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Discovery of Loureirin analogues with colorectal cancer suppressive activity via regulating cell cycle and Fas death receptor

Peng Li¹, Xiangjuan Tian¹, Die Zhang², Huiping Ou^{1,2}, Qiufeng Huang¹, Wenbin Jin^{2*} and Ran Liu^{1*}

Abstract

Chalcones and dihydrochalcones (DHCs) are important bioactive natural products (BNPs) isolated from traditional Chinese medicine. In this study, 13 chalcones were designed with the inspiration of Loureirin, a DHC extracted from *Resina Draconis*, and synthesized by classical Claisen-Schmidt reactions. Afterwards the reduction reactions were carried out to obtain the corresponding DHCs. Cytotoxicity assay indicated chalcones and DHCs possessed selective cytotoxicity against colorectal cancer (CRC) cells. The preliminary structure-activity relationships (SAR) of these compounds suggested the α , β -unsaturated ketone of the chalcones were crucial for the anticancer activity. Interestingly, compounds **3d** and **4c** exhibited selective anticancer activity against CRC cell line HCT116 with IC_{50s} of 8.4 and 17.9 μ M but not normal cell. Moreover, **4c** could also inhibit the migration and invasion of CRC cells. Mechanism investigations showed **4c** could induce cell cycle G2/M arrest by regulating cell cycle-associated proteins and could also up-regulate Fas cell surface death receptor. The virtual docking further pointed out that compounds **3d** and **4c** could nicely bind to the Fas/FADD death domain complex (ID: 3EZQ). Furthermore, silencing of Fas significantly enhanced the proliferation of CRC cells and attenuated the cytotoxicity induced by **4c**. These results suggested **4c** exerted its anticancer activity possibly regulating cell cycle and Fas death receptor. In summary, this study investigated the anticancer activity and mechanism of Loureirin analogues in CRC, suggesting these compounds may warrant further investigation as promising anticancer drug candidates for the treatment of CRC.

Highlights

- Loureirin analogues possessed selective anti-colorectal cancer activity.
- Dihydrochalcone **4c** exhibited anti-cancer activity by regulating cell cycle and Fas death receptor.
- Silencing of FAS gene promoted colorectal cancer cell growth.

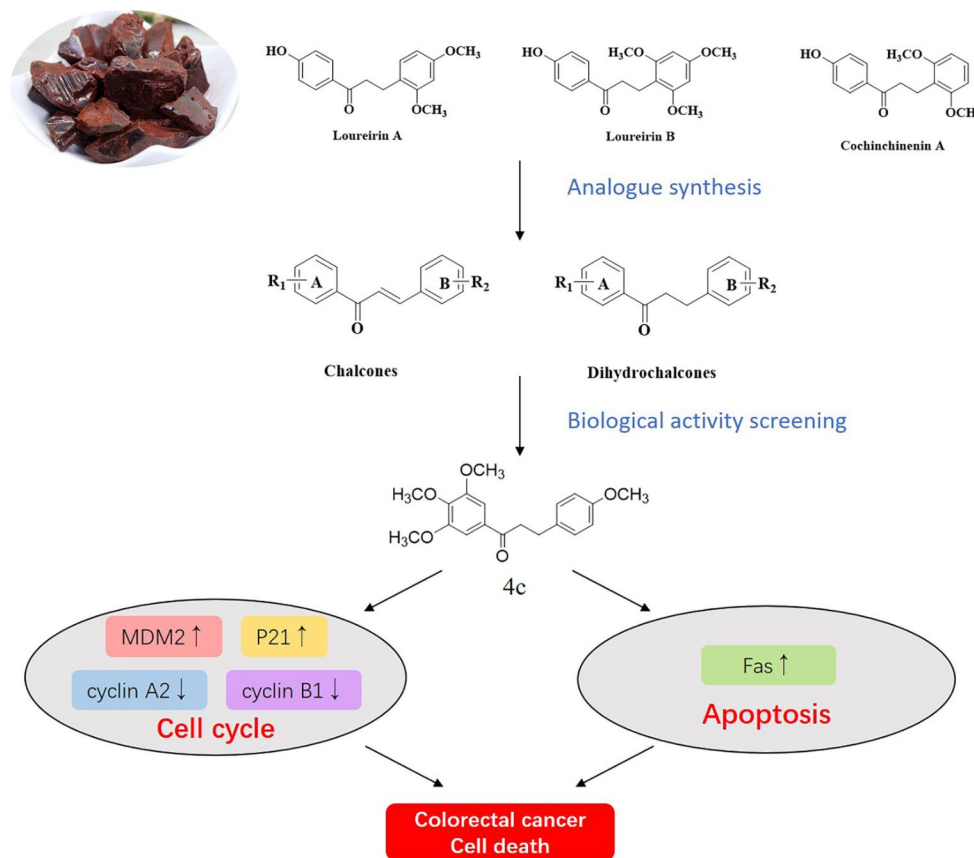
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Graphical abstract

Keywords Loureirin analogues, Chalcones and dihydrochalcones, Colorectal cancer (CRC), Cell cycle G2/M arrest, Fas

Introduction

Colorectal cancer (CRC) is the third common cancer counting for 10% of the newly diagnosed cases, leading to more than 900,000 deaths worldwide in 2020 [1]. Metastasis develops in 25–30% of CRC patients, being the leading cause of nearly 90% of the cancer-related deaths [2, 3]. Chemotherapeutic intervention coupled with surgery is the backbone of metastatic CRC treatment to increase survival [4]. Since the discovery of 5-fluorouracil (5-FU), it was the only chemotherapeutic agent available to improve 12 month survival of CRC for decades [5]. In recent years, new approaches for treating CRC have been developed including combination therapy, targeted therapy and immunotherapy. In 1998 and 2002, irinotecan and oxaliplatin were approved to combine 5-FU for the treatment of advanced CRC [6]. In 2004, cetuximab was the first approved targeted therapeutic drug as an anti-EGFR agent for the treatment of CRC, followed by the emerging of numerous agents brought

into preclinical and clinical trials until now [7]. In 2019, immunotherapeutic drug Nivolumab and Pembrolizumab have been approved for the treatment of metastatic CRC [8]. Mechanistically, CRC was reported to be caused by abnormal expression of genes related to DNA repair and cell cycle [9]. Anti-CRC reagents showed their anticancer activity by regulating cell cycle. Curcumin was reported to reverse 5-Fluorouracil resistance via inducing cell cycle arrest at G2/M phase [10]. Cyclin A2 and cyclin B1 were reported to be key regulators in controlling the cell entrance into M phase from G2 phase [11–13]. Metformin was also reported to alter the methylation status of tumor suppressor gene Ras association domain family 1 isoform A (RASSF1A) which induced cell cycle arrest [14]. Fas cell surface death receptor (also known as CD95), a member of the TNF receptor (TNFR) family, was reported to be involved in apoptosis pathway. Fas triggered apoptosis through binding with Fas ligands, followed by conformational changes and

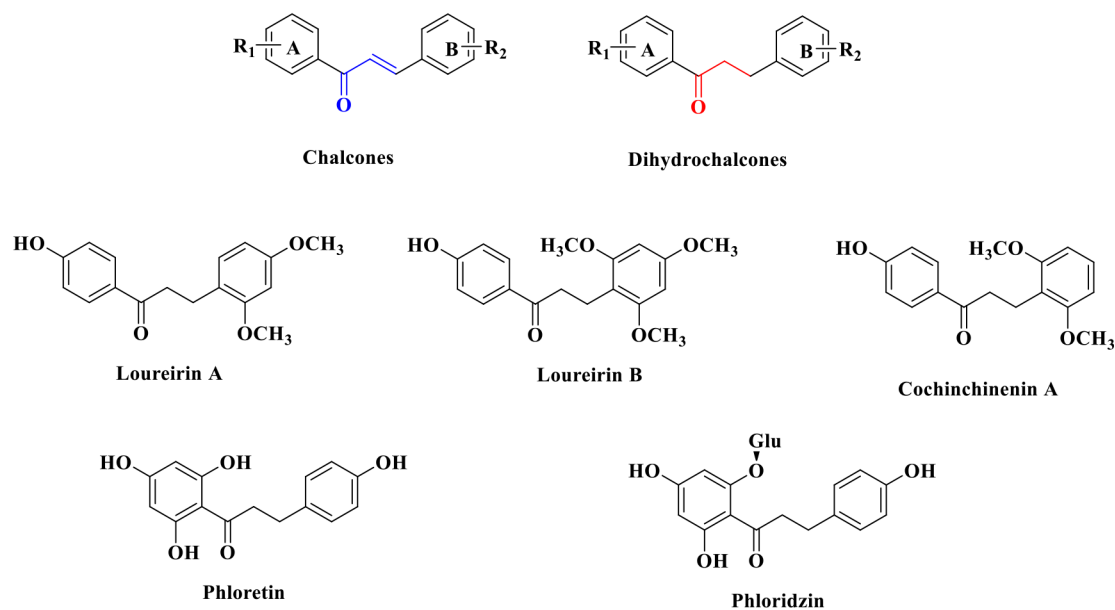


Fig. 1 Structures of chalcones and Dihydrochalcones

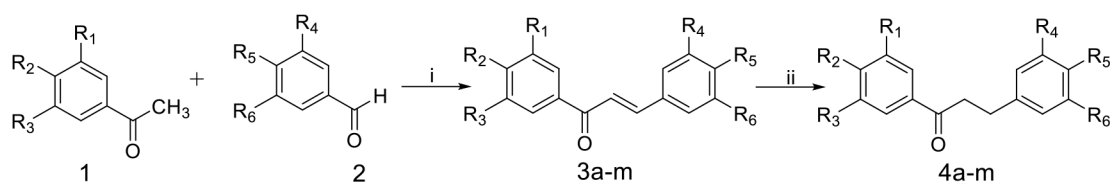
assembly of the Fas death-inducing signaling complex (DISC), which contains caspase-8 and activates the caspase cascade [15, 16]. Unfortunately, the putative clinical use of Fas ligand as anticancer reagent was troubled by severe liver toxicity related to the high expression of Fas in hepatocytes. Ligand-independent activation of Fas suggested that the death receptor could also be activated intracellularly in the absence of Fas ligand. This provided a novel framework for the development of anticancer drug targeting Fas [17]. Chalcones bearing two aromatic rings linked by an α , β -unsaturated propenone linker were considered to be primary precursors in the synthesis of flavonoids which were privileged scaffolds widely used for drug discovery in medicinal chemistry [18, 19]. The reactive α , β -unsaturated ketone was not only associated with the pharmacological properties including antimicrobial, antioxidant, anti-inflammatory and antitumor activities etc., but was also responsible for the key steps in which chalcones could be reductive to dihydrochalcones (DHCs) [20–23]. DHCs chemically distinct from chalcones also had two aryl rings connected through a saturated three-carbon bridge [24]. To date, DHCs for instance Phloretin, Phloridzin and Loureirin etc. shown in Fig. 1 have received growing attentions not only due to their convenient synthesis but also because of their broad functional and health-endorsing properties [25–27]. Loureirin A, Loureirin B, and Cochinchinenin A belonging to DHCs were important bioactive components extracted from traditional Chinese medicine *Resina Draconis* [28]. Several research results

suggested that Loureirin analogues showed promising antiproliferative activities against a collection of human cancer cell lines. In this study, we conducted the anticancer activity screening and mechanism investigation of a series of chalcones and Loureirin analogues made by ourselves in CRC.

Results

Chemistry

Claisen-Schmidt aldol condensation of acetophenone with the corresponding aromatic aldehyde in the presence of aqueous NaOH in ethanol gave the chalcone products. Dihydrochalcones were obtained by regioselective reduction of carbon-carbon double bond in α , β -unsaturated ketones. One of the methods used gaseous hydrogen, of which addition to the α , β -unsaturated olefinic bond was catalyzed by ruthenium salts in dioxane. Other methods used such common catalysts such as palladium, nickel and iridium. The chalcones **3** were prepared in good yields using the Claisen-Schmidt condensation of ketones **1** and aldehydes **2** (Scheme 1). This method for the preparation of chalcones was attractive since it predominantly generated the trans conformer from simple building blocks. A large number of these substituted benzaldehydes and acetophenones were commercially available and inexpensive. A series of DHCs **4** were prepared by base promoted reaction of chalcones **3** with palladium-carbon system in a hydrogen atmosphere. These products were easy to separate by flash chromatography (Scheme 1).



Scheme 1 Reagents and conditions: (i) EtOH, 20%NaOH, 0°C, 8 h; (ii) EtOH, Acetone, H₂, 10%Pd/C, HCO₂NH₂

Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
4a	H	OCH ₃	H	H	OCH ₃	H
4b	OCH ₃	OCH ₃	H	H	OCH ₃	H
4c	OCH ₃	OCH ₃	OCH ₃	H	OCH ₃	H
4d	F	H	F	H	OCH ₃	H
4e	CH ₃	F	H	H	OCH ₃	H
4f	H	OCH ₃	H	OCH ₃	OCH ₃	H
4g	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	H
4h	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H
4i	CH ₃	F	H	OCH ₃	OCH ₃	H
4j	H	OCH ₃	H	OCH ₃	OCH ₃	OCH ₃
4k	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	OCH ₃
4l	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃
4m	CH ₃	F	H	OCH ₃	OCH ₃	OCH ₃

Anticancer activity screening of chalcones 3a-m and DHCs 4a-m

The anticancer activity of chalcones 3a-m and DHCs 4a-m was preliminary screened by CCK-8 assay at a concentration of 20 μM. Figure 2 showed the cytotoxicity of chalcones 3a-m (A–C) and DHCs 4a-m (D–F) against CRC cell line HCT116, breast cancer cell line MCF7 and human fetal lung fibroblast HFL1, respectively. The results indicated that all chalcones 3 (Fig. 2A) with α, β-unsaturated ketone showed higher anticancer activity in HCT116 cell line compared to DHCs 4 (Fig. 2D) with saturated ketone. Similar results could also be found in MCF7 cell line as shown in Fig. 2B and E. Moreover, chalcones 3 exhibited much higher anticancer activity against CRC cell line HCT116 than breast cancer cell line MCF7 (Fig. 2A, B). Herein, we used human fetal lung fibroblast

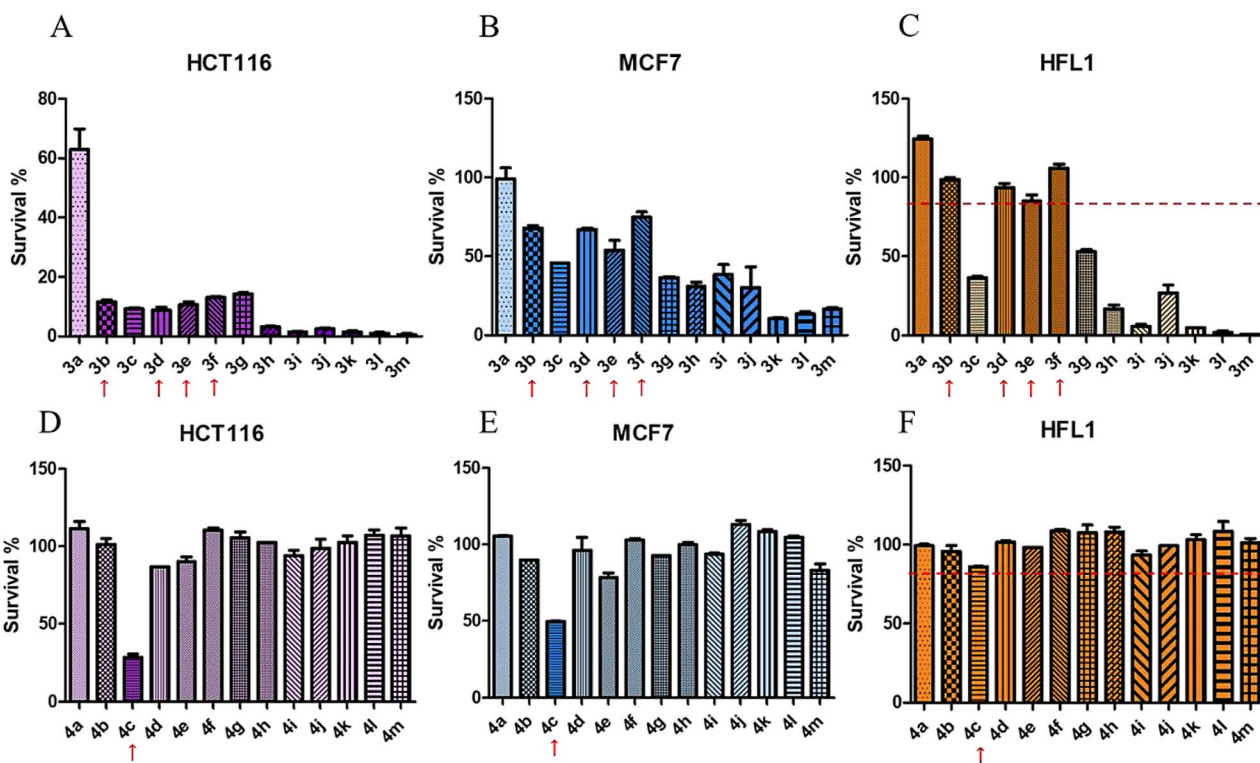


Fig. 2 Anticancer activity screening of chalcones 3a-m and DHCs 4a-m. **A–F**, CCK-8 assay of cancer cells and normal cell with 20 μM of chalcone treatment

HFL1 [29–31] to evaluate the cytotoxicity of chalcones **3** against normal cell (Fig. 2C). Surprisingly, chalcones **3a**, **3b**, **3d**, **3e** and **3f** were shown to be non-cytotoxic against HFL1 with a percentage survival rate higher than 80%. However, chalcones **3a** displayed non-cytotoxicity toward both HCT116 and MCF7 cells, either. Encouragingly, DHC **4c** was suggested to be cytotoxic to both HCT116 and MCF7, but not to HFL1, unlike other DHCs **4** without anticancer activity (Fig. 2D–F). Taken together, chalcones **3b**, **3d**, **3e**, **3f** and DHC **4c** were selected for further biological activity assay.

According to the preliminary screening assay, the anti-cancer activity of chalcones were much more potent against CRC cells compared to breast cancer cells. Therefore, we further determined the cytotoxicity of compounds **3b**, **3d**, **3e**, **3f** and **4c** against two CRC cell lines HCT116 and HT29, and human fetal lung fibroblast HFL1 as well. Among these compounds, the results suggested that the anti-proliferative activity of compounds

3d and **4c** showed much more specific to CRC cells compared to normal cell HFL1 at 10, 20 and 40 μM (Fig. 3). As shown in Fig. 3F, the anti-proliferative activity of compounds **3d** and **4c** against HCT116 with IC_{50} of 8.4 μM and 17.9 μM respectively was much more potent compared with that against HT29 and normal cell line HFL1. It also indicated that **3d** exhibited more promising anti-CRC effects than the positive control cisplatin with IC_{50} value of 14.4 μM according to the dataset provided by Genomics of Drug Sensitivity in Cancer website (<https://www.cancerxgene.org/>). However, **3d** the antitumor activity of **3d** was not as potent as that of 5-Fu as shown in Fig. 3G, H.

Compounds **3d** and **4c** inhibited the growth and migration of CRC cells

Next, we determined the effects of compounds **3d** and **4c** on colony formation, cell migration and invasion. As shown in Fig. 4A–D and Fig. 4E–H, both **3d** and **4c**

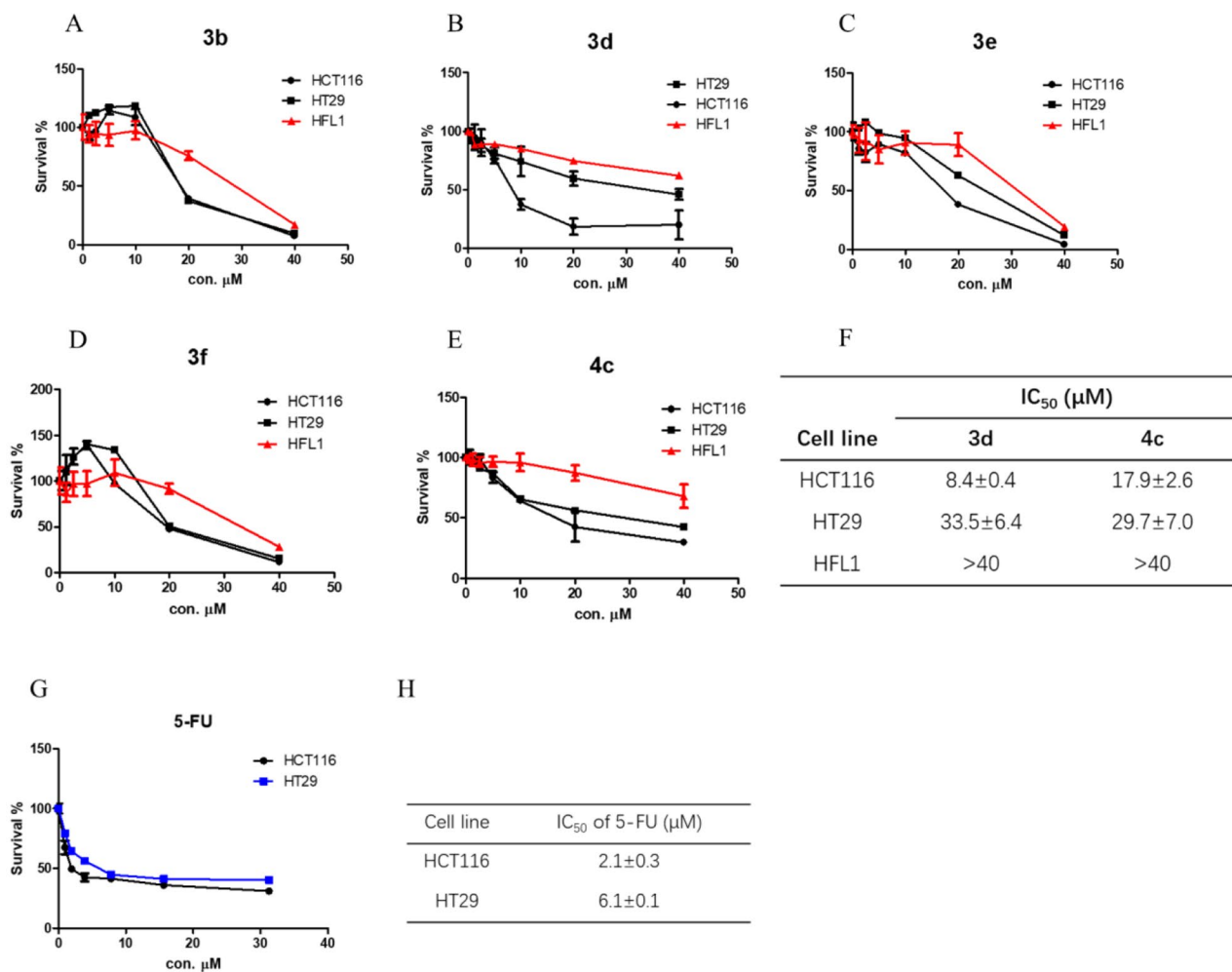


Fig. 3 Anti-proliferative activity screening of chalcones **3** and DHCs **4**. **A–E**, CCK-8 assay of CRC cells and normal cell with series dilution of chalcones. **F**, IC_{50} of **3d** and **4c** against CRC cells and normal cell. **G–H**, CCK-8 assay and IC_{50} of the positive control drug 5-FU. Data were presented as mean \pm SD

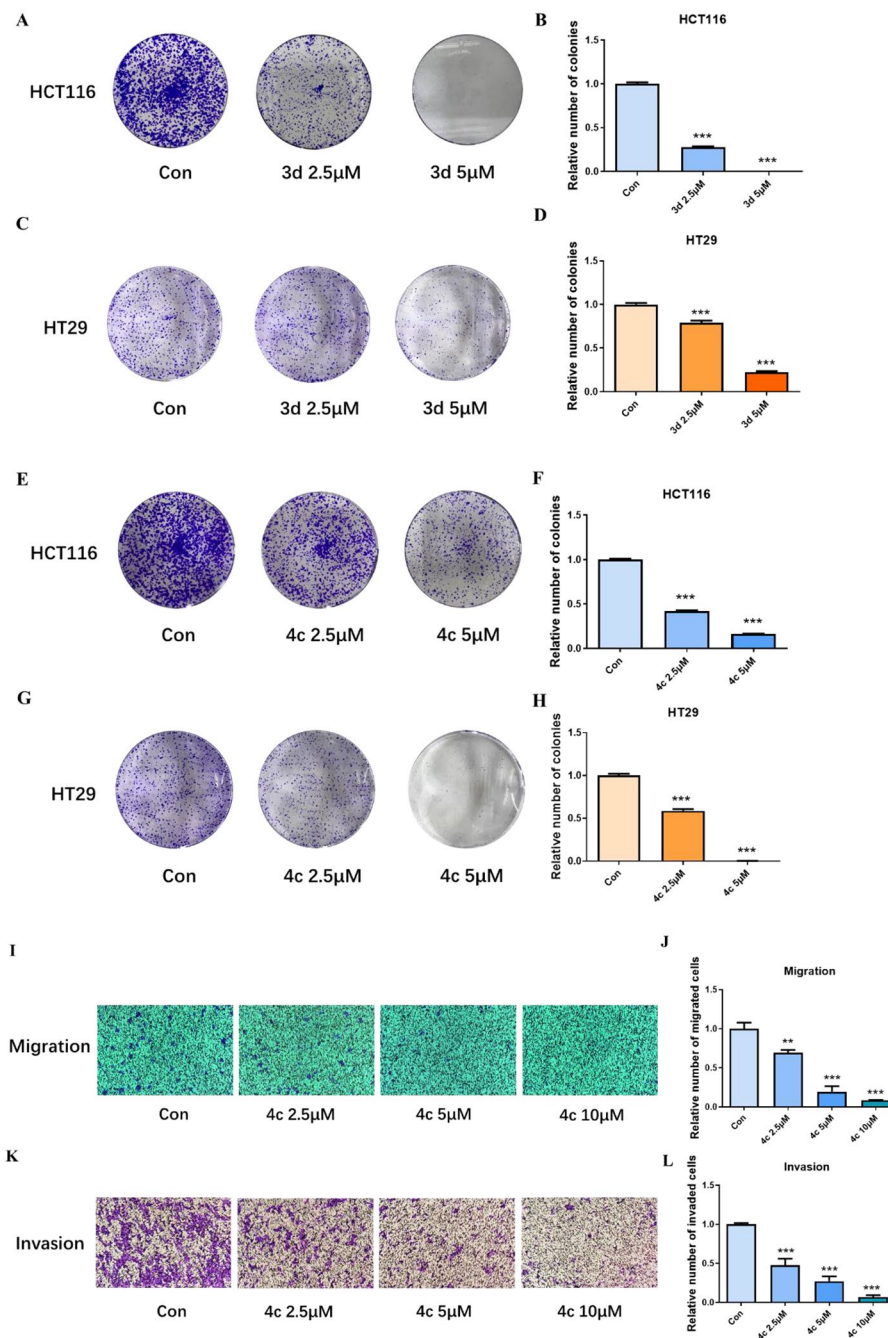


Fig. 4 Compounds 3d and 4c inhibited the growth and migration of CRC cells. **A–D**, colony formation assay of HCT116 and HT29 treated with 3d. **E–H**, colony formation assay of HCT116 and HT29 treated with 4c. **I, J**, transwell migration assay of HCT116 cell. **K, L**, transwell invasion assay of HCT116 cell. Data were presented as mean \pm SD. Statistically significant differences were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

inhibited the colony formation of HCT116 and HT29 cells, respectively. Furthermore, Fig. 4I–L indicated that 4c could inhibit the transwell migration and invasion of HCT116 cell. Compound 3d, however, showed no effect on either the migration or the invasion of HCT116 cell at the same condition (data not shown). These results indicated that compound 4c could inhibit both the cell growth and migration of CRC cell HCT116.

RNA-seq indicated compound 4c regulated p53 signaling pathway

To investigate the mechanism how 4c exhibited its anti-cancer activity in CRC cell HCT116, RNA-seq (RNA sequencing) was conducted. Figure 5A revealed that 253 genes were significantly up-regulated while 486 genes were down-regulated after treatment of 4c. Among the differentially expressed genes, the most up- and

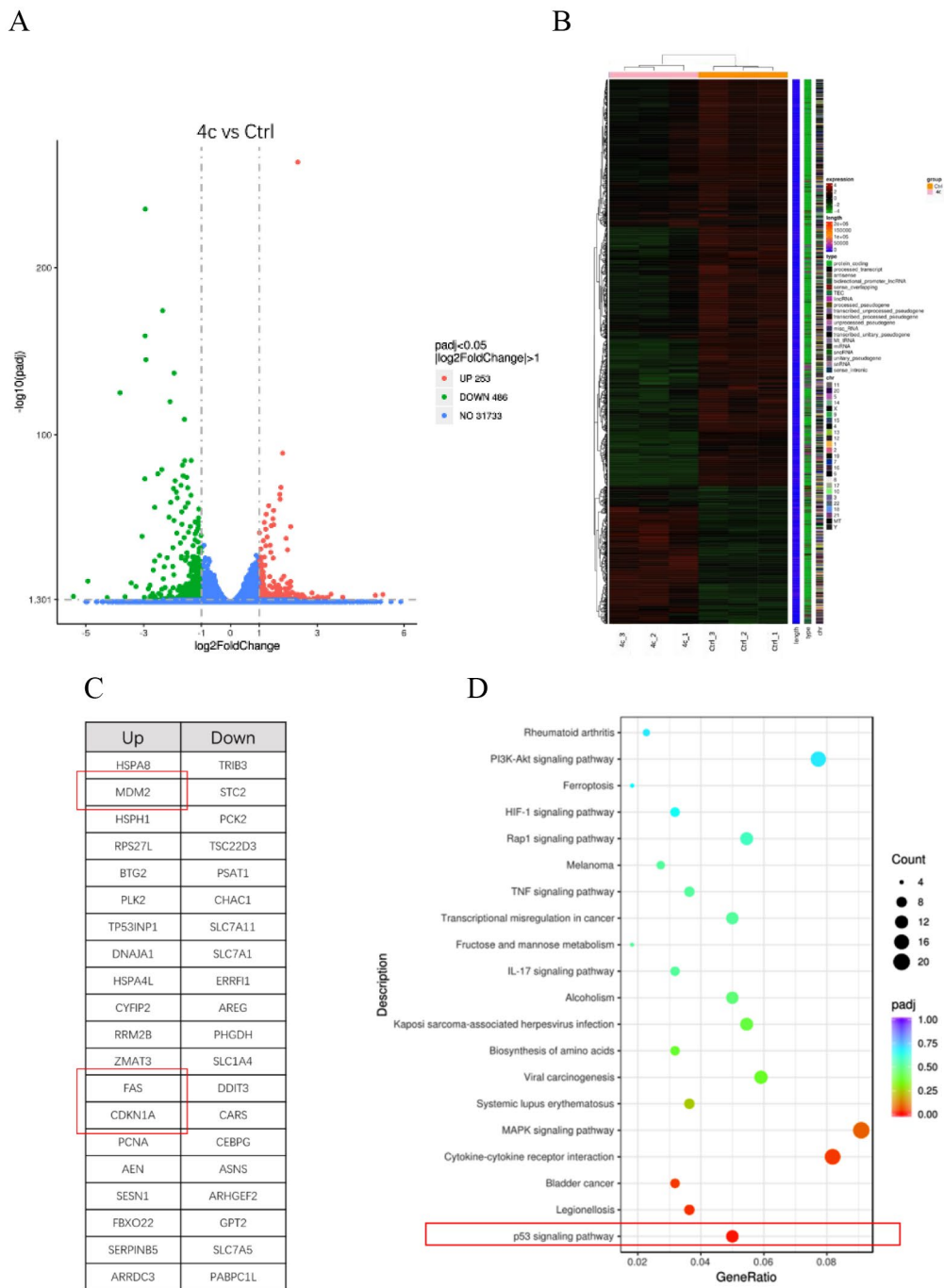


Fig. 5 Compound 4c regulated cell cycle and FAS death receptor. **A**, volcano plot showing the number of differentially expressed genes between **4c** treatment group and the control group. **B**, heat map showing differentially expressed genes between **4c** treatment group and the control group. **C**, Top 20 up- and down-regulated genes after **4c** treatment, including MDM2, CDKN1A and FAS. **D**, KEGG analysis of the differentially expressed genes after **4c** treatment

down-regulated genes were shown in Fig. 5B. The top 20 up- and down-regulated genes were listed in Fig. 5C. Focusing on cell proliferation and cell survival related genes, cell cycle-associated genes MDM2 and CDKN1A (also known as p21), as well as cell surface death receptor FAS were found to be significantly up-regulated. KEGG

analysis suggested p53 signaling pathway was the most significantly regulated pathway (Fig. 5D).

Compound 4c regulated cell cycle and FAS death receptor

According to the result of RNA-seq analysis, validation experiment was conducted. qPCR indicated that

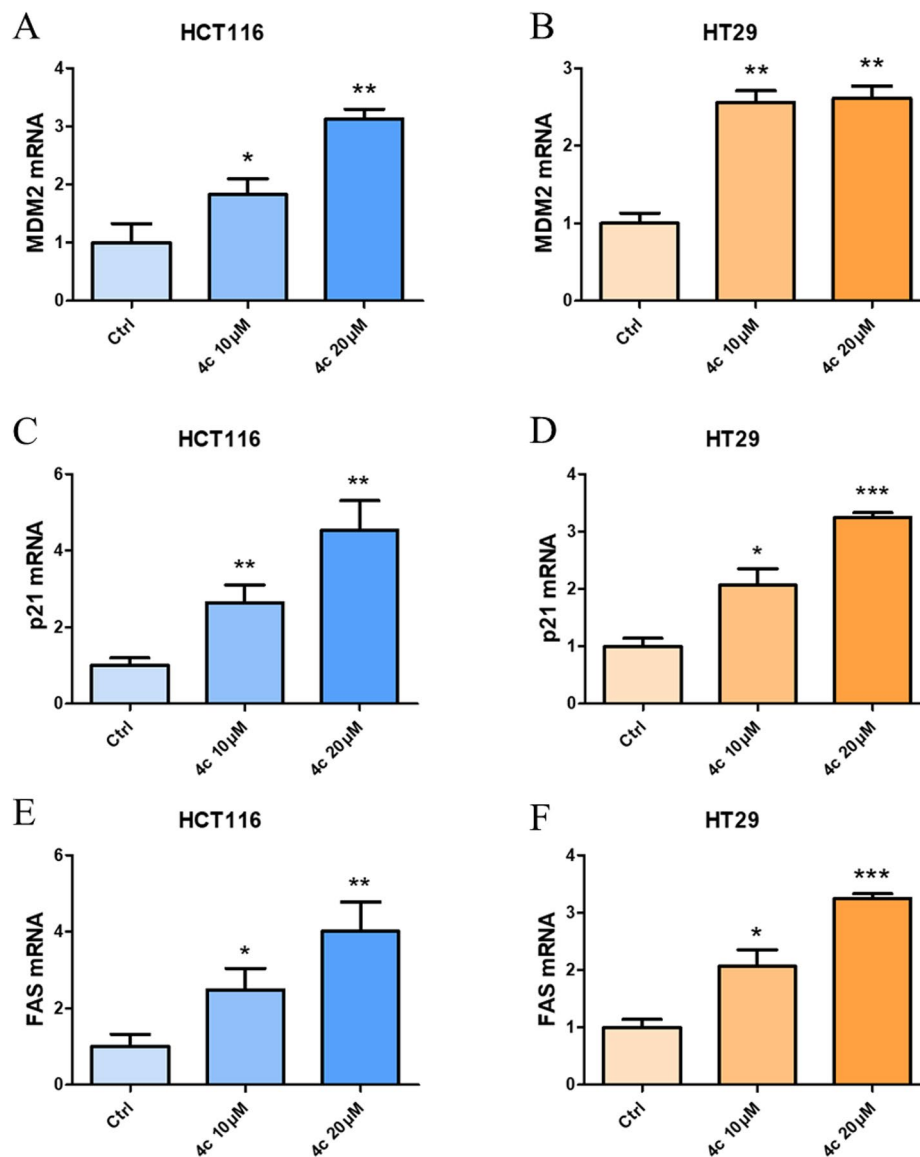


Fig. 6 qPCR validation of MDM2, p21 and FAS expression in HCT116 and HT29 cells with treatment of compound 4c at various concentrations. Data are presented as mean \pm SD. Statistically significant differences were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

the expression of cell cycle-associated genes MDM2 and p21, as well as cell surface death receptor FAS was significantly up-regulated both in HCT116 and HT29 cell lines with a dose-dependent manner (Fig. 6A–F). These results suggested 4c probably exerted its anticancer activity by regulating cell cycle and FAS death receptor.

To further confirm compound 4c could regulate cell cycle and FAS death receptor, cell cycle analysis and western blotting was performed. Results showed 4c could induce cell cycle G2/M arrest with a concentration-dependent manner (Fig. 7A–D). Meanwhile, western blotting results indicated that compound 4c could also

mediate cell cycle regulators MDM2 and p21 (Fig. 7E, F), as well as cell cycle G2/M checkpoints cyclin A2 and cyclin B1 (Fig. 7G, H). Meanwhile, 4c could also up-regulate the expression of FAS cell surface death receptor, a member of the TNF-receptor family (Fig. 7I, J).

AutoDock Vina was employed to provide more insights into molecular interactions between compound 3d and the Fas/FADD (Fas-associated death domain protein) death domain complex (ID: 3EZQ), a tetrameric arrangement of four FADD death domains bound to four Fas death domains. The Fas-FADD death inducing signalling complex (DISC) assembled by Fas receptor, FADD and

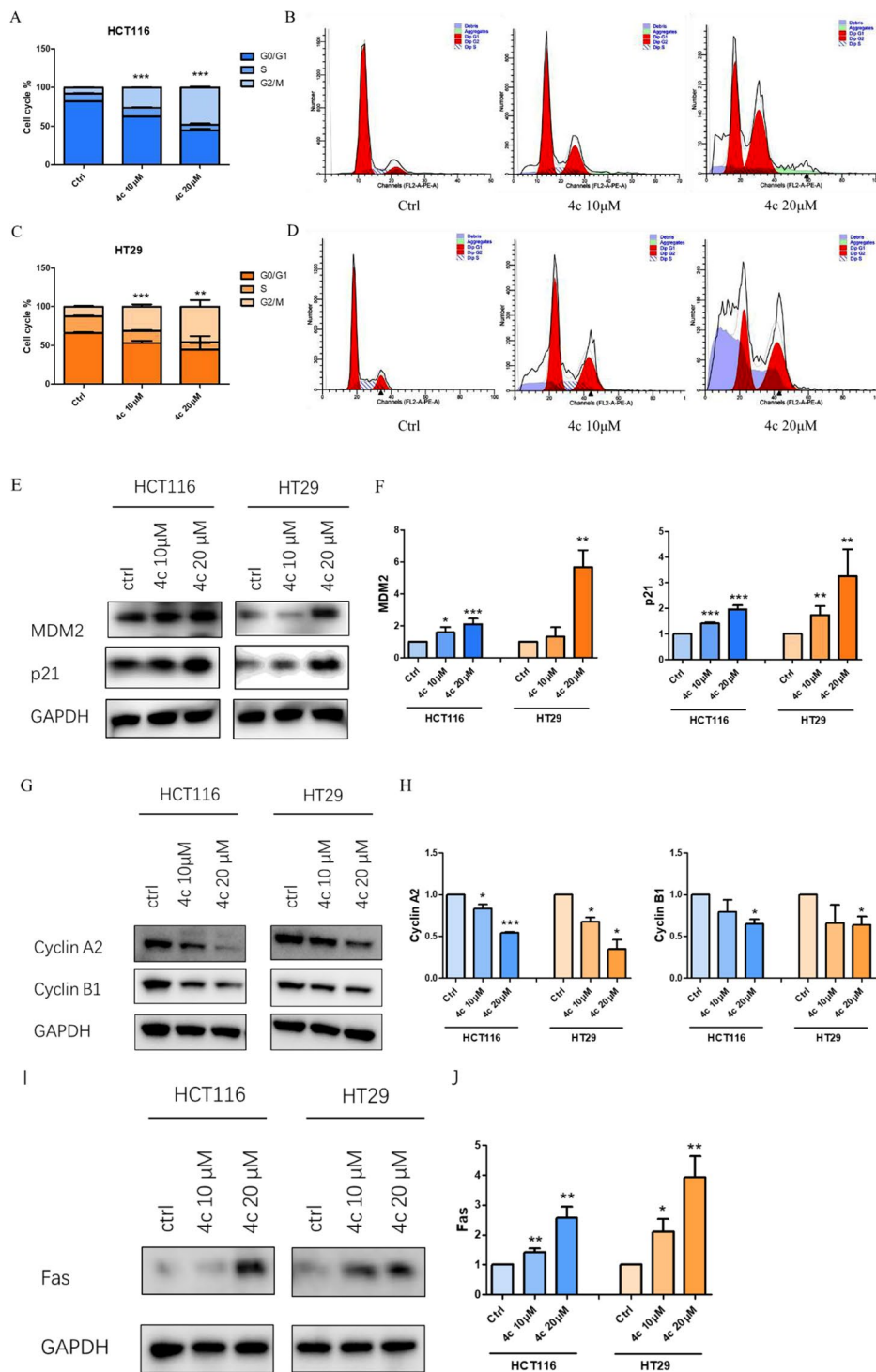


Fig. 7 Compound 4c regulated cell cycle and FAS death receptor. **A–D**, cell cycle analysis of CRC cells after 4c treatment using flow cytometry. **E, F**, 4c promoted cell cycle regulator MDM2 and p21. **G, H**, 4c suppressed cell cycle checkpoints cyclin A2 and cyclin B1. **I, J**, 4c up-regulated the expression of FAS death receptor. Data were presented as mean ± SD. Statistically significant differences were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

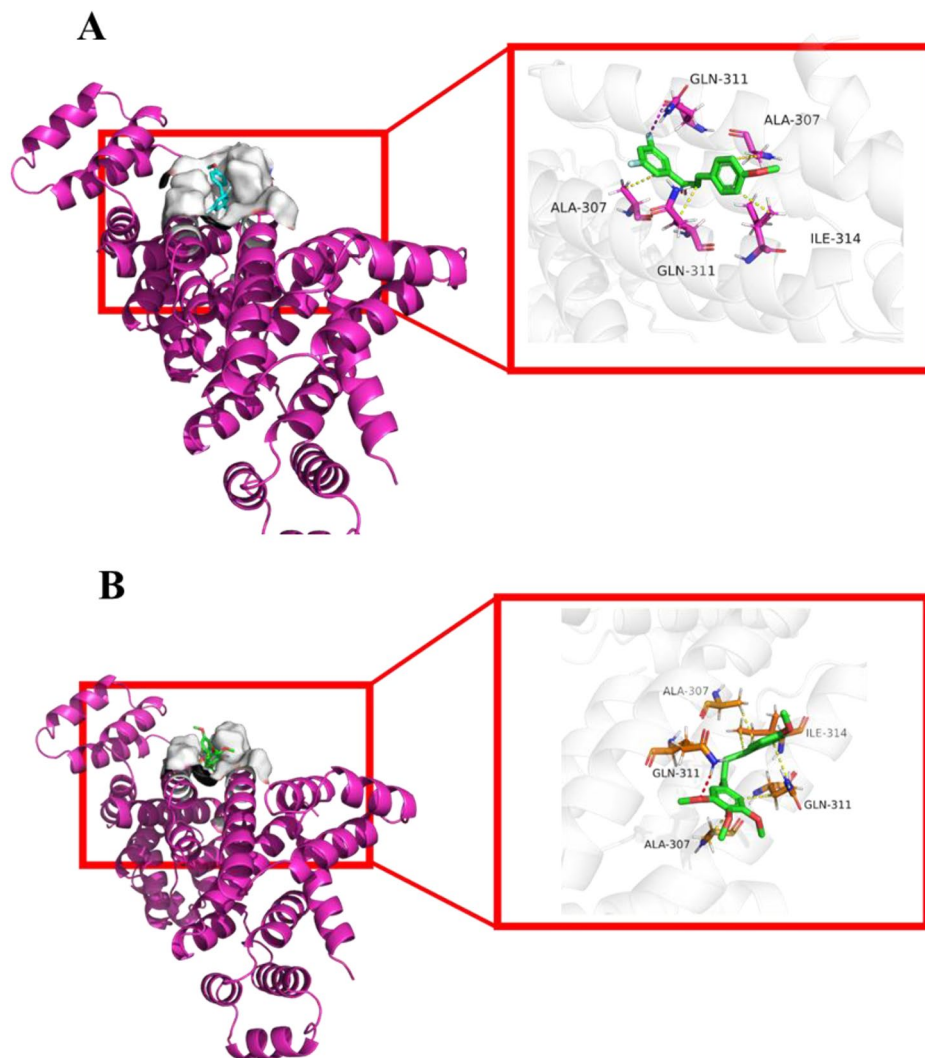


Fig. 8 Binding mode of the Fas/FADD death domain complex (PDB code: 3EZQ) in complex with compounds **3d** (A) and **4c** (B)

caspace 8 represents a receptor platform responsible for initiation of the induction of programmed cell death. The results shown in Fig. 8A revealed that the highest docking score positioned compound **3d** into the ligand-binding site of the Fas/FADD death domain complex. Hydrophobic interactions were predicted to occur between ALA-307 (A), ILE-314 (C), GLN-311 (C), ALA-307 (C) and compound **3d**. Furthermore, the docking score was -7.0 , suggesting that **3d** had better binding energy with the Fas/FADD death domain complex.

PLIP website also figured out that compound **4c** formed hydrophobic interactions with residues ALA307 (A), ALA307 (C), GLN311 (A), and ILE314 (A) of the Fas/FADD death domain complex, as well as hydrogen bonds with GLN311 (C). Additionally, the distance of

the abovementioned interactions ranged from 3.18 \AA to 3.89 \AA , suggesting strong physicochemical forces. Meanwhile, the binding affinity values of compound **4c** were -6.2 kcal/mol , indicating that **4c** nicely bound to the Fas/FADD death domain complex.

Knockdown of FAS promoted the proliferation of CRC cells

Since FAS was also known as apoptosis-associated gene, we then investigated whether **4c** could induce cytotoxicity by up-regulating FAS gene. Results showed that knockdown of FAS using small interfering RNA could significantly promote the proliferation of CRC cells (Fig. 9A, B). Moreover, HCT116 cells knocked down with FAS gene could attenuate the cytotoxicity induced by **4c** (Fig. 9C).

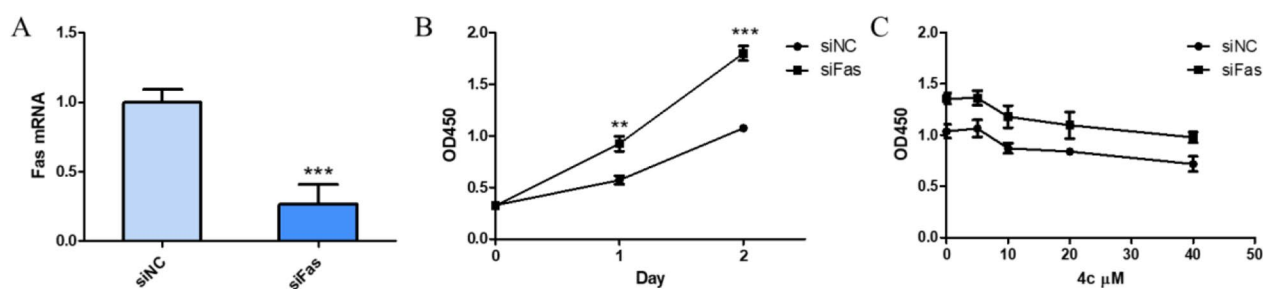


Fig. 9 Knockdown of FAS promoted the proliferation of CRC cells. **A**, qPCR assay of HCT116 cell knocked down with FAS using siRNA. **B**, CCK-8 assay of HCT116 cell knocked down with FAS. **C**, CCK-8 assay of HCT116 cell knocked down with FAS followed by **4c** treatment for 24 h. Data were presented as mean \pm SD. Statistically significant differences were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Discussion

Loueirins are important bioactive components extracted from traditional Chinese medicine *Resina Draconis*. The anticancer activity of Loueirin analogues was investigated in this study. Preliminary screening results suggested Loueirin analogues possessed anti-CRC activity. Interestingly, SAR study suggested the α , β -unsaturated ketone of the chalcones **3** was crucial for their anticancer activity. A collection of reports also supported this hypothesis as chalcones possessed a ketoethylenic moiety which could serve as Michael acceptor in Michael addition reactions with the active site residues [32], leading to manifestation pharmacological potential of chalcones including antiproliferation, antibacterial, antiviral, anti-fibrotic, and anti-leishmania, etc. [33–37].

Among the Loueirin analogues screened in this study, chalcone **3d** and DHC **4c** exhibited specific anti-CRC activity meanwhile showed no cytotoxicity against human fetal lung fibroblast HFL1. Moreover, **4c** not only inhibited the proliferation but also the migration and invasion of CRC cells, suggesting **4c** exhibited better anti-malignant properties than **3d**. RNA-seq analysis and validation experiments indicated **4c** could regulate cell cycle regulator MDM2 and p21. Subsequent flow cytometry analysis confirmed **4c** could block cell cycle at G2/M phase. Cell cycle G2/M checkpoint cyclin A2 and cyclin B1 were also found to be significantly down-regulated by **4c** [12, 38].

Additionally, FAS was also found to be significantly up-regulated after **4c** treatment. FAS, a member of the TNFR family, is widely known to be involved in apoptosis pathway. It triggers apoptosis by binding with its ligand followed by the recruitment and assembly of the FAS DISC and subsequent activation of the caspase cascade [15, 16]. Other than apoptosis, FAS was also reported to regulate cell cycle G2/M arrest in CRC [39], which raised our hypothesis that **4c** probably induced cell cycle G2/M arrest by mediating FAS. While unfortunately, inconsistent with our hypothesis, knockdown of FAS gene did not regulate MDM2 and p21 significantly, and no reversing

effect was observed even after **4c** treatment, from which we concluded that cell cycle G2/M arrest induced by **4c** was not mediated by FAS. As a result, **4c** exerted its anti-CRC activity by regulating cell cycle and possibly FAS-mediated apoptosis. Meanwhile, virtual docking also revealed that compound **4c** could nicely fit into the receptor-binding site of the Fas/FADD death domain complex (ID: 3EZQ).

Conclusion

To conclude, our research for the first time elucidated dihydrochalcone **4c**, inspired by Loueirin, important bioactive components extracted from traditional Chinese medicine *Resina Draconis*, exhibited selective anti-CRC activity via regulating cell cycle and FAS, which has the potential to be further developed into anticancer agents for the treatment of CRC.

Material and methods

Reagents, solvents and chemicals

Starting materials and reagents of commercial grade could be directly employed without further purification unless otherwise stated. All of the Loueirin analogues including chalcones **3** and DHCs **4** were synthesized according to our previous paper published [40]. All tested compounds with at least 95% purity were determined by an Agilent 1220 Infinity II. The NMR spectra, high resolution MS spectra, melting points, FT-IR spectrum and HPLC spectrum of the Loueirin analogues could be given with reference to the aforementioned paper whose DOI was 10.1039/D4MD00011K.

Cell culture and transfection

Human cancer cell line HCT116, HT29 MCF7 was kindly provided by Dr. Ouyang from Shenzhen Polytechnic University and human embryonic lung fibroblast HFL1 was obtained from Shenzhen large-scaled cell culture and cell bank in Shenzhen Polytechnic University. Cells were cultured with RPMI 1640 medium (8121465, Gibco, USA), supplemented with 10 %v/v fetal bovine

serum (FBS500-S, AusGeneX) and 1% penicillin/streptomycin (25200–056, Gibco, USA) at 37 °C in a saturated humidified atmosphere supplied with 5% CO₂. Knock-down of Fas was performed by transient transfection with siRNA (RIBOBIO, China) and lipofectamine 2000 (2067563, Invitrogen, USA) according to the manufacturer's instructions. The targeted sequence of Fas siRNA was GCAGATGTAAACCAAACCTT. Negative control siRNA was obtained from RIBOBIO with catalogue No. siN0000001-1-5.

CCK-8 assay and colony formation assay

According to our previous protocol published before, a number of 5000 cells/well was seeded in 96-well plate [41, 42]. Compounds were added after cell attachment with an incubation for 48 h. CCK-8 reagent (C6005, NCM Biotech, China) was used to determine cell proliferation according to the manufacturer's instructions. OD450 was determined after CCK-8 incubation.

Colony formation was performed by seeding 2000 cells/well in 6-well plate and allowed to grow for 7 days followed by fixing. Fixing was conducted using methanol for 20 min at room temperature and staining was conducted using crystal violet for another 10 min.

Cell migration and invasion assay

For cell migration assay, a number of 2×10^4 cells/well of HCT116 cell was seeded into the upper chamber of the transwell insert (725301, NEST) in RPMI 1640 medium without FBS. The lower chamber was filled with RPMI 1640 medium with 10% FBS. Compound was added in culture medium in both the upper and lower chambers when cells were seeded. Cells were allowed to migrate for 24 h. Cells in the upper chamber of the insert were removed with cotton swabs and the migrated cells at the bottom of the membrane were fixed with methanol and stained with crystal violet. For cell invasion assay, the membrane of the transwell insert was coated with Matrigel (354234, Corning,) on top according to the manufacturer's instructions. A number of 4×10^4 cells/well of HCT116 cells was used. Cells were allowed to invade for 48 h.

Western blot

Cells with different treatment were lysed using RIPA lysis bufer (WB3100, NCM Biotech, China) supplemented with 1×protease inhibitor (70090050, Biosharp, China) and 1×phosphatase inhibitor (70080020, Biosharp, China) at 4 °C. Western blot was performed according to the common protocol. The primary antibodies used were anti-MDM2 antibody (ab3110, abcam), anti-p21 antibody (2947T, CST), anti-Fas antibody (4233T, CST), anti-GAPDH antibody (5174S, CST), anti-cyclin A2 antibody (4656S, CST), anti-cyclin B1 antibody (12231S, CST).

The secondary antibodies used were Anti-mouse IgG, HRP-linked Antibody (7076S, CST) and Anti-rabbit IgG, HRP-linked Antibody (7074S, CST). The dilution for primary antibody was 1:1000 and the dilution for secondary antibody was 1:3000.

Flow cytometry

A number of 6×10^5 cells/well were seeded in 6-well plate for 24 h before drug treatment. HCT116 cell was treated with 4c for 48 h and HT29 cell was treated with 4c for 24 h before cell collection followed by cell fixation with 70% ethanol. Cells were stained with cell cycle analysis kit from BD (550825). Flow cytometry analysis was performed using Beckman DxFLEX flow cytometer. Data analysis was conducted using modfit.

RNA extraction and quantitative PCR (qPCR)

Extraction was conducted using RNA extraction kit (DP419, DP501, Tiangen, China). Reverse transcription was conducted using reverse transcription kit (RR037A, Takara). qPCR was performed using StepOnePlus (ABI) and SYBR green qPCR kit (A6002, Promega).

Primers used in the qPCR assays were

GAPDH forward: 5'-CGAGATCCCTCCAAAATCA
A-3',

GAPDH reverse: 5'-GGTGCTAAGCAGTTGGTGG
T-3',

FAS forward: 5'-GACTCAGAACTTGGAAGGCC-3',

FAS reverse: 5'-TCATGACTCCAGCAATAGTGG-3',

p21 forward: 5'-TAGCAGCGGAACAAGGAG-3',

p21 reverse: 5'-AAACGGGAACCAGGACAC-3',

MDM2 forward: 5'-TTATTAAGTCTGTTGGTGC
A-3',

MDM2 reverse: 5'-TGAAGTTTCTCTTCCTGAA
G-3'.

RNA-seq

HCT116 cell was seeded in 3-cm dish followed by 20 μM of 3c treatment for 24 h before RNA extraction. Samples were prepared in triplicates. Total RNA was extracted with Trizol reagent (15596026, Invitrogen) according to the manufacturer's instructions. Preparation of RNA library and transcriptome sequencing was conducted by Novogene Co., LTD (Beijing, China). Genes with adjusted p -value < 0.05 and $|\log_2(\text{FoldChange})| > 1$ were set as the threshold for significantly differential expression.

Computational docking studies

AutoDock vina 1.2.0 was employed for virtual docking [43, 44]. The 3D structures of compounds 3d and 4c were provided and conducted energy minimization by Chem-Draw 22.0 prior to importing into the AutoDock Vina.

The crystal structure of the Fas/FADD Death Domain Complex (ID: 3EZQ) downloaded from the Protein Data Bank (<https://www.rcsb.org/>) was used for docking measurements after removal of water molecules, metal ions, and small ligands bound to the protein, as well as the duplicate domains. According to the papers reported before, the ligand-binding pocket of the Fas/FADD Death Domain Complex was thought to be around the crucial residue Ile313, necessary for the Fas-opening which is a key step responsible for the formation of Fas–Fas dimer and the receptor–adaptor interactions [45, 46]. The binding results after analysis of PLIP website were visualized by PyMOL version 2.5.2 software.

Statistical analysis

All the quantitative results were presented as the mean \pm standard deviation (SD). Student's *t*-test was used to compare the Statistical significance between two groups using Prism. Differences were considered as significant when $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Abbreviations

SAR	Structure-activity relationships
DHC	Dihydrochalcone
RNA-seq	RNA sequencing
TNFR	TNF receptor
DISC	Death-inducing signaling complex

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40360-024-00758-2>.

Supplementary Material 1

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Author contributions

PL: Conceptualization, formal analysis, investigation, methodology, writing-original draft, writing-reviewing and editing, funding acquisition. XT: Investigation, methodology. DZ: Investigation (chemical synthesis), methodology. HO: Investigation. QH: Investigation. RL: Conceptualization, resources, supervision, funding acquisition. WJ: Conceptualization, resources, writing-original draft, writing-review and editing, supervision, funding acquisition.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

All authors disclosed no relevant relationships.

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