Aspirin promotes RSL3-induced ferroptosis by suppressing mTOR/SREBP-1/SCD1-mediated lipogenesis in PIK3CA-mutant colorectal cancer

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\textbf{ABSTRACT}

Ferroptosis, a new form of regulated cell death triggered by the iron-dependent peroxidation of phospholipids, is associated with cellular metabolism, redox homeostasis, and various signaling pathways related to cancer. Aspirin is a widely used non-steroidal anti-inflammatory drug (NSAID) and has been reported to show therapeutic benefit in cancers harboring oncogenic PIK3CA, which encodes the catalytic p110α subunit of phosphoinositide 3-kinase (PI3K). In this study, we found that aspirin sensitized cancer cells harboring oncogenic activation of PIK3CA to ferroptosis induction. Mechanistically, aspirin inhibited protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling, suppressed downstream sterol regulatory element-binding protein 1 (SREBP-1) expression, and attenuated stearoyl-CoA desaturase-1 (SCD1)-mediated lipogenesis of monounsaturated fatty acids, thus promoting RSL3-induced ferroptosis in colorectal cancer (CRC) cells. Moreover, genetic ablation of SREBP-1 or SCD1 conferred cancer cells greater sensitivity to ferroptosis induction. Conversely, ectopic expression of SREBP-1 or SCD1 restored ferroptosis resistance in CRC cells and abolished the effect of aspirin on RSL3-induced cytotoxicity. Additionally, the synergistic effects of aspirin and RSL3 were confirmed in a xenograft mouse model. The combined use of aspirin and RSL3 resulted in significant tumor suppression. Our work demonstrated that aspirin enhanced the cytotoxic effect of RSL3 in PIK3CA-mutant cancers, and the combination of aspirin and ferroptosis inducer displayed promising therapeutic effects in cancer treatment.

1. Introduction

CRC is a serious health burden worldwide, ranking third in incidence and second in mortality among all cancers [1]. As the dreadful disease becomes symptomatic at an advanced stage, primary prevention and organized screening programs are of great importance [2–4]. However, once the early critical stage is missed, CRC is difficult to control and caused approximately 935,000 deaths last year [5]. Despite great progress, such as the development of new targeted drugs [6] or immune checkpoint inhibitors [7,8] since the 2000s, the overall prognosis of patients with advanced or recurrent CRC is relatively unsatisfactory [9].

Aspirin (acetylsalicylic acid) is the cornerstone of modern therapies because of its important role in analgesia and cardiovascular prophylaxis [10,11]. In addition, epidemiological studies indicate that daily use of low-dose aspirin is associated with a reduced risk of cancer development [12,13], suggesting that it can be used as part of adjuvant therapy. Inhibition of cyclooxygenase (COX) enzymes, particularly COX-2, and reduced production of inflammatory mediators are thought to be the main mechanisms of tumor control [14]. In addition, other COX-independent mechanisms, such as inhibition of NF-kappaB [15], Wnt/beta-catenin [16,17], and the mTOR signaling pathway [18,19] are involved. A recent study showed that aspirin use is associated with a significant improvement in survival in patients with mutant PIK3CA CRC but not in their wild-type counterparts [20], suggesting that the benefits of aspirin treatment vary in patients with different PIK3CA statuses.

Ferroptosis is a newly discovered form of regulated cell death characterized mainly by iron accumulation, redox system imbalance and lipid peroxidation [21]. In mammalian cells, the system xc/glutathione peroxidase 4 (GPX4) axis plays a crucial role in limiting lipid...
β-elemene has been described as a novel ferroptosis inducer, and combined treatment with drugs, such as sulfasalazine or sorafenib, that target ferroptosis are used as a strategy to treat a variety of cancers. Recent evidence suggests that ferroptosis may offer a new therapeutic strategy for CRC [37–39]. Our previous study showed that cetuximab, which is approved for the treatment of metastatic CRC with wild-type Kirsten rat sarcoma (KRAS), promoted RSL3-induced ferroptosis by suppressing the NRF2/HO-1 pathway in KRAS-mutant CRC [37]. The natural product β-elemene has been described as a novel ferroptosis inducer, and combined treatment with β-elemene and cetuximab inhibits KRAS-mutant CRC cell growth by inducing ferroptosis [38]. Xia et al. reported that talaromycin A (TalaA) suppresses CRC cells via ferroptosis, and may be a new and potentially effective drug candidate for CRC therapy [39]. Recently, oncogenic activation of PI3K/AKT/mTOR signaling was reported to render cancer cells resistant to ferroptosis [40], suggesting that drugs targeting PI3K in combination with ferroptosis inducers may achieve better therapeutic outcomes in the treatment of PIK3CA-mutant CRC.

Here, we found that CRC harboring the oncogenic PIK3CA mutation might benefit from aspirin therapy in combination with the ferroptosis inducer RSL3. Mechanistically, aspirin suppressed the PI3K/AKT/mTOR pathway, thereby promoting the sensitivity of CRC cells to ferroptosis by inhibiting downstream SREBP-1/SCD1-mediated lipogenesis. Our study provides an experimental basis for the combined use of aspirin and ferroptosis inducers for the treatment of PIK3CA-mutant CRC.

2. Materials and methods

2.1. Cell lines

Human cancer cell lines, including DLD-1, HCT 116, HepG2, PANC-1, and AGS, were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS; Shanghai, China). All cells were cultured in RPMI-1640 or DMEM media supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA), 100 μg/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere with 5% CO2 at 37 °C according to the recommendations of the American Tissue Culture Collection (ATCC).

2.2. Reagents and antibodies

RSL3 (#SML2234), aspirin (#A2093), OA (#O1383), POA (#P9417), PA (#P0500) and SA (#47581) were purchased from Sigma-Aldrich (Darmstadt, Germany). Ferrostatin-1 (Fer-1, #HY100579), Z-VAD-FMK (#HY16658B), 3-Methyladenine (3-MA, #HY19312), MVIH485 (#HY-B0795) and Rapamycin (#HY10219) were obtained from MedChemExpress (NJ, USA). Primary antibodies against AKT (#4691), phosphorylated AKT (p-AKT; #4060), mTOR (#29283), phosphorylated mTOR (p-mTOR; #29831), p70S6K (#2211), phosphorylated p70S6K (p-p70S6K; #9204), 4E-BP1 (#9456), phosphorylated 4E-BP1 (p-4E-BP1; #29255) and SCD1 (#2794) were purchased from Cell Signaling Technology (MA, USA). SREBF1 antibody (#14088-1-AP) was obtained from Proteintech (IL, USA). The antibodies against Ki67 (#ab16667) and 4-HNE (#ab46545) were purchased from Abcam (MA, USA).

2.3. Cell viability assay

Cells were seeded in 96-well plates at optimal cell density (3000–5000 per well) and incubated overnight at 37 °C. After cells were subjected to various treatments as indicated, 10 μL of Cell Counting Kit-8 (CCK-8; #CK04, Dojindo Laboratories, Japan) solution was added to medium. Following 3 h incubation, absorbance was determined at 450 nm using a microplate reader (Bio-Tek, USA).

2.4. Lipid reactive oxygen species (ROS) assay

The level of Lipid ROS was detected by C11-BODIPY 581/591 (#D3861, Thermo Fisher Scientific). After cells were exposed to the indicated treatments, 5 μM of C11-BODIPY 581/591 dye was added and incubated at 37 °C for 30 min. All results were analyzed using FlowJo V10 software.

2.5. Crystal violet staining

For crystal violet staining, cells (20,000 per well) were seeded in 24-well plates and treated with aspirin or RSL3 at the indicated concentration. After 2 days of incubation, cells were stained with crystal violet staining solution (0.05%, w/v) for 30 min before washing and air-drying.

2.6. Malondialdehyde (MDA) assay

The levels of intracellular MDA were measured using a Lipid Peroxidation MDA Assay Kit (#AO03-4-1, Nanjing Jiancheng, China) according to the manufacturer’s instructions.

2.7. Intracellular iron assay

The level of intracellular Ferrous ion was determined using FerroOrange dye (#F374, Dojindo Laboratories). Cells were seeded and exposed to the indicated treatments. After treatment, FerroOrange (1:2000, v/v) was added to the cells and the cells were incubated at 37 °C for 30 min. Fluorescence images were acquired using a confocal microscope.

2.8. Western blot analysis

Cells with different treatments as described in each experiment were lysed in RIPA buffer (#89901, Thermo Fisher Scientific) supplemented with protease inhibitor. A total of 20–30 μg of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) gels and then electrochemically transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). After incubation in 5% skim milk for 2 h at room temperature, the membranes were incubated with the indicated primary antibodies at 4 °C overnight. After washing three times with Tris-buffered saline and Tween-20 (TBST) buffer, the blots were then probed with the HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were subjected to enhanced chemiluminescence and visualized using a Gel Doc 2000 (CA, USA).

2.9. Small interfering RNA (siRNA) and plasmid transfection

The siRNAs targeting the SREBF1 or SCD1 gene were purchased from GenePharma (Shanghai, China). The sequences of the siRNAs are as follows: siSREBF1 #1 (sense: 5'-GGGAGAAAGCUCCGUAUCGA-3'); siSREBF1 #2 (sense: 5'-CCACACGCCATCGAGAAA-3'); siSCD1 #1
The plasmids containing the insert SREBP-1a (pcDNA3.1-2×FLAG-SREBP-1a, #26801) or SREBP-1c (pcDNA3.1-2×FLAG-SREBP-1c, #26802) were obtained from Addgene. The plasmid containing the SCD1 insert and the control vector were purchased from GenePharma. Transfection of cells was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the instructions.

2.10. Immunohistochemistry (IHC)

Xenograft tumor tissue was fixed, dehydrated, embedded, and sectioned (3.5 μm). The slides were stained with Ki67 (1:200), 4-HNE (1:50), SREBP-1 (1:200) and SCD1 (1:200) antibodies. The staining index was calculated by multiplying the intensity value (negative = 0, weak = 1, moderate = 2, or strong = 3) by the percentage value of positively stained cells (<5% = 0, >5 to ≤25% = 1, >25 to ≤50% = 2, >50 to ≤75% = 3, >75% = 4) independently by two pathologists.

Fig. 1. Aspirin promotes RSL3-induced ferroptosis in PIK3CA-mutant CRC cells. (A) DLD-1 and HCT116 cells were treated with different concentration of RSL3 (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 μM) combined with DMSO or aspirin (2 mM) for 24 h, and cell viability was measured by CCK-8 assays. (B) Violet staining was performed with the indicated concentration of aspirin and RSL3 combination treatment. (C) The changes in cell morphology were observed after treatment with aspirin (2 mM), RSL3 (0.1 μM) or their combination in DLD-1 and HCT116 cells. Scale bar, 100 μm. (D) Cell viability was measured in DLD-1 and HCT116 cells treated with aspirin (2 mM), RSL3 (0.1 μM), or the combination in the presence or absence of Fer-1 for 24 h. (E) Cell viability was measured in DLD-1 and HCT116 cells treated with aspirin (2 mM) and RSL3 (0.1 μM) combination in the presence of with Fer-1 (1 μM), Z-VAD-FMK (10 μM), or 3-MA (1 mM) for 24 h. (F–H) The levels of lipid ROS detected with C11-BODIPY 581/591 (F), MDA detected with the MDA assay kit (G), and ferrous ion detected with FerroOrange dye (H), in DLD-1 and HCT116 cells treated with aspirin (2 mM), RSL3 (0.1 μM) or the combination for 24 h. Scale bar, 50 μm. *, P < 0.05; **, P < 0.001; ***, P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
2.11. In vivo tumor xenograft study

Male BALB/c nude mice (5–6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments were approved by the Laboratory Animal Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. An equal number (5 mice) of DLD-1 cells suspended in 200 μL phosphate-buffered saline (PBS) were injected subcutaneously into the right flank of each mouse. Six days after inoculation, the mice were randomly divided into four groups (6 mice/group): 1) the control group, 2) the RSL3 group, 3) the aspirin group, and 4) the RSL3 + aspirin group. Both RSL3 (5 mg/kg) and aspirin (100 mg/kg) were administered by intraperitoneal injection in a volume of 100 μL once per day. The mice weight and tumor volume were measured every other day. Tumor volume was calculated as 0.5 × length × width². On day 22 after treatment, all mice were sacrificed and tumors were removed and weighed.

2.12. Statistical analysis

All analyses were performed using GraphPad Prism 8.0 software (CA, USA). Data were expressed as means ± SD. Student’s t-test analysis was used to determine differences between the means of two groups, whereas one-way ANOVA analysis for multiple groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Aspirin promotes RSL3-induced ferroptosis in PIK3CA-mutant CRC cells

To validate the effect of aspirin on PIK3CA-mutant CRC cells, DLD-1 and HCT116 cells were exposed to different concentrations of aspirin. We found that aspirin inhibited cell growth in dose- and time-dependent manners (Fig. S1A). To determine the combined effect of aspirin and RSL3, a Bliss independence model was established to calculate the synergistic effect (Fig. S1B). Surprisingly, the combined administration of aspirin and RSL3 showed strong synergism in terms of anti-proliferative effects (Figs. S1B and 1A). This synergistic phenomenon was verified using crystal violet staining (Fig. 1B). Combined administration of aspirin and RSL3 also resulted in low cell viability when observed under a light microscope (Fig. 1C).

As RSL3 is a potent inducer of ferroptotic cell death, we hypothesized that the effects of ferroptosis were enhanced by the addition of aspirin. Consistent with our expectation, the inhibition of cell growth by the combination treatment was reversed by the application of the ferroptosis inhibitor Fer-1, but not by the autophagy inhibitor 3-MA or the apoptosis inhibitor Z-VAD-FMK (Fig. 1D and E). In addition, the levels of lipid ROS, intracellular ferrous ion and MDA, three widely used ferroptosis indicators, were measured in the DLD-1 and HCT116 cells. The levels of lipid ROS and MDA increased significantly after the combination treatment with aspirin and RSL3 (Fig. 1F and G). Moreover, DLD-1 and HCT116 cells showed higher fluorescence in the combination group than in the RSL3 group (Fig. 1H). Interestingly, aspirin itself did not affect the levels of lipid ROS, MDA, or iron fluorescence intensity in either DLD-1 or HCT116 cells, suggesting that aspirin may enhance RSL3-induced cell death in PIK3CA-mutant CRC cells. We also validated the combined effect of aspirin and RSL3 in PIK3CA wild type CRC cells (SW480, SW620, and Lovo). The addition of aspirin did not significantly enhance ferroptosis induced by RSL3 (Fig. S1C). Taken together, these results suggest that aspirin administration promotes RSL3-induced ferroptosis in PIK3CA-mutant CRC cells.

3.2. Combination treatment with aspirin and RSL3 suppresses the AKT/mTORC1 signaling axis in PIK3CA-mutant CRC cells

To determine whether the increased sensitivity of ferroptosis to aspirin treatment was due to the suppression of the PI3K/AKT/mTOR pathway in PIK3CA-mutant CRC cells, we analyzed the associated protein levels. Indeed, we found that the protein levels of AKT, p-AKT, p-mTOR and mTOR decreased sharply in the combination treatment group (Fig. 2A and S2A). In addition, the phosphorylation of mTORC1 substrate 4E-binding protein 1 (4E-BP1) reduced by aspirin and RSL3 (Fig. 2B and S2B). Next, the mTORC1 activator MHY1485 and inhibitor rapamycin were used to further investigate the effects of mTORC1 on the regulation of ferroptosis. The results showed that pretreatment with MHY1485 conferred DLD-1 and HCT116 cells resistance to the ferroptosis inducer even in the presence of aspirin (Fig. 2C), whereas rapamycin sensitized cancer cells to ferroptosis (Fig. 2D). Furthermore, MHY1485 significantly decreased RSL3-induced lipid ROS, MDA and Fe²⁺ levels, whereas rapamycin increased RSL3-induced lipid ROS, MDA and Fe²⁺ levels in LDR-1 and HCT116 cells (Fig. 2E–G). The above results suggest that the activity of mTORC1 is responsible for the sensitivity of ferroptosis in PIK3CA-mutant CRC cells.

3.3. Aspirin sensitizes PIK3CA-mutant CRC cells to RSL3-induced ferroptosis by inhibiting SREBP-1 activity

SREBP-1 has recently been identified as a downstream target of mTORC1 [41]. Therefore, we investigated whether SREBP-1 played a key role in modulating ferroptosis sensitivity. Consistent with our expectations, the levels of both total SREBP-1 and its mature form (mSREBP-1) decreased to a high extent by the combination treatment than by RSL3 only (Fig. 3A). Small interfering RNAs (siSREBF1 #1 and siSREBF1 #2) were used to knock down the expression of SREBP-1, and the knockdown effects were verified by western blotting (Fig. 3B). We found that inhibition of SREBP-1 expression sensitized DLD-1 and HCT116 cells to ferroptosis induction, and cell death was reversed by the ferroptosis inhibitor Fer-1 (Fig. 3C). In contrast, ectopic expression of the mature form of SREBP-1 (SREBP-1a or SREBP-1c) resulted in resistance to ferroptosis (Fig. 3D and E). The above evidence suggests that aspirin promotes ferroptosis by suppressing SREBP-1 expression.

3.4. Aspirin sensitizes PIK3CA-mutant CRC cells to ferroptosis by suppressing SCD1 expression

As SREBP-1 regulates transcription of several genes related to lipid synthesis, RT-PCR was performed to assess the mRNA expression of downstream target genes, including ACC, ACLY, SCD1 and FASN. We found that the mRNA expression of SCD1 decreased more markedly than other genes (Fig. 4A). Combination treatment with aspirin and RSL3 also significantly decreased SCD1 protein expression (Fig. 4B and S3). To investigate whether inhibition of SCD1 triggers activation of ferroptosis, siRNAs targeting SCD1 were transfected into DLD-1 and HCT116 cells and the efficacy of knockdown was verified by western blotting (Fig. 4C). Knockdown of SCD1 sensitized DLD-1 and HCT116 cells to ferroptosis induction, and the effects were abolished by the ferroptosis inhibitor Fer-1 (Fig. 4D). The pro-ferroptotic effects were confirmed by the accumulation of lipid ROS, intracellular iron, and lipid peroxides (Fig. 4E–G).

3.5. Ectopic expression of SCD1 renders PIK3CA-mutant CRC cells resistant to ferroptosis induction

To further confirm the role of SCD1 in ferroptosis process, SCD1 was overexpressed in PIK3CA-mutant CRC cells (Fig. 5A). Overexpression of SCD1 in DLD-1 and HCT116 cells reversed the inhibition of cell growth induced by the combination treatment with RSL3 and aspirin or knocking down SREBP1 (Fig. 5B). At the same time, the levels of lipid ROS, MDA, and Fe²⁺ decreased dramatically upon overexpression of SCD1 (Fig. 5C–E). Similar to Fer-1 treatment, overexpression of SCD1 restored cell viability inhibited by the combination treatment with RSL3 and aspirin and knockdown of SREBP-1 (Fig. 5F).
Fig. 2. Combination treatment with aspirin and RSL3 suppresses the AKT/mTORC1 signaling axis in PIK3CA-mutant CRC cells. (A–B) The protein levels of AKT, p-AKT, mTOR, 4E-BP1, p-4E-BP1 were detected in DLD-1 and HCT116 cells treated with aspirin (2 mM), RSL3 (0.1 μM), or their combination for 24 h. (C) DLD-1 and HCT116 cells were pretreated with MHY1485 (2 μM) for 6 h and then exposed to aspirin (2 mM), RSL3 (0.1 μM) or the combination for 24 h. Cell viability was determined by CCK-8 assays. (D) DLD-1 and HCT116 cells were pretreated with rapamycin (50 nM) for 6 h and then exposed to RSL3 (0.1 μM) or Fer-1 (1 μM) for 24 h. Cell viability was determined by CCK-8 assays. (E–G) DLD-1 and HCT116 cells were pretreated with rapamycin (50 nM) or MHY1485 (2 μM) for 6 h and then treated with RSL3 (0.1 μM) for 24 h. The levels of lipid ROS detected with C11-BODIPY 581/591 (E), MDA detected with the MDA assay kit (F), and ferrous ion detected with FerroOrange dye (G). Scale bar, 50 μm * P < 0.05; ** P < 0.001; *** P < 0.0001.
SCD1 is an important lipid desaturase that converts saturated fatty acids (SFAs) to monounsaturated fatty acids (MUFAs) [42]. Therefore, MUFA oleic acid (18:1, OA) and palmitoleic acid (16:1, POA) as well as SFA stearic acid (18:0, SA) and palmitic acid (16:0, PA) were supplemented in the experiment. We found that the addition of OA or POA rather than SA or PA protected DLD-1 and HCT116 cells from RSL3-induced ferroptosis, independent of addition of aspirin (Fig. 5G).

Taken together, these results suggest that aspirin enhances RSL3-induced ferroptosis in PIK3CA-mutant CRC cells by inhibiting SCD1-mediated MUFA production.

3.6. The antitumor efficacy of combined aspirin and RSL3 therapy in a human CRC xenograft model

To further investigate the anticancer therapeutic potential of simultaneous treatment with aspirin and RSL3 in vivo, a DLD-1 xenograft model was established in nude mice. Six days after tumor inoculation, the mice were treated with vehicle, aspirin (100 mg/kg), RSL3 (5 mg/kg), or a combination of both. As shown in Fig. 6A and B, tumor growth was significantly slower in the combination groups than in the groups treated with aspirin or RSL3 alone. The tumor weight was also significantly lower in the combination group than in the other groups (Fig. 6C). Meanwhile, MDA levels significantly increased in the tumor of the combination group (Fig. 6D). Fig. 6E shows that body weight remained stable during the treatments, indicating that the treatments were well tolerated. In addition, after simultaneous treatment with aspirin and RSL3, the Ki67 proliferation index significantly decreased compared to that in the groups treated with aspirin or RSL3 alone (Fig. 6F). The accumulation of lipid peroxides was also confirmed by IHC analysis of 4-HNE, another indicator of oxidative stress (Fig. 6F).

Moreover, the levels of SREBP-1 and SCD1 decreased significantly in the combination treatment group compared with the RSL3 group, indicating that the synergistic effects promoted the induction of tumor ferroptosis (Fig. 6F). Overall, these in vivo results showed that aspirin enhanced RSL3-induced ferroptosis via tumor suppression by inhibiting the SREBP-1/SCD1 pathway. Taken together, these results show that the combined use of aspirin and ferroptosis induction shows therapeutic potential for the treatment of PIK3CA-mutant CRC.

4. Discussion

In the current study, we found that aspirin promoted the sensitivity
of PIK3CA-mutant CRC cells to RSL3-induced ferroptosis by inhibiting the AKT/mTORC1 axis and SREBP-1/SCD1-mediated lipogenesis of MUFA (Fig. 7). Although it is used clinically as an antipyretic analgesic, aspirin has shown therapeutic effects on primary tumors and metastatic neoplasms \[43,44\]. Aspirin has been reported to directly disrupt the mTOR-Raptor interaction and enhance the anticancer effects of sorafenib \[19\]. Moreover, aspirin has shown stronger suppression of PIK3CA-mutant breast cancers than their wild-type counterparts by activating AMPK signaling, suppressing mTOR activity, and inducing autophagy \[45\].

The PI3K/AKT/mTOR pathway plays a critical role in regulating cell proliferation, survival and metabolism \[46\]. Somatic mutations in PIK3CA have been detected in approximately 20% of all CRC cases \[47\], leading to increased PI3K and downstream AKT activity and promotion of tumor progression. Although oncogenic mutations in PIK3CA or its hyperactivation are common in CRC, small molecules targeting the PI3K/AKT/mTOR pathway have yielded little benefits in clinical trials owing to the emergence of acquired resistance and dose-dependent
toxicities [48]. The goal of finding new therapeutic strategies has taken on greater significance. Our results showed that the combination treatment with aspirin and RSL3 suppressed the AKT/mTORC1 signaling axis in PIK3CA-mutant CRC cells. COX-2 expression is significantly associated with Akt phosphorylation in epithelial ovarian cancer [49] and breast cancer [50]. Uddin S et al. showed that inhibition of COX-2 impairs the phosphorylation of AKT, leading to inhibition of cell growth and induction of apoptosis, and aspirin treatment inhibits the

Fig. 5. Ectopic expression of SCD1 renders PIK3CA-mutant CRC cells resistant to ferroptosis induction. (A) The protein levels of SCD1 were detected in DLD-1 and HCT116 cells transfected with SCD1 overexpression plasmids. (B) DLD-1 and HCT116 cells with SCD1 overexpression were treated with RSL3 (0.1 μM) for 24 h. Cell viability was measured by CCK-8 assays. (C–E) The levels of lipid ROS detected by C11-BODIPY 581/591 (C), MDA detected by the MDA assay kit (D), and ferrous ion detected by FerroOrange dye (E), in RSL3 (0.1 μM)-treated DLD-1 and HCT116 cells with SCD1 overexpression. Scale bar, 50 μm. (F) DLD-1 and HCT116 cells transfected with siSREBF1 were treated with RSL3 (0.1 μM) and SCD1 overexpression plasmid. Cell viability was measured by CCK-8 assays. (G) DLD-1 and HCT116 cells were treated with RSL3 (0.1 μM), OA (100 μM), SA (100 μM), POA (100 μM) or PA (100 μM), with simultaneous treatments of Aspirin (2 mM) for 24 h. The cell viability was measured by CCK-8 assays. *, P < 0.05; **, P < 0.001; ***, P < 0.001.
growth of MDAH2774 tumor cells in nude mice via down-regulation of COX-2 and AKT activity [49].

mTORC1 is a downstream molecule of PI3K-AKT signaling and is frequently deregulated in cancer [51]. Recent studies have shown that mTORC1 acts as a negative regulator of ferroptosis [52]. It has been reported that mTORC1 protects cardiomyocytes from excessive iron accumulation and therefore inhibits ferroptosis [53]. In addition, mTORC1 mediates cystine-induced GPX4 synthesis [54]. Our work here showed that aspirin-induced suppression of mTORC1 further inhibits SREBP1/SCD1-mediated MUFA production. Aljohani et al. demonstrated that SCD1 deficiency mediates activation of mTORC1, and therefore causes endoplasmic reticulum stress [55], showing that SCD1 may in turn regulate cellular metabolism by modulating mTORC1 activity.

**Fig. 6.** The antitumor efficacy of combined aspirin and RSL3 therapy in a human CRC xenograft model. DLD-1 cells were injected subcutaneously into the right flank of nude mice. Six days after inoculation, mice received daily intraperitoneal RSL3 (5 mg/kg), aspirin (100 mg/kg), or the combination of the two drugs (n = 6). (A) Tumor images in different groups. (B) Tumor volume was measured every 2 days. (C) Tumor weight in four groups. (D) The levels of intracellular MDA were measured in different treatment groups. (E) The body weight of mice was measured every 2 days after the xenograft mouse model was established. (F) The expressions of Ki67, 4-HNE, SREBP-1, and SCD1 were determined by immunohistochemical staining, and the staining score was calculated. Scale bar, 40 μm *; P < 0.05; **, P < 0.001; ***, P < 0.001.
SREBPs are a family of transcription factors that regulate the promoter of genes involved in lipid homeostasis and glucose metabolism in response to various metabolic stress [41,56]. The three SREBP isoforms, SREBP-1a and SREBP-1c, both encoded by SREBF1, and SREBP-2, encoded by SREBF2, have different roles in lipid synthesis [57–60]. Emerging evidence suggests that SREBP-1, including SREBP-1a and SREBP-1c, is an important link between tumor metabolism and oncogenic signaling. Critical genes in the lipogenesis pathway, such as acetyl-CoA carboxylase (ACC), ATP citrate lyase (ACLY), fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD1) are the main targets of SREBP-1 [61]. Moreover, SREBP-1 has been confirmed to be a downstream protein of the PI3K/AKT/mTOR signaling pathway. In this study, we found that aspirin inhibited the activity of mSREBP-1 and SCD1 and suppressed the synthesis of MUFA, thereby curbing the occurrence of ferroptosis [62]. SCD1 has been reported to protect lung and gastric cancers from ferroptosis [63,64]. Knockdown of SCD1 sensitized CRC cells to ferroptosis induction, whereas overexpression of SCD1 rendered CRC cells resistant to ferroptosis induction and restored cell viability inhibited by the combination treatment with RSL3 and knockdown of SREBP-1. Magtanong et al. reported that exogenous MUFA inhibits ferroptosis in an ACSL3-dependent manner [65]. These results demonstrate not only the role of unsaturated fatty acids in the regulation of ferroptosis, but also the relationship between SREBP-1 and SCD1 in the development of ferroptosis.

Here, we demonstrated that aspirin promoted ferroptosis in cancer cells harboring malignant mutation of PI3K, providing valuable insights into future ferroptosis-inducing cancer therapies. Our findings provide a theoretical basis for combining aspirin with ferroptosis inducers in the treatment of CRC and highlight the need for clinical trials to evaluate their potential chemotherapeutic effects.

Declaration of competing interest

The authors have no conflicts of interest to disclose. Fig. 7 is created using Figdraw (www.figdraw.com).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102426.

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