Original Article
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type by modulation of ROS through activating the p53 signaling pathway

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Abstract: Colorectal cancer (CRC) is one of the top three most deadly cancers despite using chemotherapy based on oxaliplatin or irinotecan combined with targeted therapy. Chiauranib has recently been identified to be a promising anticancer candidate with impressive efficacy and safety. However, the role and molecular mechanisms of Chiauranib in the treatment of CRC remain to be elucidated. Our study shows that Chiauranib inhibits cell proliferation and induces apoptosis in KRAS wild-type CRC cells in a dose- and time-dependent manner, but not mutation ones. Meanwhile, Chiauranib increases ROS production in KRAS wild-type CRC cells. Moreover, Chiauranib selectively suppresses KRAS wild-type CRC cells growth in vivo. Mechanistically, Chiauranib inhibits KRAS wild-type CRC cells by triggering ROS production via activating the p53 signaling pathway. Further, KRAS mutation CRC cells are resistant to Chiauranib by increasing Nrf2 to stably elevate the basal antioxidant program and thereby lower intracellular ROS induced by Chiauranib. Our findings provide the rationale for further clinical evaluation of Chiauranib as a therapeutic agent in treating KRAS wild-type CRC.

Keywords: Colorectal cancer, chiauranib, ROS

Introduction

Colorectal cancer (CRC) ranks as the third leading cause of cancer death worldwide [1]. Despite the emergence of numerous screening methods to reduce CRC incidence, an increasing proportion of CRC patients are diagnosed at an advanced stage, which results in difficulties in curative removal of primary tumors and metastases [2]. For those patients, irinotecan- or oxaliplatin-based chemotherapy combined with targeted therapy are the leading strategies in suppressing the further growth and spread, and even killing the cancer cells [3]. However, these chemotherapies are associated with unsatisfying response rate, systemic toxicity, and acquired resistance [4]. Moreover, one of the most breakthrough targeted therapy anti-EGFR compounds are limited to being used in EGFR- and KRAS-wildtype otherwise in CRC patients with those drug-resistant mutation [5-7]. Therefore, more investments are urgent to be pledged to develop novel targets and agents.

Chiauranib is a novel oral multi-target small molecule inhibitor of select serine threonine-kinases, including the angiogenesis-related kinases (VEGFR1, VEGFR2, VEGFR3, and c-Kit), the chronic inflammation-related kinase CSF-1R, and the mitosis-related kinase Aurora B, with a variety of potential anti-tumor activities [8-10]. Chiauranib reduces hepatocellular carcinoma and gastric cancer cell proliferation by simultaneous inhibiting VEGFR2 and Aurora B [8]. Besides, Chiauranib exerts anti-tumor
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

effect against angiogenesis and mitosis in hematologic tumors, such as acute myeloid leukemia and Non-Hodgkin's lymphomas [9, 10]. The animal study results illustrate that Chiauranib has impressive efficacy and safety, verified in a phase I clinical trial (NCT03074-825) [10, 11]. Currently, Chiauranib phase Ib/II clinical trials are undergoing in multiply cancers. However, the efficacy and potential mechanism of Chiauranib in the treatment of CRC have not been investigated.

Reactive oxygen species (ROS) are essential signaling messengers of normal cells and tumor cells [12]. Moderate levels of ROS contribute to tumor development, promoting cell differentiation, activating cancer angiogenesis, and metastasis. But excessive oxidative stress causes DNA damage and abnormal stress response, thus triggering cancer cell death [13, 14]. Therefore, it might be effective to eliminate cancer cells by increasing ROS generation. Cellular redox state can be modulated by the tumor suppressor protein p53, which is an essential regulator of the DNA damage response, cell cycle, and cell apoptosis [15]. The p53 protein is tightly regulated by post-transcriptional modifications such as phosphorylation and ubiquitination [16]. Chiauranib has been reported to regulate p53 phosphorylation by inhibiting Aurora B activity [17]. However, whether Chiauranib can induce ROS is still unclear.

Our results demonstrated the anti-tumor role of Chiauranib in inhibition of cell proliferation and induction of cell apoptosis in KRAS-wildtype CRC by increasing ROS production through activating the p53 signaling pathway, indicating that Chiauranib may be a promising novel strategy to treat KRAS-wildtype CRC.

Materials and methods

Cell lines and culture
The human CRC cell lines (SW48, CaCO₂, LoVo, HCT116) were obtained from the American Type Culture Collection (Manassas, USA). Cell lines were authenticated by Cellcook Biotech Co., Ltd, (Guangzhou, China). SW48 KRASwt, SW48 KRASG13D, LoVo-shNC and LoVo-shKRAS cells were previously constructed [18]. All these cells were cultured in RPMI1640 (Gibco, New York, USA, Cat. No. 61870044) and supplementeded with 10% FBS (Gibco, Uruguay, 10270-106) at 37°C in a humidified incubator containing 5% CO₂.

Western blotting
Western blotting was performed according to a standard protocol, as described previously [19]. The total proteins were collected using SDS lysis buffer (Beyotime, P0013G), and protein concentration were determined by Bicinchoninic Acid (KeyGen, KGP902). Nuclear extracts were obtained using the NE-PER Nuclear and cytoplasmic extraction reagents (Thermo Scientific, Massachusetts, USA, 78833) according to the manufacturer's instructions. The following primary antibodies were used: CDK1 (AF1516), P21 (AP021) from Beyotime (Shanghai, China); Bcl-2 (sc-7382), Bax (sc-6236) from Santa Cruz (California, USA); P53 (CBL404) from Merck Millipore (Boston, USA); Aurora B (ab2254), p-Aurora B (ab115793), KRAS (ab180772), Nrf2 (ab-31163) from Abcam (Cambridge, UK); β-actin (A5441) from Sigma-Aldrich (St. Louis, USA). HRP-conjugated anti-rabbit IgG (Cell Signaling Tech, #7074) and anti-mouse IgG (Sigma-Aldrich, AP308P) were used as secondary antibodies. Proteins were determined using ECL Plus Reagent (Millipore, WBKLS0100).

RNA isolation and RT-qPCR
Total RNA was extracted using TRizol (Thermo Scientific, 15596026) according to the manufacturer's protocol [19]. First-strand cDNA synthesis was performed using 500 nanograms of total RNA, and the RT-qPCR analysis system was performed using iQ SYBR Green Supermix and the iCycler Real-time PCR Detection System (Bio-Rad, USA). β-actin was used for normalization. The PCR primer sequences are listed in Supplementary Table 1.

Immunofluorescence staining
After fixed in 4% paraformaldehyde, cells were blocked with goat serum at 37°C for 1 h. They were incubated with rabbit Ki67 (Millipore, AB9260) antibodies at 4°C overnight, then were incubated with FITC conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark, K500711) at 37°C for 1 h after three times washing. Finally, the cell nucleus was stained with DAPI (Sigma-Aldrich, D9542).
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

**Cell counting Kit-8 (CCK8)**

Cell proliferation was measured via cell viability with a Cell Counting Kit-8 (Dojindo, Japan). CRC cells were seeded into 96-well plates and cultured for 24, 48 and 72 h. Then, 100 μl CCK8 reagent was added to 96-well plates and incubated for 2 h. The absorbance (OD450 nm) was measured using a microplate reader (TECAN, Switzerland) and calculated.

**Colony formation assay**

CRC cells were plated in 6-well dishes (500 cell/dish) and then incubated for 2 weeks for colony formation with or without Chiauranib. After 14 days, cell colonies were then fixed in 4% polyformaldehyde and stained with 0.1% crystal violet. All colonies were counted separately for each sample, and the relative colony numbers were calculated.

**Dihydroethidium (DHE) staining**

Reactive oxygen species (ROS) were measured with DHE staining following incubation at 37°C for 30 min (Beyotime, Shanghai, China). PBS was used instead of DHE in the negative control group. The fluorescence intensity was quantified with the ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining**

Tissue sections were deparaffinized and hydrated in xylene and gradient concentrations of ethanol, then incubated in proteinase K at room temperature for 30 min and stained with TUNEL kit (Sigma-Aldrich, St. Louis, MO, USA). Label solution was used instead of TUNEL reagent in the negative control group. All the images were captured by a fluorescence microscope (DFC700T, Leica, Germany). Cells that were positive for TUNEL staining and aligned with DAPI staining were considered apoptotic cells and counted.

**Annexin V/propidium iodide flow cytometric analysis**

CRC cell staining with Annexin V and PI was carried out using an Annexin V-FITC/PI Apoptosis Detection kit (Merck, Germany). A total of 1×10⁶ cells were incubated at 37°C for 30 minutes before centrifugation to collect the cell pellet, then resuspended in a Ca²⁺-enriched binding buffer and analysed using a Beckman Coulter flow cytometry. Data were calculated using CellQuest software.

**ROS assays**

Cells were plated at a density of 20000 cells per well in 12-well plates. After 48 hours, DCF-DA (Sigma) and MitoSOX (Invitrogen) was added to each well at a concentration of 5 μM. After 30 min, the samples were run on the flow cytometer to detect fluorescence. Each sample was collected using 20,000 events.

**Tumor xenograft**

Male BALB/c nude mice (4-weeks-old, 16-18 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Based on a previously described standard protocol, the mice were randomly divided into the indicated groups. 1×10⁶ SW48 or LoVo cells were inoculated into the inguinal folds of mice (n = 8 in each cell line per group). Tumor volumes were measured with an external caliper, and calculated according to the following formula: Volume = length × width²/2. At 27 days after inoculation, the mice were sacrificed, and the tumors were dissected, weighed, taken photos, and stored at -80°C for further experiments.

**siRNA and transfection**

P53 siRNA, Nrf2 siRNA and control siRNA were purchased from RiboBio (Guangzhou, China). According to the manufacturer’s instructions, transfections were performed at approximately 60% confluency using RNAiMAX (Invitrogen, USA). After 48 hours, confirmation of interference was carried out using real-time quantitative PCR (RT-qPCR) and Western blotting.

**RNA-Seq**

RNA sequence was performed with BGISEQ-500 platform. In brief, the first step in the workflow involved purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type
divalent cations under elevated temperature. The cleaved RNA fragments were copied into
first strand cDNA using reverse transcriptase and random primers. This was followed by sec-
ond strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA frag-
ments then had the addition of a single ‘A’ base and subsequent ligation of the adapter. The
products were then purified and enriched with PCR amplification. We then quantified the
PCR yield by Qubit and pooled samples to-
ger: make a single strand DNA circle (ssDNA circle), which gave the final library. DNA
nanoballs (DNBs) were generated as the ssDNA circle by rolling circle replication (RCR)
to enlarge the fluorescent signals at the sequencing process. The DNBs were loaded
into the patterned nanoarrays and single-end read of 50 bp were read through on the
BGISEQ-500 platform for the following data analysis study. For this step, the BGISEQ-500
platform combined the DNA nanoball-based nanoarrays and stepwise sequencing using
Combinational Probe-Anchor Synthesis Sequencing Method. Tools such as HISAT2,
Bowie2, Cluster etc. were used for the following bioinformatics analysis. In the analysis of
differentially expressed genes (DEGs), the genes that had more than double fold change
and the corrected P value is less than or equal to 0.05 were defined as DEGs. With DEGs, we
performed KEGG pathway classification and Gene set enrichment analysis (GSEA) using R.
we have upload the sequence data onto
the online database SRA (Sequence Read Archive). Our sequence data is numbered
PRJNA667187.

Statistical analysis

The variability of the data is presented as the SD (mean ± SD) and was assessed with
Student’s t test between two groups. For multiple groups, significant differences were
determined using one-way ANOVA. Statistical significance was defined at P < 0.05.

Results

The anti-tumor effect of Chiauranib in KRAS wild-type CRC cells

To validate whether Chiauranib exhibited an anti-tumor effect against CRC, CCK-8 assays
were performed to evaluate the cell viability in response to Chiauranib treatment in different
human CRC cell lines (LoVo, HCT116, SW48, CaCO₂). Surprisingly, Chiauranib treatment
markedly decreased the cell viability of SW48 and CaCO₂ in a concentration- and time-de-
pendent manner, whereas LoVo and HCT116 cells were resistant to Chiauranib (Figure 1A,
1B). The IC50 values of Chiauranib in SW48 cells and CaCO₂ cells were 8.843 μM and
9.165 μM, respectively (Figure 1C). Chemotherapeutic drug resistance often occurred in
CRC, especially those with genetic mutations of KRAS, BRAF, TP53, PIK3CA. There was only
KRAS gene mutation in LoVo cells, which also occurred in HCT116 cells [20]. Meanwhile,
both SW48 and CaCO₂, which sensitive to Chiauranib, were KRAS wild-type cells [20].
Consequently, we investigated whether Chiauranib resistance of LoVo and HCT116 cells
was related to KRAS mutations? We estab-
lished SW48 KRAS G13D cell model by the
CRISPR-Cas9 system as previously described
[18], and the results indicated that KRAS
mutations could impair the sensitivity to the
anti-viability therapy of Chiauranib (Figure
1D, 1F). On the contrary, short hairpin RNA
(shRNA)-mediated KRAS knockdown up-regu-
lated the sensitivity to Chiauranib in LoVo
cells (Figure 1E, 1G). Taken together, these
results suggested that Chiauranib exhibited a
great concentration- and time-dependent anti-
tumor effect against KRAS wild-type CRC cells.

Chiauranib selectively inhibits KRAS wild-type
CRC cells proliferation

Colony formation and EdU assays were per-
formed to further validate the cytotoxic effects of Chiauranib towards CRC cells on prolif-
eration. As shown in Figure 2A, 2B, Chiauranib
concentration-dependently impaired the clon-
ing ability of KRAS wild-type and KRAS knock-
down CRC cells, whereas the cloning phenomenon did not occur in KRAS mutation cells. Consistently,
EdU experiments showed that the proliferation of SW48 KRAS WT and LoVo-shKRAS cells was
significantly inhibited under Chiauranib treat-
ment for 48 hours, an effect not seen in KRAS
mutation cells SW48 KRAS G13D and LoVo-shNC
(Figure 2C, 2D). These results indicated that
Chiauranib selectively inhibited KRAS wild-type CRC cells proliferation.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Figure 1. The anti-tumor effect of Chiauranib in KRAS wild-type CRC cells. A. 48-hour cell viability of KRASWT CRC cell lines SW48 and CaCO2, KRASG13D CRC cell lines LoVo and HCT116 were detected by CCK-8 after treatment with 0, 2, 4, 8 μM Chiauranib; ***P < 0.001 was compared with 0 μM Chiauranib. B. Cell viability of KRASWT CRC cell lines SW48 and CaCO2, KRASG13D CRC cell lines LoVo and HCT116 were detected by CCK-8 after treatment with 4 μM Chiauranib for 0, 24, 48, 72 hours; *P < 0.05, **P < 0.01 and ***P < 0.001 was compared with 0 hours. C. 48-hour cell viability of SW48 and CaCO2 cells were detected by CCK-8 after treatment with 0, 1, 2, 4, 8, 10, 12 μM Chiauranib. D. SW48 KRASG13D cells viability were determined after treatment with incremental Chiauranib for 48 hours and treatment with 4 μM Chiauranib for 0, 24, 48, 72 hours. E. LoVo-shKRAS cells viability were determined after treatment with incremental Chiauranib for 48 hours and treatment with 4 μM Chiauranib for 0, 24, 48, 72 hours; **P < 0.01, ***P < 0.001. F. 48-hour cell viability of SW48 KRASWT and SW48 KRASG13D were determined after treatment with incremental Chiauranib; ***P < 0.001 was compared with KRASWT SW48 group. G. 48-hour cell viability of LoVo-shNC and LoVo-shKRAS were determined after treatment with incremental Chiauranib; ***P < 0.001 was compared with LoVo-shNC group. All Bars represented the mean ± SD of three independent experiments.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Figure 2. Chiauranib selectively inhibits KRAS wild-type CRC cells proliferation. A. Representative images of colony formation in the indicated $KRAS^{WT}$ SW48, $KRAS^{G13D}$ SW48, LoVo-shNC and LoVo-shKRAS cells after treatment with 0, 0.5, 1, 2, 4, 8 μM Chiauranib for 14 days. B. Statistical analysis of colony formation. Bars represent the mean ± SD of three independent experiments; ***$P < 0.001$ was compared with $KRAS^{WT}$ SW48 group or LoVo-shNC group. C. Representative images of EDU staining in the indicated cells after treatment with 0, 2, 4, 8 μM Chiauranib for 48 hours. D. Statistical analysis of EDU staining. Bars represented the mean ± SD of three independent experiments; *$P < 0.05$, **$P < 0.01$ was compared with 0 μM Chiauranib.
**Chiauranib selectively promotes KRAS wild-type CRC cells apoptosis**

Previous studies have suggested that Chiauranib exerted anti-tumor activity by inducing apoptosis [9, 10]. To evaluate whether apoptosis was involved in Chiauranib-induced cytotoxicity in CRC cells, apoptosis was determined by flow cytometric analysis with Annexin V/propidium iodide (PI) double staining. As expected, Chiauranib apparently increased the apoptosis rate in KRAS wild-type and KRAS knockdown CRC cells, whereas Chiauranib did not affect KRAS mutation cells apoptosis (Figure 3A, 3B). The pro-apoptotic effect of Chiauranib in CRC cells was further evidenced by TUNEL staining. Our results showed that the TUNEL staining intensity was significantly increased in SW48 KRAS\textsuperscript{WT} and LoVo-shKRAS cells, and the phenomenon did not occur in KRAS mutation cells SW48 KRAS\textsuperscript{G13D} and LoVo-shNC (Figure 3C, 3D). Collectively, these results demonstrated that Chiauranib exhibited a pro-apoptotic effect in KRAS wild-type CRC cells.

**Chiauranib alters gene sets related to ROS in KRAS wild-type CRC cells**

To demonstrate the molecular mechanisms of Chiauranib-induced CRC suppression, we determined the global gene expression pattern for Chiauranib-treated SW48 cell and compared it with that for the controls based on RNA-Seq analysis. Using a ≥ 2-fold change (FC) and < 0.05 P-value as a cut-off, we identified a series of differential expressed genes (DEGs) when compared between Chiauranib-treated and control groups (Supplementary Figure 1). Furthermore, KEGG pathway analysis presented that these DEGs mainly participated in the p53 signaling pathway (Figure 4A). Consistently, gene set enrichment analysis (GSEA) revealed that these DEGs were enriched in oxidative phosphorylation and p53 signaling pathway (Figure 4B; Supplementary Figure 2). Meanwhile, the heat map demonstrated 44 DEGs related to oxidative phosphorylation and p53 signaling pathway (Figure 4C). To verify the accuracy of the RNA-Seq, we selected nine essential p53 signaling pathway-related genes for Q-PCR validation, including BCL2, BAX, ZMAT3, FAS, TP53AIP1, CDKN1A, CDK1, El24 and MDM2 (Figure 4D). The results revealed that the changes in the nine genes were consistent with the sequencing results.

Previous studies have proven that activation of the p53 signaling pathway led to imbalance of oxidative phosphorylation, thus inducing ROS production [21]. ROS accumulation could inhibit cell proliferation and induce cell apoptosis. Therefore, we determined to detect whether Chiauranib increased intracellular ROS levels. The results showed that Chiauranib treatment markedly increased Mitosox fluorescence (for mitochondrial O\textsubscript{2}^-) and DCF-DA fluorescence (for H\textsubscript{2}O\textsubscript{2}) of SW48 KRAS\textsuperscript{WT} and LoVo-shKRAS cells, but not mutation ones (Figure 4E, 4F; Supplementary Figure 3). Taken together, these observations indicated that Chiauranib increased the level of ROS in KRAS wild-type CRC cells.

**Chiauranib induces ROS of KRAS wild-type CRC cells by activating the p53 signaling pathway**

In the above study, RNA-Seq analysis indicated that Chiauranib significantly activated the p53 signaling pathway. To further explore the mechanism of Chiauranib activating the p53 signaling pathway in KRAS wild-type CRC cells, we detected a series of critical molecules in the p53 signaling pathway. The mRNA and protein levels of these downstream molecules in the p53 signaling pathway were substantially changed. However, the transcription level of p53 did not change, but the protein level increased significantly, indicating that Chiauranib might affect the post-transcriptional regulation of p53. It has been reported that Chiauranib could inhibit the phosphorylation of Aurora B, which participated in regulating p53 phosphorylation [8]. Indeed, our results showed that Chiauranib could inhibit Aurora B phosphorylation in KRAS wild-type CRC cells, thus inducing p53 increasing (Figure 5A). Silencing p53 inhibited the p53 signaling pathway activated by Chiauranib, while it did not affect the phosphorylation of Aurora B (Figure 5B, 5C). These results indicated that Chiauranib inhibited Aurora B phosphorylation, resulting in an increased in p53 expression.

Next, we investigated whether ROS accumulation caused by Chiauranib was through activa-
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Figure 3. Chiauranib selectively promotes KRAS wild-type CRC cells apoptosis. A. Flow cytometric analysis of apoptosis in the indicated cells after treatment with 0, 2, 4, 8 μM Chiauranib for 48 hours. B. Statistical analysis of apoptotic cells detected by flow cytometry. Bars represent the mean ± SD of three independent experiments; *P < 0.05, **P < 0.01 and ***P < 0.001 was compared with 0 μM Chiauranib. C. Representative images of TUNEL staining in the indicated KRAS\textsuperscript{W13}, SW48, KRAS\textsuperscript{G13D} SW48, LoVo-shNC and LoVo-shKRAS cells after treatment with 4 μM Chiauranib for 48 hours. Scale bar, 50 μm. D. Statistical analysis of TUNEL staining. Bars represented the mean ± SD of three independent experiments; *P < 0.05 was compared with KRAS\textsuperscript{W13} SW48 group or LoVo-shNC group.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

**Figure 4.** Chiauranib alters gene sets related to ROS in KRAS wild-type CRC cells. (A) KEGG pathway enrichment between DMSO and Chiauranib groups, based on RNA-Seq analysis. (B) Gene set enrichment analysis (GSEA) analysis
showed that DEGs were positively correlated with oxidative phosphorylation and the p53 signaling pathway. (C) Heat map of 44 DEGs related to oxidative phosphorylation and the p53 signaling pathway. (D) Q-PCR of genes related to the p53 signaling pathway of SW48 cells after treatment with 4 μM Chiauranib for 48 hours. (E, F) KRAS WT SW48, KRAS G13D SW48, LoVo-shNC and LoVo-shKRAS cells were incubated for 30 min in the presence of Mito-SOX or DCH-DA. Representative flow cytometry plots showed the separate analysis of (E) mitochondrial O$_2^\cdot$ and (F) H$_2$O$_2$ in SW48 cells after treatment with 4 μM Chiauranib for 48 hours.

Figure 5. Chiauranib induces ROS of KRAS wild-type CRC cells by activating the p53 signaling pathway. (A) Western blot of proteins related to the p53 signaling pathway in SW48 cells after treatment with 4 μM Chiauranib for 48 hours. (B, C) SW48 cells were infected with siRNA to knock down p53 and treatment with 4 μM Chiauranib for 48 hours, followed by (B) Western blot and (C) Q-PCR analysis. (D) Representative flow cytometry plots showed the separate analysis of mitochondrial O$_2^\cdot$ and (F) H$_2$O$_2$ in SW48 cells after treatment with 4 μM Chiauranib for 48 hours.
tion of the p53 signaling pathway. The increased ROS levels induced by Chiauranib were dramatically abrogated by knockdown of p53 (Figure 5D, 5E). Moreover, silencing p53 substantially increased the cell viability reduction by Chiauranib treatment in KRAS wild-type CRC cells (Figure 5F). These data collectively suggested that Chiauranib induced intracellular ROS levels via activating the p53 signaling pathway in KRAS wild-type CRC cells.

**KRAS mutation CRC cells are resistant to ROS induced by Chiauranib via upregulating Nrf2**

Although we found the role and mechanism of ROS production induced by Chiauranib in KRAS wild-type CRC cells, the mechanism of Chiauranib resistance in KRAS mutation cells were not precise. Impressively, Chiauranib treatment inhibited phosphorylation of Aurora B in LoVo-shNC cells, but p53 was not affected. However, both of Aurora B phosphorylation and p53 expression could be inhibited in LoVo-shKRAS cells (Figure 6A). Recently, several studies have suggested that KRAS maintained low p53 levels by activating Nrf2 expression [22]. Meanwhile, western blot results revealed that Nrf2 was downregulated and p53 was upregulated in KRAS-silenced LoVo cells, and reverse changes in KRAS mutation LoVo cells (Figure 6B). Therefore, we hypothesized that KRAS mutation promoted the expression of Nrf2 and thus inhibited the increase of the p53 signaling pathway, making KRAS mutation cells resistant to Chiauranib.

To test this hypothesis, we further detected the p53 signaling pathway and cell viability in Nrf2 knockdown cell after treatment with Chiauranib. Interestingly, our results suggested that silencing Nrf2 activated the p53 signaling pathway and inhibited cell viability in KRAS mutation LoVo cells with Chiauranib treatment (Figure 6C-E). Moreover, silencing Nrf2 promoted ROS production in LoVo cells with Chiauranib treatment (Figure 6F, 6G). Above all, KRAS mutation CRC cells were resistant to ROS induced by Chiauranib through upregulating Nrf2.

**Chiauranib selectively inhibits the growth of KRAS wild-type CRC cells in vivo**

To further verify whether pharmacological Chiauranib was sufficient to mediate anti-tumor effects in a preclinical model, we generated mouse Xenograft Models by subcutaneous injection of SW48 and LoVo cells. One week after injection, tumor-bearing mice were randomized into two groups and administered intragastrically with vehicle or Chiauranib (40 mg/kg) once every three days. While treatment with Chiauranib in LoVo bearing Xenograft Models had little anti-tumor effects than the vehicle control, Chiauranib treatment significantly inhibited SW48 bearing Xenograft Models tumor growth (Figure 7A-C; Supplementary Figure 4). No remarkable weight loss or signs of morbidity were observed in the mouse Xenograft Models treated with Chiauranib during the experiment (Supplementary Figure 5). Moreover, Ki67 staining and TUNEL staining of the SW48 Xenograft Models tumors showed that Chiauranib inhibited KRAS wild-type CRC cells proliferation and induced apoptosis (Figure 7D-G). Noticeably, DHE staining samples showed Chiauranib increased accumulation of superoxide anion radicals in SW48 Xenograft Models tumors consistent with in vitro experimental results (Figure 7H, 7I). Our findings provided direct evidence of the safety and efficacy of Chiauranib for KRAS wild-type CRC treatment in preclinical models (Figure 8).

**Discussion**

Abnormal KRAS signaling played a crucial role in tumor growth and was usually associated with resistance to anti-EGFR therapy. Our recent research found that metformin selectively inhibited metastatic KRAS mutation CRC by decreasing excretion through silencing channel protein MATE1 [18]. On the other hand, 50% of KRAS wild-type CRC patients failed to respond to the anti-EGFR antibody cetuximab treatment, which has been proven effective in the treatment of KRAS wild-type CRC patients [7]. Therefore, it is imperative to find new drugs targeting KRAS wild-type CRC. Recently, the anti-tumor activity of Chiauranib has been explored in various cancers, including Non-Hodgkin’s lymphomas, acute myeloid leukemia, hepatocellular carcinoma, and gastric cancer [9, 10]. Surprisingly, in our experiments, we found for the first time that Chiauranib selectively inhibited the growth of KRAS wild-type CRC cells. This dramatic effect was further confirmed in mouse...
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type
Figure 6. KRAS mutation CRC cells are resistant to ROS induced by Chiauranib via upregulating Nrf2. (A) Western blot of proteins related to the p53 signaling pathway in LoVo-shNC and LoVo-shKRAS cells after treatment with 4 μM Chiauranib for 48 hours. (B) Western blot of Nrf2 in the indicated cells. (C-E) LoVo cells were infected with siRNA to knock down Nrf2, followed by (C) Western blot and (D) Q-PCR analysis and (E) cell viability detection. (F) Representative flow cytometry plots show the separate analysis of mitochondrial O$_2^•^−$ and H$_2$O$_2$ in LoVo cells after infected with siRNA to knock down Nrf2, and treatment with 4 μM Chiauranib for 48 hours. (G) Statistical analysis of mitochondrial O$_2^•^−$ and H$_2$O$_2$. Bars represented the mean ± SD of three independent experiments; *P < 0.05, **P < 0.01 and ***P < 0.001.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Xenograft Models. Our study has put forward the possibility of a therapeutic strategy for KRAS wild-type CRC patients by using Chiauranib.

Given the complex landscape of genetic alterations in cancer, it does not mean that chemotherapy drugs are useful for every patient. In our initial experiment, we tested the effect of Chiauranib on four CRC cell lines (LoVo, HCT116, SW48, CaCO₂). Surprisingly, only SW48 and CaCO₂ cells were sensitive to Chiauranib, while LoVo and HCT116 cells were resistant to Chiauranib (Figure 1). After carefully searching, we found that only the KRAS gene mutated in LoVo cells among the common CRC mutation genes of KRAS, BRAF, PIK3CA, PTEN, and TP53 [20]. KRAS mutation also occurred in HCT116 cells, whereas both of SW48 and CaCO₂ cells were KRAS wild-type cells. Further experiments showed that Chiauranib selectively inhibited proliferation and induced apoptosis of KRAS wild-type CRC cells (Figures 2, 3). Previous studies on various types of tumors have not found that some tumor cells are resistant to Chiauranib, possibly because KRAS gene mutation was not common in these tumors. However, the rate of KRAS mutation in CRC patients was as high as 30-50% [23]. It is possible that Chiauranib resistance may also exist in other types of tumors with frequent KRAS mutations, such as lung cancer and pancreatic cancer, which is worthy of further exploration.

To further explore the molecular mechanisms of Chiauranib inhibiting KRAS wild-type CRC, we determined the overall gene expression pattern of SW48 cells treated with Chiauranib by RNA sequence analysis (Supplementary Figure 1). Through KEGG and GSEA analysis, we found that the p53 signaling pathway and oxidative phosphorylation pathway were most significantly regulated, respectively (Figure 4A, 4B; Supplementary Figure 2). P53 signaling pathway played an essential role in DNA damage response, cell cycle, and apoptosis [15]. ROS was a direct representation of the activation of oxidative phosphorylation pathway, which could be modulated by the p53 signaling pathway [24, 25]. Activation of the p53 signaling pathway led to the accumulation of ROS, thus inhibiting cell proliferation and inducing apoptosis [25].

Figure 7. Chiauranib selectively inhibits the growth of KRAS wild-type CRC cells in vivo. (A-C) (A) The representative morphology, (B) tumor growth rate, and (C) tumor weight were shown as a result of treatment with Chiauranib (40 mg/kg) once every three days in SW48 Xenograft Models. Data are shown as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001. (D) Representative images of Ki67 staining in Xenograft Models tumors. Scale bar, 50 μm. (E) Statistical analysis of Ki67 staining. (F) Representative images of TUNEL staining in Xenograft Models tumors. Scale bar, 50 μm. (G) Statistical analysis of TUNEL staining. (H) Representative images of DHE staining in Xenograft Models tumors. Data were expressed as mean ± SD; *P < 0.05, **P < 0.01.

Figure 8. Simplified model depicting the pathway through how Chiauranib regulates KRAS wild-type CRC growth.
Some studies also suggested that ROS could regulate the p53 signaling pathway, indicating that ROS and p53 signaling pathways have positive feedback [26]. In our research, we found that the p53 signaling pathway and ROS related molecules were significantly changed after Chiauranib treatment, but the mRNA level of p53 did not change (Figure 4C, 4D). These results indicated that Chiauranib activated ROS through direct stimulation of p53 signaling pathway via p53 post-transcriptional regulation. Aurora B was a member of the chromosomal passenger complex, which a key regulator of mitosis and cell cycle [27]. Several lines of evidence suggested that Aurora B was prominently overexpressed in CRC and positively correlated with advanced tumor stages [28-30]. In addition, Aurora B has been shown to block the anti-tumor function of p53 through promoting its degradation, and otherwise, promote malignant transformation via activating RAS signaling [31, 32]. While whether the novel serine-threonine-kinases-targeted agent Chiauranib can be considered as an alternative choice at all times should be illustrated. In this study, our results demonstrated that Chiauranib inhibited Aurora B phosphorylation, resulting in an increased in p53 expression, thus inducing ROS production in KRAS wild-type CRC cells (Figure 5). Notably, p53 mutation also occurs frequently in many types of tumors, including CRC [33]. Whether p53 mutation could also lead to resistance to Chiauranib needs further study, However, as we known, Chiauranib is a multi-target small molecule inhibitor, which was very likely to play an anti-tumor role through other pathways in p53 mutant cells.

Nrf2 pathway was closely related to antioxidation, lowering intracellular ROS levels, and reducing intracellular environment [34]. The increase of Nrf2 expression induced by KRAS was a new mechanism of Nrf2 antioxidant program activation, which can steadily improve Nrf2 antioxidant basic program by increasing Nrf2 transcription [22, 35]. KRAS-directed increased expression of Nrf2 was evident in tissues of KRAS mutation CRC patients consistent with our in vitro results (Figure 6B). Moreover, genetic targeting of the Nrf2 pathway impaired KRAS120R-induced tumorigenesis and development in vivo [36, 37]. Therefore, Nrf2 antioxidant and cellular detoxification programs were crucial mediators of Chiauranib resistance in KRAS mutation CRC.

Impressively, the results of our animal experiments showed that Chiauranib has significant efficacy and safety, consistent with the phase I clinical trial (NCT03074825) (Figure 7). During the investigation, no significant weight loss or signs of disease were observed in the xenograft model treated with Chiauranib (Supplementary Figure 5). However, the commonly used chemotherapy drugs such as oxaliplatin and irinotecan have apparent side effects [38, 39]. Chiauranib may replace these chemotherapeutic drugs and become the new choice for the treatment of KRAS wild-type CRC. Notably, we found that Chiauranib slightly inhibited tumor growth on day 27, but it was not observed at any previous point (Supplementary Figure 4). This result suggested that long-term Chiauranib treatment may also inhibit KRAS mutation CRC growth in vivo, which needed to be confirmed by observation for a longer time. Recent findings provided evidence that Chiauranib could not only directly cause tumor cell death, but also restore anti-tumor immunity by altering some crucial components of the tumor microenvironment, which might build up the foundation for combined immunotherapies [40]. Therefore, it is worthy to obtain more convincing data to see whether Chiauranib can be therapeutically employed in KRAS mutation CRC treatment by regulating tumor microenvironment.

In conclusion, precision medicine is an emerging method that guides patients to receive more effective treatment strategies according to individual differences. We reveal that Chiauranib induces p53 upregulation, resulting in ROS accumulation, thus inhibiting cell proliferation and inducing apoptosis. This study suggests that KRAS wild-type status could be a potential biomarker for using Chiauranib in CRC patients. Further clinical trials are necessary to verify efficacy and safety from Chiauranib treatment in CRC patients.

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Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

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Disclosure of conflict of interest

None.

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Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Supplementary Table 1. Primer sequence

| Primer     | Sequence (5’ to 3’)                  |
|------------|-------------------------------------|
| BCL2-F     | GGGGGGGTCATGTGTGTGG                 |
| BCL2-R     | CGGTCAGTAGTCAGTCATCC                |
| BAX-F      | CCGAGAGGCTTTTTTCCGAG                |
| BAX-R      | CCAGCCCATGTGGGTCTCTGAT              |
| ZMAT3-F    | AGAAGCCTTTTGGGGAGAGGAGG            |
| ZMAT3-R    | TGCTGCATAGTAATTTCCGGAGTT           |
| FAS-F      | TCTGGTCTTACGCTCTGTG                |
| FAS-R      | CTGGCAGTCCCTACGCTTCC               |
| TP53AIP1-F | GGTGCCCAAGTGTCAGGGGAGG            |
| TP53AIP1-R | CTGGAGAGACCTAGACCCAAGG             |
| CDKN1A-F   | TGTTCCTCAGCAACCACATGC             |
| CDKN1A-R   | AAAGTGAAGTTTCACTCGCTC             |
| CDK1-F     | AAACATAGCTCAAGTGAGGCC             |
| CDK1-R     | TCTGCAAGCAATACATCTGA              |
| EI24-F     | TGCCAGAGGAATCAAAAGACTCC           |
| EI24-R     | TCTCTGCTTCCGCTCTACTACT            |
| MDM2-F     | GAATCATCGGACTCAGGACCATC           |
| MDM2-R     | TCTGTCTCAATTGCCTCTCT              |
| TP53-F     | CAGCACAGTGACGGAGGTGTG             |
| TP53-R     | TCTCCAGAGCCTTCCCTTC              |
| β-actin-F  | ACTCTGCAGCCTTCCCTTC              |
| β-actin-R  | ATCTCCTCAGCATTGCTGTG              |
| Nrf2-F     | TCAGCAGGAGAAGGATGATGA            |
| Nrf2-R     | CACTGTTTCCGTAGCATGGATG            |

Supplementary Figure 1. Volcanic map of DEGs between DMSO and Chiauranib groups. DEGs between DMSO and Chiauranib groups were displayed by volcanic map. The X-axis represented the difference multiples after log2 conversion, and the Y-axis represented the significant value after log10 conversion. The blue one represented down-regulated DEGs, the red one represented up-regulated DEGs, and the gray one represented non-DEG. log_2 |FC| ≥ 1 and P < 0.05.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Supplementary Figure 2. Gene set enrichment analysis (GSEA) of DEGs DMSO and Chiauranib groups. The significantly enriched hallmark pathways in GSEA were listed.

| Rank | Hallmark name                                      | NES  | p-value |
|------|---------------------------------------------------|------|---------|
| 1    | Oxidative phosphorylation                        | 1.898| 0.000   |
| 2    | Drug metabolism - cytochrome P450                 | 1.807| 0.001   |
| 3    | p53 signaling pathway                            | 1.750| 0.002   |
| 4    | Lysosome                                         | 1.729| 0.000   |
| 5    | Hedgehog signaling pathway                       | 1.670| 0.006   |
| 6    | Proteasome                                       | 1.670| 0.006   |
| 7    | Antigen processing and presentation              | 1.653| 0.004   |
| 8    | Glycosphingolipid biosynthesis - ganglio series   | 1.584| 0.020   |
| 9    | N-Glycan biosynthesis                            | 1.567| 0.010   |
| 10   | Arachidonic acid metabolism                      | 1.544| 0.016   |
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Supplementary Figure 3. Statistical analysis of ROS levels in CRC cells after treatment with Chiauranib. (A, B) Statistical analysis of (A) mitochondrial O$_2^•$ and (B) H$_2$O$_2$ in KRAS$^\text{WT}$ SW48, KRAS$^{G13D}$ SW48, LoVo-shNC and LoVo-shKRAS cells. Bars represented the mean ± SD of three independent experiments; *$P$ < 0.05, **$P$ < 0.01 and ***$P$ < 0.001.
Chiauranib selectively inhibits colorectal cancer with KRAS wild-type

Supplementary Figure 4. The antitumor effects of Chiauranib in LoVo Xenograft Models. (A-C) (A) The representative morphology, (B) tumor growth rate, and (C) tumor weight were shown as a result of treatment with Chiauranib (40 mg/kg) once every three days in LoVo Xenograft Models. Data are shown as mean ± SD; *P < 0.05.

Supplementary Figure 5. The body weight of SW48 Xenograft mice between DMSO and Chiauranib groups. The body weight of SW48 Xenograft mice was measured at 27 days after inoculation.