-FU, notably compromising its therapeutic efficacy. The present study aimed to investigate whether loperamide (LOP) can enhance the sensitivity of colorectal cancer cells to 5-FU and to elucidate the potential underlying molecular mechanism. First, the IC₅₀ values of LOP were determined in the 5-FU-sensitive HCT8 and 5-FU-resistant HCT8R colorectal cancer cell lines, using the Cell Counting Kit-8 assay to evaluate LOP-induced alterations in 5-FU sensitivity. The effects of LOP on cell proliferation were subsequently analyzed using 5-ethynyl-2'-deoxyuridine and colony formation assays. Cell migration was assessed through wound healing and Transwell migration assays, and apoptosis was evaluated using flow cytometric analysis with PI/Annexin V staining. Western blot analysis was performed to measure the expression levels of the autophagy-associated proteins microtubule-associated protein 1 light chain 3 (LC3) and Beclin, and autophagosome

formation following LOP treatment was visualized. The role of autophagy in LOP-mediated reversal of drug resistance was further examined using autophagy inhibitors. Finally, xenograft experiments in nude mice were performed to investigate the *in vivo* effects of LOP on the 5-FU sensitivity of HCT8R cells. Compared with in the parental cell line, HCT8R cells exhibited enhanced migratory capabilities and resistance to 5-FU. Notably, LOP was revealed to potentiate the sensitivity of HCT8R cells to 5-FU, as evidenced by reduced rates of cell proliferation, suppressed migratory ability, increased levels of apoptosis, and decreased tumor weight and volume in subcutaneous xenografts in mice. LOP was also shown to induce upregulation of autophagy marker proteins, leading to the accumulation of autophagosomes within the cells. Blocking autophagy with 3-methyladenine led to a reversal of the inhibitory effect of LOP on HCT8R cell migration. LOP was also shown to enhance the sensitivity of HCT8R cells to 5-FU by activating cellular autophagy, thereby suppressing resistant cell proliferation and migration, promoting apoptosis and reversing drug resistance. Taken together, these findings provide novel insights into the mechanisms underlying 5-FU resistance, thereby highlighting potential therapeutic strategies for colorectal cancer.

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Abbreviations: 5-FU, 5-fluorouracil; TS, thymidylate synthase; LOP, loperamide; FBS, fetal bovine serum; EdU, 5-ethynyl-2'-deoxyuridine; LC3, microtubule-associated protein 1 light chain 3; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8; 3-MA, 3-methyladenine; DPD, dihydropyrimidine dehydrogenase; MDR, multidrug resistance; PDAC, pancreatic ductal adenocarcinoma

Key words: LOP, autophagy, 5-FU, chemotherapy resistance

Introduction

According to the International Agency for Cancer Research, the incidence of colorectal cancer has been steadily increasing over the past two decades (1). In 2020, colorectal cancer ranked as the third most common cancer among men and the second most common among women, with its mortality rate ranking second among all malignancies (2,3).

5-Fluorouracil (5-FU) is a key chemotherapeutic agent that has been used to treat colorectal cancer, exerting its effects primarily through inhibiting thymidylate synthase (TS); this inhibition subsequently disrupts RNA and DNA synthesis, thereby suppressing tumor cell proliferation (4). Although 5-FU has demonstrated significant efficacy against various types of solid tumor, especially those of the gastrointestinal tract, its therapeutic success in advanced colorectal

cancer has been shown to be limited, with response rates of 10-15% (5). The emergence of 5-FU resistance substantially diminishes its clinical effectiveness, posing a major challenge in the management of colorectal cancer; therefore, strategies to enhance the sensitivity of colorectal cancer cells to 5-FU, and to mitigate chemotherapy resistance, remain urgent priorities for improving patient outcomes.

Loperamide (LOP) is a United States Food and Drug Administration-approved antidiarrheal medicine (6) with a well-established safety profile, which has been shown to induce autophagy and reverse multidrug resistance (MDR) in several types of cancer, including glioblastoma and breast cancer (7,8). The safety profile of LOP, combined with its efficacy at therapeutic dose (2-8 mg/day) makes it a promising candidate for drug repurposing in oncology. Its potential to overcome 5-FU resistance in colorectal cancer is supported by its ability to modulate cellular stress pathways and enhance chemosensitivity. These effects have been shown to be mediated through the induction of autophagy and the down-regulation of MDR1 gene expression (7-9). However, despite these promising findings, the impact of LOP on colorectal cancer, in particular its potential to overcome 5-FU resistance and the associated underlying molecular mechanisms, remains poorly understood.

Autophagy is a highly regulated cellular process in which eukaryotic cells utilize lysosomes to degrade cytoplasmic proteins and damaged organelles, and it can be characterized into basal physiological autophagy and stress-induced autophagy (9). Basal autophagy serves as a protective mechanism, maintaining cellular homeostasis and protecting cells from stress or oxidative damage induced by external stimuli, whereas excessive autophagy can lead to metabolic stress, extensive degradation of essential cellular components, and, ultimately, cell death.

In the context of colorectal cancer drug resistance, numerous studies have suggested that autophagy serves a critical role in enhancing resistance to various chemotherapeutic agents (10,11). Consequently, targeting autophagy represents a potential strategy for modulating the sensitivity of gastrointestinal tumors to 5-FU treatment.

In the present study, the 5-FU-sensitive colorectal cancer cell line HCT8 and its 5-FU-resistant counterpart, HCT8R, were employed in a series of *in vitro* and *in vivo* experiments. The current study investigated whether LOP potentiates 5-FU sensitivity in colorectal cancer cells and elucidated the underlying molecular mechanisms.

Materials and methods

Cell lines and culture conditions. The human colorectal cancer cell line HCT8 (cat. no. 1101HUM-PUMC000104; Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College) and its 5-FU-resistant derivative, HCT8R (cat. no. MXC149; Shanghai MEIXUAN Biological Science and Technology Ltd.), were kindly provided by the research group of Professor Xi (Hubei University of Medicine, Shiyan, China). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures

were maintained in a humidified incubator at 37°C containing 5% CO₂ to support optimal cell growth. To inhibit autophagic activity, the HCT8R cells were co-incubated with an autophagy inhibitor, 3-methyladenine (3-MA; 5 mM), 5-FU (20 µg/ml) and LOP (20 µM) for 24 h at 37°C.

RPMI-1640 medium was purchased as a powder from Gibco; Thermo Fisher Scientific, Inc., FBS was obtained from Zhejiang Tianhang Biotechnology Co., Ltd., the antibiotics were sourced from Beyotime Institute of Biotechnology, and LOP, 5-FU and 3-MA were purchased from MedChemExpress.

Cell counting kit-8 (CCK-8) viability assay. HCT8 and HCT8R cell metabolic activity was assessed using the CCK-8 kit (cat. no. C0038; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, cells in the logarithmic growth phase were seeded at 1×10³ cells/well in 96-well plates (100 µl/well). After 24 h of adhesion, the medium was replaced with fresh medium containing 5-FU (0, 10, 20, 40 and 80 µg/ml) and LOP (0, 10, 20, 40 and 80 µM). To evaluate the combined effects of 5-FU and LOP on cell viability, HCT8R cells were treated with 5-FU (20 µg/ml), dimethyl sulfoxide (DMSO, 0.2% v/v) or LOP (10 and 20 µM). The cells were then incubated for 24 h, followed by the addition of 10 µl CCK-8 reagent/well and further incubation for 2 h at 37°C. Culture medium without cells served as the blank control. Absorbance at 450 nm was measured using a microplate reader (BioTek Synergy H1; Biotek; Agilent Technologies, Inc.).

5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay. Exponentially growing HCT8R cells were counted and seeded into 48-well plates at a density of 10,000 cells/well. After 24 h of adherence to the plates, the cells were treated with 5-FU (20 µg/ml), DMSO (0.2% v/v) or LOP (20 µM) for 48 h at 37°C. To assess cell proliferation, the cells were incubated with EdU diluted in culture medium at a 100:1 ratio (200 µl/well) for 6 h at 37°C. Following EdU labeling, the medium was subsequently removed and the cells were fixed with 4% paraformaldehyde (Biosharp Life Sciences) for 30 min at room temperature. Fixed cells were then incubated with 2 mg/ml glycine for 5 min to neutralize residual paraformaldehyde, followed by permeabilization with 0.5% Triton X-100 for 20 min at room temperature. The EdU reaction solution was prepared according to the manufacturer's protocol (Beyotime Institute of Biotechnology) and applied to the cells (100 µl/well) for 30 min in the dark to ensure precise labeling at room temperature. Subsequently, the cells were counterstained with DAPI (Beyotime Institute of Biotechnology) for 10 min to visualize the nuclei at room temperature. Finally, fluorescent images were acquired using an inverted fluorescence microscope.

Colony formation assay. Exponentially growing HCT8R cells were seeded into 6-well plates at a density of 1,000 cells/well. Once the cells had formed small colonies, they were treated with 5-FU (20 µg/ml), DMSO (0.2% v/v) or LOP (20 µM) for 48 h at 37°C. After 6-8 days, colony formation was assessed under a microscope. When distinct colonies were visible in the control group, the culture medium was aspirated and the cells were washed three times with PBS. Cells were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature, and colonies were then stained with crystal violet for

30 min at room temperature to enhance visualization. Excess stain was removed with multiple PBS washes and the plates were left to air-dry prior to imaging. Crystal violet-stained colonies (>50 cells) were visualized using an inverted light microscope (IX71; Olympus Corporation). Colony counts per group were manually quantified in triplicate and averaged.

Wound healing assay. Exponentially growing HCT8R cells were treated with trypsin until they formed a single-cell suspension, and $\sim 5 \times 10^5$ cells/well were seeded into 6-well plates. The cells were subsequently cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h to allow them to reach 100% confluence. A uniform scratch was introduced using a 200- μ l pipette tip and the cells were subsequently washed three times with PBS to remove any detached cells. Fresh RPMI-1640 medium supplemented with 2% FBS was then added to each well. Initial images were captured to confirm that the scratch that had been made was uniform, centered and perpendicular to the plane of the plate. Following treatment with 5-FU (20 μ g/ml), DMSO (0.2% v/v) or LOP (20 μ M) for 24 h at 37°C, additional images were acquired under a light microscope. Cell migration into the scratch area was quantified using ImageJ (version 4.1; National Institutes of Health), and the wound healing rate was calculated as: (0 h scratch width -24 h scratch width)/0 h scratch width x100 to assess cell motility.

Transwell assay. For the Transwell assay experiments in HCT8 and HCT8R cells, 600 μ l RPMI-1640 medium supplemented with 20% FBS was added to the lower chamber, with or without 3-MA (5 mM), 5-FU (20 μ g/ml) and LOP (10 and 20 μ M). Subsequently, 200 μ l suspension containing 10^5 cells/ml in FBS-free medium was added to the upper chamber (pore size, 8 μ m; Corning, Inc.), which was placed into a 24-well plate. The cells were cultured for 48 h to allow for migration. After the incubation period, the insert was carefully removed and washed once with PBS. The cells that had migrated to the lower surface of the insert were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times with PBS. The interior of the insert was gently swabbed with a soft cotton swab to remove non-adherent cells. Finally, staining was performed using crystal violet for 20 min at room temperature, and excess stain was removed by multiple washes with PBS. Images were captured under a light microscope.

Observation of autophagosomes by confocal microscopy. Exponentially growing HCT8R cells were plated in 6-well plates, and cultured for 24 h until they reached 70-80% confluence. Transfection was subsequently performed using Lipo8000™ reagent (Beyotime Institute of Biotechnology) in combination with 2 μ g pEGFP (cat. no. 165830; Addgene, Inc.) or pEGFP-microtubule-associated protein 1 light chain 3 (LC3) plasmid (cat. no. 24920; Addgene, Inc.) and Opti-MEM™ serum-free medium (Gibco; Thermo Fisher Scientific Inc.). After 36 h of transfection at 37°C, the cells were subsequently transferred to confocal dishes, and allowed to continue growing for an additional 24 h. Once cells had reached $\sim 50\%$ confluence, they were incubated with either 5-FU (20 μ g/ml), DMSO (0.2% v/v) or LOP (20 μ M) for 24 h at 37°C. Imaging

was performed using an Olympus FV3000RS super-resolution laser confocal microscope (Olympus Corporation). All samples were analyzed under identical imaging conditions to ensure consistency and reproducibility.

Western blot analysis. HCT8R cells were harvested into 1.5-ml Eppendorf tubes containing RIPA lysis buffer (Beyotime Institute of Biotechnology) for protein extraction at 4°C. The protein concentration was subsequently quantified using a bicinchoninic acid protein assay kit, and 20 μ g protein samples were separated by SDS-PAGE on 12% gels and transferred to a PVDF membrane (MilliporeSigma). Non-specific binding was blocked using PBS containing 5% nonfat milk for 2 h at room temperature. The membrane was subsequently incubated overnight at 4°C with primary antibodies (diluted with PBS) raised against LC3 (1:1,000; cat. no. 18725-1-AP), Beclin (1:1,000; cat. no. 11306-1-AP) (both from Proteintech Group, Inc.) and β -actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Following incubation with the primary antibodies, the membrane was washed and incubated with horseradish peroxidase-conjugated anti-rabbit (diluted with PBS, 1:5,000; cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.) and anti-mouse secondary antibodies (diluted with PBS, 1:5,000; cat. no. 31430; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. Finally, the blots were visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.). Images were semi-quantified using ImageJ (version 4.1; National Institutes of Health).

Apoptosis detection. For apoptosis detection, the HCT8R cells were seeded in 6-well plates at a density of 4×10^5 cells/well. Cells were treated with 5-FU (20 μ g/ml) and LOP (10 or 20 μ M) for 48 h at 37°C. Both adherent and floating cells were then harvested and washed with ice-cold PBS. Cells from different experimental groups were then digested with trypsin (without EDTA), combined with the culture supernatant and centrifuged at $112 \times g$ for 4 min at 4°C to pellet the cells. Subsequently, the pellet was gently resuspended in pre-chilled binding buffer and adjusted to a concentration of $1-5 \times 10^6$ cells/ml. Aliquots of the cell suspension (100 μ l) were then mixed with 5 μ l FITC-Annexin V and 5 μ l PI, gently mixed, and incubated in the dark at room temperature for 8-10 min. Subsequently, 400 μ l binding buffer was added, and the mixture was gently mixed on a vortex mixer. Finally, apoptosis was assessed using a flow cytometer (SA3800; Sony Biotechnology Inc.) within 1 h, with an Annexin V-PI reagent kit purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Xenograft tumor study in nude mice. HCT8R cells (5×10^6 cells/100 μ l PBS) were subcutaneously injected into the lateral abdominal region of female nude BALB/c mice (age, 4 weeks; weight, 20-24 g; n=3/group; total n=9) that were housed under the following specific pathogen-free conditions: Temperature, $23 \pm 1^\circ\text{C}$; humidity, $55 \pm 5\%$; 12-h light/dark cycle, and with free access to standard chow and drinking water. The mice were randomly divided into the control (vehicle: 0.9% NaCl + 0.1% DMSO, i.p. daily), 5-FU monotherapy (25 mg/kg i.p., three times per week) and combination (5-FU as above + LOP 2 mg/kg/day i.p., three times per week) groups. All procedures followed protocols approved

by the Hubei University of Medicine Animal Research Committee (approval no. 2023-034). Treatment was initiated at day 7 post-inoculation when tumors reached $100 \pm 10 \text{ mm}^3$ and continued for 21 days. The humane endpoints requiring euthanasia included $\geq 20\%$ body weight loss within 48 h, tumor diameter $> 1.5 \text{ cm}$, impaired mobility or signs of distress (labored breathing/vocalization). Animal health and behavior were assessed twice daily (8:00 a.m./6:00 p.m.) using validated scoring sheets, and tumor volume ($V = L \times W^2/2$) and weight were measured every 48 h, with the endpoint being day 28 or when tumor volume exceeded $1,500 \text{ mm}^3$. Welfare provisions included: Individual ventilated cages with environmental enrichment (paper tunnels, chew blocks), and euthanasia by cervical dislocation under deep anesthesia (5% isoflurane for induction and 2% for maintenance). Death was verified by the cessation of respiration, an absent heartbeat, fixed dilated pupils and bilateral diaphragmatic transection. All 9 mice were sacrificed, with no unexpected deaths recorded and no mice excluded from the analysis.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 9 (Dotmatics). Comparisons between two groups were made using unpaired Student's t-test, whereas comparisons among multiple groups were performed by one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LOP enhances the sensitivity of colorectal cancer cells to 5-FU. To evaluate the malignant potential of HCT8 and HCT8R cells, Transwell assays were performed, which revealed that the migratory capability of the drug-resistant HCT8R cells was significantly higher compared with that of HCT8 cells (Fig. 1A). The preliminary dose-response assessments revealed marked insensitivity of HCT8R cells to 5-FU monotherapy, with conventional therapeutic concentrations ($10\text{--}80 \mu\text{g/ml}$) failing to induce significant inhibition of cell viability (Fig. S1). Subsequently, the inhibitory effects of LOP on cell viability were assessed using the CCK-8 assay. The results demonstrated that LOP inhibited the viability of both HCT8 and HCT8R cells, with IC_{50} values of ~ 24.74 and $34.29 \mu\text{M}$, respectively. These findings suggested that LOP was able to effectively suppress the viability of both the parental and 5-FU-resistant colorectal cancer cell lines (Fig. 1B and C).

To further examine the impact of LOP on the sensitivity of HCT8R cells to 5-FU, additional CCK-8 assays were performed. The results showed that the HCT8R cells exhibited resistance to 5-FU, as increasing concentrations of the drug did not significantly inhibit cell proliferation; however, treating the cells with $20 \mu\text{M}$ LOP in addition to 5-FU led to a marked enhancement of the inhibitory effect of 5-FU on cell proliferation (Fig. 1D). Further analysis revealed that treating the cells with 5-FU ($20 \mu\text{g/ml}$) alone did not substantially alter HCT8R cell proliferation; by contrast, compared with in the 5-FU group, the addition of LOP caused a significant improvement in the sensitivity of these cells to 5-FU in a concentration-dependent manner (Fig. 1E).

LOP enhances the 5-FU-induced inhibition of HCT8R cell proliferation in vitro and in vivo. The EdU assay results demonstrated that the proliferative activity of the HCT8R cells remained unaffected by the addition of 5-FU or DMSO alone; however, treatment with LOP led to a significant reduction in the number of proliferating HCT8R cells (Fig. 2A). Colony formation assays were subsequently performed, which further demonstrated that treatment with LOP caused a marked decrease in the number of colonies formed by HCT8R cells, whereas treatment with 5-FU alone or DMSO did not lead to any significant inhibitory effects (Fig. 2B).

To confirm the ability of LOP to enhance the inhibitory effect of 5-FU on HCT8R cell proliferation *in vivo*, a xenograft model was generated using nude mice. HCT8R cells were implanted into nude mice, which were then randomly divided into control, DMSO and LOP treatment groups. With the exception of the control group, the other groups were injected intraperitoneally with 5-FU (25 mg/kg per week, three times per week), whereas the LOP treatment group was injected intraperitoneally with LOP (2 mg/kg per day). The results demonstrated that treatment with LOP led to a significant enhancement of the inhibitory effect of 5-FU on tumor growth (Fig. 2C), as evidenced by a marked reduction in tumor weight and volume in the LOP treatment group (Fig. 2D and E). Compared with in the 5-FU group, the 5-FU and LOP co-treatment group demonstrated a stronger tumor growth inhibitory effect. Collectively, these findings suggested that LOP is able to effectively suppress cell proliferation both *in vitro* and *in vivo*.

LOP augments the inhibition of HCT8R cell migration mediated by 5-FU. Tumor cell migration is a critical factor for both tumor invasion into normal tissues and subsequent metastasis. After performing wound healing assays, it was observed that treatment with 5-FU or DMSO alone did not significantly affect the migratory capabilities of drug-resistant HCT8R cells; however, the addition of $20 \mu\text{M}$ LOP led to a marked inhibition of cell migration compared with in the 5-FU group (Fig. 3A). These findings were further validated by employing Transwell assays, which demonstrated that LOP was able to effectively suppress HCT8R cell migration in a dose-dependent manner (Fig. 3B).

LOP enhances 5-FU-induced apoptosis in HCT8R cells. The balance between tumor cell proliferation and apoptosis is a crucial determinant of drug sensitivity. The aforementioned experiments demonstrated that LOP can effectively inhibit the proliferation, invasion and migration of 5-FU-resistant cells; however, its impact on apoptosis has not yet been fully elucidated. To investigate this possibility, Annexin V-PI staining experiments were performed for apoptosis detection. Flow cytometric analysis revealed that treatment with 5-FU alone did not cause any significant induction of apoptosis; by contrast, the addition of LOP markedly increased the proportions of late apoptotic cells compared with in the 5-FU group (Fig. 3C).

LOP reverses 5-FU resistance through the activation of autophagy. To elucidate the molecular mechanism underlying the ability of LOP to reverse 5-FU resistance, the expression

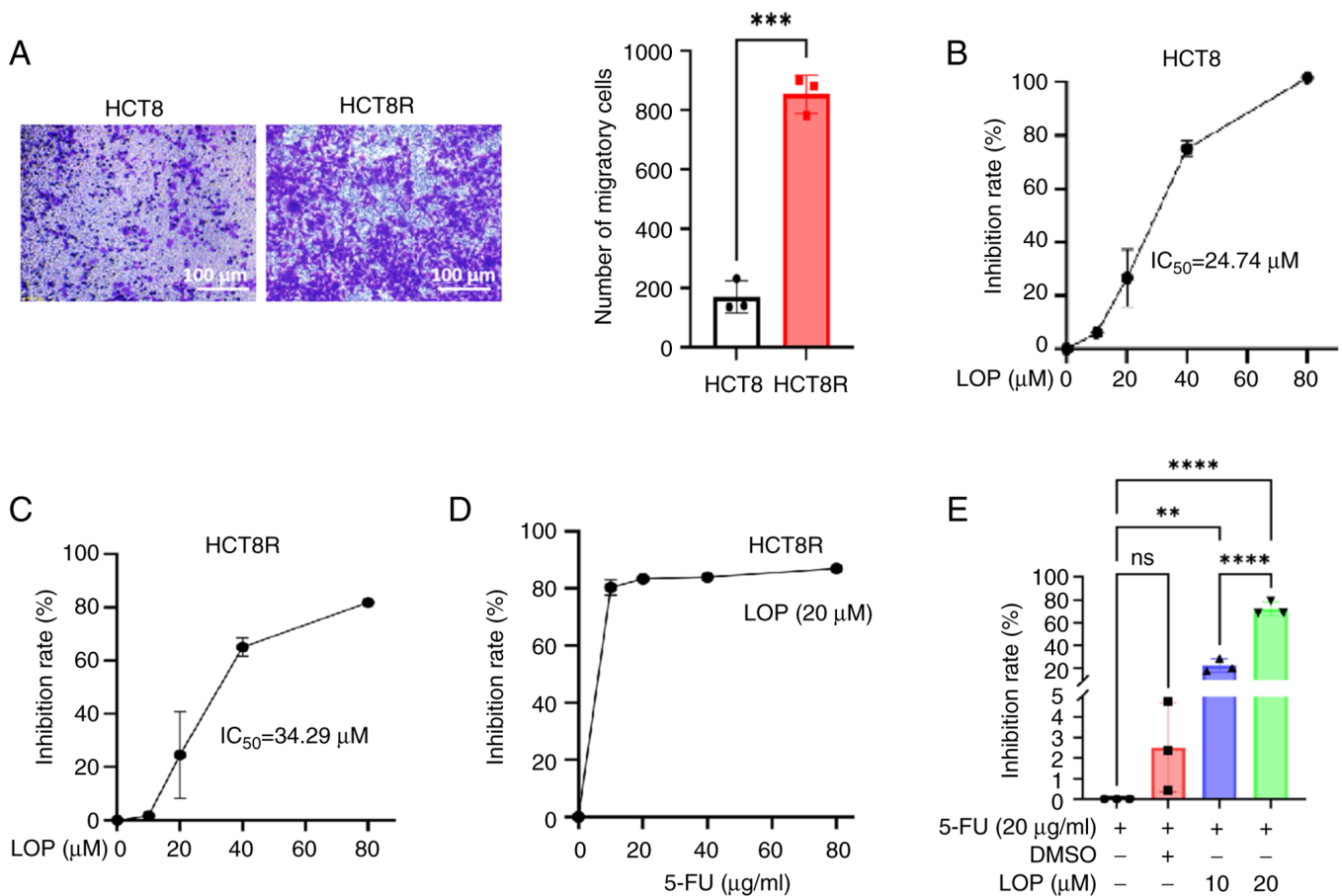


Figure 1. LOP enhances the sensitivity of colorectal cancer cells to 5-FU. (A) Transwell assay was performed, assessing the migratory capability of HCT8 and HCT8R cells. (B) CCK-8 assay was performed, evaluating the inhibitory effect of LOP on HCT8 cell proliferation. CCK-8 assay was performed to (C) evaluate the inhibitory effect of LOP on HCT8R cell proliferation, (D) examine the effects of LOP combined with various concentrations of 5-FU on HCT8R cell proliferation, and (E) evaluate the effects of 5-FU combined with different concentrations of LOP on HCT8R cell proliferation. ** P <0.01, *** P <0.001, **** P <0.0001 (n =3). 5-FU, 5-fluorouracil; CCK-8, Cell Counting Kit-8; DMSO, dimethyl sulfoxide; LOP, loperamide; ns, not significant.

levels of the autophagy markers LC3 and Beclin were assessed using western blot analysis. The results demonstrated that LOP treatment led to an upregulation of Beclin expression and an increase in the LC3-II/I ratio compared with in the 5-FU group (Fig. 4A), indicating the activation of autophagy.

To further investigate this phenomenon, HCT8R cells were transfected with EGFP or EGFP-LC3 plasmids, and autophagic vesicles were visualized using confocal microscopy. The results obtained showed that treatment with LOP in combination with 5-FU resulted in dispersed, dense fluorescent spots in the cytoplasm of resistant cells, characteristic of autophagic vesicles; by contrast, cells treated with 5-FU or DMSO alone exhibited diffuse fluorescence without the presence of autophagic vesicles (Fig. 4B).

To confirm that the reversal of HCT8R cell resistance by LOP was associated with autophagy activation, the autophagy inhibitor 3-MA was used to block autophagy. Western blot analysis confirmed a significant downregulation of Beclin following 3-MA treatment (Fig. 4C). Moreover, Transwell assays indicated that treatment with 3-MA led to a reversal of the inhibition of migration caused by LOP (Fig. 4D) compared with in the group not treated with 3-MA, highlighting the importance of autophagy as a target of the action of LOP.

Taken together, these findings suggested that LOP may enhance autophagy to inhibit cell proliferation, invasion and migration, while also promoting apoptosis, thereby reversing 5-FU resistance in HCT8 colorectal cancer cells.

Discussion

Research has highlighted several key mechanisms contributing to tumor resistance to 5-FU. A notable factor is the rapid degradation of 5-FU mediated by dihydropyrimidine dehydrogenase (DPD). The liver, which is rich in DPD, performs a crucial role in the rapid catabolism of 5-FU, leading to reduced bioavailability and efficacy (5). The expression of TS is another critical determinant of 5-FU sensitivity. In 5-FU-resistant cell lines, gene amplification and increased protein expression of TS are commonly observed (12,13). Furthermore, polymorphisms in the TS gene promoter have been shown to result in elevated TS expression, contributing to decreased responsiveness to 5-FU (4). Additionally, microsatellite instability, along with other factors, including the MDR genes MDR3 and MDR4, the polyamine degradation enzyme spermidine/spermine N1-acetyltransferase, heat shock protein co-chaperone Hsp10, chloride transporter MAT8 and Yamaguchi sarcoma

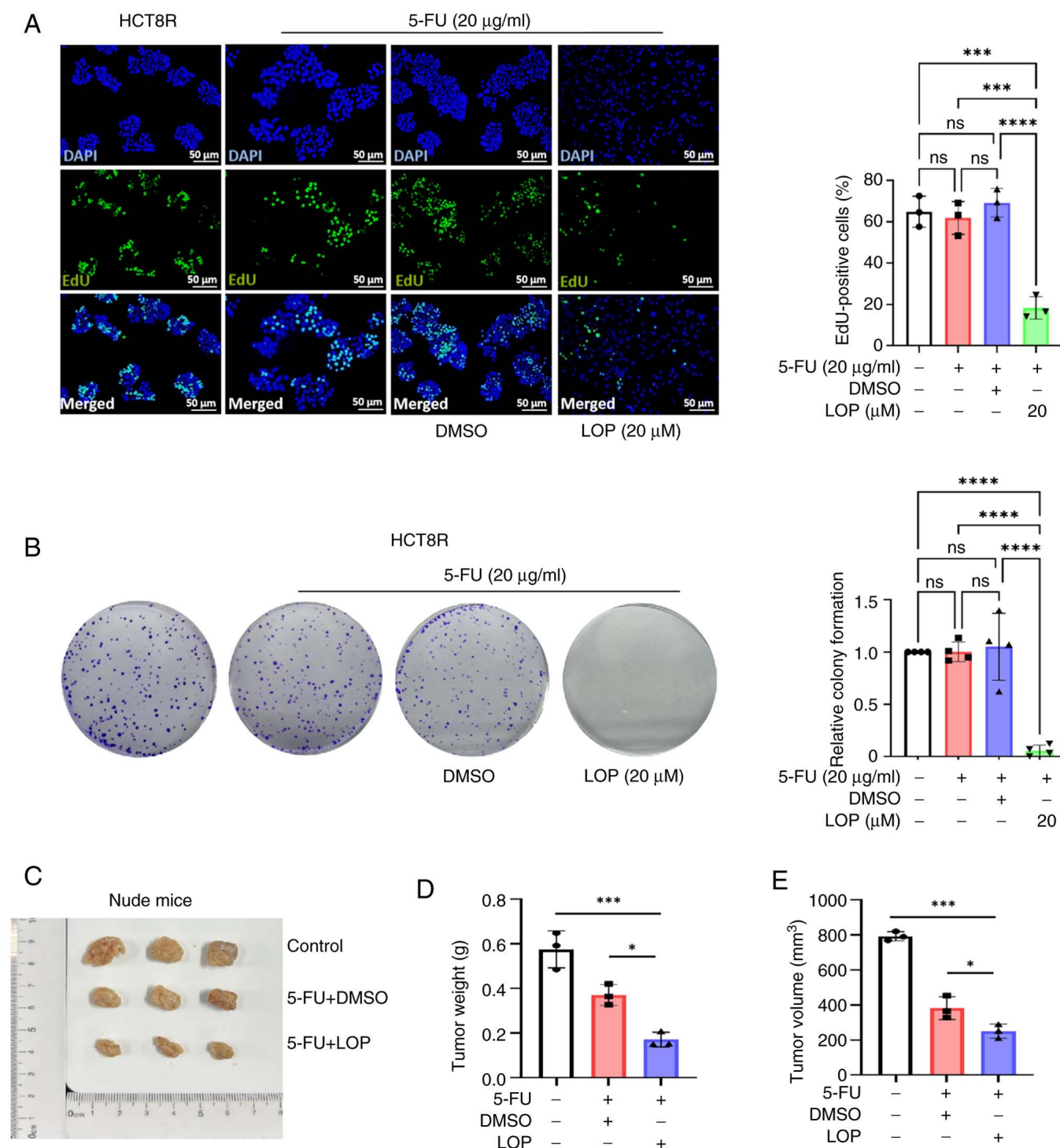


Figure 2. LOP inhibits the proliferation of 5-FU-treated HCT8R cells *in vitro* and *in vivo*. (A) EdU assay was performed, demonstrating the effect of LOP combined with 5-FU on the proliferation of HCT8R cells. (B) Colony formation assay was performed, showing the impact of LOP combined with 5-FU on the number of colonies formed by HCT8R cells. (C) Subcutaneous tumor transplantation experiments were performed to assess the *in vivo* effect of LOP on the 5-FU sensitivity of HCT8R cells. (D) Tumor weight differences and (E) Tumor volume differences among the groups are shown. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 3$). 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; EdU, 5-ethynyl-2'-deoxyuridine; LOP, loperamide; ns, not significant.

viral oncogene homolog 1, have been associated with 5-FU resistance (5).

LOP is a widely used antidiarrheal agent that acts as a peripheral μ -opioid receptor agonist. LOP exhibits minimal systemic absorption (0.3% bioavailability) due to its charged state at physiological pH, with >90% excreted unchanged in feces. A typical dosing regimen starts with 4 mg, followed by

2 mg after each diarrheal episode (14). Due to its inability to cross the blood-brain barrier, LOP is considered a safe drug for clinical use, with minimal central nervous system side effects (15,16). Previous studies have explored its potential beyond gastrointestinal applications, particularly in cancer research, where LOP has demonstrated notable anti-proliferative and pro-apoptotic effects on bladder cancer (17), glioma (9),

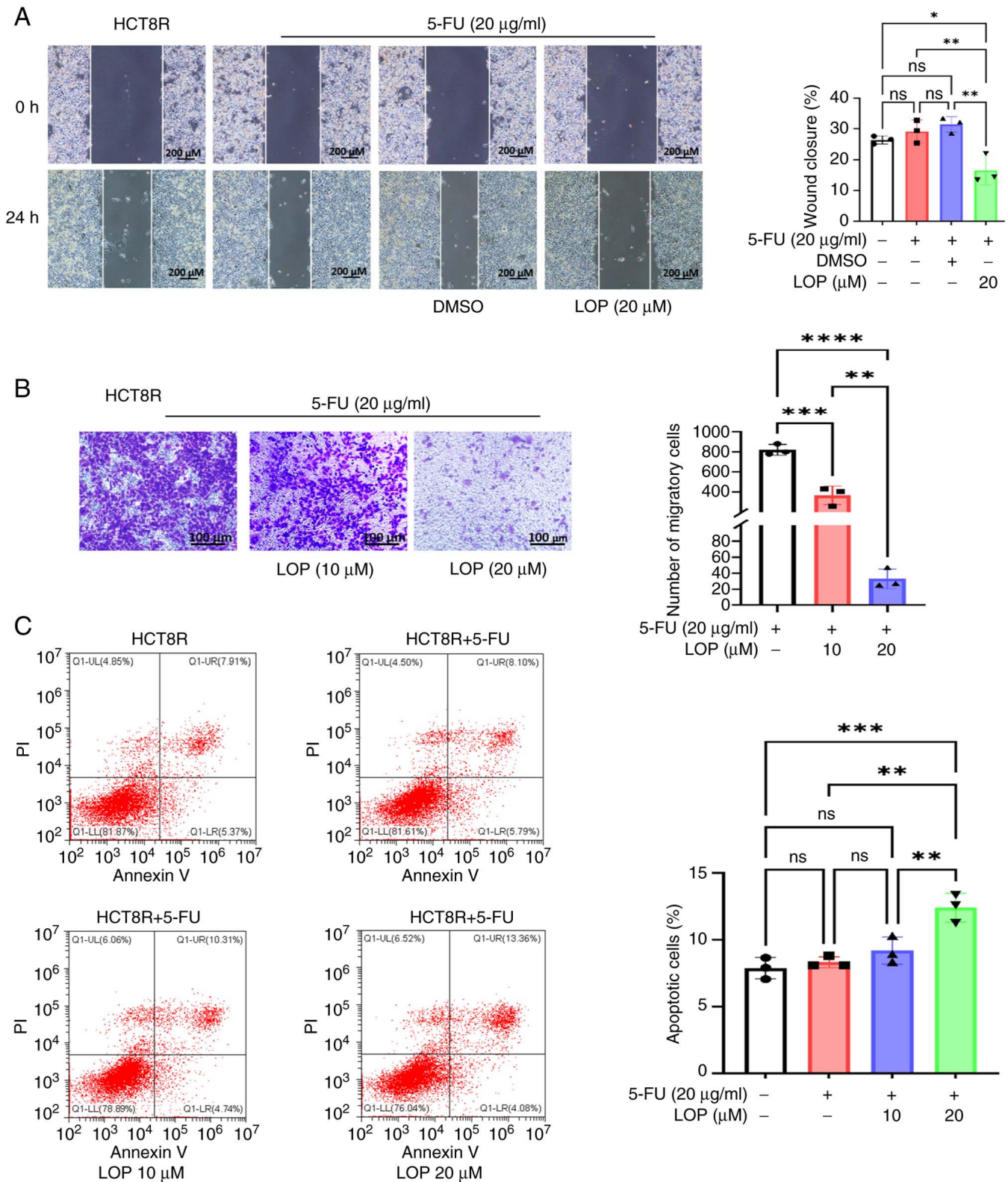


Figure 3. LOP enhances the inhibition of HCT8R cell invasion and migration, and induces apoptosis mediated by 5-FU. (A) Wound healing assay was performed, assessing the effects of LOP combined with 5-FU on the migration of HCT8R cells. (B) Transwell assay was performed to evaluate the impact of LOP combined with 5-FU on the migration of HCT8R cells. (C) Flow cytometric analysis was performed to evaluate the effects of LOP on 5-FU-induced apoptosis in HCT8R cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 3$). 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; LOP, loperamide; ns, not significant.

different types of canine cancer (18), colon cancer (19) and NSCLC cells (20). *In vitro* studies have consistently employed LOP at concentrations ranging between 20 and 100 μ M to investigate its cellular mechanisms. Specifically, 20, 40 and 60 μ M

concentrations of LOP have been shown to effectively inhibit the proliferation of bladder cancer cells (17). These concentrations have been reported not only to suppress cell proliferation, but also to promote apoptosis and induce autophagy, suggesting

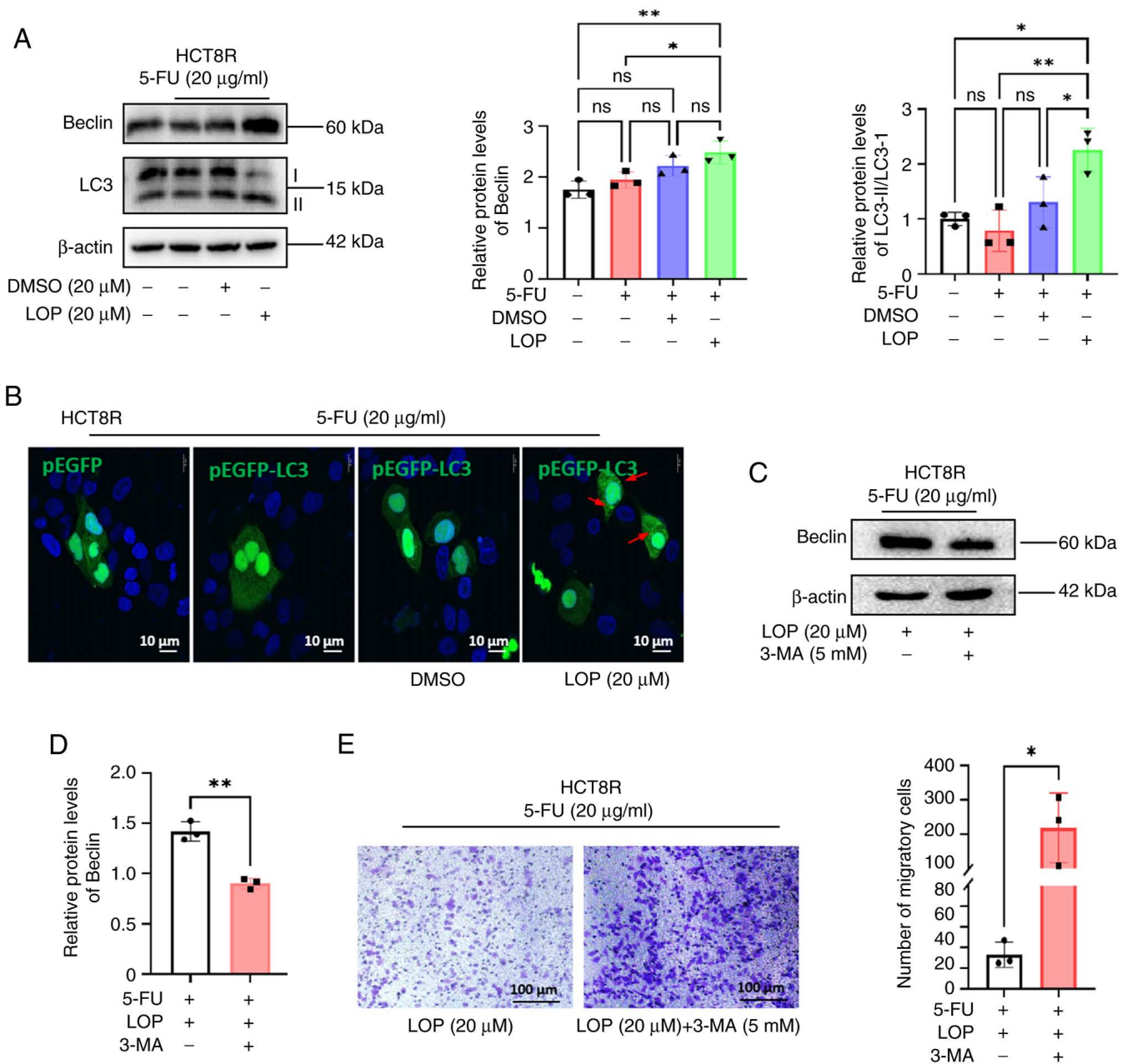


Figure 4. LOP enhances the inhibitory effects of 5-FU on HCT8R cells through autophagy activation. (A) Western blot analysis of changes in LC3 and Beclin protein expression in HCT8R cells treated with LOP and 5-FU. (B) Confocal microscopy images, showing the generation of autophagic vesicles in HCT8R cells transfected with the EGFP-LC3 plasmid, and treated with LOP and 5-FU. (C) Western blot analysis of Beclin protein expression in HCT8R cells treated with or without 3-MA. (D) Statistical analysis of changes in Beclin expression in HCT8R cells treated with or without 3-MA. (E) Transwell assays were performed, assessing the migratory capability of HCT8R cells treated with LOP, 5-FU, with or without 3-MA. * $P < 0.05$, ** $P < 0.01$ ($n = 3$). 3-MA, 3-methyladenine; 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; LC3, microtubule-associated protein 1 light chain 3; LOP, loperamide; ns, not significant.

a multifaceted role of LOP in cancer cell regulation (21,22). The induction of autophagy, in particular, highlights the potential of LOP to modulate cellular stress responses, which may be leveraged in therapeutic strategies targeting cancer cell survival pathways. However, the impact of LOP on 5-FU-resistant colorectal cancer has yet to be fully investigated.

In the present study, the colorectal cancer cell line HCT8R, which is resistant to 5-FU, was observed to exhibit significantly enhanced invasion and migration compared with the parental HCT8 cell line, indicating a higher level of malignancy. It was revealed that LOP effectively inhibited the proliferation of both HCT8 and HCT8R cells, underscoring its potential as a

therapeutic agent in colorectal cancer. Notably, treatment with LOP was shown to markedly sensitize 5-FU-resistant HCT8R cells to 5-FU.

Using CCK-8 and EdU assays (to assess cell viability and cell proliferation, respectively) and clonogenic assays, and an *in vivo* nude mouse tumorigenic model the present study demonstrated that a combination of 5-FU and LOP significantly suppressed HCT8R cell proliferation, invasion and migration compared with treatment with 5-FU alone or the control group. This effect was found to be dose-dependent. Additionally, LOP treatment was associated with a substantial increase in apoptosis in 5-FU-resistant cells.

The association between autophagy and drug resistance has garnered notable attention from researchers. The role of autophagy modulation in cancer treatment, whether via its inhibition or activation, has been shown to yield different outcomes, depending on the type of tumor (23-26). Hu *et al* (27) demonstrated that IL-6 promoted chemotherapy resistance in colorectal cancer by activating autophagy through the IL-6/JAK2/Beclin 1 signaling pathway. Additionally, Reyes-Castellanos *et al* (28) identified dysregulated metabolic pathways in pancreatic ductal adenocarcinoma (PDAC), with autophagy being a pivotal factor. This process supports tumor survival through facilitating a continuous supply of intracellular components via both autonomous and non-autonomous mechanisms. An elevated level of autophagy in established PDAC tumors may allow for abnormal proliferation and growth, even under nutrient-limited conditions.

On the other hand, enhancing cellular autophagy to induce cell death has been shown to overcome doxorubicin resistance (29). In the context of colorectal cancer, numerous studies (30-32) have suggested that autophagy contributes to resistance against various chemotherapeutic agents. Moreover, excessive autophagy-induced cell death has been shown to potentially be an effective strategy to overcome MDR in tumor cells (33). Therefore, autophagy may serve as a crucial target for modulating the sensitivity of gastrointestinal tumors to 5-FU treatment (10,34).

In conclusion, the present study demonstrated that LOP may activate cellular autophagy, leading to the formation of autophagosomes. This effect was not observed following treatment with 5-FU alone or in combination with DMSO, underscoring the important role of autophagy in enhancing the sensitivity of HCT8R cells to 5-FU when LOP is present. Additionally, the inhibition of autophagy reversed the suppressive effect of LOP on the proliferation of resistant cells, suggesting that LOP could enhance the sensitivity of 5-FU-resistant colorectal cancer cells through autophagy activation. This mechanism involved the suppression of cell proliferation, invasion and migration, as well as the induction of apoptosis, ultimately overcoming drug resistance. However, one limitation of the present study is that it lacked *in vivo* pharmacokinetic data for LOP, and there is therefore a need to explore additional mechanisms beyond autophagy, including apoptosis and metabolic reprogramming. Further studies are also needed to elucidate the precise molecular mechanisms underlying these effects, and to explore the potential of LOP in combination therapies for colorectal cancer and other malignancies.

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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

JY was responsible for devising the methodology, choosing the software, performing the investigation, formal analysis, writing the original draft of the manuscript and funding acquisition. XA was responsible for devising the methodology, choosing the software, performing the investigation and funding acquisition. XQ, JK, HR and YL were responsible for devising the methodology, choosing the software and performing the investigation. ZL and DL were responsible for conceptualization of the study and providing resources. JJ was responsible for conceptualization of the study, providing resources and supervision of the project. SL was responsible for conceptualization of the study, funding acquisition, and writing, reviewing and editing the manuscript. JY and XA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were performed according to the guidelines and with non-retrospective ethics approval from the Hubei University of Medicine Institutional Animal Care and Use Committee (approval no. 2023-034).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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