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Original research paper

A novel 4-(1,3,4-thiadiazole-2-ylthio)pyrimidine derivative inhibits cell proliferation by suppressing the MEK/ERK signaling pathway in colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) is one of the most common types of malignant cancers worldwide. Although molecularly targeted therapies have significantly improved treatment outcomes, most of these target inhibitors are resistant. Novel inhibitors as potential anticancer drug candidates are still needed to be discovered. Therefore, in the present study, we synthesized a novel 4-(1,3,4-thiadiazole-2-ylthio)pyrimidine derivative (compound **4**) using fragment- and structure-based techniques and then investigated the anticancer effect and underlying mechanism of anti-CRC. The results revealed that compound **4** significantly inhibited HCT116 cell proliferation with IC_{50} values of 8.04 ± 0.94 μmol L^{-1} after 48 h and 5.52 ± 0.42 μmol L^{-1} after 72 h, respectively. Compound **4** also inhibited colony formation, migration, and invasion of HCT116 cells in a dose-dependent manner, as well as inducing cell apoptosis and arresting the cell cycle in the G2/M phase. In addition, compound **4** was able to inhibit the activation of the MEK/ERK signaling in HCT116 cells. And compound **4** yielded the same effects as the MEK inhibitor U0126 on cell apoptosis and MEK/ERK-related proteins. These findings suggested that compound **4** inhibited cell proliferation and growth, and induced cell apoptosis, indicating its use as a novel and potent anticancer agent against CRC *via* the MEK/ERK signaling pathway.

Keywords: colorectal cancer, proliferation, apoptosis, metastasis, MEK/ERK signaling pathway

Colorectal cancer (CRC) is one of the most common malignant cancers and the leading cause of cancer-related morbidity and mortality worldwide (1, 2). According to the World Health Organization's International Agency for Research on Cancer (IARC), the 10 most common cancer types accounted for more than 60 % of newly diagnosed cancer cases and more than 70 % of cancer-related deaths, colorectal cancer was the third most commonly occurring

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cancer worldwide (10.0 % of the total new cases) (3). Long-term trends in incidence and mortality rates have significantly increased for CRC in China. And there will be approximately 592,232 new CRC diagnoses and 309,114 deaths in 2022 (4). At the time of diagnosis, approximately 25 % of CRC patients had distant metastases. The poor prognosis of patients with metastatic CRC resulted in a 5-year survival rate of 10–30 % (5–7). Chemotherapy remains an essential treatment for CRC. Traditional therapy, on the other hand, ignores the differences between individual patients, and the treatment effect is insufficient (8–11). Therefore, molecularly targeted therapies such as VEGFR and EGFR inhibitors have been developed, and have significantly improved treatment outcomes. Unfortunately, the efficacy of these inhibitors is limited by mutations that activate downstream signaling pathways, leading to resistance to targeted therapy (12). Therefore, one of the critical strategies for long-term CRC treatment is the development of novel, effective anti-CRC drugs.

Previous research has shown that the mitogen extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway is essential for cell physiology control, as it regulates cellular proliferation, differentiation, apoptosis, angiogenesis, metastasis, and drug resistance (13–15). Its dysregulation has been linked to various cancers, including colorectal cancer (16, 17). Furthermore, activation of ERK can activate downstream protein kinases or transcription factors, promoting tumor development. It is also important in the development of drug resistance and the onset of CRC metastatic events (18, 19). Specific pharmacological inhibitors that target the ERK pathway produce significant antitumor effects, indicating that these inhibitors have therapeutic potential against CRC (20–22). To overcome resistance in CRC research, finding prospective anticancer drugs with improved bioactivities in suppressing proliferation and inducing apoptosis in CRC cells *via* deactivation of the MEK/ERK signaling pathway appears to be a promising strategy for drug discovery.

Pyrimidine, as a structural analog of purines, is a useful scaffold widely used in synthesizing various effective anticancer drugs (23–26). We previously synthesized a series of 4-(1,3,4-thiadiazole-2-ylthio)pyrimidine derivatives using pyrimidine scaffolds and fragment grafting approaches based on the structural characteristics of marketed inhibitors. In the previous preliminary experiments, these compounds exhibited antiproliferative activity against multiple tumor cells and significantly reduced protein phosphorylation within the MEK/ERK signaling pathway (27). To determine the inhibitory efficacy and underlying molecular mechanisms in the human CRC cell HCT-116, we screened a novel small-molecule compound, 1-(5-((6-((3-morpholinopropyl)amino)pyrimidine-4-yl)thio)-1,3,4-thiadiazol-2-yl)-3-(4-(trifluoromethyl)phenyl)urea (compound **4**) with better bioactivities from these derivatives. The purpose of this study was to investigate the possible anti-CRC ability of compound **4**, so as to explore a potential and effective antitumor drug-like agent.

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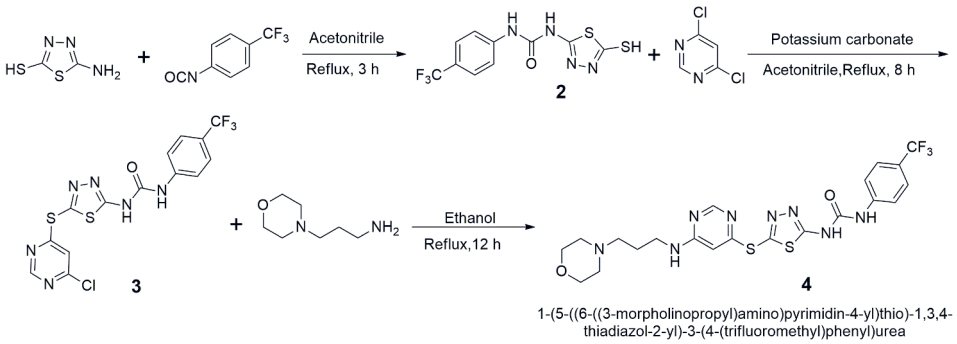
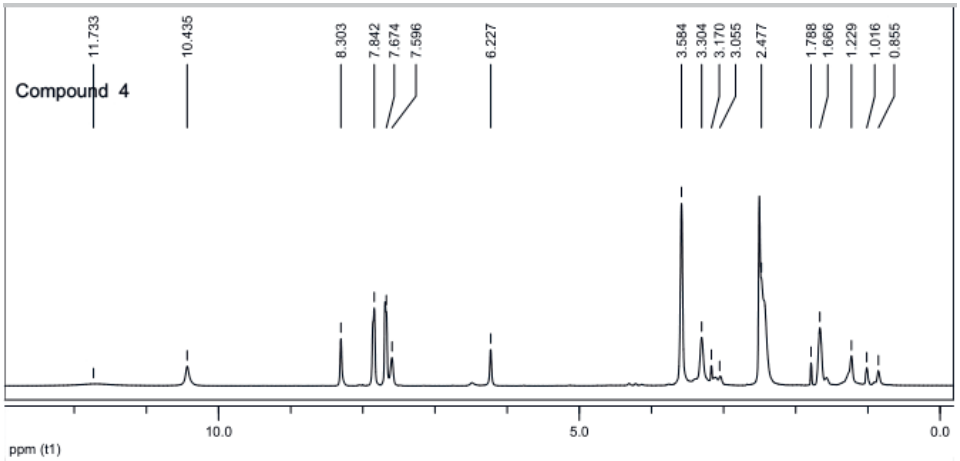
*Synthesis of compound **4***

The synthetic route of compound **4** is depicted in Scheme 1 (27). The intermediate 1-(5-mercapto-1,3,4-thiadiazol-2-yl)-3-(4-(trifluoromethyl)phenyl)urea (compound **2**) was obtained by reaction of commercially available 5-amino-1,3,4-thiadiazole-2-thiol with 1-isocyanato-4-(trifluoromethyl)benzene under reflux for 3 h in acetonitrile. Then compound





Scheme 1. Synthetic route and structure characterization of compound **4**.

2 and potassium carbonate were dissolved in acetonitrile and then reacted with commercially available 4,6-dichloropyrimidine under reflux for 8 h to produce 1-(5-((6-chloropyrimidin-4-yl)thio)-1,3,4-thiadiazol-2-yl)-3-(4-(trifluoromethyl)phenyl)urea (compound **3**). Finally, compound **3** was reacted with 3-morpholinopropan-1-amine in ethanol under reflux for 12 h. Compound **4** was isolated as a white powder with the following properties: ESI-MS (*m*/*z*, %) 539.01 $(M-H)^-$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.86–1.79 (m, 8H), 3.06–3.30 (m, 2H), 3.58 (br s, 4H), 6.23 (s, 1H), 7.60–7.67 (m, 3H), 7.84 (br s, 2H), 8.30 (s, 1H), 10.44 (s, 1H), 11.73 (br s, 1H) ppm.

Cells and treatment

The ATCC provided the human CRC HCT116 and HT-29 cells, human gastric carcinoma SGC7901 cells, human breast cancer MDA-MB-231 cells, human glioblastoma A172 cells, and normal control NCM460 cells. These cells were routinely cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin. All cell cultures were carried out in a 5 % CO_2 incubator at 37 °C.

MTT assay

Sigma-Aldrich provided the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. Following the manufacturer's instructions, the standard MTT assay was used to determine cell proliferation. All cell lines were cultured overnight in 96-well plates (5000 cells/well). The cells were then treated with various concentrations of compound **4** (2.5, 5, 10, 20, and 40 μmol L^{-1}) for 48 h (final volume of 200 μL). HCT116 cells were treated for 24, 48 and 72 h. Following drug administration at 2.5–40 μmol L^{-1} concentration, 5 mg mL^{-1} MTT reagent was added to each well (20 μL/well) and incubated for 4 h at 37 °C. Finally, all wells were treated with 150 μL of DMSO, and the absorbance at 490 nm was measured. 0.1 % DMSO served as vehicle control. The cell viability = absorbance of the treated groups/absorbance of the control group) × 100 %. The GraphPad Prism 5 software was used to calculate the IC_{50}.

Colony formation assay

HCT116 cells were seeded in 6-well plates (500 cells/well) and incubated for 24 h before being treated with various concentrations of compound **4** (2.5, 5, and 10 μmol L^{-1}) for 2 weeks to form colonies. The colonies were washed three times with phosphate-buffered saline (PBS), fixed with methanol for 15 min, and stained for 10 min with 0.1 % crystal violet at room temperature. Finally, colonies with more than 50 cells were counted using a microscope (Olympus FV500, Japan).

Cell cycle assay

HCT116 cells were seeded in 6-well plates (5×10^5 cells/well) and treated for 24 h with compound **4** (10 and 20 μmol L^{-1}) or 0.1 % DMSO, then extracted and rinsed twice with cold PBS before being fixed with 70 % ethanol overnight at 4 °C for cell cycle analysis. The cells were incubated in the dark for 30 min with a mixture containing 40 μg mL^{-1} propidium iodide (PI) and 100 μg mL^{-1} RNase A at 37 °C. A flow cytometer was used to detect cells in the G0/G1 phase, the S phase, and the G2/M phase (Cytomics^{TM}FC500, Beckman Coulter, USA).

Cell apoptosis assessment

HCT116 cells were seeded in 6-well plates (5×10^5 cells/well) and treated for 24 h with compound **4** (10 and 20 μmol L^{-1}) or 0.1 % DMSO. Apoptotic cells were collected and resuspended at a cell density of 10^6 cells/mL after trypsinization (no EDTA) and then stained in the dark with 5 μL of Annexin V-FITC (positive) and 5 μL of PI solution (negative) for 10 min at room temperature. A flow cytometer was used for cell apoptosis analysis.

Western blot analysis

HCT116 cells were seeded in 100-mm diameter dishes (2×10^6 cells/dish) and treated with various concentrations of compound **4** (10 and 20 μmol L^{-1}) or 0.1 % DMSO for 24 h, followed by further incubation with the MEK inhibitor U0126 (10 μmol L^{-1}) for 24 h, and then harvested and lysed for 30 min in a lysis buffer containing 1 mmol L^{-1} phenyl-

methanesulfonyl fluoride (PMSF). The cells were centrifuged for 10 min at 4 °C. The protein concentrations were determined using a BCA protein kit (Beyotime, China). Equal amounts of total protein were loaded onto 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDE) membranes. The PVDE membranes were then blocked for 1 h at 20 °C in a blocking solution before being blotted overnight at 4 °C with primary antibodies against Bax (#2772), Bcl-2 (#3498), cleaved caspase 3 (#9664), p-ERK1/2 (#4376), ERK1/2 (#4695), p-MEK (#86128), and MEK (#9126) (all used at a dilution of 1:1000). Before incubating with secondary antibodies for 1 h at room temperature, the membranes were washed three times with 5 % blocking buffer. Protein bands were visualized using chemiluminescence (ECL), and protein amounts were calculated using Image J. Cell Signaling Technology supplied all of the reagents.

Cell migration and invasion assays by transwell

Cell migration and invasion assays were carried out using a 24-well transwell assay with or without Matrigel according to the manufacturer's instructions. After treatment with or without compound **4** (10 and 20 μmol L^{-1}) for 48 h, HCT116 cells (2×10^4 cells *per* well) were resuspended in serum-free medium and seeded into the upper chamber of the transwell. The lower chamber was filled with a chemoattractant medium containing 10 % FBS, which was then cultured in the incubator for 24 h at 37 °C. The cells that had migrated and invaded were then fixed for 30 min with methanol, stained for 20 min with crystal violet solution, and washed with PBS for cell counting. Microscopic images were used to observe cell migration and invasion, and visual fields were randomly selected and photographed. The average cell count of the three stained membrane images was calculated using Image J software. The following steps were identical to those described above for the migration assay. The data were presented in the form of relative migration or invasion rates.

Statistical analyses

Data analysis was carried out using GraphPad Prism 5. Data from at least three independent experiments were presented as mean ± standard deviations (SD) and analyzed by the *t*-test or one-way analysis of variance. $p < 0.05$ denoted a statistical significance (indicated by *).

RESULTS AND DISCUSSION

The antiproliferative activity of compound **4** against human CRC HCT116 and HT29 cells, human gastric carcinoma SGC7901 cells, human breast cancer MDA-MB-231 cells, and human glioblastoma A172 cells was tested using the MTT assay. Compound **4** inhibited cell proliferation in a dose-dependent manner (Fig. 1). It had IC_{50} values of 8.04 ± 0.94 μmol L^{-1} in HCT116 cells, 17.71 ± 1.63 μmol L^{-1} in HT29 cells, 17.84 ± 0.70 μmol L^{-1} in SGC7901 cells, and 28.18 ± 8.88 μmol L^{-1} in MDA-MB-231 cells, respectively. Compound **4**, on the other hand, had a minor inhibitory effect on human glioblastoma A172 cells with an IC_{50} value of more than 200 μmol L^{-1}. Compound **4** had no effect on the viability of normal NCM460 cells derived from the human intestinal epithelium. Given that compound **4** had the

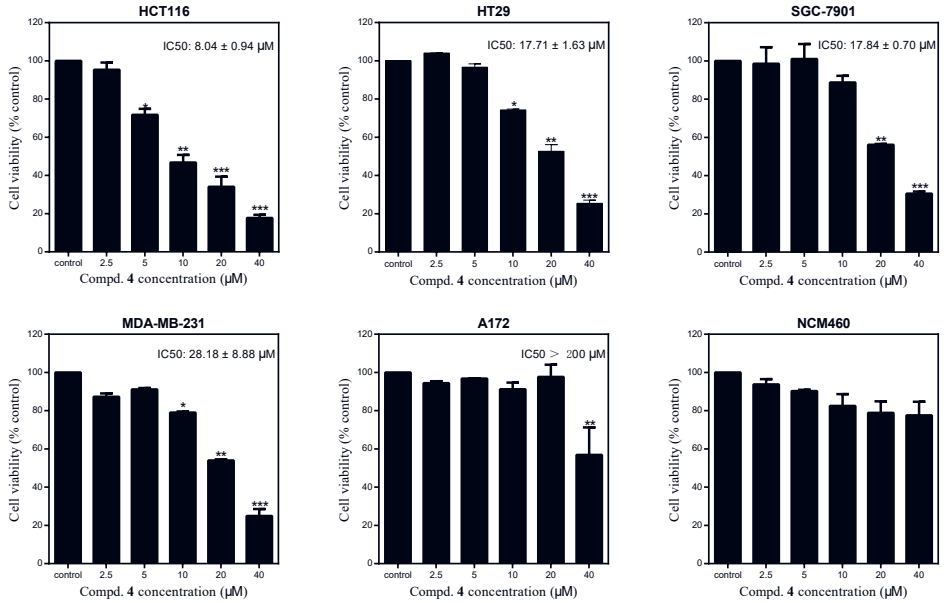


Fig. 1. The effect of compound **4** on tumor cell proliferation. HCT116, HT-29, SGC7901, MDA-MB-231, A172, and NCM460 cell lines were treated with compound **4** at various concentrations for 48 h. Data are represented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to the control group.

highest activity to HCT cells, the following experiments looked into the antitumor mechanism of compound **4** on human CRC HCT cells.

Compound **4** effectively inhibited HCT116 cell proliferation in a dose- and time--dependent manner (Fig. 2a). It had IC_{50} values of 21.64 ± 1.73 after 24 h, 8.04 ± 0.94 after 48 h, and 5.52 ± 0.42 μmol L^{-1} after 72 h for HCT116 cells, respectively. The colony formation assay was then used to determine the effect of compound **4** on the independent survival and environmental adaptation of cancer cells. The results showed that compound **4** inhibited the formation of CRC HCT116 cell colonies in a concentration-dependent manner (Fig. 2b).

To see if compound **4** inhibited CRC cell proliferation by regulating the cell cycle, the number of cells in the G0/G1 phase, S phase, and G2/M phase were counted using a flow cytometry method after cells were treated with compound **4** for 24 h. As shown in Fig. 3a, compound **4** treatment for 24 h significantly increased the G2/M phase cell population in HCT116 cells (from 18.50 ± 2.56 % to 28.97 ± 3.37 % at 10 μmol L^{-1}, and 30.51 ± 4.05 % at 20 μmol L^{-1}, $p < 0.05$). This suggested that compound **4** inhibited HCT116 cell proliferation by regulating the cell cycle so that it advanced from the S phase to the G2/M phase and then stopped.

Furthermore, compound **4** had the potential to significantly induce cellular apoptosis (Fig. 3b). Similarly, increasing the concentration of compound **4** increased the apoptotic rate significantly. The apoptotic rates in the 10 μmol L^{-1} (16.03 ± 3.11 %, $p < 0.01$) and 20 μmol

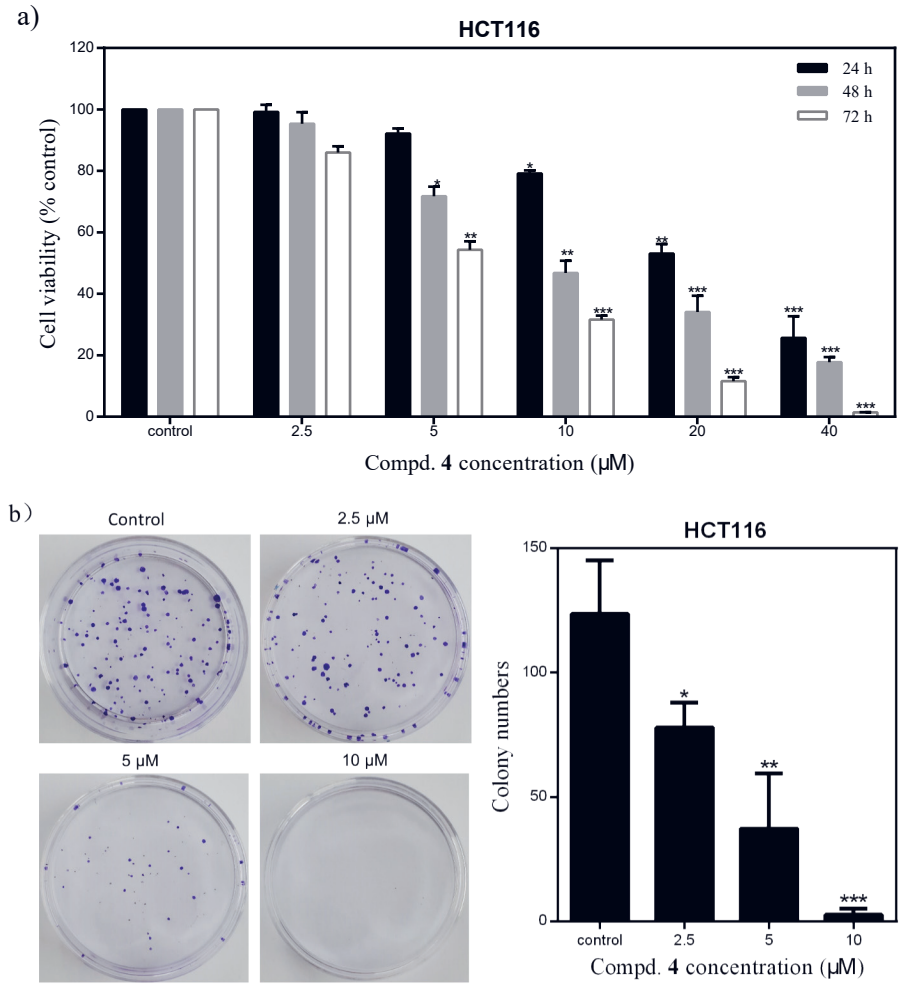


Fig. 2. The effect of the compound on HCT116 cell proliferation and growth. a) HCT116 cells exposed to compound **4** at various concentrations for 24, 48, or 72 h; b) HCT116 cell growth was assessed using colony formation assays. Data are represented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to the control group.

L^{-1} (38.71 ± 7.46 %, $p < 0.0001$) groups were considerably higher than in the control group (4.12 ± 0.96 %). Mitochondria plays an important role in cell apoptosis regulation, and the mitochondria-dependent process is regulated by the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 (28, 29). As shown in Fig. 3c, Compound **4** decreased the expression of Bcl-2 and the Bcl-2/Bax ratio, which determines the direction of apoptosis, while increasing the expression levels of Bax and cleaved caspase-3 in a dose-dependent manner when compared to the control group. These findings suggested that compound **4** induced

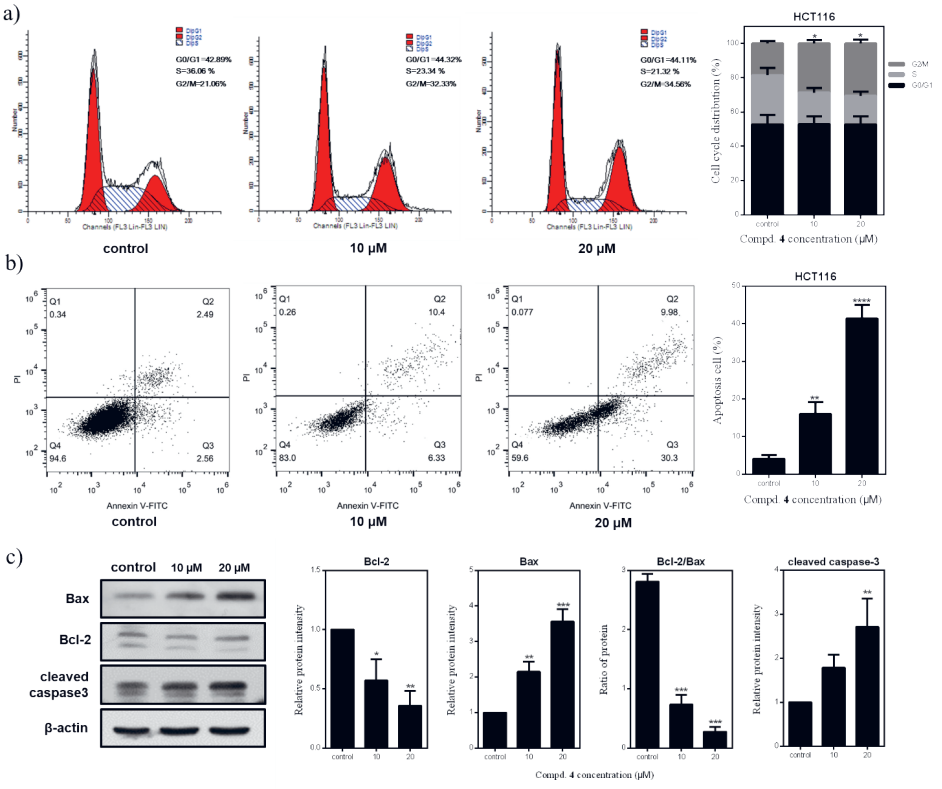


Fig. 3. Compound **4** induces G2/M arrest and promotes cell apoptosis in CRC HCT116 cells. a) The cell cycle distribution of HCT116 cells was assessed using flow cytometry after 24 h with compound **4** (10 and 20 μmol L^{-1}); b) apoptotic rates of HCT116 cells were analyzed by flow cytometry and Annexin V-FITC/PI staining after 24 h with compound **4** (10 and 20 μmol L^{-1}); c) the effect of compound **4** on the mitochondria-mediated apoptotic pathway. Apoptosis-associated protein levels were detected by Western blot analysis with antibodies against Bad, Bcl-2, and cleaved caspase-3. The protein intensities of the bands were quantified by the Image J program. Protein β-actin was used as an internal control. Data are represented as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ when compared to the control group.

apoptosis in human CRC cells by activating apoptosis-related proteins *via* the mitochondrial-dependent apoptotic pathway.

After treatment of HCT116 cells with compound **4** for 48 h, their ability to migrate decreased progressively when compared to the control group (Fig. 4a, $p < 0.001$). The relative migration rate of HCT116 cells at 10 μmol L^{-1} compound **4** was comparable to or close to that at 20 μmol L^{-1} compound **4**, which was 32.60 ± 6.25 % and 25.30 ± 4.41 %, respectively. Figure 4b depicted the relative invasion rates of HCT116 cells at 10 and 20 μmol L^{-1} compound **4**. With increasing concentrations, compound **4** exerted a greater effect on reducing the relative rate of invading cells. Therefore, compound **4** could inhibit HCT116 cell migration and invasion *in vitro* significantly.

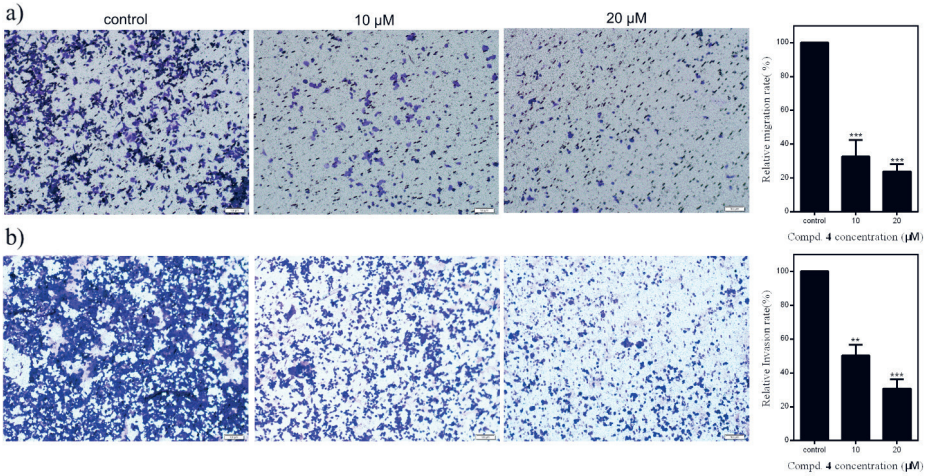


Fig. 4. Compound **4** inhibited HCT116 cell a) migration and b) invasion. Transwell assay was used to determine the migration and invasion capability of HCT116 cells treated with compound 4 (10 and 20 μmol L^{-1}) for 48 h. Data are represented as mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control group.

To better understand the regulatory mechanisms of compound **4** in HCT116 cells *via* the MEK/ERK pathway, MEK and ERK protein phosphorylation and expression levels were investigated. The results showed that after treatment with compound **4** (10 and 20 μmol L^{-1}) for 24 h, the expression ratios of p-MEK/MEK and p-ERK/ERK in HCT116 cells were significantly reduced in a dose-dependent manner (Fig. 5a). Compound **4** inhibited the expression of phosphorylated MEK and ERK proteins more effectively than unphosphorylated versions. To further confirm that cell apoptosis caused by compound **4** was linked with MEK/ERK signaling pathways, the MEK inhibitor U0126 was administered to HCT116 cells. The results showed that U0126 could increase the effects of compound **4** on the downregulation of Bcl-2 and upregulation of Bax in HCT116 cells. Accordingly, combined treatment with U0126 and compound **4** enhanced the phosphorylation inhibition of MEK and ERK1/2 in HCT116 cells when compared with the compound **4** only treated group (Fig. 5b). These results suggested that the anticancer effect of compound **4** on HCT116 cell growth was related to apoptosis induction *via* downregulation of the MEK/ERK signaling pathway.

Drug resistance and metastasis are the leading causes of treatment failure in CRC patients, and traditional treatments make it difficult to eradicate all aggressive CRC cells. Because of their broad bioactivities, exploring potent small-molecule inhibitors, particularly pyrimidine scaffolds (for instance, in-market pazopanib (30), infigratinib (31), and clinical surufatinib (32)), is a constant priority for research and development of new anticancer drugs. The active fragments of these marketed tyrosine kinase inhibitors with pyrimidine scaffolds were incorporated into the design using Docking Scores, and then a series of 4-(1,3,4-thiadiazole-2-ylthio) pyrimidine derivatives were synthesized (27). This study examines the antiproliferative activity of compound **4** against human tumor cells and shows that it has potent anticancer activity in human CRC HCT116 cells. Furthermore,

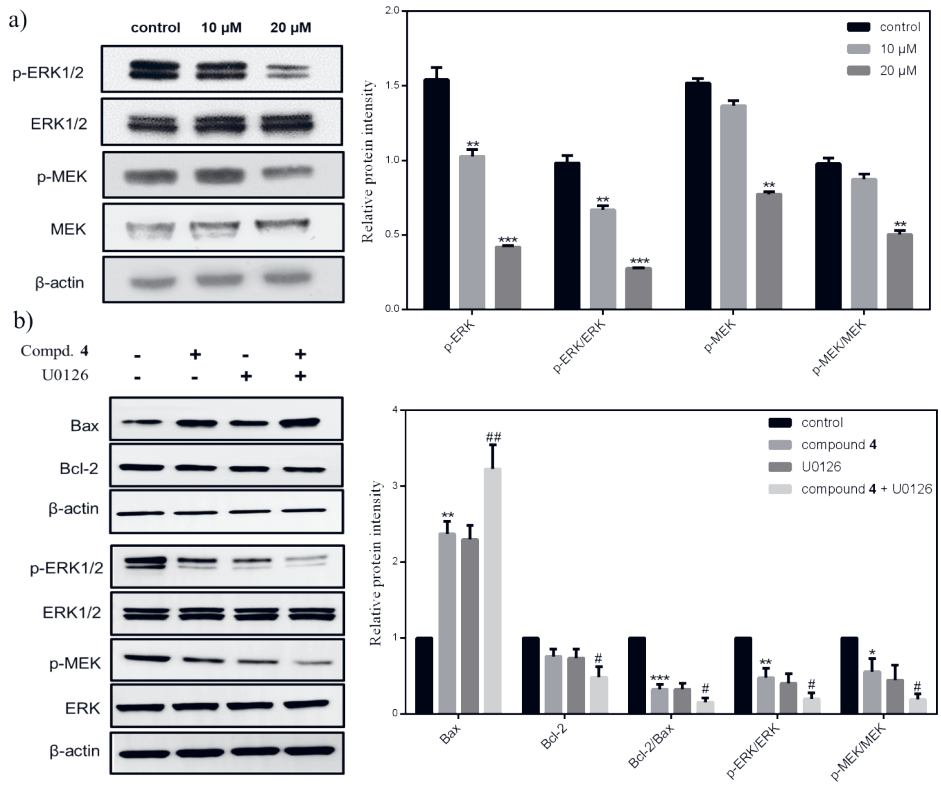


Fig. 5. Compound **4** inhibited the MEK/ERK signaling pathway in HCT116 cells. a) The expressions of MEK and ERK1/2 proteins were analyzed by western blot. Cells treated with compound 4 (10 and 20 μmol L^{-1}) for 24 h; b) effect of U0126 treatment as a specific inhibitor on the expression of apoptotic-related proteins and MEK/ERK pathway-related proteins. Protein β-actin was used for normalization. Data are represented as mean ± SD, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to the control group. #$p < 0.05$, ##$p < 0.01$ when compared to the compound **4** group.

compound **4** has low cell toxicity and enhances the therapeutic efficacy against tumor resistance. Therefore, we are further looking into the anticancer effects and underlying molecular mechanisms of compound **4** in HCT116.

CRC develops as a result of an imbalance in cell growth caused by excessive proliferation or a lack of apoptosis (33, 34). This study shows that compound **4** effectively reduces cell viability and growth of HCT116 cells in a dose- and time-dependent manner, as well as significantly arresting the cell cycles in the G2/M phase. In addition, compound **4** reduces the expression of Bcl-2 protein while increasing the expression of Bax and cleaved caspase-3. The most important proteins regulating apoptosis were Bcl-2 and Bax (29). Bcl-2 activation can promote cell growth and resistance to cell death, leading to abnormal increases in cell number and tumor growth. Bax may also stimulate apoptosis (35, 36). Therefore, the Bcl-2 to Bax ratio is critical. We discovered that compound **4** could significantly reduce the Bcl-2/Bax

ratio. Furthermore, one of the major biological characteristics of CRC formation is tumor cell metastasis (37). Compound **4** inhibited the migration and invasion of HCT116 cells *in vitro* in a dose-dependent manner. These findings explained how compound **4** suppressed CRC development and metastasis by inhibiting cell proliferation, growth, migration, and invasion and inducing mitochondria-mediated HCT116 cell apoptosis.

According to various studies (13, 15, 16, 19, 38–40), the MEK/ERK signaling pathway is a classic signal pathway in tumorigenesis and is also involved in cancer cell activities. It encourages cancer growth and metastasis, leading to poor therapeutic outcomes and tumor recurrence. The present study shows that compound **4** inhibits MEK and ERK phosphorylation levels in HCT116 cells, and MEK inhibitor U0126 could greatly intensify the apoptotic effects and phosphorylation inhibition of compound **4** on HCT116 cells compared to compound **4** single treatments, indicating that compound **4** exerts the anticancer effect on CRC cells by regulating the MEK/ERK signaling pathway.

CONCLUSIONS

Finally, the results show that compound **4** has anticancer activity against CRC HCT116 cells. It inhibits CRC cell proliferation and growth by inducing G2/M cell cycle arrest and mitochondria-mediated cell apoptosis. *In vitro*, it can also inhibit cell migration and invasion. Furthermore, compound **4** significantly inhibits the MEK/ERK signaling pathway in CRC HCT116 cells. These findings elucidate the molecular mechanism of compound **4** and suggest that compound **4** could be a novel potential lead small-molecule inhibitor of CRC.

Supplemantary material is available upon request.

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Conflicts of interest. – The authors declare no conflict of interest.

Author's contributions. – Conceptualization, W.L., M.Y. and J.W.; methodology, L.D. and M.Y.; analysis, W.L., Z.Y. and Y.W.; investigation, X.Z., J.C. and Q.J.; writing, original draft preparation, review and editing, W.L. and M.Y.; W.L. obtained the funding and supervised the whole project. All authors have read and approved to the published version of the manuscript.

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