A combinatorial strategy for overcoming primary and acquired resistance of MEK inhibition in colorectal cancer

Junjun Chen  
*Shanxi Medical University*

Jie Dai  
*Shanxi Medical University*

Zhiming Kang  
*Shanxi Medical University*

Ting Yang  
*Shanxi Medical University*

Qi Zhao  
*Shanxi Medical University*

See next page for additional authors

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Keywords
Colorectal cancer; Combination therapy; Targeted therapy resistance; Trametinib

Authors
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A combinatorial strategy for overcoming primary and acquired resistance of MEK inhibition in colorectal cancer

Junjun Chen¹,†, Jie Dai¹,†, Zhiming Kang¹†, Ting Yang¹, Qi Zhao², Jinxu Zheng², Xinxin Zhang³, Jisheng Zhang⁴, Jun Xu⁵, Gongqin Sun⁶*, Lijun Yang²*, Tao Yang¹*  

¹Department of Biochemistry & Molecular Biology, Shanxi Medical University, Taiyuan 030001, Shanxi, China  
²Department of Pharmacology, Shanxi Medical University, Taiyuan 030001, Shanxi, China  
³Department of Medical Experimental Techniques, Shanxi Medical University, Taiyuan 030001, Shanxi, China  
⁴Department of Otolaryngology-Head and Neck Surgery, Key Laboratory, The Affiliated Hospital of Qingdao University, Qingdao University, Qingdao 266003, Shandong, China  
⁵Department of General Surgery, Shanxi Bethune Hospital, Taiyuan 030001, Shanxi, China  
⁶Department of Cell and Molecular Biology, University of Rhode Island, Kingston 02881, RI, USA
† These authors contributed equally to this work.

*Corresponding Author: Tao Yang
Postal Address: No.56 Xinjian South Road, Taiyuan, Shanxi, 030001, China
Tel & Fax: +86-351-3985139
E-mail: yangtao056cn@126.com

*Co-corresponding Author: Lijun Yang
Postal Address: No.56 Xinjian South Road, Taiyuan, Shanxi, 030001, China
Tel & Fax: +86-351-3985139
E-mail: yanglijunmm@126.com

*Co-corresponding Author: Gongqin Sun
Postal Address: 120 Flag Road, Kingston, RI, 02881
Tel & Fax: +01-401-874-5937
E-mail: gongqinsun@uri.edu
Abstract

Compared with traditional chemotherapeutic drugs, targeted therapeutic medicine has the advantages of high efficacy and less toxic side effects. However, in clinical practice for treatment of colorectal cancer, the primary and acquired resistance of these medicines limits their effectiveness in targeted therapy, therefore impedes the development of precision medicine and personalized therapy. Currently, there are limited number of drugs for targeted therapy of colorectal cancer, mainly monoclonal antibodies against EGFR or VEGFR inhibitors. Trametinib, a MEK inhibitor, has been applied in melanoma patient successfully, but not been used in clinical treatment of colorectal cancer because of its drug resistance. To identify the resistance mechanism of colorectal cancer cells to trametinib and find useful chemical combination to overcome the resistance, we screened primary and acquired cell line first and then tested multiple synergistic drug combinations by using the Chou-Talalay method. We obtained the primary resistant cell lines SW480, CW-2 and the acquired drug-resistant cell line RKO-R as well as a synergistic combination of trametinib and GSK2126458. This combination inhibits the colony formation of colorectal cancer cells and the growth of xenograft tumors in nude mice. Mechanistic analysis showed that trametinib can activate the alternative PI3K-AKT signaling pathway while inhibiting the MAPK pathway, which may be one of the molecular mechanisms of primary and acquired trametinib tolerance in colorectal cancer cells. Importantly, this bypass...
activation can be blocked by GSK2126458. These results suggest that a combination of trametinib and GSK2126458 is an effective approach for treating colorectal cancer resistance to trametinib.

**Keyword:** Colorectal cancer, Trametinib, targeted therapy resistance, combination therapy
Colorectal cancer (CRC) is a major gastrointestinal malignancy, whose incidence and mortality ranks third in common cancers\(^1\). The therapeutic methods for CRC include surgery, radiation, traditional chemotherapy, and targeted therapy\(^2\). Targeted therapy or molecular targeted therapy is one of the primary ways for cancer treatment. Most of the targeted drugs for clinical treatment to CRC have largely relied on EGFR and VEGFR monoclonal antibodies\(^3\), including cetuximab, panitumumab and ramucirumab\(^5\). In clinical cancer practice, these targeted agents have achieved certain therapeutic effects. For instance, the overall survival of patients with stage IV colorectal cancer is extended to more than 30 months through targeted therapy\(^8\). However, inter-tumor and intra-tumor heterogeneity, primary drug-resistance and acquired resistance caused by long-term treatment severely limit the efficacy of targeted therapy\(^9\). The mechanisms of drug resistance are complex, and usually involve mutations in the target gene or activation of the bypass signaling pathway or downstream signaling molecules\(^11\).

The Ras-Raf-MEK-ERK (MAPK) and PI3K-AKT are two important signaling pathways that control cell proliferation. Constitutive activation of any of these pathways would result in sustained cell proliferation, leading to tumorigenesis and progression\(^13\). The RAS-MAPK signaling is downstream of EGFR. Clinical research has shown that about 40% of CRC patients have KRAS mutations, and 5% to 10% of patients have BRAF mutations, making these
patients unresponsive to EGFR monoclonal antibodies\textsuperscript{5,6}. This situation has prompted the search of new CRC-specific targeted drugs or more effective combinations to overcome the resistance\textsuperscript{16,17}. Trametinib (GSK1120212) is a second generation of MEK inhibitor\textsuperscript{18,19} that has been approved by the US Food and Drug Administration for treating metastatic melanoma patients with BRAF V600E/K mutations. This agent can inhibit the proliferation of B-Raf mutant CRC cell line HT-29 and Colo205 with IC\textsubscript{50} of 0.48 and 0.52 nM, but there are few reports of Trametinib for CRC treatment\textsuperscript{20,21}. Besides, clinical trials have shown that melanoma patients with resistance to trametinib monotherapy will appear within 6 to 7 months\textsuperscript{22}. These data indicate that the resistance to MEK inhibitors may also be a major clinical challenge.

Therefore, to explore the possibility of trametinib in treatment of CRC, in the present study, we screen the sensitivity of CRC cell lines to trametinib, select and set up the primary and acquired resistant CRC cell lines to this agent, and then test the effective combinative drug with trametinib to overcome its resistance \textit{in vitro} and \textit{in vivo}. Furthermore, we will also investigate the mechanism of trametinib resistance \textit{in vitro}.

\textbf{Materials and methods}

\textbf{Cell Culture}

The RKO resistant cell line was generated by treating parental cell with increasing concentrations of Trametinib as previously described\textsuperscript{23}. Other
human colorectal cancer cell lines were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). CW-2 and RKO-R were maintained in a humidified incubator at 37°C with 5% CO₂, and respectively grown in RPMI 1640 (Hyclone, USA) and MEM (Sigma, UK), while SW480 was cultured in L-15 medium (Gibco, USA) at 37°C with 100% air. The medium was supplemented with 10% FBS and 100 IU ml⁻¹ penicillin/ streptomycin. All cell lines were detected for the presence of mycoplasma using Detection Kit (Genechem, China).

Reagents

All the targeted inhibitors were purchased from Selleck (Houston, USA). The inhibitors were dissolved in DMSO to a final concentration of 20 mmol/L and stored at -20°C. The stock solution was diluted to working concentrations with culture medium just before each experiment.

MTT

Cancer cell lines were seeded in 96-well plates (1-2×10³ cells per well) and were treated with different concentrations of inhibitors. After 72h, cell viabilities were measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay. Results of IC₅₀ and the synergistic combination were determined according to the method of Chou and Talalay by using the CompuSyn software program.²⁴ CompuSyn software was used to generate combination index (CI) values, where CI<1, =1, and >1 indicate synergism, additive effect, and antagonism,
respectively.

**Colony Formation**

For the assays of colony formation, cells were seeded into 6-well plates (1 \( \times 10^3 \) to 2\( \times 10^3 \) cells per well) and allowed to adhere over 12 hours in a regular culture medium. Then cells were cultured in the absence or presence of drugs as indicated in complete medium for 10–15 days, and the medium were replaced every other day. After the culturing, cells were fixed with methanol, stained with 10% Giemsa solution, and photographed. The number of colonies was count by Image J software, and each experiment was repeated in triplicate at least. Clonal formation rate = number of clones / number of cells inoculated \( \times 100 \). Inhibition rate of colony formation (%) = \( (1 - \text{clone formation rate of experimental group} / \text{clone formation rate of control group}) \times 100\% \).

**Cell cycle assay**

For cell cycle analysis, RKO and RKO-R cells were respectively treated with 0.1\( \mu \)M trametinib for 24 hours. Cells were fixed by 70% ethanol for 4 hours and then stained with propidium iodide (KenGen BioTECH, Jiangsu) according to the manufacturer’s instructions. Proportions of the cell cycle were performed by a BD Biosciences FACS Calibur flow cytometer and analyzed using FlowJo software.

**Immunoblotting**

Proteins of whole cells were obtained by a lysis buffer (Beyotime, Shanghai) supplemented with Protease Inhibitor Cocktail (Sigma, USA) and
the concentrations of proteins estimated by a modified Bradford assay (Bio-Rad). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA), while anti-rabbit IgG secondary Abs were from Sangon (Shanghai, China). The specific bands were visualized by enhanced chemiluminescence (ECL plus, Thermo Fisher Scientific). Each experiment was done in triplicate.

**Xenograft Studies**

Cells were subcutaneously injected (5 × 10^6 cells per injection) into BALB/c nude male mice (4–5 weeks old) which were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). When tumors reached an average volume of approximately 100 mm³, mice were randomized into control and three different medicated groups. Trametinib and GSK2126458 were suspended in 1% Cellulose Sodium which was purchased from Chron Chemicals (Chengdu, China). Trametinib was administered through oral gavage daily at 0.3 mg/kg dose for 21 days, and GSK2126458 was orally administered at 0.3 mg/kg alone or in the indicated combination daily for 21 days. Tumor volume was assessed and calculated using the following formula: \( \text{Volumes} = \frac{\text{Length} \times \text{Width}^2}{2} \). All experiments were performed according to the protocols approved by the Animal Care and Use Committee guidelines of Shanxi Medical University.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on all the tissues of xenograft
tumor using biotin-streptavidin HRP detection systems. Briefly, paraffin sections were subjected to deparaffinization and antigen retrieval. Then the slides were incubated with Ki67 (1:400), Cleaved caspase-3 (1:200) antibodies (Cell Signaling Technology, USA). Biotinylated secondary antibody (Zhongshan Golden Bridge Biotechnology Co. Ltd., China) was performed according to the manufacturer’s recommendations.

Results

Screening the cell model for primary trametinib resistance

A panel of human colorectal cancer cell lines with different gene mutation types (Supplementary Table 1) was used to test their sensitivity to 30 targeted inhibitors. Relative cell viability and IC$_{50}$ were measured after treatment of cells with 16 different concentration gradients. Results showed that eight out of nine cell lines were sensitive to only one to three targeted drugs (Figure S1-S9, Supplementary Table 2), which indicates strong resistance characteristic. It is noteworthy that seven out of nine CRC cells are bearing KRAS mutations, but these cell lines showed different responses to inhibitors. For instance, SW480 and SW620 cell line have the same KRAS G12V mutation, but SW620 was sensitive to trametinib. SW480 was tolerant to all targeted inhibitors, including the MEK inhibitor trametinib. Therefore, SW480 and CW-2 cells were selected as primary trametinib resistance cell model for further investigation of combination therapy. SW480 cells were PI3KCA wild type, and CW-2 cells have P283S mutation.
Establishment of the cell model of acquired trametinib resistance

An acquired resistance cell model was established, which is resistant to trametinib by long-term treatment of colorectal cancer cell line RKO (designated as RKO-R). MTT assay showed that the parental cell was sensitive to trametinib (IC$_{50}$ 0.01-0.05 $\mu$M). However, the RKO-R cell line required higher doses of this inhibitor for partial growth inhibition (IC$_{50}$ 0.5-1.0$\mu$M) (Figure 1A). We also observed that 0.2 $\mu$M trametinib treatment can only inhibit 1/3 colony formation in RKO-R cells in comparison with parental RKO cells (Figure 1B). Flow cytometry analysis showed that after treatment with 0.1 $\mu$M of trametinib, RKO-R cells had a decreased percentage of G0/G1 phase and an increased S phase (P < 0.05) (Figure 1C). The inverted behavior indicated that trametinib did not retard cell cycle in G0/G1, which showed RKO-R cells were resistant to trametinib’s killing.

Identification of drug combination on primary and acquired resistance to trametinib

To explore new therapeutic strategies for overcoming primary resistance to trametinib, we first performed MTT assay to detect inhibitors synergistic with it in SW480 cells. Of the 30 drug combinations, only three pairs showed robust inhibition of cell proliferation, among them, trametinib combination with PI3K inhibitor GSK2126458 (Figure S10, Supplementary Table 3) is most efficient. We next analyzed IC$_{50}$ and combination index (CI) of trametinib combination with GSK2126458 according to the CompuSyn
MTT assay showed that SW480, CW-2, and RKO-R were highly sensitive to trametinib and GSK2126458 combo (Figure 2A-C). The IC_{50} of combination therapy in three cells was 2.86, 13.8, and 7.36 nM, respectively, which was robustly lower than 1199.0, 478.7 and 303.4 nM of trametinib and 177.2, 185.5 and 134 nM of GSK2126458. (Figure 2D). CI values for these three cell lines treated in combination were significantly less than one at the indicated concentrations (CI values ranging between 0.01 and 0.5) (Table 1).

Then we analyzed the effects of trametinib and GSK2126458 on colorectal cancer cells via colony formation assay. Although trametinib alone resulted in 57% inhibition of colony formation, cotreatment with GSK2126458 dramatically increased to 91% inhibitory in SW480 cells (Figure 3A). The significant effects of combination therapy against colony formation were also observed in CW-2 and RKO-R cells (Figure 3A). In addition, trametinib plus GSK2126458 promoted the expression of the apoptosis-related protein cleaved-PARP compared with monotherapy, thus stimulating cell apoptosis (Figure 3B). Taken together, these data indicate that this combination has a synergistic effect in colorectal cancer cells of primary and acquired resistant to trametinib.

Trametinib combination with GSK2126458 inhibits tumor growth in colorectal cancer xenograft models

To verify the synergistic effect of drug combo, we next established SW480 and RKO-R xenografts and treated them with vehicle control, trametinib alone,
GSK2126458, or the combination. SW480 and RKO-R xenografts were daily initiated administration with 0.3 mg/kg trametinib alone or combo with GSK2126458 (0.3 mg/kg) when tumors reached ~100 mm$^3$. Of interest, combined treatment showed a significant antitumor effect compared with control and single-agent groups in RKO-R xenograft (Figure 4). We also observed that in SW480 xenograft model, the tumor sizes have less difference between mice receiving trametinib alone and combination groups. This could be attributed to the likelihood that trametinib exhibited more inhibition effects in mice than in cultivated cells. However, there is still a statistical difference between combination group and trametinib group. H&E analysis indicated that the tumor cells were abnormally shaped, and the nuclei were deeply stained and arranged in an irregular cell structure. IHC analysis on tumor tissue revealed that Ki-67 expression level was significantly reduced in mice treated with trametinib plus GSK2126458. Besides, cleaved caspase-3 level was higher in combination- than in single-agent-treated tumors (Figure 5). Thus, our data indicated that trametinib plus GSK2126458 had strong antitumor activity.

**Effects of trametinib alone or in combination with GSK2126458 on intracellular signaling pathways**

To identify molecular modifiers that influence the sensitivity of colorectal cancer cells to MEK inhibition, we first profiled SW480 cells to determine their response to the MEK1/2 inhibitor trametinib. As the downstream molecule of
MEK, ERK phosphorylation was inhibited after 6 to 8 h treatment with 0.1 μM trametinib. Unexpectedly, AKT, another protein kinase associated with tumor growth was activated, especially after 24 h treatment with trametinib. The same occurrence was also observed in CW-2 cells (Figure 6A). In RKO-R cells, trametinib was unable to inhibit the phosphorylation of ERK, which might be related to the fact that this cell line has tolerated this inhibitor (Figure 6A).

These findings provide evidence that activation of the PI3K-AKT pathway renders KRAS mutant colorectal cancer cells less sensitive to MEK inhibitor trametinib. With combination of PI3K inhibitor GSK2126458, we observed phosphorylation levels of ERK and AKT in SW480, CW-2, and RKO-R cells were simultaneously blocked by combination therapy (Figure 6B). This indicated that the coordinate repression was due to concurrently disruption of MAPK and PI3K pathway signaling.

Inhibition of IGF1R sensitizes SW480 cells to MEK pathway inhibition

Others have demonstrated previously that FGFR1 can mediate adaptive resistance to trametinib in KRAS-mutant lung cancer and MEK inhibition led to autocrine activation of STAT3 via the FGF receptor and JAK kinases in EGFR mutant lung cancer 12,25. It was reported that intrinsic resistance to MEK inhibitor selumetinib was through upregulation of ERBB3 in KRAS mutant lung and colon cancer 26. To further explore the mechanism of AKT feedback activation, we examined phosphorylation changes of several upstream molecules that regulate AKT in SW480 cells upon trametinib treatment. We
found a significant increase in p-IGF1R in a manner similar to that of phosphorylated AKT (Figure 6A).

We then determined whether IGF1R inhibitor could block trametinib-induced AKT activation and thereby sensitize SW480 cells to trametinib. MTT assay showed that when combined with trametinib and BMS-754807 (an IGF1R inhibitor), the cell activity was lower than monotherapy (Figure 7A). The CI value was less than 1 which indicated that the two agents had a synergistic effect (Table 2). Consistently, trametinib significantly prevented colonies when combined with BMS-754807 (Figure 7B).

Although activated AKT was partly inhibited when trametinib was combined with BMS-754807, it could not be restored to normal levels (Figure 7C).

Discussion

We have several findings in this study: First, nine CRC cells showed different responses to 30 targeted inhibitors. One (SW480) of the nine cell lines did not respond to any of the inhibitors. Second, the trametinib resistance of SW480, CW-2, and RKO-R cells could be reversed by combination with PI3K inhibitor GSK2126458. Third, Western blotting analyses indicated that the bypass activation of PI3K-Akt signaling pathway was contributing to the trametinib resistance. This activation was partly due to IGF1R upregulation with trametinib treatment.

Our data showed that none of the 30 targeted inhibitors could block the proliferation of all nine CRC cells. Six of the nine CRC cells bear KRAS
mutations at codon 12 and 13, which indicates constitutively activation of MAPK pathway. These cells are supposed to be blocked by trametinib. But only three of them were sensitive to trametinib. KRAS mutation cell line HCT15 and LoVo were resistant to trametinib. However, they were sensitive to GSK2126458, which indicates that the cellular proliferation was driven by PI3K-AKT pathway. As we focused on GSK2126458 plus trametinib to overcome resistance, we did not select these two cell lines for further investigation.

The different responses of CRC cell lines indicate the complex molecular heterogeneity in tumor. Activation of tumor compensatory signaling pathways, changes in the tumor microenvironment, and adaptation of tumors to inhibitors are the main resistance mechanisms to targeted therapy. Recent reports showed that microRNAs are also a factor influencing the acquired drug resistance of cancer cells. Resistance to MEK inhibitors has been observed in various human tumors, including lung cancer, colorectal cancer, and ovarian cancer, etc. Considering that resistance to monotherapy frequently occurs in colorectal cancer and other tumors, and more effective antitumor effect can be obtained by combination therapy. Therefore, combinations of the inhibitors are much more effective in enhancing anticancer activity and overcoming drug resistance.

We find that one mechanism of colorectal cancer cells resistance to trametinib is due to the activated PI3K-AKT bypass pathway. This finding can
also be verified by the phenomenon that the MAPK signal pathway in RKO-R
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cells cannot be blocked under long-term MEK inhibition. This may be due to
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the bypass activation PI3K-AKT pathway under trametinib treatment. It has
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been reported that there is certain interaction between the PI3K-AKT and
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MAPK pathways, which not only have common upstream molecules, but they
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can all be activated by oncogenic RAS\(^3^8\). And some compensatory signals
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may appear when one of the two signal pathways is inhibited\(^3^9\). It has been
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shown that MAPK is activated by PI3K via RAS when the downstream target
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mTOR of AKT is restrained\(^4^0\). It is also found that these two pathways are
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simultaneously activated in many different tumor types including melanoma,
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prostate cancer, and colorectal cancer, suggesting that they work through
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complex signaling networks\(^4^1,^4^2\). So blocking both pathways at the same time
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may be an effective approach to reverse the resistance of a single targeted
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drug. Clinical trials have shown that dual inhibition of these two pathways may
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have potential advantages than monotherapy, especially in patients with
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coexisting PI3K genetic alterations and KRAS/BRAF mutations\(^4^3\).
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To further explain the upstream molecule that activates PI3K-AKT, we have
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found that MEK inhibition can up-regulate the upstream protein IGF1R of PI3K
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in SW480 cells. It has been reported that cetuximab resistance is associated
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with increased expression of insulin-like growth factor 1 receptor (IGF1R) and
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tyrosine-protein kinase Met (c-MET) in patients with metastatic CRC\(^4^4\). To
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verify IGF1R upregulation is responsible for PI3K-AKT activation, we treated
trametinib resistant CRC cells with combined MEK and IGF1R inhibition. As expected, this combo displayed a synergistic effect, but could not completely prevent the activation of \( p\)-AKT caused by trametinib. These findings suggest that inhibition of IGF1R sensitizes cells to trametinib, but not as effectively as directly block PI3K-AKT, perhaps in connection with IGF1R regulation of other signaling pathways\(^{45}\). Therefore, in subsequent xenograft studies, we still used the combination therapy of trametinib and GSK2126458.

In initial xenograft experiments, mice were treated daily with trametinib at 1mg/kg, a dose used commonly in xenograft studies\(^{19}\), and GSK2126458 was at the dose of 1mg/kg\(^{46}\), too. Vehicle and monotherapy were well-tolerated, but mice receiving the combination therapy began to lose weight (>10%) and lassitude and started to die on the seventh day after administration. Although it has been reported that trametinib was administered to mice even at a dose of 3 mg/kg body weight\(^{25}\), the mouse allometric equivalent of the maximum tolerated dose (MTD) in humans is about 0.3 mg/kg. Then we administrated the light weight group of mice with the reduced daily dose of trametinib and GSK2126458 (0.5 mg/kg respectively). Mice with a dose halved lived longer than before; the combination still led to weight loss and death (data not shown). Exploratory dose finding resulted in a tolerable schedule, and we administrated the mice with a daily dose of 0.3 mg/kg trametinib and GSK2126458 respectively, so they showed stable weight and normal behavior. Finally, in the absence of significant side effects, experiments also verified the
effect of this combination therapy in vivo. The combination of trametinib and
GSK2126458 inhibited tumor growth in SW480 and RKO-R cell xenografts.

In summary, our study provides a molecular explanation for the primary
and acquired resistance of colorectal cancer cells to MEK inhibitor trametinib.
Our findings provide a reasonable rationale for the combination of MEK and
PI3K inhibitors in colorectal cancers with existing feedback activation of PI3K.
It will be crucial in future studies to explore whether combination therapy with
trametinib and GSK2126458 is effective for treatment of colorectal cancer in
clinic.

Acknowledgements

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Conflict of interest

All the authors declare that they have no conflict of interest.

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Table 1. Combination Index (CI) values of drug combinations in CRC cells.

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<th>GSK2126458 (nM)</th>
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Note: Cells were treated with the combination of trametinib and GSK2126458 at indicated doses. The CI value was analyzed using CompuSyn software and represented synergistic effect when it was less than 1.

Table 2. Combination Index (CI) values of drug combinations in SW480 cells.
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Figure legends

Figure 1. Development of acquired trametinib resistant colorectal cancer cell line RKO-R.
(A) Sensitivity of trametinib treatment on RKO (blue) and RKO-R (red) cells were assessed by MTT assays. Cell viability was calculated as the ratio of treated to untreated cells at each dose for each replicate. Data are represented as mean ± SD (n = 3). (B) Clonogenic assays and quantitative analysis of the RKO and RKO-R cells with trametinib treatment for 15 days. The growth inhibition rates of clone formation were calculated (right panel). (C) Cell cycle analysis of RKO and RKO-R cells with treatment of 0.1 μM trametinib for 24 h.

Figure 2. Cell viability of trametinib and GSK2126458 treatment on human colorectal cancer cell lines.
Combination index method was used for experimental design and data analysis. (A-C) Cells were treated with gradient concentrations of trametinib and GSK2126458 (range, 0.01-2.5 μM) for 72 hours and cell viability was evaluated by MTT assay. (D) The IC50 values were determined by the CompuSyn software, each experiment performed in triplicate. Error bars represents SD.

Figure 3. Combination of trametinib and GSK2126458 suppressed colorectal
cancer cells growth and promoted cell apoptosis.

(A) Clonogenic assay of SW480, CW-2 and RKO-R cells treated with trametinib, GSK2126458, or their combination as indicated (left). Percentage inhibition at each concentration of the inhibitors in the three cells is presented (right). Data presented as mean of three independent experiments. (B) Immunoblot of SW480, CW-2 and RKO-R cells treated with trametinib (100 nM) alone or in combination with GSK2126458 (100 nM) for 24 h.

Figure 4. Effects of combination with Trametinib and GSK2126458 on the growth of SW480 cell and RKO-R cell xenografts.

(A, D) Relative tumor growth curve on each day compared with day 0. (B, E) Subcutaneous xenografts of each groups. Data are expressed as means ± SD. N= 5(SW480) and N = 6 (RKO-R). (C, F) Relative tumor volume on day 21 compared to day 0 in xenograft model. (*p<0.05, **p<0.01).

Figure 5. Representative hematoxylin and eosin (HE) staining of neoplasms and immunohistochemical staining of Ki-67 and Cleaved caspase-3 in tumors. Data are expressed as means ± SD. n = 3. (*p<0.05, **p<0.01). Original magnification, ×40

Figure 6. Effects of trametinib alone or in combination with GSK2126458 on intracellular signaling pathways in colorectal cancer cell lines with primary and
acquired resistance to trametinib.

(A) Western blot analysis of the effect of trametinib on the related signaling pathway of indicated cell lines. Cells were treated with trametinib at a dose of 0.1 μM for different time point. Total cell protein extracts (40-80 μg) were subjected to immunoblotting with the indicated antibodies. (B) Effects of pharmacological inhibition of MEK, PI3K and their combinations. Cells were treated with trametinib, GSK2126458 and their combinations as indicated for 24 h. Responses of cells were examined by western blot analysis with the indicated antibodies.

Figure 7. Effects of trametinib alone or in combination with BMS-754807 of IGF1R inhibitor in CRC SW480 cells.

(A) Cells were treated with gradient concentrations of trametinib and BMS-754807 (range, 0.01-2.5 uM) for 72 hours and cell viability was evaluated by MTT staining. Error bars represents SD (n=3). (B) Clonogenic assay of SW480 cells treated with increasing concentrations of trametinib, IGF1R inhibitor BMS-754807, or their combination as indicated (upper). Percentage inhibition at each concentration of the drugs were calculated by relevant formula (lower). Data presented as mean of three independent experiments (n = 3). (C) Immunoblot analysis of SW480 cells treated with trametinib (0.05 μM), BMS-754807 (0.05 μM), or their combination for 24 h. Then the expression levels and activation of ERK and AKT were detected by indicated antibodies.
Figure 1

A

Trametinib

Relative cell viability

\[ \text{log(drug concentration, \( \mu M \))} \]

RKO

RKO-R

B

Trametinib(nM)

0 50 100 200

TRAMETINIB

RKO

RKO-R

C

RKO

RKO-R

Cells population (%)

G0/G1 S G2/M

Figure 2

A

SW480

Relative cell viability

\[-2 -1 0 \text{ log (drug concentration, \( \mu M \))} \]

Trametinib

GSK2126458

Combo

B

CW-2

Relative cell viability

\[-2 -1 0 \text{ log (drug concentration, \( \mu M \))} \]

Trametinib

GSK2126458

Combo

C

RKO-R

Relative cell viability

\[-2 -1 0 \text{ log (drug concentration, \( \mu M \))} \]

Trametinib

GSK2126458

Combo

D

IC_{50} (nM)

SW480 CW2 RKO-R

Trametinib

GSK2126458

Combo

P values for IC_{50}:

Table

- SW480
- CW2
- RKO-R

- Trametinib
- GSK2126458
- Combo
Figure 3

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Growth inhibition (%)
Figure 4

(A) SW480 xenograft

(B) SW480 xenograft

(C) SW480 xenograft

(D) RKO-R xenograft

(E) RKO-R xenograft

(F) RKO-R xenograft
Figure 5

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![Graph A](image)

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![Graph B](image)
Figure 6

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Figure 7

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