Combined HASPIN and mTOR inhibition is synergistic against KRAS-driven carcinomas

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ABSTRACT

Background: Oncogenic mutations in the KRAS gene are very common in human cancers, resulting in cells with well-characterized selective advantages. For more than three decades, the development of effective therapeutics to inhibit KRAS-driven tumorigenesis has proved a formidable challenge and KRAS was considered ‘undruggable’. Therefore, multi-targeted therapy may provide a reasonable strategy for the effective treatment of KRAS-driven cancers. Here, we assess the efficacy and mechanistic rationale for combining HASPIN and mTOR inhibition as a potential therapy for cancers carrying KRAS mutations.

Methods: We investigated the synergistic effect of a combination of mTOR and HASPIN inhibitors on cell viability, cell cycle, cell apoptosis, DNA damage, and mitotic catastrophe using a panel of human KRAS-mutant and wild-type tumor cell lines. Subsequently, the human transplant models were used to test the therapeutic efficacy and pharmacodynamic effects of the dual therapy.

Results: We demonstrated that the combination of mTOR and HASPIN inhibitors induced potent synergistic cytotoxic effects in KRAS-mutant cell lines and delayed the growth of human tumor xenograft. Mechanistically, we showed that inhibiting of mTOR potentiates HASPIN inhibition by preventing the phosphorylation of H3 histones, exacerbating mitotic catastrophe and DNA damage in tumor cell lines with KRAS mutations, and this effect is due in part to a reduction in VRK1.

Conclusions: These findings indicate that increased DNA damage and mitotic catastrophe are the basis for the effective synergistic effect observed with mTOR and HASPIN inhibition, and support the clinical evaluation of this dual therapy in patients with KRAS-mutant tumors.

Introduction

KRAS mutations occur in approximately 30% of tumors, making it one of the most common genetic mutations associated with cancer. They are frequent triggers for lung, colorectal and pancreatic cancers. KRAS is mutated in 32% of lung cancers, 40% of colorectal cancers (CRC), and...
90% of pancreatic ductal cancers (PDAC) [1]. KRAS remains a pharmacologic challenge in direct inhibition due to its structure as well as picomolar affinity to GDP/GTP, except for recent advances in selective inhibitors targeting the G12C variant with AMG 510 [2–6]. Patients with tumors harboring KRAS mutations are among the most difficult to treat. Individual inhibitors targeting mutated RAS downstream signaling pathways (such as PI3K/akt/mTOR and RAF/MEK/ERK, etc) have achieved limited response rates of less than 20% in clinical trials [7]. This suggests that RAS is related to cascading crosstalk of signals that leads to a high degree of complexity and redundancy in bypass pathways and negative feedback loops [8]. Given the greater molecular diversity of tumors with KRAS mutations compared with other operable oncogenic targets in tumors, one way to improve the clinical efficacy of inhibitors is to identify drug combinations that either target multiple RAS-driven pathways or circumvention resistance.

mTOR (Mammalian target to rapamycin) is a critical downstream effector of KRAS and plays an important role in the occurrence and development of a variety of tumors [9]. The hyper-activated mTOR pathway is a characteristic hallmark after chemotherapy in KRAS-mutant lung adenocarcinoma and mTOR inhibition circumvents the refractory phenotype and restored the sensitivity of drug-resistant KRAS mutated lung cancer cells to chemotherapy [10]. In pancreatic ductal adenocarcinoma, KRAS remains a difficult target to suppress pharmacologically [11–13]. Pharmacological inhibition of mutation-activated KRAS or MEK leads to rapid adaptive activation of mTORC1/2 pathways, leading to tumor regeneration after initial regression. A combined approach of co-targeting KRAS or MEK and mTORC1/2 complexes to overcome adaptive responses and achieve sustained PDAC tumor growth inhibition [14]. Clinical trials of mTOR inhibitors in colorectal cancer with high KRAS activation rates have been investigated in CRC [15]. mTOR inhibitors may represent an attractive anti-tumor target, combined with strategies to target other pathways that may overcome resistance [9,16–18]. Although mTOR inhibitors have been widely used in clinical studies, there is limited efficacy in treating tumors with mTOR inhibitors alone in KRAS mutated tumors. In KRAS-mutant lung cancer, the mTOR inhibitor Ridaforolimus as a monotherapy showed little clinical benefit in a phase II trial, resulting in only a modest increase in PFS (Progression-free survival) [19]. Likewise, early phase II studies of mTOR inhibition alone in KRAS-dependent PDAC subtypes showed no improvement in overall survival [13]. In KRAS-mutant metastatic colorectal cancer, the mTOR inhibitor temsirolimus (CCI-779) or everolimus alone has no significant anti-tumor activity in phase II studies [20–22]. Therefore, it is particularly important to find synergistic lethal targets of mTOR inhibitor for KRAS-mutant tumors.

HASPIN (Haploid Germ Cell-Specific Nuclear Protein Kinase) is a recently discovered mitotic kinase that phosphorylates histone H3 at threonine 3 (H3-T3). Phosphorylation of H3-T3-T3 promotes inner centromeric localization of the chromosome passenger complex (CPC) during mitosis and is essential for the function of Aurora B at the centromere [23–25]. Consumption of HASPIN by siRNA results in defective mitosis, characterized by chromosome misalignment, premature loss of cohesion between sister chromatids, and the formation of multipolar spindles [26]. mTOR plays an important role in the normal mitosis of cells. The report has shown that Mio (a highly conserved member of the SEACAT/GATOR2 complex) possibly by linking Plk1 and Aurora A to mTOR signaling in a pathway to promote faithful mitotic progression of mTOR cells. Reduced mTOR activity causes the mitotic defects observed upon Mio depletion [27]. mTORC1 cooperates with nuclear RNAPII-CTD kinase CDK12 through phosphorylation of 4E-BP1, and plays a vital role in maintaining the stability of mitotic chromosomes [28]. Although previous studies have shown that HASPIN inhibitors such as CHR-6494, augment the effects of chemotherapy by driving transformed cells to mitotic catastrophe [29], it is unclear whether HASPIN inhibition potentiates the effects of mTOR.

Here, we investigated the mechanism underlying KRAS-mutant cell sensitivity to dual HASPIN and mTOR inhibition. We show that HASPIN inhibition in KRAS-mutant cells induces mitotic catastrophe accumulation, which is further compounded by simultaneous preventing phosphorylation of histone H3, mediated by mTOR inhibition. Importantly, intrauterine administration of mTOR and HASPIN inhibitors at clinically relevant doses caused a significant reduction in human xenografted tumor growth. Our results support further clinical investigation of combined HASPIN/mTOR inhibition as a potential KRAS-driven carcinomas therapy.

Materials and methods

Cell lines and reagents

Human tumor cell lines HCT116, A549, LOVO, SW480, SW620, HPAF-II, MDA-MB-231, HT29, BXPC3, H446, and H1688 were purchased from the American Type Culture Collection (ATCC). Each tumor cell line was cultured in its standard medium as recommended by the ATCC. Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics. All cells were cultivated at standard tissue culture conditions (37°C and 5% CO2/95% air). Fresh medium was added every 2 to 3 days. For in vitro studies, the HASPIN inhibitor (CHR-6494) and mTOR inhibitor (CCI-779) were ordered from MCE (MedChem Express, Shanghai, China), and dissolved in dimethyl sulfoxide (DMSO).

Stable cell pools generation

Vectors cloned with cDNA of KRAS-G12V, Flag-Aurora B, and Flag-Survivin, and sgRNA targeting VRK1 were constructed. Retroviral or lentiviral expressing systems were used to generate stable over-expression or knockout cells lines. cDNA encoding Flag-Aurora B and Flag-Survivin were cloned into pCDH (Supplemental Methods). KRAS-G12V cDNA was cloned into pBabe (Addgene, #46746). sgRNAs targeting VRK1 and HASPIN were designed using ChOPChOP (https://chopchop.rc.fas.harvard.edu) and cloned into LentiCRISPRv2 vector (Addgene, #52961) digested with BsmB1 and ligated with annealed oligonucleotides acid. In brief, retrovirus particles were produced in HEK293T cells using Polyethylenimine (PEI) (Beyotime, Shanghai, China) transfection. Lentiviruses were prepared by transfecting two packaging plasmids into HEK293T cells using PolyJet (SignaGen; Shandong, China). After filtration with a Milex-HV sterile 0.45μm filter (Merck Millipore, Shanghai, China) and titration, viruses were added to cells in presence of polybrene (10μgml−1). The medium was replaced 24–48h after infection, followed by selection with puromycin (5μgml−1) for 1–2 weeks. Protein overexpression or knockdown efficiency was confirmed by quantitative real-time PCR or western blot. The VRK1 knockout target sequences are as follows: 5′-GACGGCAGGATCATGATGCCACAA-3′. The HASPIN knockout target sequences are as follows: 5′-GACGGCAGGATCATGATGCCACAA-3′ and sgRNA2 5′-GACGGCAGGATCATGATGCCACAA-3′

Proliferation and colony formation

Cell proliferation was measured by using the Cell-Counting kit 8 (CCK8) assay. Cells were seeded in 96-well plates (1,000 cells/well) with 10% FBS in culture medium, and treated with indicated agents for 72 h. CCK8 (Dojindo Molecular Technologies, Rockville, USA) was added to the wells prior to incubation for 2 h at 37°C. The assay was performed according to the manufacturer’s instructions. Cell viability of the treated group was normalized to the vehicle control. IC50 values were determined using the Prism 7 Software (LaJolla, California, USA). The combination indices (CI) were calculated by CompuSyn software using the Chou-Talalay method (Biosoft, Palo Alto, CA); additive effect (CI−1), synergism (CI<−1), and antagonism (CI>1) [30]. For colony formation assays, cells were seeded in triplicate in 6-well
dishes (1,000 cells/well) and allowed to adhere overnight in culture media. The cells were then cultured with the drug(s) alone or in combination, or with the vehicle in complete media for 14 days. Observed colonies were fixed with 0.4% buffered paraformaldehyde and then stained with crystal violet for 20 min. The colonies were enumerated using Image J software (NIH, Maryland, USA). Image J filters scored colonies that were ≥100μm in size.

**Human xenograft models**

BALB/C nude male mice, aged 4–5 weeks, were obtained from Shanghai Slack Laboratory Animal Co. Ltd (Shanghai, China). Animals were maintained in a sterile environment; their cages, food, and bedding were sterilized by autoclaving. All manipulations were performed under sterile conditions following procedures approved by the Experimental Animal Management and Ethics Committee of University. A549 (1×10⁴), HCT116 (7.5×10⁶), HPAF-II (1×10⁵) was injected subcutaneously in the flanks of all experimental nude mice. For treatment, mice were randomized into 4 groups (n = 5–6 per group) with similar mean tumor volumes of approximately 40–50 mm³. Mice were treated with vehicle, 50 mg/kg CH-6494 (in 0.5% Sodium carboxymethyl cellulose), 20 mg/kg CCI-779 (in 0.5% Sodium carboxymethyl cellulose), or a combination of CH-6494 and CCI-779 via intraperitoneal injection.

MDA-MB-231 (5×10⁶) cells were injected subcutaneously in each animal. 25 tumor-bearing mice (50 mm³ tumor volume) were randomized into 4 groups and exposed to vehicle, 50 mg/kg CH-6494, 20 mg/kg CCI-779 or CH-6494/CCI-779 in combination via intraperitoneal injection in four cycles of three consecutive days for 21 days, or 20 mg/kg paclitaxel (in 0.5% sodium carboxymethyl cellulose) (MedChem Express, Shanghai, China) via intraperitoneal injection for every other day.

Tumor growth was measured every 3 days using a digital caliper and volume was assessed as (length x width²)/2. Bodyweight was measured every 3 days as an indicator of toxicity. Mice were euthanized when the tumor volume reached the best contrast effect. At sacrifice, tumors were excised and weighted. Upon killing mice, portions of tumors were snap-frozen and stored in liquid nitrogen or were fixed in 10% buffered formalin for routine histopathologic processing.

**Western blot analyses and antibodies**

Total cell lysates were prepared from the cells after treatment with the drug(s) or vehicle with RIPA Buffer (Beyotime, China) supplemented with protease and phosphatase inhibitor cocktail (Roche) and 1% sodium dodecyl sulfate (SDS). The protein concentration was determined with protease and phosphatase inhibitor cocktail (Roche) and 1% so-

**Quantitative real-time PCR**

Total RNA was extracted from A549 and SW480 cells with RNA Isolation Kit (Vazyme, Shanghai, China). cDNA was synthesized by a reverse transcription kit (Vazyme, China). Amplification was performed with iQ™ Universal SYBR Green Supermix (BIO-RAD, USA) under universal cycling conditions using the ABI 7500 system (Applied Biosystems, CA, USA). Relative mRNA expression was calculated using the 2-ΔΔCt method. GAPDH was used as the housekeeping gene. The primer sequences were as follows: VRK1 (forward, 5′-CCTCGTGTAAAGAACGCTCA-3′; reverse, 5′-GCCAATGGAATCC-TACTCC-3′), KRAS (forward, 5′-CGAAAGTTGCTGTAGGATA-3′; reverse, 5′-CCTCATGCTGACTTCTCC-3′) and GAPDH (forward, 5′-GGAGGAGATCCCTCCTCAAAAT-3′; reverse, 5′-GCGTTTGTCA-TCTCTCATGG-3′).

**Immunohistochemistry (IHC)**

Tissues were fixed with 10% buffered formalin at 4°C for overnight and embedded in paraffin (FFPE). FFPE were sectioned at 4-μm thick-

**TUNEL and H&E staining**

TUNEL staining was performed on paraffin sections using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, #S7100). TUNEL-positive cell staining was quantified in 5 regions of each tumor section using Image J software with a minimum sample size of 5 animals per cohort. Hematoxylin and eosin (H&E) staining was done by standard techniques.

**Immunofluorescence staining**

Cells were placed on the slides treated with the indicated concentrations for 72 h and followed fixation with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 5% Triton X-100 in PBS for 30 min. Blocked cells with 3% bovine serum albumin (BSA) were incubated with primary antibodies γ-H2AX (Cell Signaling, #9718), p-AKT (Cell Signaling, #4060), p-PI3K (Cell Signaling, #20201), p-HER (Cell Signaling, #1648), p-EGFR (Cell Signaling, #2285), p-ERK (Cell Signaling, #4370) or anti-Flag (Cell Signaling, #2197) in 5% bovine serum albumin for 1 h at room temperature. Stained cells were mounted with DAPI (Beyotime, China). The Nikon microscope was used to image the fluorescently-stained slides and analysis. We acquired a range of 10–20 fields per treatment using oil 100x objective magnification and at least 120 cells were analyzed using Image J software for data analysis.
Flow cytometric analysis for cell cycle and apoptosis

A549 and HCT116 cells were treated with CHR-6494 (600 nM) and/or CCI-779 (3 μM) for 72 h. Floating and trypsin-detached cells (1 × 10⁶) were collected and fixed in 70% cold ethanol for 2 h. Subsequently, the cells were washed once with PBS, resuspended in PBS containing 200 μg/ml RNase A, incubated at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (PI, BD Biosciences, New Jersey, UAS). Cell cycle distribution was acquired by BD LSRFortessa flow cytometry (BD Biosciences, USA), and the data were analyzed using FlowJo 10 software used for data analysis.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 7 (La Jolla, California, CA) software. Results are expressed as mean ± SD of the indicated number of independent experiments. The P values were calculated using the Student t-test for comparison of two groups. For multiple comparisons, two-way ANOVA was performed. P values of < 0.05 were considered statistically significant.

Results

Inhibition of HASPIN sensitized KRAS-mutant cancer cell lines to mTOR inhibitor treatment in vitro

We first evaluated the effect of combination treatment with HASPIN inhibitor (CHR-6494) and mTOR inhibitor (CCI-779) to inhibit cell proliferation of a panel of KRAS-mutant (A549, SW480, SW620, HPAF-II, LOVO, and HCT116) and KRAS-wildtype (HT29, BXPC3, H446, and H1668) cell lines (Fig. 1A-1C, Supplementary Table 1). The proliferation assay showed the drugs affected all the KRAS-mutant cell lines tested, both single agents and in combination after 72 h treatment (Fig. 1A). However, all cell lines were more sensitive to combination treatment compared to single therapy (Fig. 1A). Synergism was determined by the combination indices (CI) calculated using the Chou-Talalay method [30]. Strikingly, the combination indices analysis demonstrated that dual inhibition of HASPIN and mTOR synergistically suppressed cell viability of all KRAS-mutant cells (Fig. 1B). The synergistic cell suppression we observed with HASPIN and mTOR inhibitor is unlikely due to off-target effects because knockout of HASPIN with sgRNA shows cooperative inhibition as well (Supplementary Fig. 1). In contrast, no stronger inhibitory activity was observed in any of the KRAS-wildtype cell lines treated with the drug combination, compared to the single-agent (Fig. 1C). IC50 values for CHR-6494 and CCI-779 were established (Supplementary Table 2). These results suggested that the two targeted drugs may have a complementary mechanism of action in KRAS-mutant cell lines.

Further evaluation of the colony growth ability of KRAS-mutant cells (A549 and HPAF-II) and KRAS-wildtype cells (HT29 and H446) under long-term treatment showed that compared with monotherapy or vehicle control, the colony formation of dual inhibitory treatment was significantly reduced in KRAS-mutant but not in KRAS-wildtype cells (Fig. 1D-H). Taken together, the combinatorial inhibition of HASPIN and mTOR was synergistically superior to either single drug in inhibition of cell viability and anchorage-dependent growth in KRAS-mutant cells.

Antitumor activity of combination treatment in human KRAS-driven tumor xenografted models

Given the biological implications of our in vitro data, we further explored the combinatorial anti-tumor activity of CHR-6494 and CCI-779 in HCT116 and A549 cells xenografted in vivo mouse models. According to the aforementioned animal experiment protocol and schematic depiction of Fig. 2A, when the tumor reached 40–50 mm³, HCT116 or A549 tumor-bearing mice were grouped (n = 5) and administered i.p. with CHR-6494, CCI-779 or combination treatment. In HCT116 xenografts, although no tumor completed regression was observed in any groups with different treatment, tumor growth was significantly retarded in the group with combined CHR-6494 and CCI-779, with essentially no increase in tumor size (Fig. 2B-2C). Combination treatment for 27 days suppressed tumor progression (baseline 56.2 ± 20 mm³; post-treatment 77 ± 35 mm³), whereas the vehicle-treated tumors progressed from 58 ± 24 to 1460 ± 56 mm³ (Fig. 2C). At the time of sacrifice, tumors treated with the combination weighed nearly 97% less than the vehicle-treated group (Fig. 2D). As seen before, CHR-6494 and CCI-779 single treatment led to tumor growth delay, but combined treatment led to tumor shrinkage in mice bearing A549 xenografts (Fig. 2E-F). At the endpoint, tumors treated with the combination weighed significantly lower than vehicle-treated tumors (Fig. 2G). To further verify the efficacy of combination therapy, we expanded the type of KRAS mutant HPAF-II cancer cells xenografted mice models. Consistent with the antitumor effect of the combination in the HCT116 and A549 xenograft model, we observed similar effects on tumor size, tumor growth, and tumor weight in HPAF-II xenografts as well (Supplementary Fig. 2A-2C).

To evaluate the ability of combined therapy to induce apoptosis and DNA damage in vivo, cells staining positively for the DNA fragmentation marker TUNEL (Terminal deoxynucleotidyl transferase DUTP nick end labeling) and the DNA damage marker γ-H2AX were quantified in HCT116 tumors after combined treatment and compared to vehicle controls (Fig. 2H-2K). We detected significantly increased numbers of TUNEL and γ-H2AX-positive tumor cells in the combination-treated tumors compared to controls (Fig. 2H-2I and 2K). It also reduces the fraction of proliferating tumor cells, as measured by Ki-67 staining (Fig. 2H and 2J). Histological evaluation of HPAF-II dissected tumor, the same results as HCT116 xenograft were obtained (Supplementary Fig. 2D-2G). To further verify the ability of combined treatment to induce both apoptotic and DNA damage response in vivo, xenograft-bearing mice were treated for 72 h. Western blot analysis showed an increase in γ-H2AX and levels of cleaved PARP (cPARP) (Fig. 2L). Of note, monotherapy with either CHR-6494 and CCI-779 alone failed to induce significant detectable accumulation of γ-H2AX or cleaved PARP (Fig. 2L).

Combination treatment with HASPIN and mTOR inhibitors leads to mitotic catastrophe in cells by preventing H3 phosphorylation

Given the potent in vitro and in vivo treatment response, we next examined downstream effectors of HASPIN and mTOR inhibition. We found that treated with HASPIN or mTOR inhibitor alone in A549 cells, the phosphorylation level of H3 histones in Thr3 (H3T3ph) was slightly reduced, or even negligible, compared with the significant reduction in combination therapy (Fig. 3A). Consistent with this finding in SW480 cells, the combined treatment strongly suppressed the phosphorylation of histone H3 (Fig. 3A).

Aurora B forms a complex with a histone H3, which has been phosphorylated in Thr3, and this binding of AURKB is mediated by Survivin [31]. Insufficient H3 phosphorylation sites will prevent chromosomal passenger complex (CPC) binding to histones [32]. Thus, we next confirmed that combination therapy resulted in the loss of Survivin and Aurora B interaction with H3 without any effect on the expression of the total CPC (Aurora B and Survivin) protein in nocodazole-treated
Fig. 1. Inhibition of HASPIN sensitized KRAS-mutant cancer cell lines to mTOR inhibitor treatment in vitro.

(A–C) Synergistic interaction between HASPIN inhibitor (CHR-6494) and mTOR inhibitor (CCI-779) was measured. (A) KRAS-mutant (KRAS-Mut) tumor cell lines were treated with CHR-6494 (600nM) or/and CCI-779 for 72 h. (B) Compusyn combination indices (CI) were derived from seven-point concentration proliferation experiments. The cutoff for additive effect (CI: 1) is marked by a dashed line. (C) KRAS-wildtype (KRAS-WT) tumor cell lines were treated for three days with either single agents or combined agents. (D–H) Representative images of KRAS-Mut tumor cell lines (A549 and HPAF-II) and KRAS-WT tumor cell lines (HT29 and H446) colony formation, untreated or treated with either CHR-6494 (600nM), CCI-779 (3μM), or in combination for 2 weeks. (E–H) Mean number of colonies formed after treating cells for 2 weeks. Data represent mean ± SD (n=3). ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Fig. 2. Antitumor activity of combination treatment in human KRAS-driven tumor xenografted models.

(A) Schematic depicting experimental plans with the timeline for HCT116, A549, and HPAF-II cells injection with single agents or combination treatment using nude mice xenograft models. Representative image of xenografts formed in nude mice treated with either single agents or combined agents using HCT116 cells (B) and A549 cells (E). (C and F) Effect of single-agent and dual treatment on tumor growth of HCT116 (C) and A549 (F) cells in nude mice dosed at 50 mg kg$^{-1}$ (CHR-6494) and 20 mg kg$^{-1}$ (CCI-779). (D and G) Final HCT116- and A549-treated tumor weights at time of sacrifice compared to vehicle-treated tumors. (H) Representative histologic sections of xenografts from HCT116 tumors were immunostained with γ-H2AX, Ki-67, and TUNEL. (I-K) The percentage of positive γ-H2AX (I), Ki-67 (J), and TUNEL (K) cells in HCT116 tumor sections were scored at 5 high-power fields (n=5/group). (L) Western blot analysis of changes in protein levels of γ-H2AX and cleaved PARP (cPARP) expression in HCT116 tumors mentioned in (B). Scale bars: 20μm. Data represent mean ± SD (n=3), ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Fig. 3. Combination treatment with HASPIN and mTOR inhibitors leads to mitotic catastrophe in cells by preventing H3 phosphorylation. (A) Representative western blots showing protein levels of downstream target engagement (H3T3ph and p70S6K) in A549 and SW480 cells treated for 72 h with DMSO, CHR-6494 (600nM), CCI-779 (3μM), or combination. (B) SW480 cells were synchronized and treated with indicated drugs for 72 h and harvested in mitosis by mechanical shake-off. Western blots showing CPC protein levels (Aurora B and Survivin) and total Histone 3 levels. (C) Immunoblot analysis of the strength of the interaction between CPC protein (Aurora B and Survivin) and Histone 3 with immunoprecipitated equal protein of Flag- aurora B (left) and Flag-Survivin (right) after 72 h treatment with either 600nM CHR-6494, 3μM CCI-779, or both in nocodazole-treated SW480 cells. (D) SW480 cells were synchronized and exposed to indicated drugs for 72 h. The level of CPC protein concentration on centromeres was analyzed by immunofluorescence. The quantification of the CPC and ACA signals, and the DAPI signal (DNA) were quantified and their overlap is shown in the graphs below. A total of 100 cells were counted taking into account the distribution of CPC on centromeres and chromosome arm or spread on the chromatin. Scale bar: 10 μm. (E) SW480 cells with features of mitotic catastrophe (MC), such as micronuclei, multilobular, fragmented nuclei (left), and containing >2 centrosomes (right) treated with the indicated drug singly or in combination for 72 h. Scale bar: 10μm. (F and G) MC and abnormal centrosome amplification were quantitated to HCT116 cells. (H and I) MC and abnormal centrosome amplification were quantitated to SW480 cells. Data are expressed as means ± SD (n=3) of the three experiments, ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001.
synchronized SW480 cells (Fig. 3B-3C). The expression of H3T3ph is required for the recruitment of CPC to centromeres in mitosis to perform normal mitotic regulation functions [23]. In this context, we examined the colocalization of ACA (anti-centromere antibody) and CPC on centromeres in SW480 cells treated with single agents or the combination by immunofluorescence. Control-treated cells displayed a typical localization of ACA and CPC, whereas slight delocalization of CPC protein on ACA was observed in single-treated cells (Fig. 3D). Moreover, the combination treatment of CHR-6494 and CCI-779 resulted in dramatically delocalization of CPC proteins (Fig. 3D). Furthermore, we examined the effect of indicated drugs treatment on mitotic catastrophe. Compared to control and CCI-779 treatment, in CHR-6494-treated SW480 and HCT116 cells, DAPI staining cell nuclei and immunostaining with p-Aurora A/B/C displayed an increased number of cells with features of mitotic catastrophe (MC) such as micronuclei, multilobular nuclei, and fragmented nuclei, and cells containing more than two centrosomes, respectively (Fig. 3F-3I). Typical microphotographs were shown in Fig. 3e. Interestingly, quantitative analysis showed that the number of cells having the characteristics of MC was significantly increased at dual treatment in SW480 (Fig. 3F) and HCT116 cells (Fig. 3H). On the other hand, cells containing more than two centrosomes were also highly increased after combination therapy in SW480 cells (Fig. 3G) and HCT116 cells (Fig. 3I). Together, these results indicate that the HASPIN and mTOR have a synergistic effect on molecular functions to affect the process of cell mitosis by regulating the phosphorylation of histone H3.

On-target inhibition of HASPIN and mTOR synergistically trigger enhanced DNA damage and apoptosis in KRAS-mutant cells

Mitotic catastrophe is an important mechanism for the death of cancer cells induced by anti-neoplastic agents that damage DNA [33]. Given that the combinatorial inhibition of HASPIN and mTOR resulted in much greater abnormal mitoses compared to either control or single
agent in KRAS-mutant cells, we next examined whether the combination synergistically triggers DNA damage and cell death. Treatment of exponentially proliferating SW480, HPAF-II, and A549 cells for 72-h with CHR-6494 and CCI-779 led to the accumulation of γ-H2AX, a marker for double-stranded DNA breaks (Fig. 4A). Consistent with this finding, we showed significantly elevated γ-H2AX foci in cells treated with both CHR-6494 and CCI-779 by immunofluorescence (Fig. 4C). This triggered the DNA damage response (DDR), as shown by increased phosphorylation of classical DNA damage pathway indicators (P53, CHK2) (Fig. 4D and Supplementary Fig. 3). We next asked if the observed synergistic activity of the combination treatment would result in apoptosis in KRAS-mutant cancer cell lines. Western blot results indicated that the intensity of cleaved PARP (cPARP) signal was markedly enhanced when employing CHR-6494 and CCI-779 in combination (Fig. 4A). Flow cytometry analysis showed that the apoptosis rate in the drug combination-treated cells was higher than that in the HASPIN-treated cells, while no obvious apoptosis was observed in DMSO- or CCI-779-treated cells (Fig. 4B).

We confirmed that CDK1, another DNA damage pathway indicator, was further phosphorylated with dual treatment (Fig. 4D and Supplementary Fig. 3). Phosphorylation of CDK1, resulting in a blockade of mitotic entry [34]. We, therefore, hypothesized that the observed synergistic effect of HASPIN and mTOR inhibitors were associated with a perturbed cell cycle progression. Flow cytometry analysis was performed after the cells were treated with CHR-6494, CCI-779, or their combination, and a significant cell cycle arrest at the G2/M phase was found with combination treatment in A549 and HCT116 cell lines (Fig. 4E). Collectively, these data suggest the enhanced anticancer activity of combination drug therapy in KRAS-mutant cell lines may due to the more effective induction of apoptosis levels and DNA damage-induced cell cycle arrest.

mTOR cooperatively activates H3T3ph via VRK1 in the context of mutant KRAS

Since the HASPIN or mTOR inhibitor alone inhibits the phosphorylation of histone H3 to a less extent than the combined inhibition, we hypothesized that H3 is the common signaling molecule downstream of both mTOR and HASPIN. As HASPIN and Vaccinia-related kinase 1 (VRK1) are the two major kinases responsible for phosphorylation of H3 [24], we next attempted to clarify whether mTOR regulates histone H3T3ph via a VRK1-dependent manner. The results of real-time PCR and Western blot revealed that inhibition of mTOR down-regulated the expression of VRK1 in A549 and/or SW480 cells (Fig. 5A-B). Inhibiting HASPIN can almost eliminate the phosphorylation of H3 histones (Fig. 5D) in VRK1 knockout SW480 cells (Fig. 5C).

We further examined the relationship between the expression of VRK1 and the KRAS signaling pathway. Two KRAS wild-type cell lines (HT29, H1688) and two KRAS mutant cell lines (SW480, A549) were used in this verification. The results showed that the KRAS mutation caused the activation of mTOR and the expression of VRK1, and the mTOR inhibition partially offset VRK1 expression caused by KRAS mutation (Fig. 5E). VRK1 and H3T3ph expression were up-regulated in KRAS-wildtype HT29 cells overexpressing mutant KRAS-G12V (Fig. 5F-5G), and mTOR suppression partly abrogates this phenomenon (Fig. 5G). In VRK1 knockout SW480 cells, activation of mTOR by

![Graphical representation](image-url)
Leucine failed to rescue the level of VRK1 expression and H3T3ph (Fig. 5H). Taken together, our results indicate that KRAS may regulate H3T3ph through the mTOR-VRK1 axis, which is not activated in KRAS wild-type cells.

**VRK1-depletion similarly sensitizes KRAS-mutant cells to CHR-6494 treatment as mTOR inhibition**

As shown above, mTOR affects the phosphorylation of histone H3 by positively regulating the expression of VRK1. Therefore, we concluded that VRK1 depletion can similarly sensitize cells to HASPIN inhibition. Knockout of VRK1 led to a significant dissociation of CPC protein and H3 after CHR-6494 treatment in SW480 cells (Fig. 6B), without affecting the total CPC protein expression (Fig. 6A). In addition, we observed a significant increase in DNA damage (Fig. 6C), apoptosis rate (Fig. 6D), and mitotic catastrophe (Fig. 6E-F) in VRK1-deficient cells treated with CHR-6494. Knockout of VRK1 alone cannot lead to massive dephosphorylation of H3 and cell apoptosis (Figs. 5C and 6D). These results indicate that VRK1 compensates for phosphorylation of H3 when HASPIN is inhibited. Taken together, our results suggest that inhibition of HASPIN potentiates mTOR inhibition by abrogating compensatory phosphorylation of histone H3 (Fig. 6G).

**Combined HASPIN/mTOR inhibition causes tumor growth delay in MDA-MB-231 xenografts**

We further extended to speculate whether the dual inhibition of HASPIN and mTOR could have a synthetic inhibitory effect on other KRAS-mutant tumors. MDA-MB-231 tumor cells are typical triple-negative breast cancer (TNBC) cells and have KRAS G13D mutations [35]. PTX (paclitaxel) is commonly used as the first-line treatment drug for TNBC patients [36]. HASPIN plays an important role in the regulation of normal mitosis of cells through the regulation of H3 histones. It has been shown that HASPIN inhibitors have potent anti-tumoral effects [24]. HASPIN inhibition leads to the dephosphorylation of the third threonine of H3 histones, which in turn affects the binding of CPC components to histones, and ultimately leads to the disorder of the normal mitotic process of cells. Hence, the main mechanism of HASPIN inhibition leading to tumor cell apoptosis is the occurrence of mitotic catastrophe [29,38]. Consistent with the report, we have shown in our research that a single HASPIN inhibition can also lead to an increase in the rate of tumor cell apoptosis and the probability of mitotic catastrophe (Figs. 3-4). Mitotic catastrophe is the response of mammalian cells to mitotic DNA damage [39]. Studies have confirmed that CPCR plays an important role in the process of mitosis, involving many important functions such as spindle formation, chromosome arrangement, sister chromatid separation, spindle checkpoint signaling, and cytokinesis [40]. We reasonably believe that the abnormal positioning of CPC simultaneously causes DNA damage to a certain extent, and then cumulatively leads to the complete disintegration of cells.

The mammalian target of rapamycin (mTOR) regulates cell proliferation, autophagy, and apoptosis by participating in multiple signaling pathways in the body [41]. Inhibition of mTOR/PI3K signaling pathway makes Aurora A-deficient precancerous keratinocytes occur in mitotic catastrophe characterized by multinuclei and polymorphonuclei [42]. The mTORC1 complex components have been shown to be related to mitotic spindles in mouse oocytes [43], and overexpression of mTORC1 leads to impaired spindle formation and aneuploidy [44]. Taken together, these data suggest that mTOR has a regulatory role in cell mitosis. However, how mTOR is involved in regulating the proper cell mitosis process has not been reported. In our study, we found that mTOR inhibition alone could not cause an increase in the number of apoptotic tumor cells, but it enhanced cell death induced by a single HASPIN inhibition (Fig. 4). We also found that combined mTOR inhibition increased the incidence of HASPIN-induced diffused CPC and mitotic catastrophe in tumor cells (Fig. 3). These data suggest that mTOR may be involved in the correct regulation of the cell mitosis process. We reported for the first time that in KRAS-mutant tumors, mTOR is involved in the regulation of VRK1 kinase expression and affects the phosphorylation of H3 histone (Fig. 5), thus coordinating with HASPIN to regulate the correct localization of CPC components during cell mitosis (Fig. 3). Given that VRK1 is a downstream target of mTOR signaling, we verified that depletion of VRK1 expression was sufficient to override CCI-779-mediated potentiation to CHR-6494 (Fig. 6). We consider that in KRAS-mutant tumors, due to the redundancy between mTOR and HASPIN, activation of the mTOR pathway plays a major role in the regulation of H3 phosphorylation in HASPIN-inhibited tumor cells, but the dual inhibition leads to complete dephosphorylation of H3T3ph and ultimately leads to the mitotic catastrophe death in the cell.

Triple-negative breast cancer (TNBC) is a highly malignant subtype of breast cancer with a poor prognosis [45]. TNBC accounts for approximately 20% of breast cancer cases. Although conventional chemotherapy regimens have shown some effectiveness in early TNBC cases, in advanced stages the outcome is poor. The PI3K/AKT/mTOR pathway is one of the important and active pathways involved in TNBC chemoresistance and survival. However, PI3K/AKT/mTOR targeted therapy has not been successfully developed for TNBC [46,47]. Here, we
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proved that the combined inhibition of HASPIN/mTOR is more effective than monotherapy and the traditional chemotherapy drug paclitaxel in MDA-MB-231 xenograft tumors. It is possible that there is an over-activated mTOR pathway in TNBC, and the mechanism described above exists in cells. It is noteworthy that apart from KRAS mutations, EGFR and BRAF, which lie upstream and downstream of Kras respectively, are also mutated with high frequency in various tumors. Therefore, whether the synergistic effect of simultaneously inhibiting mTOR and HASPIN can also be observed in tumor patients harboring EGFR or/and BRAF mutations warrants further investigations.

In summary, the present study is the first to show that CHR-6494 potentiates CCI-779 by abrogating the compensatory phosphorylation pathway of H3 histones, resulting in a synergistic effect in KRAS-mutant tumors. Given the absence of targeted therapies available for KRAS-driven tumors and the high rate of activation of the mTOR signaling pathway in cancers, dual inhibition of mTOR and HASPIN should be investigated as a potential strategy.

Fig. 6. VRK1-depletion similarly sensitize KRAS-mutant cells to CHR-6494 treatment as mTOR inhibition. (A) Synchronized-treated vector control or VRK1 KO SW480 cells were treated with or without CHR-6494 for 72 h and harvested in mitosis by mechanical shake-off. Western blots showing CPC protein levels (Aurora B and Survivin) and total Histone 3 levels. (B) Detection of the ability of the interaction between CPC protein (Aurora B and Survivin) and Histone 3 by Western blot with immunoprecipitated equal protein of Flag- aurora B (left) and Flag-Survivin (right) in SW480 cells expressing sgRNA targeting VRK1, untreated or treated with CHR-6494 at 600nM. Representative foci-containing cells are shown at high power magnification (100x). At least 120 nuclei were analyzed over three experiments. Scale bar: 10μm. Right: Statistical analysis of the mean number of γ-H2AX-focus in left. (D) Apoptotic cells detected with AnnexinV/PI staining, and analyzed by flow cytometry in SW480 cells expressing either VRK1 sgRNA or control guide, untreated or treated with 600nM CHR-6494 for three days. Representative images (left) and percentage of apoptotic cells (right) of the apoptosis-inducing response determined by flow cytometry analysis. (E and F) MC and abnormal centrosome amplification were quantitated to SW480 cells expressing either VRK1 sgRNA or control guide, untreated or treated with 600nM CHR-6494. (G) A simplified model showing cross-talk and compensation by mTOR and HASPIN-mediated regulation of H3T3ph. The results are mean ± SD (n=3), ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Fig. 7. Combined HASPIN/mTOR inhibition causes tumor growth delay in MDA-MB-231 xenografts. (A) Representative image of xenografts formed in nude mice using MDA-MB-231 cells exposed to vehicle, CHR-6494, CCI-779, CHR-6494/CCI-779 combination or Paclitaxel. (B) Tumor volume was monitored every three day. (C) Final tumor weight of (A) mentioned tumors at the time of sacrifice. (D) Body-weight changes were measured in tumor-bearing nude mice in each treatment mode. (E) Western blot analysis of changes in protein levels of γ-H2AX and cleaved PARP (cPARP) expression in MDA-MB-231 tumors mentioned in (A). Data represent mean ± SD (n=3), ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
The in vivo assay using nude mice was approved by the Experimental Animal Management and Ethics Committee of Fudan University (Project#20180302-003).

Supplementary Fig. 1 HASPIN and mTOR inhibition synergistically suppresses KRAS-mutant cancer cells.

(A) Western blots demonstrated HASPIN protein knockout (KO) after treatment with sgRNA in KRAS-mutant cell lines (SW480 and HCT116) and KRAS-wildtype cell (HT29). (B) Control or cells treated with CCI-779 at indicated concentrations were transfected with sgRNA against HASPIN and assayed for viability after 3 days. Data are expressed as means ± SD (n=3) of the three experiments, ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Supplementary Fig. 2 Combination treatment suppresses HPAF-II tumor xenografted growth

(A) Representative image of xenografts formed in nude mice using HPAF-II cells exposed to indicated drugs. (B) Tumor volume was measured every 3 days after implantation of (A) mentioned tumors. (C) Tumor weight of (D) control or cells treated with CCI-779 at indicated concentrations were transfected with sgRNA against HASPIN and assayed for viability after 3 days. Data are expressed as means ± SD (n=3) of the three experiments, ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Supplementary Fig. 3 Inhibition of HASPIN and mTOR results in activation of DNA damage pathway

Dual therapy dose- and time-dependently enhanced phosphorylation of CHK2, PS3, and CDK1 in SW480 cells after indicated treatment.

CRediT authorship contribution statement

Chenyue Xu: Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing. Qiongmei Gao: Conceptualization, Methodology, Project administration, Writing – review & editing. Zhengming Wu: Conceptualization, Data curation, Formal analysis. Weijuan Lou: Conceptualization, Data curation, Formal analysis. Xiaoyan Li: Conceptualization, Data curation, Formal analysis. Menghui Wang: Data curation, Formal analysis. Xiaohong Wang: Validation, Writing – review & editing. Qingquan Li: Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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