Inhibiting Tankyrases Sensitizes KRAS-Mutant Cancer Cells to MEK Inhibitors via FGFR2 Feedback Signaling

Marie Schoumacher1, Kristen E. Hurov1, Joseph Lehár1, Yan Yan-Neale1, Yuji Mishina1, Dmitry Sonkin1, Joshua M. Korn1, Daisy Flemming1, Michael D. Jones1, Brandon Antonakos1, Vesselina G. Cooke1, Janine Steiger2, Jebediah Ledell2, Mark D. Stump1, William R. Sellers1, Nika N. Danial3, and Wenlin Shao1

Abstract

Tankyrases (TNKS) play roles in Wnt signaling, telomere homeostasis, and mitosis, offering attractive targets for anticancer treatment. Through unbiased combination screening in a large panel of cancer cell lines, we have identified a strong synergy between TNKS and MEK inhibitors (MEKi) in KRAS-mutant cancer cells. Our study uncovers a novel function of TNKS in the relief of a feedback loop induced by MEK inhibition on FGFR2 signaling pathway. Moreover, dual inhibition of TNKS and MEK leads to more robust apoptosis and antitumor activity both in vitro and in vivo than effects observed by previously reported MEKi combinations. Altogether, our results show how a novel combination of TNKS and MEK inhibitors can be highly effective in targeting KRAS-mutant cancers by suppressing a newly discovered resistance mechanism. Cancer Res; 74(12): 1–12. ©2014 AACR.

Introduction

Poly(ADP-ribose) polymerases (PARP) constitute a family of enzymes that use NAD⁺ to catalyze the addition of poly(ADP-ribose) chains to target proteins (1–3). Through this posttranslational modification, PARP enzymes regulate the function of a wide range of cellular responses. Recent identification and characterization of two family members, PARP5A (tankyrase 1) and PARP5B (tankyrase 2), have fueled increased interest in the field of PARP biology. These tankyrase (TNKS) isoforms share a high degree of identity and redundant functions (3, 4). TNKS differ from other PARP enzymes by the presence of ankyrin repeats that could mediate their interaction with multiple proteins (4–6). Several TNKS substrates have been reported that link TNKS to a variety of biologic roles including mitosis, telomere homeostasis, and proteasome regulation (4, 7–12). Studies from our group have also demonstrated that TKNS positively regulate Wnt signaling through PARsylation of AXIN1, leading to AXIN1 degradation and β-catenin stabilization (8).

Discovery of roles for TNKS in cellular processes involved in proliferation and survival has raised the possibility of using TNKS inhibitors as novel anticancer therapeutics. Highly selective and potent inhibitors of TNKS (TNKSi) have been developed and characterized in different cancer models (8, 13–17), including colorectal cancer in which constitutive Wnt activity is a major driver of tumor maintenance (18, 19). However, inhibition of TNKS is not sufficient to fully suppress Wnt signaling, resulting in only partial tumor growth inhibition (15). Several reports have also shown that the antitumor effect following TNKS inhibition is more robust under serum-deprived conditions (8, 15, 20, 21), raising the possibility that TNKS inhibition may be more effective when combined with other therapies. This idea is supported by two studies reporting that TNKS inhibition could alleviate resistance to EGFR inhibitors in non–small lung cancer cell lines and potentiates colorectal cancer cell response to PI3K/AKT pathway inhibitors (22, 23). Importantly, these two combination activities were shown to be associated with the role of TNKS in modulating Wnt signaling.

Here, we asked if we could identify additional combination partners of TNKSi using an unbiased screening approach. The discovery of key genes or pathways that cooperate with a TNKSi in suppressing tumor growth will not only bring additional insights on the biologic functions of TNKS, but will also more importantly provide basis for effective therapeutic combination strategies using TNKSi. In this study, we found a robust combination activity between TNKS and MEK inhibitors (MEKi) in multiple cancer cell lines and identified KRAS-mutant cells as the best responders. The combination activity correlates with the ability of TNKSi to release a feedback loop on FGFR2 signaling induced by MEK inhibition. Moreover, dual inhibition of TNKS and MEK induces cell death and tumor regression. Together, our results provide a strong rationale to test combination therapies with TNKSi and MEKi in KRAS-mutant cancers.

Materials and Methods

Cell lines

All cell lines were provided and cultured as recommended by American Type Culture Collection (ATCC). All lines were
authenticated by single-nucleotide polymorphism fingerprinting, and typically used within 20 passages.

Cell viability assay
Cell viability was determined by CellTiter-Glo Luminescence Assay (Promega). One day before compound addition, cells were seeded in triplicate in 96-well plates and incubated for three days with various concentrations of compounds. Luminescence was recorded on an EnVision plate reader (PerkinElmer) and the inhibition of viability relative to DMSO-treated cells was calculated.

High-throughput combination testing
Combination testing was performed using a large-scale compound screening platform (24). Cells were plated in triplicate into 384-well plates that have been arrayed with compounds serially diluted at five concentrations. Cell viability was determined as described above. Compound combination effects were scored relative to Loewe dose additivity using a weighted "Synergy Score" calculation (24).

Partition analysis
Two-population differential analyses were performed to discover features associated with synergy. The data used were derived from the Cancer Cell Line Encyclopedia project (25). The examined feature types included continuous value data such as gene expression, gene copy number and Amax derived from the Cancer Cell Line Encyclopedia project (25). The data used were scored relative to mutation and categorical data sets from mutation and such as gene expression, gene copy number and Amax. The examined feature types included continuous value data such as gene expression, gene copy number and Amax compound response, and categorical data sets from mutation and cell line lineage. Continuous and categorical data P values were calculated using a two-sided Wilcoxon test and a two sided Fisher exact test respectively. Multiple hypothesis adjustment of P values was done using the Benjamini–Hochberg approach within the context of each feature type. Only features with a FDR P < 0.05 were considered significant.

Apoptosis assay
Apoptosis was measured using the Caspase-Glo 3/7 assay (Promega). All conditions were done in triplicate.

SuperTopFlash assay
SuperTopFlash (STF) reporter containing 12 T-cell factor binding sites and luciferase sequence was cloned into pLenti6/V5-DEST (Life Technologies), and used to generate stable SW480-STF cells. Cells were plated in triplicate in 96-well plates. Luminescence was measured 24 hours after compound treatment using the Bright-Glo Luciferase Assay (Promega).

Immunoblotting
Immunoblot analysis was performed using standard methods. Cell pellets were lysed using Cell Signaling Technology Cell Lysis Buffer supplemented with EDTA-free protease inhibitor tablets (Roche) and phosphatase inhibitor cocktails (Sigma). Antibodies are listed in Supplementary Material.

siRNA transfection and sensitivity to AZD6244
SW480 cells were reverse transfected using Lipofectamine RNAiMAX reagent (Life Technologies). siRNAs were purchased from Thermo Scientific (sequences listed in Supplementary Material) and used at a 50 nmol/L final concentration. Two days after transfection, AZD6244 was added in increasing dose concentrations. Cell viability was measured 3 days later.

Microarray analysis
SW480 cells were treated in duplicate with DMSO, NVP-TK656, AZD6244 or the combination of both for 4 and 16 hours. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Gene expression profiling was performed using Affymetrix U133Plus2 Arrays and data were analyzed as in (26). Differential analysis was performed by computing fold changes of treatment to DMSO. The data have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus with the accession number GSE55624.

RNA extraction and quantitative RT-PCR
Real-time PCR was performed as described in (8). Probes for FGFR2 and the housekeeping gene GUSB (beta-glucuronidase) were purchased from Life Technologies. All experiments were performed in triplicates and normalized to GUSB transcript levels (comparative CT method).

Xenograft studies
Mice were handled in accordance with the Novartis Institutes for BioMedical Research (NIBR) Animal Care and Use Committee protocols and regulations. A total of 10 × 106 cells were inoculated subcutaneously into female athymic nude mice (20–25 grams; 6- to 8-weeks old; n = 7). Compound treatment started 10 days after implant when average tumor volume reached approximately 200 mm3. Animals were administered twice daily with vehicle, NVP-TK656 (100 mg/kg, subcutaneous injection) or AZD6244 (45 mg/kg, oral) for the duration of the study. Tumor volume was measured using calipers and calculated as (length × width × width)/2. Tumor growth inhibition was determined as the percentage change in tumor volume of treated over control animals (% T/C) at the end of the study. The percentage regression was determined as the percentage change in the final tumor volume over the starting tumor volume. Five days after the first treatment and 2 hours after the last dose, tumor tissues were excised and snap frozen in liquid nitrogen for biomarker analyses.

Results
Identification of TNKS and MEKi combination activity in KRAS-mutant cancer cells
To identify novel combination partners of TNKS in an unbiased fashion, we probed the dataset of a large-scale compound screen in which all pairwise combinations of 107 compounds were screened in 138 cancer cell lines representing mixed lineages and tumor types (manuscript in preparation). Each pairwise combination was tested at multiple concentrations using a matrix in which each drug was added either as single agent or in combination. Combination activity of compound pairs was measured after a 3-day treatment and compared with untreated controls (24). Significant synergy scores were determined on the basis of synergy score P value better than a formal significance of $P_{\text{sign}} = 0.001$, and $P_{\text{fdr}}$ (false
Figure 1. Identification of TNKSi and MEKi combination activity in a large-scale unbiased compound screen. A, combination effects of different compounds with TNKSi across 138 cancer cell lines. P < \text{P}_{\text{fdr}} \text{ and } P < \text{P}_{\text{signif}}, \text{ statistically significant}; P > \text{P}_{\text{signif}}, \text{ nonstatistically significant.} \text{ B, TNKSi/MEKi combination compared with other reported TNKSi combinations, including erlotinib, NVP-BKM120, and NVP-BYL719 that are inhibitors targeting EGFR, pan-PI3K and PIK3CA, respectively.} \text{ C, combination effects of different compounds with MEKi across 138 cancer cell lines.} (\text{Continued on the following page.})
discovery rate), the probability adjusted for multiple hypotheses using Benjamini–Hochberg with FDR = 0.01.

Among the different combinations tested containing a TKNSi, previously reported combinations between inhibitors of TNKS/EGFR and TKNSi/PIK3CA scored as synergistic combinations, thus validating the data generated by the screen (Fig. 1A and B). Interestingly, MEK1 was identified as the second most synergistic combination partner with TKNSi, after only dasatinib, a multi-kinase inhibitor (Fig. 1A). Of the 138 cancer cell models tested for TKNSi/Meki combination, 35 cell lines were identified with significant synergy scores (Supplementary Table S1). Importantly, dual inhibition of TKNSi and MEK led to a higher synergy score when compared with reported TKNSi combinations with EGFRi or PI3KCAi (Fig. 1B; refs. 22, 23). Because MEKis are being investigated in the clinic in multiple tumor types, we next asked how robust this novel TKNSi/MEKi combination was when compared with other known MEK combinations. Among the 107 combinations tested with MEKi, TKNSi was ranked among the top MEK combination partners (Fig. 1C). The overall synergy score between TKNSi/MEKi was similar to combinations between MEK and receptor tyrosine kinase (RTK) inhibitors, such as EGFRi (27), but superior to the reported combinations between MEK and PI3K (28), BCL-xL (29), or RAF inhibitors (Fig. 1D; ref. 30).

To explore in more detail which cancer models were more likely to respond to TKNSi/MEKi combination, we searched for genetic features that could associate with combination sensitivity. We separated cell lines that demonstrated strong combination activity (P < P_{\text{inf}}) from those that did not (P > P_{\text{inf}}), and performed partition analyses (25). We identified three significant features enriched in the sensitive group: colorectal cancer lineage, KRAS mutation, and partial sensitivity to MEKi (Fig. 1E and Supplementary Table S2). It is worth noting that both colorectal cancer lineage and KRAS mutation are associated with synergy to TKNSi/MEKi combination. Heat maps display values of significance features, each column representing a cell line and each row representing a feature. The effect size for the mutation and lineage features is the odds ratio of the features present in synergistic versus nonsynergistic cell lines. The effect size for each compound is the average of Amax values for synergistic cell lines minus the one for nonsynergistic cell lines. F, MEK combination effects in KRAS-mutant colorectal cancer lines. CRC, colorectal cancer.

Validation of the TKNSi and MEKi combination activity in KRAS-mutant cancer cells

To further validate the results of the compound screen, we carried out combination viability assays in a panel of 18 KRAS-mutant cancer cell lines of colorectal or pancreatic lineages using two highly selective compounds NVP-TNKS656 and AZD6244 to inhibit TKNSi and MEK activity respectively (Supplementary Table S4; refs. 17, 31). Cells were treated for three days with increasing doses of inhibitor alone or in combination. Synergy scores and isobolograms were generated to quantify the combination strength. A synergy score higher than 2 was considered as significant when compared with the variation of synergy scores seen within self-crosses (drug-within-self, theoretical synergy score of 0). Of the 18 lines tested, nine showed combination activity with a >2 synergy score (Fig. 2A and Supplementary Table S5). Dose matrices and isobolograms in five most synergistic and five negative cell lines are represented in Supplementary Fig. S2. It is important to note that only a fraction of the compound dilution curve was tested in the large-scale screen, leading to incomplete dose matrices, whereas in the validation studies full dose matrices were tested using a wider range of compound concentrations. This may account for the higher synergy scores obtained in our validation studies as compared with those measured in the screen. Altogether, data from both studies demonstrated that a subset of KRAS-mutant cancer cells exhibited strong response to TKNSi/MEKi combination.

Consistent in both studies, the colorectal cancer SW480 model showed the highest level of synergy in response to combination treatment (Fig. 2B and Supplementary Fig. S3A and S3B). In this cell line, treatment with MEKi led to a growth inhibition of approximately 50% at the highest concentration tested, whereas single TKNSi treatment had minimal effect in inhibiting cell growth. Combination of the two agents led to an enhanced antiproliferative effect at all doses tested (Fig. 2B, left). The corresponding isobologram demonstrates that the combination activity was the consequence of a strong synergy between both compounds rather than a dose-additive effect of the single agents, as illustrated by the blue curve of the combination response deviating strongly below the red line of additivity (Fig. 2B, right). In addition, we found that the TKNSi/MEKi combination activity was associated with marked induction of apoptosis as measured by caspase assay. Neither single agent altered caspase activity, whereas dual inhibition of TKNSi and MEK strongly induced an apoptotic response in a time-dependent manner (Fig. 2C).

To confirm the on-target effect of TKNSi, we asked whether depletion of TKNSi via genetic knockdown could phenocopy the effect of its pharmacologic inhibition. SW480 cells were transfected with nontargeting siRNA, single siRNAs targeting TKNSi1 or TKNSi2, or a mixture of both. We have previously
Figure 2. Validation of TNKSi and MEKi combination activity in KRAS-mutant cancer cells. A, synergy scores for all KRAS-mutant cell lines tested for TNKSi (NVP-TNKS656)/MEKi (AZD6244) combination after dose matrix viability assays. The red line represents a synergy score of 2, the chosen cutoff to select strongly synergistic lines. B, validation of TNKSi/MEKi activity in SW480 using viability assay. Left, dose matrix representing percentages of growth inhibition relative to DMSO. Right, isobologram illustrating the combination effect; blue, data points; red line, additivity. C, induction of apoptosis measured by the caspase assay. SW480 cells were treated for the indicated duration with DMSO or 1 μmol/L inhibitors. Data are represented as mean ± SEM. *, P < 0.01 by t test. D, validation of the on-target effect of TNKSi using nontargeting siRNA (siCtrl), siRNAs targeting TNKS1 (siTNKS1), TNKS2 (siTNKS2), or both (siTNKS1+2). Cell viability was measured after a 3-day treatment and is represented as mean ± SEM. *, P < 0.01 by t test. Right, relative mRNA expression of TNKS1 and TNKS2 at 2 days (d2) and 5 days (d5) after siRNA transfection, represented as mean ± SEM.
demonstrated that these siRNAs lead to specific TNKS targeting, and depletion of both TNKS1/2 is required to fully suppress the TNKS function (8). In this study, both siRNAs led to effective reduction of TNKS transcripts by 65% to 80% (Fig. 2D, right). Effect of TNKS depletion on cell viability was assessed following treatment with DMSO or MEKi. Cell growth was not significantly affected by depletion of either TNKS (Fig. 2D, MEKi at 0 μmol/L). Importantly, depletion of both TNKS but not of single isoforms enhanced the antiproliferative effect of MEKi at all concentrations, supporting that TNKS inhibition potentiated MEKi activity (Fig. 2D, left). In comparison, MEKi combination with the PARP1/2 inhibitor olaparib did not show any enhanced activity compared with single agents, further demonstrating the specificity of TNKS inhibition in the regulation of MEKi activity (Supplementary Fig. S3C).

In conclusion, our data strongly support a robust and specific combination activity between TNKSi and MEKi in a subset of KRAS-mutant cancer models.

TNKSi and MEKi synergy is independent of TNKS role in Wnt signaling

The TNKSi/MEKi combination activity in several colorectal cancer lines led us to ask whether modulation of Wnt pathway activity by TNKS inhibition was required for the synergy, as it was observed with two previously reported TNKSi combinations (22, 23).

We measured Wnt pathway activity in SW480 cells stably expressing a Wnt luciferase reporter (STF). Cells were treated with DMSO or inhibitors at 1 and 10 μmol/L (Fig. 3A). Inhibition of TNKS decreased Wnt signaling activity by approximately 20%, consistent with the previous findings that TNKS inhibition was not sufficient to fully block Wnt pathway activity in APC-mutant cancer cells (8, 15). Notably, MEK inhibition increased Wnt signaling by 30%, a result that might reflect a cross-talk between the MAPK and Wnt pathways (32, 33). As a consequence, the combined treatment did not further inhibit Wnt activity as compared with TNKSi alone.

TNKS inhibition leads to reduced Wnt pathway activity through AXIN stabilization and the consequent reduction in β-catenin levels (8). Therefore, we asked whether β-catenin depletion would recapitulate the effect of TNKS inhibition and sensitize cells to MEK inhibition. SW480 cells were transfected with nontargeting siRNA or two different siRNAs targeting β-catenin that have been validated in our previous studies (8). Cell viability was assessed after treatment with DMSO or increasing concentrations of MEKi. In contrast with what was observed with TNKS depletion (Fig. 2D), depletion of β-catenin had no effect on the cellular response to MEK inhibition (Fig. 3B and C).

In line with these data, the partition analysis did not identify enrichment of Wnt-related features (e.g., mutations in APC or β-catenin) in TNKSi/MEKi sensitive cell lines (Supplementary Table S2). We also did not observe any combination activity between MEKi and the Porcupine inhibitor NVP-LGK974 that blocks Wnt ligand secretion and signaling activity (Supplementary Fig. S3D; ref. 34).

Taken together, these results indicate that TNKS role in Wnt signaling is not required for TNKSi/MEKi combination activity.

Inhibition of TNKS potentiates MEKi activity by releasing a FGFR2 signaling feedback loop

In KRAS-mutant cancer cells, MEK inhibition can induce feedback mechanisms, resulting in enhanced PI3K/AKT signaling and resistance to MEKi treatment (28, 35–37). To
determine whether derepressed AKT activity might be linked to TNKSi/MEKi synergy, we compared the effects of TNKSi/MEKi and PI3KCAi/MEKi combinations on AKT signaling activity and cell proliferation (Supplementary Fig. S4A and S4B). MEK inhibition increased phosphorylated AKT levels as expected. Interestingly, dual inhibition of TNKS and MEK led to a more sustained inhibition of AKT signaling, increased apoptosis, and more robust synergy compared with PI3KCAi/MEKi combination, consistent with the large combination screen data (Fig. 1D).

The more marked synergy between TNKSi and MEKi raised the possibility that TNKS inhibition may potentiate MEKi activity via additional mechanisms. To investigate this, we analyzed the transcriptional response compared with DMSO in SW480 cells treated with TNKSi, MEKi, or both. To control for secondary effects arising from impaired cell proliferation, samples were collected at early time points (4 and 16 hours; Supplementary Fig. S5A). We observed that inhibition of TNKS alone did not significantly affect gene expression, whereas significant differences were observed at 16 hours after MEKi or TNKSi/MEKi treatment. To identify genes differentially expressed between single agents and the combination, we selected those with a significant fold change of expression in the combination compared with the additive effect of TNKSi and MEKi as single agents (Fig. 4A). This filtering analysis highlighted one interesting pattern of gene regulation that included a gene cluster upregulated after MEKi treatment but not in the combination. Of the five genes in this cluster, FGFR2 was of particular interest, as several RTKs have been implicated in feedback loops induced by MEK inhibition (27, 38). To validate the microarray results, we measured FGFR2 mRNA and observed that MEKi increased FGFR2 mRNA expression as early as 4 hours after treatment, and combined inhibition of TNKS and MEK blocked this upregulation by 16 hours (Fig. 4B). We observed similar responses for both FGFR2 isoforms, IIIb and IIc (Supplementary Fig. S5B).

To determine whether upregulation of FGFR2 transcript level was accompanied by increased FGFR2 protein and induction of downstream signaling, we measured protein levels of FGFR2 as well as phospho-FRS2 (pFRS2), a direct downstream effector of FGF receptors (Fig. 4C). MEKi treatment increased FGFR2 protein level that was reduced by the combination in a time-dependent manner. Consistent with the modulation of FGFR2 mRNA and protein levels, phosphorylation of FRS2 was induced by MEK inhibition, which was modestly decreased following the combination treatment at 24 hours (Fig. 4C), and

**Figure 4.** TNKSi potentiates MEKi activity by suppressing a FGFR2 signaling feedback loop. A, gene expression profiling of SW480 cells after 16 hours of treatment with DMSO or the indicated inhibitors at 1 μmol/L. Heat map shows genes expressed >2.8× in the combination group as compared with DMSO or single agents. Unbiased clustering was used to classify genes according to their expression level changes in each treatment condition. Genes upregulated with MEKi and suppressed by TNKSi/MEKi are highlighted on the right. B, relative mRNA expression of FGFR2 at 4 and 16 hours after treatment, represented as mean ± SEM. **P < 0.001 by t test. C, inhibitor effects on FGFR2 signaling in SW480 cells evaluated by immunoblot.**

www.aacrjournals.org Cancer Res; 74(12) June 15, 2014 OF7

Published OnlineFirst April 18, 2014; DOI: 10.1158/0008-5472.CAN-14-0138-T
more strongly by 48 hours (Fig. 6B). This regulation of FGFR2 protein and signaling was also observed after TNKS1/2 depletion (Supplementary Fig. S5C). Notably, MEKi-induced AKT signaling activity and its repression by the combination correlated with FGFR2 downregulation (Supplementary Fig. S5D). We also did not observe any changes in the expression levels of other RTKs following MEKi or combined treatment (Supplementary Fig. S5E).
Altogether, our findings suggest a novel feedback loop of cellular response to MEK inhibition through FGFR2 regulation. MEKi treatment leads to upregulation of FGFR2 expression and signaling that are suppressed by the addition of TNKSi. Downregulation of FGFR2 signaling by TNKS inhibitor correlates with TNKSi and MEKi combination activity

Following our findings in SW480, we examined whether the ability of TNKS inhibition to downregulate MEKi-induced FGFR2 and pAKT expressions were common mechanisms mediating the synergy in other cancer cell models. We analyzed FGFR2 mRNA expression as well as FGFR2 and AKT pathway activities in a larger panel of synergistic and nonsynergistic cell lines (Fig. 5 and Supplementary Fig. S6).

All cell lines tested, with the exception of SW403, showed an increase of FGFR2 expression at both mRNA and protein levels, as well as elevated pFRS2 following MEKi treatment (Fig. 5 and Supplementary Fig. S6). This indicates that upregulation of FGFR2 signaling could be a common feedback mechanism in response to MEK inhibition. What seemed to be distinct between the synergistic and nonsynergistic cell lines was their response to TNKSi/MEKi combination. In the four synergistic models tested, the combined treatment led to significant decreases in FGFR2 at both mRNA and protein levels, and correspondingly in pFRS2 (Fig. 5A and B and Supplementary Fig. S6A). In contrast, in the nonsynergistic cell lines, combination of MEKi and TNKSi showed no or little effect in reducing FGFR2 or pFRS2 levels (Fig. 5C and D). In addition, the synergistic cell lines showed a trend toward higher induction of apoptosis after inhibition of TNKS and MEK compared with the nonsynergistic ones, as measured by both increased expression of cleaved PARP and caspase activity (Fig. 5B and D and Supplementary Fig. S6C).

We also measured AKT signaling following inhibitor treatment (Supplementary Fig. S6A and S6B). In contrast with FGFR2 signaling, there was no consistent correlation between suppression of AKT pathway activity and sensitivity to TNKS/Meki combination, indicating that inhibition of PI3K/AKT signaling was not sufficient to drive synergy between TNKS and MEK inhibitors in these additional models.

Our results show that the ability of TNKSi to suppress MEKi-induced FGFR2 upregulation correlates with cancer cell sensitivity to TNKSi/MEKi treatment.

FGFR2 upregulation mediates resistance to MEKi treatment

We next asked whether inhibition of FGFR2 using a FGFR inhibitor (FGFRi) would potentiate MEKi activity as does TNKS inhibition. The two synergistic cell lines, SW480 and CL11, and the nonsynergistic cell line SW837 were treated with specific inhibitors (Supplementary Table S4), either as single agents or in combination. Cell growth was measured after a 3-day treatment. Our data showed that FGFRi treatment effectively blocked FGFR2 signaling and significantly enhanced MEKi activity only in the synergistic cell lines, supporting that activation of FGFR signaling could be a resistance mechanism to MEK inhibition (Fig. 6A). The TNKSi/MEKi combination
showed a more robust activity compared with the FGFRi/MEKi in the synergistic cell lines, possibly due to higher induction of apoptosis as measured by PARP cleavage (Fig. 6A and B).

In conclusion, our data support that FGFR2 signaling is a novel resistance mechanism to MEK inhibition, and suppression of this upregulation leads to enhanced cancer cell response to MEKi treatment.

**TNKSi and MEKi combination leads to enhanced antitumor activity in vivo**

Having observed a robust synergy for TNKSi/MEKi combination in vitro, we examined whether this effect could be observed in vivo. Mice bearing SW480 xenograft tumors were treated twice daily with vehicle, single agents, or the combination. Tumor growth was monitored for 14 days (Fig. 7A). Consistent with a lack of cellular efficacy, TNKSi treatment did not show significant effect on tumor growth. In agreement with in vitro results, MEKi led to a significant but incomplete tumor growth inhibition (T/C = 19%), whereas dual inhibition of TNKS and MEK led to tumor regression (Regression = 29%). We also measured the tumor pharmacodynamics response to inhibitor treatment. TNKSi and MEKi treatment led to AXIN stabilization and decreased ERK phosphorylation, confirming target inhibition in vivo (Fig. 7B). More importantly, MEK inhibition led to an induction of FGFR2 mRNA expression, which was abrogated by the combined treatment, similar to what has been observed in vitro (Fig. 7C).

This result demonstrated that the TNKSi/MEKi combination activity could be recapitulated in the SW480 model in vivo, showing tumor regression and suppression of FGFR2 feedback.

**Discussion**

Alterations in key signaling pathways regulating cell proliferation and survival are a hallmark of cancer. Better understanding of the genetic abnormalities that drive tumor formation and progression has brought more effective targeted therapeutics. However, despite the development of highly selective inhibitors targeting these genetic drivers, the efficacy of single-agent therapies has been hampered by tumor heterogeneity and development of resistance (39, 40). Delineation of these mechanisms is necessary to pave the way for rationally designed and more effective combination therapies. In this report, through both unbiased large-scale compound combination screen and mechanistic delineation, we demonstrated a novel and robust combination activity between TNKS and MEK inhibitors via modulation of FGFR2 signaling in a subset of RAS-mutant cancer cells.
RAS mutations occur at high frequency in cancers, notably colorectal, pancreatic, and lung lineages (41). However, targeting RAS directly has been met with drugability challenges. Inhibition of MEK, the most proximal downstream effector of RAS, has not shown the anticipated antitumor activity in mutant RAS tumors, due to cross-talks with other signaling pathways and feedback mechanisms. Many efforts have been made to understand the mechanisms of resistance and to develop more robust targeted therapies for these cancers, as exemplified by promising results seen in several KRAS-mutant cancer models using combination of MEKi with PIK3CA or BCL-xl inhibitors (28, 29, 36). Nevertheless, this is still not curative; combined inhibition of MEK and PIK3CA leads to tumor stasis rather than regression and shows toxicity at efficacious doses (36, 42). Our study has led to the identification of a novel combination activity between TNKS and MEK inhibitors that lead to more robust anticancer activities than the aforementioned MEKi combinations. We plan to further assess the efficacy of TNKSi/MEKi combination in vivo using xenograft models of primary tumors derived from patients with colorectal cancer carrying oncogenic KRAS mutations.

Our data show that TNKSi/MEKi combination activity does not involve TNKS role in modulating Wnt signaling activity. Our findings in multiple KRAS-mutant models rather highlight a novel function of TNKS in relieving a feedback loop induced by MEK inhibition on FGFR2 signaling cascade. It remains to be determined how TNKS inhibition leads to suppression of FGFR2 transcription. The molecular mediators of TNKS effect on FGFR2 expression, including the potential role of TNKS substrates in this pathway, await future studies. A better understanding of this new role of TNKS could help to further dissect TNKS biology and bring opportunities to target FGFR2-dependent cancers and other diseases.

The more robust synergy observed between TNKSi and MEKi combination may be attributed to a stronger induction of cell death. MEK inhibition upregulates the expression of the proapoptotic protein BIM, providing rationale for combination between BCL-xl and MEK inhibitors in KRAS-mutant cells (29, 43). We also observed that MEKi treatment increased BIM expression in the cellular models tested. However, we did not observe any effect of TNKSi on BIM, MCL-1, or BCL-xl expression or interaction. We are currently exploring whether other mediators in the apoptotic pathway may be modulated by TNKSi/MEKi combination.

In conclusion, our work has identified a novel feedback regulatory mechanism upon MEKi treatment in KRAS-mutant cells through induction of FGFR2 expression and signaling. More importantly, TNKS inhibition greatly potentiates MEKi activity by relieving this feedback loop in a subset of KRAS-mutant cancer cells. Overall, our findings not only provide an additional therapeutic opportunity for treating KRAS-mutant tumors, but also shed new light on TNKS biology.

Disclosure of Potential Conflicts of Interest
W.R. Sellers is employed as Vice President/Global Head of Oncology at Novartis Pharmaceuticals and has ownership interest (including patents) in Novartis Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: M. Schoumacher, K.E. Hurov, W. Shao
Development of methodology: M. Schoumacher, K.E. Hurov, J. Lehár, Y. Mishina, W. Shao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Schoumacher, K.E. Hurov, Y. Yan-Neale, Y. Mishina, D. Flemming, M.D. Jones, B. Antonakos, V.G. Cooke, J. Steiger, J. Ledell, M.D. Stump
Analysis and interpretation of data (e.g., statistical analysis, biosciences, computational analysis): M. Schoumacher, K.E. Hurov, J. Lehár, Y. Mishina, D. Sonkin, J.M. Korn, D. Flemming, M.D. Jones
Writing, review, and/or revision of the manuscript: M. Schoumacher, K.E. Hurov, M.D. Stump, W.R. Sellers, N.N. Danial, W. Shao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Schoumacher
Study supervision: M. Schoumacher, W.R. Sellers, W. Shao

Acknowledgments
The authors thank Thomas Horn-Spirohn for sharing data and Yun Feng, HoMan Chan, Frank Stegmeier, Earl McDonald III, and Nicolas Keen for inputs and discussions.

Grant Support
All studies were funded by Novartis Institutes for BioMedical Research (NIBR). M. Schoumacher is a recipient of the presidential postdoctoral fellowship from NIBR.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 22, 2014; revised March 13, 2014; accepted April 7, 2014; published OnlineFirst April 18, 2014.

References
Cho-Park PF, Steller H. Proteasome regulation by ADP-ribosylation.


Inhibiting Tankyrases Sensitizes KRAS-Mutant Cancer Cells to MEK Inhibitors via FGFR2 Feedback Signaling

Marie Schoumacher, Kristen E. Hurov, Joseph Lehár, et al.

Cancer Res  Published OnlineFirst April 18, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0138-T

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/04/22/0008-5472.CAN-14-0138-T.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.