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Abstract

KRAS mutation is the most common type of mutation in human cancers. However, the direct pharmacological inhibition of KRAS has not been clinically successful. Trametinib (GSK1120212, Tram), a newer MEK inhibitor, inhibits RAS signaling through mitogen-activated protein kinase (MAPK) cascade suppression. The effectiveness of Tram in clinical practice is limited in KRAS mutant tumors compared to that in BRAF mutant tumors. Here, we found that Tram treatment provoked feedback activation of upstream RAS, thus causing an induction of phosphorylated MEK (pMEK) and phosphorylated ERK (pERK) rebound in KRAS mutant tumors. This failure of persistent ERK inhibition led to drug resistance. Zoledronic acid (ZA), a nitrogen-containing bisphosphonate, disrupts the biological activity of RAS by inhibiting its isoprenylation. Surprisingly, ZA overcame Tram resistance, and augmented antitumor activity was observed in KRAS mutant tumors both *in vitro* and *in vivo*. Furthermore, ZA enhanced the effect of Tram partially through the mevalonate pathway. In summary, the combination of the two FDA-approved drugs Tram and ZA may represent a novel therapeutic strategy for the treatment of KRAS mutant cancers.

Zoledronic Acid Enhances the Efficacy of the MEK Inhibitor Trametinib in KRAS Mutant Cancers

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Running title: COMBINED TRAM AND ZA TREATMENT IN KRAS MUTANT CANCERS

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Keywords: RAS, MEK, trametinib, zoledronic acid, mevalonate pathway

Abbreviations: Tram, trametinib; ZA, zoledronic acid; pERK, phosphorylated ERK; pMEK, phosphorylated MEK; FTIs, farnesyltransferase inhibitors; GGTIs, geranylgeranyltransferase I inhibitors; GGPP, geranylgeranyl pyrophosphate; RTKs, receptor tyrosine kinases.

1. Introduction

KRAS is the primary isoform of RAS small guanine nucleotide binding proteins. Mutations in KRAS, which occur in approximately 30% of all human cancers, are related to poor prognosis and resistance to targeted therapies [1-3]. So far, pharmacologically targeting KRAS has failed clinically [4,5]. Alternative approaches to inhibiting KRAS downstream effectors (e.g., RAF, MEK) have proven marginally effective or ineffective in KRAS mutant tumors [6-9].

Although both BRAF and KRAS mutant cancer cells present persistent activation of MEK-ERK signaling, MEK inhibitors have a better response in BRAF mutant cancer cells than KRAS mutant cancer cells [10]. MEK inhibitor monotherapy showed limited efficacy in preclinical studies and clinical trials in KRAS mutant cancers [8,11]. Trametinib (Tram) is the first MEK inhibitor approved for the treatment of advanced BRAF (V600E) mutant melanoma. However, Tram has modest clinical activity in KRAS mutant tumors. A phase II study showed that Tram did not demonstrate superiority to docetaxel in patients with previously treated KRAS mutant stage IV non-small cell lung cancer (NSCLC). Moreover, patients treated with Tram had more clinically significant adverse events [12]. The mechanisms underlying the resistance to MEK inhibitors remain elusive and need further investigation [13-20].

In this study, we found that Tram induced phosphorylated ERK (pERK) rebound associated with the induction of phosphorylated MEK (pMEK) in KRAS but not BRAF mutant tumors. Next, studies revealed that RAS reactivation might mediate the pERK rebound induced by Tram. Further KRAS suppression could reverse the Tram-induced signaling reactivation and enhance the cytotoxicity of Tram against KRAS mutant tumors. Notably, we found that zoledronic acid (ZA), which has been widely used in the clinical treatment of bone metastasis, could inhibit the activity of RAS and enhance the sensitivity of Tram to KRAS mutant tumors. Taken together, the present data indicate that the combined use of Tram and ZA presents a promising therapeutic approach to KRAS mutant cancers.

2. Materials and methods

2.1 Cell culture and reagents

HCT 116 (KRAS G13D), SW480 (KRAS G12V), A549 (KRAS G12S), MDA-MB-231 (KRAS G13D, BRAF G464V), HT-29 (BRAF V600E), A375 (BRAF V600E), and COLO320 (KRAS WT) cell lines were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Gibco, USA) in 5% CO_2 at 37°C. Tram (GSK1120212), geranylgeranyltransferase I inhibitor (GGTI)-298, farnesyltransferase inhibitor (FTI)-277 (all from Selleckchem, USA), geranylgeranyl pyrophosphate (GGPP; Cayman Chemical, USA) and rigosertib，PD0325901 (MedChem Express, USA) were dissolved in dimethyl sulfoxide (DMSO). ZA (Selleckchem) was dissolved in 0.1 M sodium hydroxide (NaOH, aq).

2.2 Transfection

CRAF siRNAs (5′-GGAUGUUGAUGGUAGUACATT-3′), BRAF siRNAs (5′-GCAUAAUCCACCAUCAAUATT-3′), KRAS siRNAs (5′-GCCUUGACGAUACAGCUAATT-3′) and negative control siRNAs were designed and generated by GenePharma (China). siRNA transfections were performed by using Lipofectamine 2000 RNAiMAX transfection reagents (Invitrogen, USA) according to the manufacturer's recommendations.

2.3 Western blotting analysis

Treated cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl PH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA) with protease inhibitors and phosphatase inhibitors (Calbiochem, Germany). Equal amounts of protein sample (30-50 μg) were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA) using the Bio-Rad semidry transfer system. The primary antibodies included MEK, ERK1/2, ERK1, pERK (T202/Y204), c-Myc, cyclin D1 (all from Abcam, UK), pMEK1 (S221), pMEK2 (T394) (both from Sangon Biotech, China), PARP1, Bcl-xL (both from CST, USA), CRAF, BRAF (both from Zen BioScience, China), GAPDH (Santa Cruz, USA), KRAS

(Proteintech, USA), and pan-RAS (HuaAn Biotechnology, China).

2.4 Pull-down assay

Expression of the GST-RAF-RAS-binding domain fusion proteins was induced by 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Transgene, France) for 6 hr at 30°C in *Escherichia coli*. Then, affinity purification of fusion proteins in bacterial lysates was performed using glutathione agarose beads (Thermo Scientific, USA). Equilibrated isolated fusion proteins were incubated with equal amounts of whole-cell protein sample on an end-over-end rotator for 2 hr at 4°C. The fusion protein binding beads were collected by centrifugation and washed with phosphate-buffered saline (PBS) three times and then boiled with SDS buffer. After centrifugation, the supernatants were subjected to western blot. KRAS antibody (Proteintech, USA) and RAS antibody (HuaAn Biotechnology, China) were used to detect the corresponding proteins. Equal amounts of whole-cell protein sample were immunoblotted with the above two antibodies as loading controls.

2.5 Cell proliferation and colony formation assays

Cells were seeded in a 96-well plate at 3000-7000 cells/well and cultured overnight at 37°C. After treatment with indicated reagents or transfection for 48 hr, cell proliferation was determined using the Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Japan). For the colony formation assay, a total of 8×10^3-80×10^3 cells were seeded in a 35-mm dish. After overnight incubation, cells were treated with drugs for 5-7 days. Cell colonies were fixed with 4% paraformaldehyde for 15 min, stained with 0.5% crystal violet for 15 min and then photographed. We used the Annexin V-FITC Apoptosis Detection Kit (Dojindo Molecular Technologies, Japan) to assess cell apoptosis. Cells were seeded at 60% confluence/well in 6-well plates and cultured overnight. Then, the cells were treated with drugs for 24 hr. After trypsin digestion, cells were harvested and washed with PBS. Thereafter, cells were stained with propidium iodide and annexin V-FITC and subjected to flow cytometry using a Navios flow cytometer (Beckman Coulter, USA).

2.6 In vivo study

Five-week-old female nude mice (Beijing HFK BioScience, China) were subcutaneously injected with 5×10^6 HCT 116 cells (100 μL in PBS) in the right flank. After the tumor volumes exceeded approximately 100 mm^3, mice were randomized into four groups (n=6 per group): Tram (2 mg/kg, i.g.) every other day, ZA (2 mg/kg, i.p.) once every 5 days, a combination of Tram (2 mg/kg, i.g.) every other day and ZA (2 mg/kg, i.p.) once every 5 days, and vehicle control every other day for 14 days. Tumor size was measured every 3 days. After 14 days, mice were sacrificed, and tumors were resected and weighed. The resected tumors were minced and lysed in RIPA buffer and subjected to western blot to detect the effects on MEK/ERK signaling and cell proliferation and apoptosis. The animal experiments were approved by the institutional review board of West China Hospital of Sichuan University (Item number: 2016017A).

2.7 Statistical analysis

The statistical significance of the differences between two groups was calculated by Student's t test using GraphPad Prism version 5.01. Statistical significance was set at *$p<0.05$, **$p<0.01$, or ***$p<0.001$. All statistical tests were two-sided. An unpaired t test with Welch's correction was applied when the homogeneity of variance assumptions were not satisfied.

3. Results

3.1 pERK rebound induced by Tram occurs in KRAS mutant tumors but not in BRAF mutant tumors

To investigate the association between the sensitivity to Tram and the magnitude of ERK signaling inhibition in different genotypes, we examined protein expression after Tram treatment in a number of colorectal cancer (C), lung cancer (L) and melanoma (M) cell lines. Among them, HCT 116 (C), SW480 (C) and A549 (L) harbor KRAS mutations, while HT-29 (C) and A375 (M) have BRAF

(V600E) mutations.

As shown in Figure 1A, Tram inhibited ERK phosphorylation both in KRAS and BRAF mutant cells in a dose-dependent manner. After 24 hr of treatment, 50-60 nM Tram significantly suppressed pERK in KRAS mutant tumors. Meanwhile, the levels of pMEK were increased, especially in SW480 and A549 cells. In contrast, the same dose of Tram almost completely inhibited pERK and produced a remarkable decrease in pMEK in BRAF mutant cell lines. We then examined the influence of Tram treatment on the durability of ERK signaling inhibition. The results showed that there was a rebound in pERK after 24-48 hr, and pMEK increased gradually with time in KRAS mutant tumors. In contrast, this increase was not observed in BRAF mutant malignancies (Figure 1B). Dr. Rosen's lab has reported that in contrast to the MEK inhibitor PD0325901, Tram reduced both the induction of MEK phosphorylation and the rebound in ERK phosphorylation observed in KRAS mutant tumors, and induced a durable inhibition on pMEK and pERK. However, our results showed that Tram could still induce the rebound of pERK and the upregulation of pMEK in the KRAS mutant cancer cells, though it was not as significant as that with PD0325901 in the KRAS mutant A549, the cells which has been used in Rosen's research (Figure S6A). The induction of pMEK in KRAS mutant cells exposed to Tram might resulted from inhibition of ERK-dependent inhibitory phosphorylation of the RAF kinases, which was partly in agreement with previous data [20]. Together, these results suggested that the low susceptibility of KRAS mutant cells to Tram might be due to the failure of sustained inhibition of ERK signaling.

3.2 KRAS suppression overcomes Tram resistance in KRAS mutant tumors

Previous studies have indicated that the RAF-MEK complex formation induced by the MEK allosteric inhibitors (including PD0325901 and selumetinib) mainly mediated the rebound of pERK in the KRAS mutant cancer cells [20]. Therefore, we investigated the effect of RAF (mainly focused on BRAF and CRAF) knockdown on the Tram-induced rebound of pERK and pMEK in A549 and SW480 cells, respectively. The results showed that CRAF SiRNA attenuated the rebound in ERK phosphorylation,

whereas knockdown of BRAF had a small effect on the rebound of pERK in the two KRAS mutant cells. The RAF inhibition caused a slight reduction on the pMEK in the A549 cells with BRAF knockdown and SW480 cells with CRAF knockdown, whereas the RAF inhibition had nearly no effect on the pMEK in the SW480 with BRAF knockdown and A549 cells with CRAF knockdown. Our results were consistent with the published data, indicating that the RAF-MEK complex, but not pMEK, might mediate the rebound of pERK in the KRAS mutant cancer cells [20]. Both BRAF and CRAF siRNAs attenuated cell proliferation, as indicated by the reduced expression of cyclin D1 and c-Myc (Figure 2A).

The activated RAS–GTP drives the formation of high activity homodimers or heterodimers of the RAF protein kinases (including the ARAF, BRAF and CRAF), thus activates the downstream MEK-ERK signaling [3], so we predicted that the failure of sustained inhibition of ERK signaling was due to the activation of RAS in the KRAS mutant cells with Tram treatment. We then measured the activity of pan-RAS using a pull-down assay in HCT 116 and SW480 cell lines. As shown in Figure 2B, the levels of RAS-GTP were significantly increased upon Tram treatment in a time-dependent manner. We also measured the pan-RAS activity in A549 cells. Although there was no obvious increase in RAS-GTP, total RAS expression decreased remarkably (Figure S1), indicating an increasement of RAS activity. Because KRAS is the primary isoform of the RAS family members, we confirmed that KRAS-GTP was also increased after Tram treatment (Figure 2B). To test the effect of hyperactivation of KRAS on Tram resistance, we combined KRAS knockdown with Tram treatment. The combination therapy resulted in a significant decrease in cell proliferation and induced a marked inhibition of cell proliferation-related proteins (Figure 2C and 2D). In addition, KRAS suppression reversed the reactivation of MEK/ERK signaling induced by Tram treatment (Figure 2C). And these results were consistent with previous reports that the MEK inhibitors inhibited the ERK activation and so relieved its negative feedback, and consequently induced stronger activation of upstream pathway components, including RAS and RAF (represented by active the RAS molecules and active RAF dimers), and the activated RAS promoted the RAS-RAF-MEK complex formation, thus ultimately induced the pERK rebound [3,7]. Rigosertib, a

recently reported RAS inhibitor, was used in combination with Tram, and this approach induced an augmented lethality in HCT 116 and SW480 cells (Figure 2E). Taken together, the above data indicated that the dual blockade of MEK and RAS might present a novel therapeutic strategy in KRAS mutant tumors.

3.3 ZA enhances the cytotoxicities of Tram in KRAS mutant tumors

Next, we determined whether any FDA-approved inhibitors exist that could inhibit RAS activity. Through literature review, we found two types of drugs, statins and bisphosphonates. Further screening of the FDA-approved Drug Library also validated that bisphosphonates could enhance the cytotoxicities of Tram in the Tram resistant SW1116 cells (data not shown). Here, we mainly focused on ZA, the most intensively studied bisphosphonate, because ZA has already been clinically used in metastatic bone diseases, while statins are primarily used in cardiovascular diseases.

First, we investigated the sensitivity of different genotypes to ZA and found that there was no obvious difference between the KRAS- and BRAF- mutant cells, while wild-type COLO320 was the most sensitive cell line to ZA (Figure S2). CCK8 assays indicated that ZA significantly enhanced the cytotoxicity of Tram in HCT 116, SW480, A549 and MDA-MB-231 KRAS mutant cancer cells (Figure 3A). In contrast, ZA reversed the lethal effect of Tram in BRAF mutant and KRAS wild-type cells (Figure S3). Further clonogenic assays were performed to assess the long-term effects of combined Tram and ZA on cell survival. The results showed that colony formation was significantly inhibited in cells treated with the combination for 5-7 days compared to that in the Tram or ZA alone groups (Figure 3B). In addition, FACS analysis showed that combined therapy significantly induced cell apoptosis compared to that in each monotherapy group (Figures 3C and S4).

Further western blot analysis demonstrated the enhanced anti-proliferative and proapoptotic effects of combined Tram and ZA in KRAS mutant cancer cells. The combinatorial treatment dramatically reduced the expression of proliferative c-Myc and cyclin D1, whereas Tram or ZA alone produced a marginal decrease. Downregulation of anti-apoptotic Bcl-xL and total PARP1, together with upregulation

of cleaved PARP1, indicated that apoptosis was augmented in the combined treatment groups (Figure 3D). The above data indicated that ZA could enhance the cytotoxicities of Tram in KRAS mutant tumors.

3.4 ZA decreases the hyperactivation of RAS and the rebound of pERK induced by Tram in KRAS mutant tumors

Next, we investigated the underlying mechanisms of the combination strategy. As shown in Figure 4A, ZA significantly reduced the rebound of pERK induced by Tram at 48 hr in the 4 cell lines, while the reversal effects were not remarkable at 24 hr. In addition, the magnitude of decreased pMEK varied in the 4 cell lines. We have also compared the effect of ZA combined with Tram or PD0325901 on the expression of pMEK and pERK, the results showed that the combination of ZA and Tram produced more thorough inhibition of pERK and pMEK than that of the combination of ZA and PD0325901(Figure S6B), and the cell morphology assay also indicated that ZA combined with Tram produced more effective inhibition on the cell survival of A549 cells than that of ZA combined with PD0325901(Figure S6C) . The pull-down results showed that the increased RAS-GTP induced by Tram was reduced by the combination treatment, while ZA treatment alone partially reduced the level of RAS-GTP (Figures 4B and S5A). A similar phenomenon in KRAS was also observed in HCT 116 cells (Figure S5B). The above data illustrated that ZA could enhance the effects of Tram in KRAS mutant cancer cells through suppressing RAS activity, thus prolonging the duration of ERK signaling inhibition.

3.5 ZA synergizes with Tram mainly through the mevalonate pathway

Protein isoprenylation, a lipid modification including farnesylation and geranylgeranylation, is required for RAS biological activity [13]. Given that ZA suppresses RAS prenylation through the mevalonate pathway, we assumed that the same pathway is involved in mediating the effects of the ZA and Tram combinatorial treatment [14,15].

FTIs and GGTIs suppress farnesylation and geranylgeranylation, respectively. Neither FTIs nor GGTIs alone can completely inhibit the prenylation of KRAS or NRAS [16-18]. We tested the effect of

FTI-277 and GGTI-298 on the lethality of Tram in HCT 116, SW480 and MDA-MB-231 cells. The results showed that the cytotoxicity of Tram was slightly enhanced with the addition of FTI-277 or GGTI-298 (Figure 5A and 5B). Farnesyl pyrophosphate (FPP) and GGPP, intermediates of isoprenylation, can restore the isoprenylation of RAS. Further studies showed that the addition of FPP and GGPP could partially reverse the efficacy of ZA treatment alone and in combination therapy (Figure 5C and 5D). Moreover, the western blot results were in line with the above functional data (Figure 5E and 5F). In sum, we supposed that the mevalonate pathway may be involved in mediating the effects of the ZA and Tram combinatorial treatment to some extent.

3.6 Combined Tram and ZA suppresses the growth of KRAS mutant tumors in vivo

We used a mouse xenograft model with HCT 116 cells to investigate the effects of the combination strategy *in vivo*. The results showed that Tram alone significantly inhibited tumor growth, while there were no obvious differences between the ZA-treated group and the vehicle-treated control group (Figure 6A). Notably, after treatment with Tram (2 mg/kg, i.g.) every other day and ZA (2 mg/kg, i.p.) once every 5 days for 14 days, tumors were significantly reduced both in weight and in size compared to those in the vehicle-treated controls or the monotherapy groups (Figure 6B).

To further verify our previous *in vitro* studies, western blot was performed to investigate the expression of proliferative and anti-apoptotic proteins in lysates from the xenografts. In contrast to the monotherapy groups, the combined Tram and ZA treatment downregulated the expressions of c-Myc, cyclin D1, Bcl-xL and total PARP1 (Figure 6C). Although the combination treatment inhibited the rebound of pERK induced by Tram, it had little effect on the changes in pMEK (Figure 6C). This was in line with our *in vitro* data, which showed that the expression of pMEK changed minimally in HCT 116 cells compared to that in the other tested cell lines. Taken together, the above results indicated that the combined Tram and ZA treatment could exert a better tumor suppression efficacy in xenograft models. These effects might be the result of sustained inhibition of ERK signaling and suppression of anti-apoptosis- and proliferation-related proteins.

4. Discussion

The failure in directly pharmacologically targeting RAS has forced an emerging need for alternative approaches to target other effectors in the RAS-RAF-MEK-ERK pathway. However, the effectiveness of the available inhibitors is limited when a single agent is used. Previous studies have revealed many mechanisms of MEK inhibitor monotherapy resistance, including the relieved negative feedback loops between ERK and RAF, upregulation of receptor tyrosine kinases (RTKs), and activation of compensatory or parallel pathways [19-26]. Based on these mechanisms, researchers have proposed several combinational regimens to overcome the drug resistance, but the outcomes have varied [27-33]. Further clinical and preclinical studies are still needed to evaluate the efficacy of these MEK inhibitor-based combinatorial strategies.

The mechanisms of resistance to different allosteric MEK inhibitors are distinct from each other [34]. Here, we focused on Tram, the first FDA-approved MEK inhibitor for the treatment of unresectable or metastatic melanoma with a BRAF V600E or V600K mutation. We compared the changes in MEK-ERK signaling after Tram treatment between KRAS and BRAF mutant tumors and found that the inhibition of ERK signaling cannot be sustained in the former. With the increase in Tram concentration and treatment duration, the levels of pMEK are significantly increased in KRAS mutant but not in BRAF mutant cells. These results suggest that overcoming MEK inhibition and reactivating ERK signaling might be important causes of Tram resistance. At this point, the current data are in consistent with previous studies, which have showed that Tram could induce more durable inhibition on the expression of pERK than PD0325901. Our data indicate that Tram treatment could still induce the rebound of pERK and the upregulation of pMEK in the KRAS mutant A549 cells. In RAS-activated cells, activated ERK directly phosphorylates and inhibits CRAF kinase activity. After treatment with a MEK inhibitor, this feedback inhibition is relieved, and CRAF is reactivated, thus inducing MEK phosphorylation. Conversely, RAF kinase is active and insensitive to this negative feedback in BRAF V600E cells, so the induction of pMEK

by MEK inhibitors does not appear in this type of cells [7,35-37].

The selective induction of pMEK indicates that upstream RAF might be involved in the resistance of KRAS mutant cells to Tram. Therefore, we knocked down CRAF or BRAF in KRAS mutant cells and then treated cells with Tram. We found that the efficacy of pERK suppression is enhanced with a decrease in pMEK to some extent. These results are in accordance with a previous report showing that BRAF-RAF1 dimerization, induced by MEK inhibitors, is responsible for the drug resistance of KRAS mutant tumors. Combined RAF and MEK suppression presents a synthetic, lethal effect on KRAS mutant tumors [36]. In addition, MEK inhibitors were reported to induce RAF-MEK complexes, thus leading to MEK inhibition resistance in KRAS mutant tumors. This CRAF-mediated MEK reactivation can be impaired in new-generation catalytic MEK inhibitors, such as Tram [20]. However, from our experimental results, Tram can still induce pERK rebound and pMEK induction in KRAS mutant tumors to a relatively lesser extent than PD0325901.

Feedback loop reactivation after MEK inhibition occurs at multiple levels of the RTK-RAS-MAPK pathway, and the precise mechanisms are still elusive. In addition to RAF proteins, selumetinib, an allosteric MEK inhibitor, has demonstrated transcriptional upregulation of RTK ERBB3 and triggers hyperactivation of KRAS in KRAS mutant cancers. Dual blockade of RTKs and MEK or RAF and MEK could produce a synthetic lethality in KRAS mutant tumors [23,36]. Here, we found that Tram induces RAS hyperactivation in the KRAS mutant cell lines HCT 116, SW480 and A549. KRAS knockdown combined with Tram has an augmented lethal effect. The KRAS suppression may overcome the rebound of pERK and pMEK induction that are induced by Tram. In addition, rigosertib, a recently reported RAS inhibitor [38], enhanced the cytotoxicity of Tram against KRAS mutant cells. The presented data suggested that concomitant blockade of RAS and MEK would produce synthetic lethality in KRAS mutant tumors.

To translate the above theory into a potential clinical therapy, we screened the FDA-approved drugs that have the potential to inhibit RAS activity through combined review of the literature and the drug bank (unpublished data). Among these drugs, we mainly focused on ZA, which had demonstrated

anti-tumor activity, independent of its effect on bone density as a single agent or in combination with anti-neoplastic drugs [39-41]. As expected, ZA and Tram cooperate to inhibit tumor growth both *in vitro* and *in vivo*. Functional studies revealed that the combination of ZA and Tram produced a more significant inhibition on the survival of KRAS mutant A549 cells than that of the combination of ZA and PD0325901, the mechanism might be that the combination of ZA and Tram induced more thorough inhibition on the expression of pERK and pMEK, and their downstream effectors, including c-Myc, cyclin D1, Bcl-xL and the pro-PARP1 than that of ZA combined with PD0325901(Figure S6B, S6C). In addition, inhibition of RAS prenylation might be involved in this process to some extent (Figure 6A,B, C). Simvastatin, mainly used in cardiovascular diseases, could also inhibit the mevalonate pathway through suppressing a rate-limiting enzyme, HMG-CoA reductase. Our recent published work revealed that simvastatin enhanced the anti-tumor activity of Tram in multiple KRAS mutant cells [42] . This was in line with a recent report that combined Tram and statins have a synergistic anti-tumor effect in drosophila lung cancer models [43]. This further demonstrated that ZA synergizes with Tram to inhibit KRAS mutant tumors, possibly through the mevalonate pathway. However, the precise underlying mechanisms still need further investigation.

In conclusion, we provided a promising novel strategy for treating KRAS mutant tumors through combined Tram and ZA. Further preclinical studies are still required to reveal the precise mechanisms, and clinical studies are warranted to evaluate the clinical application in KRAS mutant colorectal cancer, lung cancer, breast cancer and pancreatic cancer.

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Author contributions

Feng Bi and Hongwei Xia designed and provided guidance for the study. Xinyu Dai and Hongwei Xia performed the experiments, analyzed the data, and wrote the manuscript. Sheng Zhou and Qiulin Tang participated in some of the experiments.

Declarations of Interest: The authors declare that no competing financial interests exist.

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References

[1] A. Lièvre, J.B. Bachet, D. Le Corre, V. Boige, B. Landi, J.F. Emile, J.F. Côté, G. Tomasic, C. Penna, M. Ducreux, P. Rougier, F. Penault-Llorca, P. Laurent-Puig, KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer, Cancer Res. 66 (2006) 3992–3995.

[2] W. Pao, T.Y. Wang, G.J. Riely, V.A. Miller, Q. Pan, M. Ladanyi, M.F. Zakowski, R.T. Heelan, M.G. Kris, H.E. Varmus, KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib, PLoS Med. 2 (2005) e17.

[3] Y. Pylayeva-Gupta, E. Grabocka, D. Bar-Sagi, RAS oncogenes: weaving a tumorigenic web, Nat. Rev. Cancer 11 (2011) 761–774.

[4] S. Lu, H. Jang, S. Gu, J. Zhang, R. Nussinov, Drugging Ras GTPase: a comprehensive mechanistic and signaling structural view, Chem. Soc. Rev. 45 (2016) 4929–4952.

[5] F. McCormick, KRAS as a therapeutic target, Clin. Cancer Res. 21 (2015) 1797–1801.

[6] G. Hatzivassiliou, K. Song, I. Yen, B.J. Brandhuber, D.J. Anderson, R. Alvarado, M.J.C. Ludlam, D. Stokoe, S.L. Gloor, G. Vigers, T. Morales, I. Aliagas, B. Liu, S. Sideris, K.P. Hoeflich, B.S. Jaiswal, S. Seshagiri, H. Koeppen, M. Belvin, L.S. Friedman, S. Malek, RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth, Nature 464 (2010) 431–435.

[7] S.J. Heidorn, C. Milagre, S. Whittaker, A. Nourry, I. Niculescu-Duvas, N. Dhomen, J. Hussain, J.S. Reis-Filho, C.J. Springer, C. Pritchard, R. Marais, Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF, Cell 140 (2010) 209–221.

[8] G. Migliardi, F. Sassi, D. Torti, F. Galimi, E.R. Zanella, M. Buscarino, D. Ribero, A. Muratore, P. Massucco, A. Pisacane, M. Risio, L. Capussotti, S. Marsoni, F. Di Nicolantonio, A. Bardelli, P.M. Comoglio, L. Trusolino, A. Bertotti, Inhibition of MEK and PI3K/mTOR suppresses tumor growth but does not cause tumor regression in patient-derived xenografts of RAS-mutant colorectal carcinomas, Clin. Cancer Res. 18 (2012) 2515–2525.

[9] H. Singh, D.L. Longo, B.A. Chabner, improving prospects for targeting RAS, J. Clin. Oncol. 33 (2015) 3650–3659.

[10] D.B. Solit, L.A. Garraway, C.A. Pratilas, A. Sawai, G. Getz, A. Basso, Q. Ye, J.M. Lobo, Y. She, I. Osman, T.R. Golub, J. Sebolt-Leopold, W.R. Sellers, N. Rosen, BRAF mutation predicts sensitivity to MEK inhibition, Nature 439 (2006) 358–362.

[11] J.R. Infante, L.A. Fecher, G.S. Falchook, S. Nallapareddy, M.S. Gordon, C. Becerra, D.J. DeMarini, D.S. Cox, Y. Xu, S.R. Morris, V.G.R. Peddareddigari, N.T. Le, L. Hart, J.C. Bendell, G. Eckhardt, R. Kurzrock, K. Flaherty, H.A. Burris, W.A. Messersmith, Safety, pharmacokinetic, pharmacodynamic, and efficacy data for the oral MEK inhibitor trametinib: a phase 1 dose-escalation trial, Lancet Oncol. 13 (2012) 773–781.

[12] G.R. Blumenschein, E.F. Smit, D. Planchard, D.W. Kim, J. Cadranel, T. De Pas, F. Dunphy, K. Udud, M.J. Ahn, N.H. Hanna, J.H. Kim, J. Mazieres, S.W. Kim, P. Baas, E. Rappold, S. Redhu, A. Puski, F.S. Wu, P.A. Jänne, A randomized phase II study of the MEK1/MEK2 inhibitor trametinib (GSK1120212) compared with docetaxel in KRAS-mutant advanced non-small-cell lung cancer (NSCLC)†, Ann. Oncol. 26 (2015) 894–901.

[13] A.D. Cox, C.J. Der, M.R. Philips, Targeting RAS membrane association: back to the future for anti-RAS drug discovery? Clin. Cancer Res. 21 (2015) 1819–1827.

[14] F. Coxon, K. Thompson, M. Rogers, Recent advances in understanding the mechanism of action of bisphosphonates, Curr. Opin. Pharmacol. 6 (2006) 307–312.

[15] S.P. Luckman, D.E. Hughes, F.P. Coxon, R.G.G. Russell, M.J. Rogers, Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras, J. Bone Miner. Res. 13 (1998) 581–589.

[16] G. James, J.L. Goldstein, M.S. Brown, Resistance of K-RasBV12 proteins to farnesyltransferase inhibitors in Rat1 cells, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 4454–4458.

[17] C.A. Rowell, J.J. Kowalczyk, M.D. Lewis, A.M. Garcia, Direct demonstration of geranylgeranylation and farnesylation of Ki-Rasin vivo, J. Biol. Chem. 272 (1997) 14093–14097.

[18] D.B. Whyte, P. Kirschmeier, T.N. Hockenberry, I. Nunez-Oliva, L. James, J.J. Catino, W.R. Bishop, J.K. Pai, K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors, J. Biol. Chem. 272 (1997) 14459–14464.

[19] C.J. Caunt, M.J. Sale, P.D. Smith, S.J. Cook, MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road, Nat. Rev. Cancer 15 (2015) 577–592.

[20] P. Lito, A. Saborowski, J. Yue, M. Solomon, E. Joseph, S. Gadal, M. Saborowski, E. Kastenhuber, C. Fellmann, K. Ohara, K. Morikami, T. Miura, C. Lukacs, N. Ishii, S. Lowe, N. Rosen, Disruption of CRAF-mediated MEK activation is required for effective MEK inhibition in KRAS mutant tumors, Cancer Cell 25 (2014) 697–710.

[21] R. Mandal, S. Becker, K. Strebhardt, Stamping out RAF and MEK1/2 to inhibit the ERK1/2 pathway: an emerging threat to anticancer therapy, Oncogene 35 (2016) 2547–2561.

[22] P. Pettazzoni, A. Viale, P. Shah, A. Carugo, H. Ying, H. Wang, G. Genovese, S. Seth, R. Minelli, T. Green, E. Huang-Hobbs, D. Corti, N. Sanchez, L. Nezi, M. Marchesini, A. Kapoor, W. Yao, M.E.D. Francesco, A. Petrocchi, A.K. Deem, K. Scott, S. Colla, G.B. Mills, J.B. Fleming, T.P. Heffernan, P. Jones, C. Toniatti, R.A. DePinho, G.F. Draetta, Genetic events that limit the efficacy of MEK and RTK inhibitor therapies in a mouse model of KRAS-driven pancreatic cancer, Cancer Res. 75 (2015) 1091–1101.

[23] C. Sun, S. Hobor, A. Bertotti, D. Zecchin, S. Huang, F. Galimi, F. Cottino, A. Prahallad, W. Grernrum, A. Tzani, A.S. Lodewyk, F.A. Wessels, Egbert F. Smit, E. Thunnissen, P. Halonen, C.L. Roderick, L. Beijersbergen, F. Di Nicolantonio, A. Bardelli, L. Trusolino, R. Bernards, Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3, Cell Rep. 7 (2014) 86–93.

[24] S. Van Schaeybroeck, M.Kalimutho, P.D. Dunne, R. Carson, W. Allen, P.V. Jithesh, K.L. Redmond, T. Sasazuki, S. Shirasawa, J. Blayney, P. Michieli, C. Fenning, H.J. Lenz, M. Lawler, D.B. Longley, P.G. Johnston, ADAM17-dependent c-MET-STAT3 signaling mediates resistance to MEK inhibitors in KRAS mutant colorectal cancer, Cell Rep. 7 (2014) 1940–1955.

[25] S. Wee, Z. Jagani, K.X. Xiang, A. Loo, M. Dorsch, Y.M. Yao, W.R. Sellers, C. Lengauer, F. Stegmeier, PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers, Cancer Res. 69 (2009) 4286–4293.

[26] Y.K. Yoon, H.P. Kim, S.H. Song, S.W. Han, D.Y. Oh, S.A. Im, Y.J. Bang, T.Y. Kim, Down-regulation of mitogen-inducible gene 6, a negative regulator of EGFR, enhances resistance to MEK inhibition in KRAS mutant cancer cells, Cancer Lett. 316 (2012) 77–84.

[27] P.L. Bedard, J. Tabernero, F. Janku, Z.A. Wainberg, L. Paz-Ares, J. Vansteenkiste, E. Van Cutsem, J. Perez-Garcia, A. Stathis, C.D. Britten, N. Le, K. Carter, D. Demanse, D. Csonka, M. Peters, A. Zubel, H. Nauwelaerts, C. Sessa, A Phase Ib dose-escalation study of the oral pan-PI3K inhibitor buparlisib (BKM120) in combination with the oral MEK1/2 inhibitor trametinib (GSK1120212) in patients with selected advanced solid tumors, Clin. Cancer Res. 21 (2015) 730–738.

[28] H. Kitai, H. Ebi, S. Tomida, K.V. Floros, H. Kotani, Y. Adachi, S. Oizumi, M. Nishimura, A.C. Faber, S. Yano, Epithelial-to-mesenchymal transition defines feedback activation of receptor tyrosine kinase signaling induced by MEK inhibition in KRAS-mutant lung cancer, Cancer Discov. 6 (2016) 754–769.

[29] E. Manchado, S. Weissmueller, J.P. Morris, C.C. Chen, R. Wullenkord, A. Lujambio, E. de Stanchina, J.T. Poirier, J.F. Gainor, R.B. Corcoran, J.A. Engelman, C.M. Rudin, N. Rosen, S.W. Lowe, A combinatorial strategy for treating KRAS-mutant lung cancer, Nature 534 (2016) 647–651.

[30] K.S. Park, B. Oh, M.H. Lee, K.Y. Nam, H.R. Jin, H. Yang, J. Choi, S.W. Kim, D.H. Lee, The HSP90 inhibitor, NVP-AUY922, sensitizes KRAS-mutant non-small cell lung cancer with intrinsic resistance to MEK inhibitor, trametinib, Cancer Lett. 372 (2016) 75–81.

[31] M. Schoumacher, K.E. Hurov, J. Lehar, Y. Yan-Neale, Y. Mishina, D. Sonkin, J.M. Korn, D. Flemming, M.D. Jones, B. Antonakos, V.G. Cooke, J. Steiger, J. Ledell, M.D. Stump,

W.R. Sellers, N.N. Danial, W. Shao, Inhibiting tankyrases sensitizes KRAS-mutant cancer cells to MEK inhibitors via FGFR2 feedback signaling, Cancer Res. 74 (2014) 3294–3305.

[32] A. Spreafico, J.J. Tentler, T.M. Pitts, A.C. Tan, M.A. Gregory, J.J. Arcaroli, P.J. Klauck, M.C. McManus, R.J. Hansen, J. Kim, L.N. Micel, H.M. Selby, T.P. Newton, K.L. McPhillips, D.L. Gustafson, J.V. DeGregori, W.A. Messersmith, R.A. Winn, S.G. Eckhardt, Rational combination of a MEK inhibitor, selumetinib, and the Wnt/calcium pathway modulator, cyclosporin A, in preclinical models of colorectal cancer, Clin. Cancer Res. 19 (2013) 4149–4162.

[33] A. Zaanan, K. Okamoto, H. Kawakami, K. Khazaie, S. Huang, F.A. Sinicrope, The mutant KRAS gene Up-regulates BCL-XL protein via STAT3 to confer apoptosis resistance that is reversed by BIM protein induction and BCL-XL antagonism, J. Biol. Chem. 290 (2015) 23838–23849.

[34] G. Hatzivassiliou, J.R. Haling, H. Chen, K. Song, S. Price, R. Heald, J.F.M. Hewitt, M. Zak, A. Peck, C. Orr, M. Merchant, K.P. Hoeflich, J. Chan, S.M. Luoh, D.J. Anderson, M.J.C. Ludlam, C. Wiesmann, M. Ultsch, L.S. Friedman, S. Malek, M. Belvin, Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers, Nature 501 (2013) 232–236.

[35] B.B. Friday, C. Yu, G.K. Dy, P.D. Smith, L. Wang, S.N. Thibodeau, A.A. Adjei, BRAF V600E disrupts AZD6244-induced abrogation of negative feedback pathways between extracellular signal-regulated kinase and Raf proteins, Cancer Res. 68 (2008) 6145–6153.

[36] S. Lamba, M. Russo, C. Sun, L. Lazzari, C. Cancelliere, W. Grernrum, C. Lieftink, R. Bernards, F. Di Nicolantonio, A. Bardelli, RAF suppression synergizes with MEK inhibition in KRAS mutant cancer cells, Cell Rep. 8 (2014) 1475–1483.

[37] C.A. Pratilas, B.S. Taylor, Q. Ye, A. Viale, C. Sander, D.B. Solit, N. Rosen, V600EBRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 4519–4524.

[38] Sai K. Athuluri-Divakar, R.C. Vasquez-Del, K. Dutta, S.J. Baker, S.C. Cosenza, I. Basu, Yogesh K. Gupta, M.V.R. Reddy, L. Ueno, Jonathan R. Hart, P.K. Vogt, D. Mulholland, C. Guha, A.K. Aggarwal, E.P. Reddy, A small molecule RAS-mimetic disrupts RAS association with effector proteins to block signaling, Cell 165 (2016) 643–655.

[39] D. Santini, M. Caraglia, B. Vincenzi, I. Holen, S. Scarpa, A. Budillon, G. Tonini, Mechanisms of disease: preclinical reports of antineoplastic synergistic action of bisphosphonates, Nat. Clin. Pract. Oncol. 3 (2006) 325–338.

[40] A. Stachnik, T. Yuen, J. Iqbal, M. Sgobba, Y. Gupta, P. Lu, G. Colaianni, Y. Ji, L.L. Zhu, S.M. Kim, J. Li, P. Liu, S. Izadmehr, J. Sangodkar, T. Scherer, S. Mujtaba, M. Galsky, J. Gomez, S. Epstein, C. Buettner, Z. Bian, A. Zallone, A.K. Aggarwal, S. Haider, M.I. New, L. Sun, G. Narla, M. Zaidi, Repurposing of bisphosphonates for the prevention and therapy of nonsmall cell lung and breast cancer, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 17995–18000.

[41] H.H. Van Acker, S. Anguille, Y. Willemen, E.L. Smits, V.F. Van Tendeloo, Bisphosphonates for cancer treatment: mechanisms of action and lessons from clinical trials, Pharmacol. Ther. 158 (2016) 24–40.

[42] S Zhou, H Xia, H Xu, Q Tang, Y Nie, QY Gong, Bi F. J Exp Clin Cancer Res. (2018) 37:218.

[43] B.D. Levine, R.L. Cagan, Drosophila lung cancer models identify trametinib plus statin as candidate therapeutic, Cell Rep. 14 (2016) 1477–1487.

Figure captions

Figure 1. Tram induced pERK rebound and pMEK induction in KRAS mutant tumors but not in BRAF mutant tumors

(A) KRAS and BRAF (V600E) mutant cells were treated with increasing doses of Tram for 24 hr. Whole-cell lysates were evaluated by western blot analysis. The phosphorylation levels of MEK and ERK were detected with the indicated antibodies.

(B) KRAS and BRAF (V600E) mutant cells were treated with Tram (60 or 80 nM) at the indicated time points. Whole-cell extracts were evaluated by western blot analysis to determine the phosphorylation levels of MEK and ERK.

Figure 2. RAS hyperactivation mediated Tram resistance in KRAS mutant tumors

(A) KRAS mutant cell lines A549 and SW480 were transfected with siRNAs targeting BRAF or CRAF and then treated with 60 and 80 nM Tram, respectively, for 48 hr. Whole-cell extracts were assayed by western blot analysis. The effect on MEK/ERK signaling and cell proliferation was detected with the indicated antibodies.

(B) HCT 116 and SW480 cells were treated with Tram (60 and 80 nM, respectively) for the indicated time, and then whole-cell lysates were subjected to pull-down assays. Indicated antibodies were used to detect the activity of pan-RAS-GTP and KRAS-GTP.

(C) A549 and SW480 cell lines were transfected with KRAS siRNA and then treated with Tram (60 and 80 nM, respectively) for 48 hr. Whole-cell extracts were subjected to immunoblotting to detect the effects on MEK/ERK signaling and cell proliferation.

(D) HCT 116 cells were transfected with KRAS siRNA or negative control siRNA and then treated with Tram (30 and 60 nM) or DMSO control for 48 hr. CCK8 assays were performed to assess cell proliferation and growth. SW480 cells (80 and 160 nM Tram) were treated as described for HCT 116. The data from a representative example of three independent experiments are presented as the mean±SD.

(E) HCT 116 and SW480 cells were treated with 50 nM rigosertib and vehicle control, respectively, and then treated with Tram (0, 30, and 60 nM; and 0, 80, and 160 nM, respectively). CCK8 assays were performed to assess cell proliferation and growth. The data from a representative example of three independent experiments are presented as the mean±SD.

Figure 3. ZA enhances Tram cytotoxicities against KRAS mutant tumors *in vitro*

(A) Four representative KRAS mutant cell lines were treated with increasing concentrations of Tram combined with the treatment of ZA or vehicle control for 48 hr. Cell proliferation and growth were evaluated by CCK8 assays. The data from a representative example of three independent experiments are presented as the mean±SD.

(B) Indicated cells were treated with Tram, ZA, Tram and ZA, or vehicle control for 5-7 days to determine the long-term effect on cell viability by using colony assays.

(C) HCT 116 cells were treated with 50 nM Tram, 20 μM ZA, the combination, or vehicle control for 24 hr and then subjected to flow cytometry after stained with propidium iodide and annexin V-FITC. SW480 cells (100 nM Tram, 80 μM ZA) and A549 cells (60 nM Tram, 45 μM ZA) were treated as described for HCT 116 cells. The data from a representative example of three independent experiments are presented as the mean±SD.

(D) HCT 116 cells were treated with 50 nM Tram, 20 μM ZA, the combination, or vehicle control for 24 and 48 hr, and then whole-cell lysates were subjected to western blot and incubated with the indicated antibodies. SW480 cells (100 nM Tram, 80 μM ZA), MDA-MB-231 cells (40 nM Tram, 12.5 μM ZA), and A549 cells (60 nM Tram, 45 μM ZA) were treated as described for HCT 116 cells.

Figure 4. ZA decreases the rebound of pERK induced by Tram

(A) HCT 116 cells were treated with 50 nM Tram, 20 μM ZA, the combination, or vehicle control for 24 and 48 hr, and then whole-cell lysates were subjected to western blot and incubated with the indicated antibodies to evaluate the changes in MEK/ERK signaling. SW480 cells (100 nM Tram, 80 μM ZA), MDA-MB-231 cells (40 nM Tram, 12.5 μM ZA), and A549 cells (60 nM Tram, 45 μM ZA) were treated

as described for HCT 116 cells.

(B) HCT 116 and SW480 tumor cells were treated with Tram, ZA, the combination, or vehicle control for 48 hr, and then whole-cell lysates were subjected to pull-down assays. Active RAS-GTP was detected by using the GST-RAF-RAS-binding domain.

Figure 5. ZA synergizes with Tram mainly through the mevalonate pathway

(A) HCT 116 cells were treated with Tram (0, 30, or 60 nM), and then treated with 10 μM FTI-277/4 μM GGTI-298 or vehicle control for 48 hr. CCK8 assays were performed to assess cell proliferation and growth. The data from a representative example of three independent experiments are presented as the mean±SD.

(B) SW480 and MDA-MB-231 cells were treated with Tram (0, 80, 160 nM), and then treated with 10 μM FTI-277/4 μM GGTI-298 or vehicle control for 48 hr. CCK8 assays were performed to assess cell proliferation and growth. The data from a representative example of three independent experiments are presented as the mean±SD.

(C) HCT 116 cells were treated with 40 μM ZA or 40 μM ZA combined with 100 nM Tram in the presence or absence of 2 μM FPP/4 μM GGPP for 48 hr. CCK8 assays were performed to assess cell proliferation and growth. The data from a representative example of three independent experiments are presented as the mean±SD.

(D) SW480 cells were treated with 80 μM ZA, or 80 μM ZA combined with 100 nM Tram in the presence or absence of 2 μM FPP/4 μM GGPP for 48 hr. MDA-MB-231 cells (30 μM ZA, 80 nM Tram) were treated as described for SW480 cells. CCK8 assays were performed to assess cell proliferation and growth. The data from a representative example of three independent experiments are presented as the mean±SD.

(E) Western blot was performed to evaluate the effect of the addition of FPP/GGPP on KRAS mutant cell lines. HCT 116 cells were treated with 20 μM ZA, 50 nM Tram, the combination, or vehicle control in the presence or absence of 2 μM FPP/4 μM GGPP for 24 hr. Whole-cell lysates were subjected to western

blot and incubated with antibodies for proliferation- and apoptosis-related proteins.

Figure 6. Combined Tram and ZA suppress tumor growth in a nude mouse xenograft model

(A) Mice injected with HCT 116 cell line xenografts were treated with vehicle control every other day, 2 mg/kg Tram every other day, 2 mg/kg ZA once every 5 days, or the combination of Tram and ZA. There were 6 nude mice in each group. At the end of the experiment, the mice were sacrificed, and the tumors were resected as shown.

(B) The weights and sizes of the resected tumors. The inhibitory effects of different treatments on tumors are shown as the mean±SEM.

(C) Western blot assay of xenografts. Indicated antibodies were used to detect the corresponding proteins.

Figure 7. ZA synergizes with Tram mainly through the mevalonate pathway

(A) The mevalonate pathway causes isoprenylation of RAS, which is required for RAS biological activity.

(B) ZA suppresses RAS prenylation by inhibiting FPP synthase, thus leading to inhibition of RAS hyperactivation that is induced by Tram in KRAS mutant tumors.

Figure1

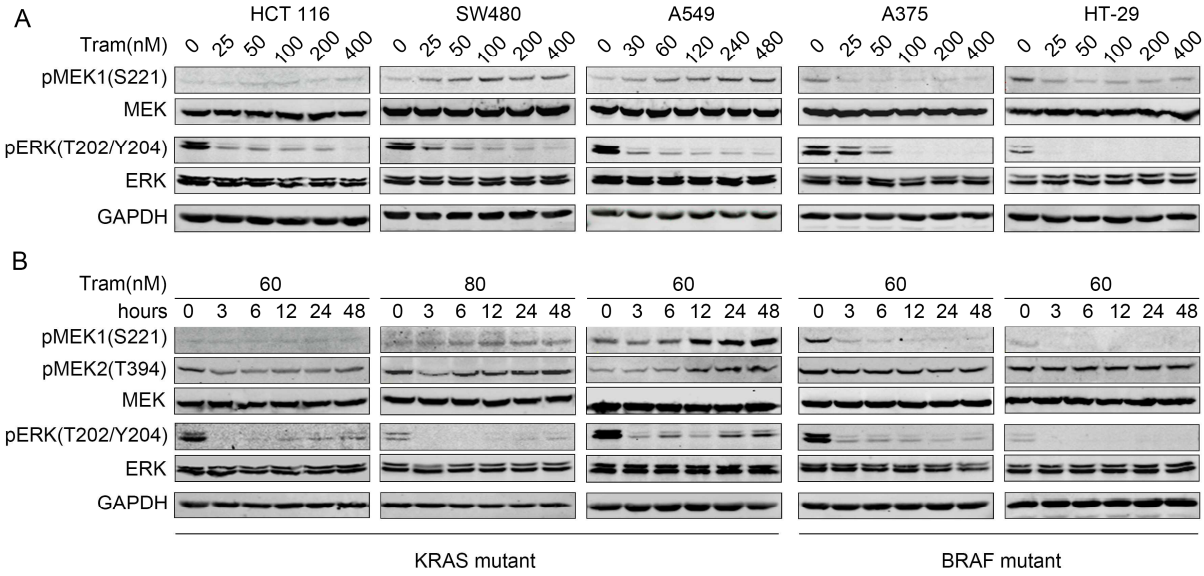

Figure2

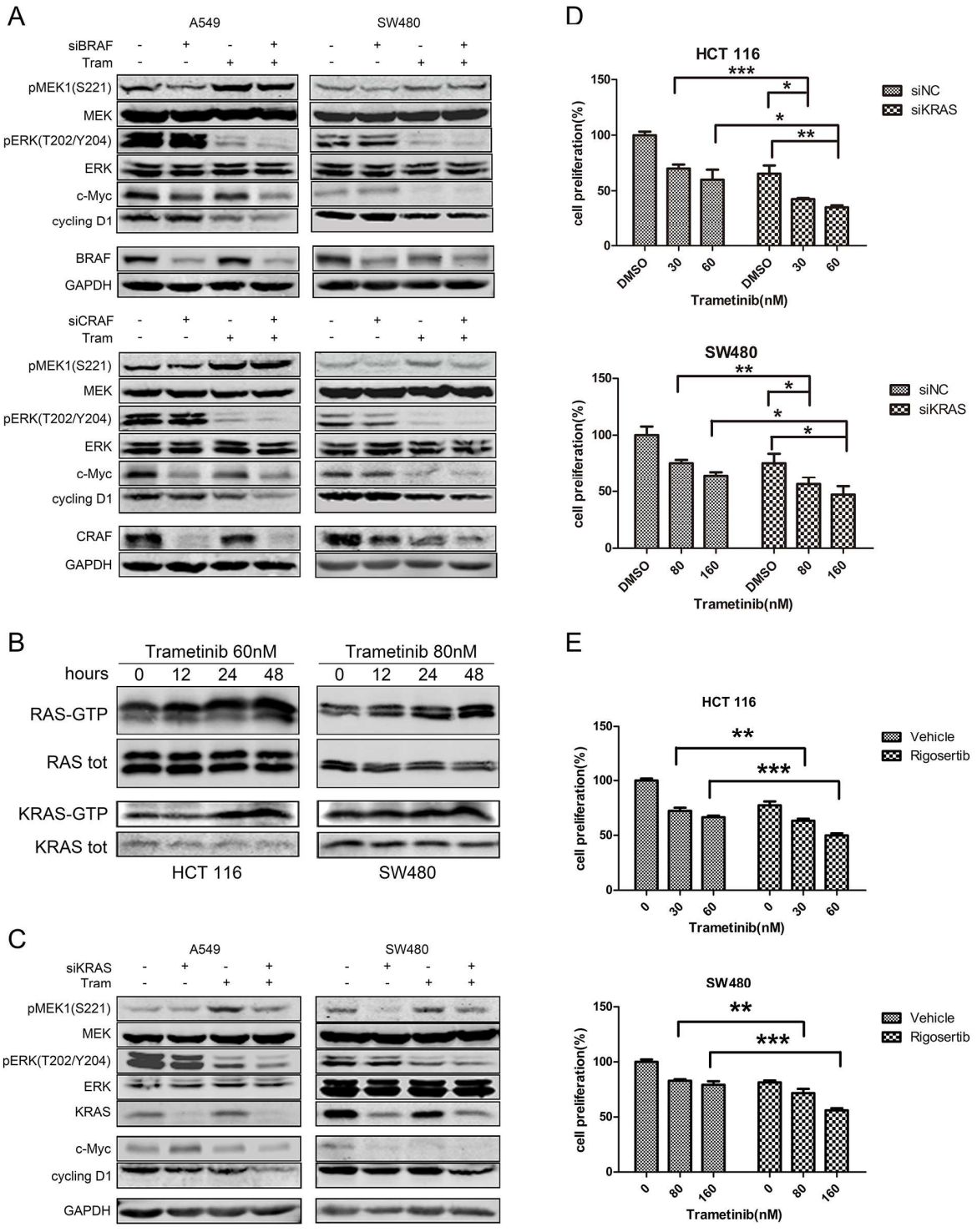
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Figure3

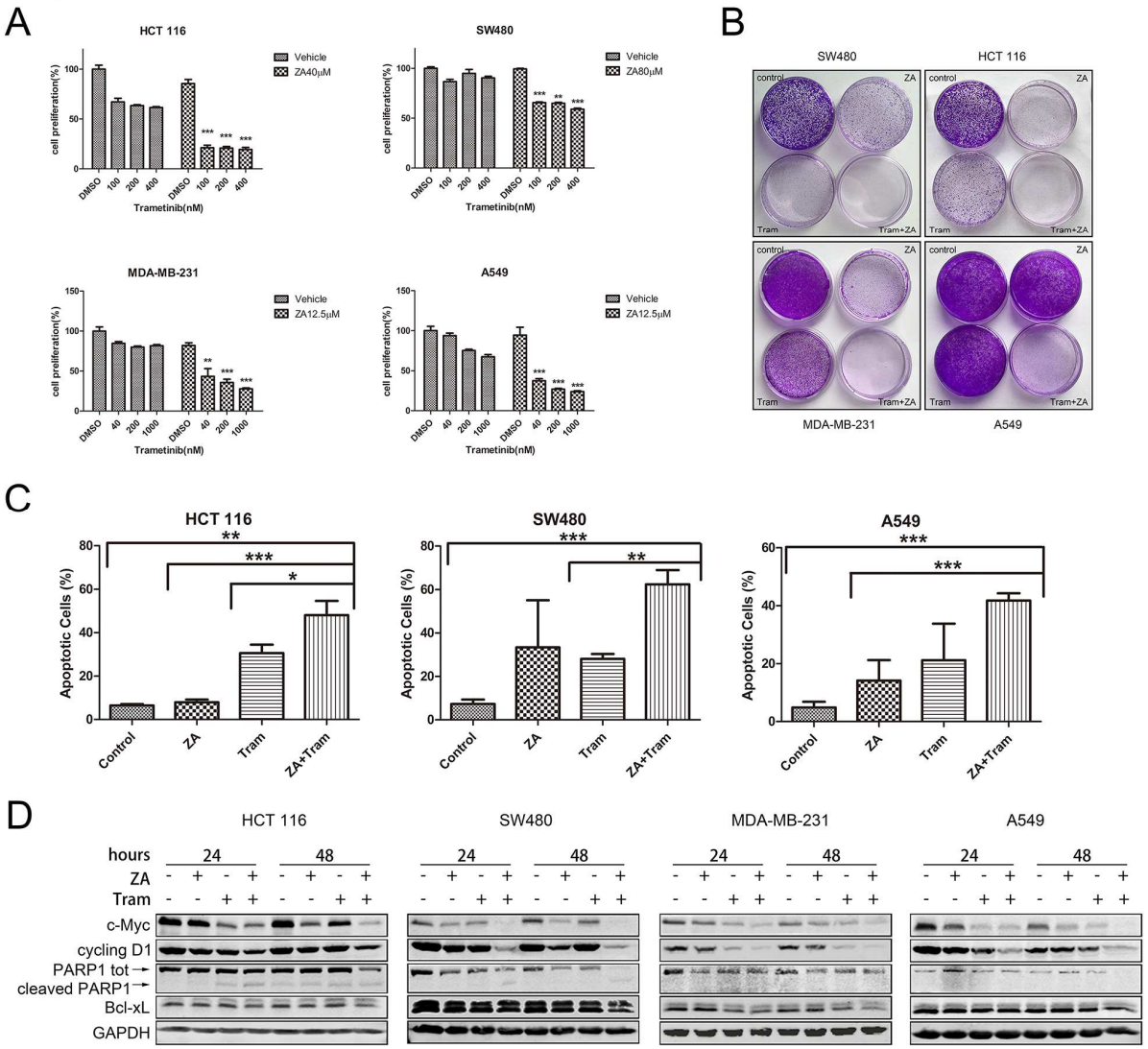
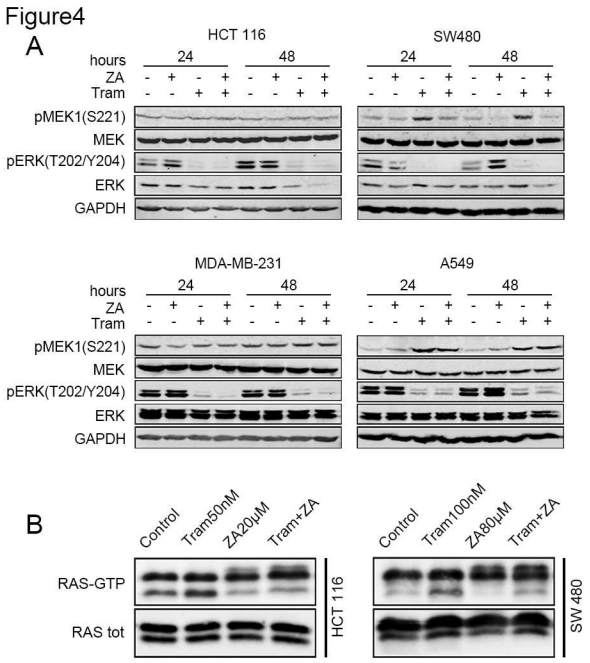


Figure4



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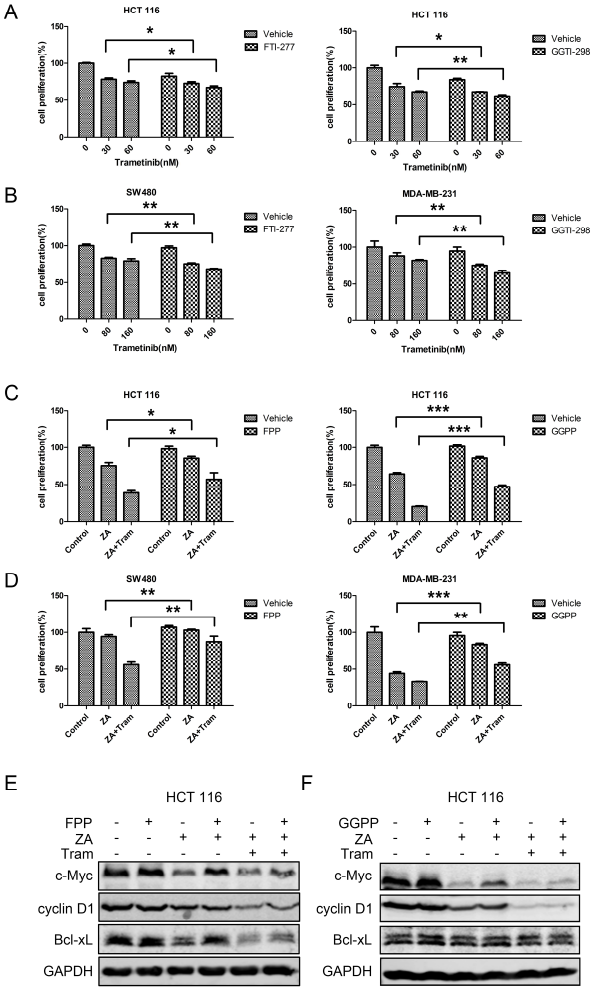


Figure6

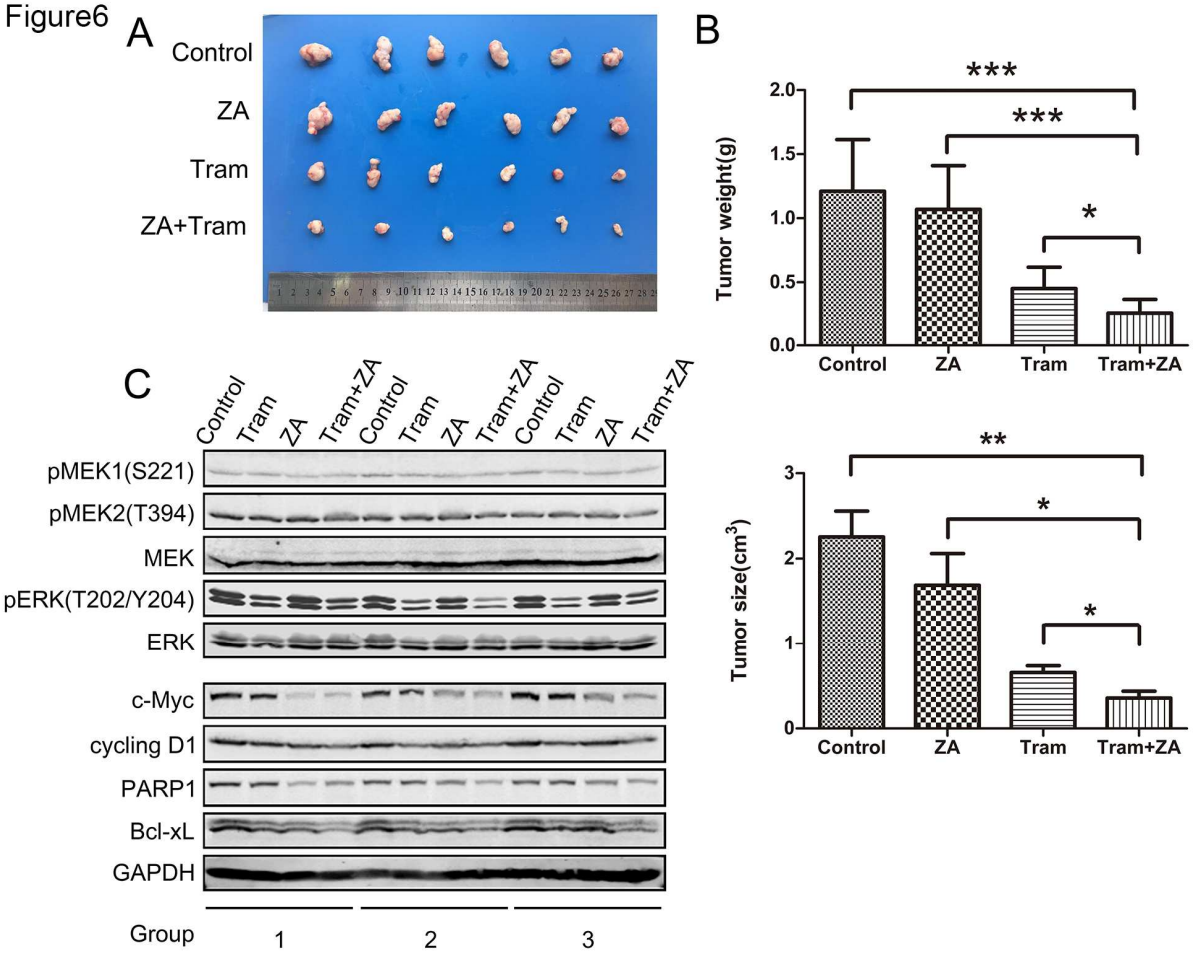
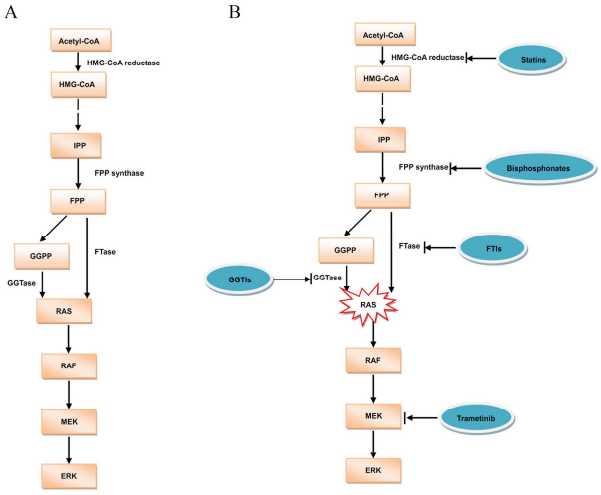


Figure7



Highlights

1. The combined use of the FDA-approved inhibitors trametinib and zoledronic acid suppresses the growth of KRAS mutant tumors both *in vivo* and *in vitro*.

2. Zoledronic acid decreases the hyperactivation of RAS and the rebound of pERK induced by trametinib in KRAS mutant tumors

3. Zoledronic acid synergizes with trametinib mainly through the mevalonate pathway

Conflicts of Interest: The authors declare that no competing financial interests exist.