TANKYRASE Inhibition Enhances the Antiproliferative Effect of PI3K and EGFR Inhibition, Mutually Affecting β-CATENIN and AKT Signaling in Colorectal Cancer


Abstract

Overactivation of the WNT/β-CATENIN signaling axis is a common denominator in colorectal cancer. Currently, there is no available WNT inhibitor in clinical practice. Although TANKYRASE (TNKS) inhibitors have been proposed as promising candidates, there are many colorectal cancer models that do not respond positively to TNKS inhibition in vitro and in vivo. Therefore, a combinatorial therapeutic approach combining a TNKS inhibitor (G007-LK) with PI3K (BKM120) and EGFR (erlotinib) inhibitors in colorectal cancer was investigated. The data demonstrate that TNKS inhibition enhances the effect of PI3K and EGFR inhibition in the TNKS inhibitor–sensitive COLO320DM, and in the nonsensitive HCT-15 cell line. In both cell lines, combined TNKS/PI3K/EGFR inhibition is more effective at reducing growth than a dual TNKS/MEK inhibition. TNKS/PI3K/EGFR inhibition affected in a context-dependent manner components of the WNT/β-CATENIN, AKT/mTOR, EGFR, and RAS signaling pathways. TNKS/PI3K/EGFR inhibition also efficiently reduced growth of both COLO320DM and HCT-15 tumor xenografts in vivo. At the highest doses, tumor xenograft growth was halted without affecting the body weight of the tested animals.

Implications: Combining TNKS inhibitors with PI3K and EGFR inhibition may expand the therapeutic arsenal against colorectal cancers.

Introduction

Colorectal cancers are some of the most common cancers worldwide and are frequently initiated by mutations in the adenomatous polyposis coli (APC) tumor suppressor gene or the gene encoding β-CATENIN (CTNNB1; ref. 1). Subsequent mutations in KRAS and TP53 and deregulation of signaling pathways like PI3K/PROTEIN KINASE B (AKT) and TGFβ are further hallmarks for colorectal cancer development and progression (2, 3).

Targeted therapy against a number of molecular targets is being explored for treatment of colorectal cancer. EGFR is an upstream regulator of two central pathways: the MAPK pathway and the PI3K/AKT pathway. Both pathways regulate cellular proliferation, migration, differentiation, and apoptosis (4, 5). Several inhibitors targeting EGFR have been developed, including erlotinib (6) and gefitinib (7), and a number of PI3K inhibitors are in clinical studies (8, 9). TANKYRASE (TNKS) is a central cytoplasmic biotarget in the WNT/β-CATENIN signaling pathway, where it controls the turnover of AXIN1/2 and thereby prevents degradation of β-CATENIN (10–12). Experimental TNKS inhibitors, such as XAV939 and G007-LK, have gained increasing attention as inhibitors for WNT induced colorectal cancer (10, 12). However, despite long-term tolerability of TNKS treatment in mice (13), the advance of TNKS inhibitors to clinical trials is currently hampered by cytotoxicity issues (12, 14).

Multiple interactions have been mapped between the WNT, PI3K/AKT, and EGFR signaling pathways. In lung cancer cells, TNKS activity was shown to protect cells from EGFR inhibition, an effect that was counteracted by combined TNKS/EGFR inhibition (15). In colorectal cancer, high levels of nuclear β-CATENIN confer resistance to apoptosis-inducing signals mediated by PI3K/AKT inhibition, an effect that can be reversed by exposure to TNKS inhibition (16). Furthermore, depending on the mutational background, EGFR, PI3K in addition to MEK inhibitors have shown to enhance the effect of TNKS inhibitors in colorectal cancer cell lines (17).

In this study, we use the highly specific TNKS 1/2 inhibitor G007-LK (18), the pan-class I PI3K inhibitor BKM120 (8), and the EGFR inhibitor erlotinib (6) to investigate the effect of single and combined inhibition in two representative colorectal cancer cell lines. Using TNKS inhibitor–sensitive COLO320DM, and insensitive HCT-15, we show that TNKS inhibition can be potentiated by EGFR and PI3K inhibition both in vitro and in vivo. At the tested doses, combined TNKS/PI3K/EGFR inhibition was more efficacious than combined TNKS/MEK inhibition in vitro.

Materials and Methods

Cell culture

COLO320DM, COLO205, HCT-8, and HCT-15 cells were cultured in RPMI1640 (Sigma-Aldrich), WiDr, and RKO in Eagle...
Minimum Essential Medium (LGC standards), containing 10% FBS, and 1% penicillin/streptomycin at 37°C and 5% CO2. SW403 and SW480 cells were cultured in Leibovitz’s L-15 with l-glutamine (Sigma-Aldrich), 10% FBS, and 1% penicillin/streptomycin at 37°C and 0% CO2. Cell lines were obtained from ATCC and routinely tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza). Cell line authentication was performed on COLO320DM, HCT-15, SW403, and SW480 cell lines.

Inhibitors
Inhibitors were dissolved in DMSO (Sigma-Aldrich), which was also used as vehicle control. The following inhibitors were used: G007-LK (ChemRoyal), BKM120 (NVP-BKM120; Chemietek), API-2 (TOCRIS), and GDC-0941 (Selleckchem), erlotinib (Chemietek), gefitinib (Selleckchem), and GDC-0973 (MedChem Express).

Incucyte proliferation assay
A total of 1,000 COLO320DM or HCT-15 cells, 7,000 SW403 cells, or 1,200 SW480 cells were seeded in 96-well plates (minimum 3 replicates). The next day, culture media were replaced with drug-containing media. Cell confluence was quantified with the Incucyte live-cell analysis system (Essen BioScience).

Cell-cycle analysis
After 72 hours of treatment, cells were fixed in EtOH for 2 hours at −20°C, and propidium iodide (PI) for 30 minutes at room temperature. Flow cytometric analysis (Attune), at least 10,000 cells were analyzed.

Apoptosis analysis
After 72 hours of treatment, trypsinized cells were incubated in Annexin V binding buffer containing 1:500 Annexin V-FTTC, Apoptosis Detection Reagent (abcam) and PI for 5 minutes prior to flow cytometric analysis (Attune).

Immunoblotting
After 72 hours of treatment, total protein extracts were obtained with RIPA buffer (Millipore), and nuclear/cytoplasmic protein fractions were obtained with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Equal amounts of protein were denatured, separated on SDS-PAGE gels (Bio-Rad Laboratories), and transferred to polyvinylidene difluoride membranes (Millipore). After blocking in 5% skim milk (AppliChem)/TBS-T membranes were probed with primary antibody overnight at 4°C. Following secondary antibody incubation, proteins were visualized with chemiluminescent substrate (ECL prime Western Blotting Detection Reagent, Sigma-Aldrich).

esiRNA-mediated knockdown
A total of 8 × 10⁴ COLO320DM and 5 × 10⁴ HCT-15 cells were transfected with 50 nmol/L EGFP (EHUJEGFP, Sigma) or TP53 (EHUJ123221, Sigma) esiRNA using Lipofectamine RNAiMax Transfection Reagent (Thermo Fisher Scientific, 13778075) in culture medium without antibiotics.

qRT-PCR
After 72 hours of treatment, total RNA was obtained using the GenElute Miniprep Kit (Sigma-Aldrich). cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Life Technologies).

Xenografts
Female CB17SCID mice were implanted subcutaneously with 4 × 10⁶ cells. After tumor development, animals were randomized, 10 mice per group, and desired treatment was administered orally as described in details in Supplementary Materials and Methods. Animal experiments were approved by local ethics authorities at Norwegian Food Safety Authority (Norway) or Ethics Committee for Animal Experimentation (Germany) and carried out following accepted ethical standards.

Results
G007-LK enhances the effect of PI3K/AKT and EGFR inhibition on growth reduction in colorectal cancer cells
To investigate the benefit of combining TNKS inhibition with PI3K and EGFR signaling inhibition in human colorectal cancer, four APC-mutant colorectal cancer cell lines were initially selected: COLO320DM and SW403 (TNKS inhibitor sensitive), HCT-15, and SW480 (TNKS inhibitor insensitive). Somatic mutations are listed in Supplementary Fig. S1. Inhibitor doses providing moderate growth inhibition in COLO320DM were selected (1 μmol/L G007-LK, 0.5 μmol/L BKM120, and 5 μmol/L erlotinib) and used on all cell lines (dose–response curves in Supplementary Fig. S2). Results regarding SW403 and SW480 cell lines are reported in Supplementary Fig. S3–S9).

At the selected doses, G007-LK significantly reduced growth in COLO320DM (Fig. 1A; Supplementary Fig. S10), while HCT-15 cells were insensitive to single G007-LK treatment (Fig. 1B; Supplementary Fig. S10). Both BKM120 and erlotinib significantly reduced growth in both COLO320DM and HCT-15 cell lines. Combining G007-LK with either BKM120 or erlotinib further reduced proliferation in COLO320DM. In HCT-15, G007-LK

Statistical analysis
SigmaPlot11 (Systat Software Inc.) was used to perform statistical analyses. P < 0.05 was regarded as a statistically significant difference when using Student t test and Mann–Whitney rank sum tests. Gene probes, antibodies, and further details are listed in Supplementary Materials and Methods.
insignificantly enhanced the effect of BKM120 and erlotinib. BKM120 enhanced the effect of erlotinib treatment in COLO320DM, while in HCT-15, the effect was highly augmented. Adding G007-LK to BKM120/erlotinib further reduced proliferation both in COLO320DM and HCT-15 cells, leading to a total of 83% and 92% growth reduction compared with control, respectively. Biotarget-specific inhibition was confirmed with alternative PI3K/AKT (GDC-0941 and API-2; Supplementary Fig. S7) and EGFR (gefitinib, Supplementary Fig. S8) inhibitors. The combined treatment effect of G007-LK/BKM120/erlotinib was further confirmed in COLO205, HCT-8, WiDr, and RKO colorectal cancers (Supplementary Fig. S11).

MEK inhibition has been identified as a sensitizing factor for TNKS inhibition, in particular in KRAS-mutant colorectal cancer (17) and was therefore compared with the G007-LK/BKM120/erlotinib treatment regime. MEK inhibition (1 μmol/L GDC-0973) slightly (not significant) enhanced growth in KRAS wild-type COLO320DM cells and moderately (significant) reduced growth in KRAS-mutant HCT-15 cells (Fig. 1C and D). Furthermore, GDC-0973 significantly sensitized HCT-15 cells to G007-LK treatment, and G007-LK significantly sensitized COLO320DM cells to GDC-0973 treatment. However, combined G007-LK/BKM120/erlotinib treatment was more effective on growth inhibition than combined G007-LK/GDC-0973 treatment at the selected doses in both cell lines, regardless of the KRAS mutation status (molecular responses in Supplementary Fig. S12).

Combination of inhibitors reduces the colony-forming ability of colorectal cancer and induces molecular characteristics of differentiation

The colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. We tested the colony-forming ability of the colorectal cancer in the presence of inhibitors in media containing low serum (1% FBS). COLO320DM cells showed reduced ability to form colonies in response to inhibitor treatments (not significant with G007-LK), while both G007-LK and BKM120 significantly enhanced erlotinib-mediated colony reduction (Fig. 2A; Supplementary Fig. S9). In accordance with the proliferation assay, combined G007-LK/BKM120/erlotinib reduced colony numbers to 2% compared with control. In contrast, HCT-15 cells showed increased colony formation with single inhibitor treatments (significantly with erlotinib; Fig. 2B; Supplementary Fig. S9). Combining G007-LK with BKM120 significantly enhanced the numbers of colonies, while combining G007-LK with erlotinib attenuated erlotinib-mediated induction in colony-forming ability. Only combined BKM120/erlotinib treatment reduced colony formation compared with the control, and a significant reduction to 35% of the control was seen with G007-LK/BKM120/erlotinib treatment.

In both cell lines, combined G007-LK/BKM120/erlotinib treatment most efficiently reduced both proliferation and colony formation.

As the colony formation may be influenced by the expression of stem cell and differentiation markers, we analyzed the expression of LEUCINE-RICH REPEAT-CONTAINING HETEROTRIMERIC GLIANYNE NUCLEOTIDE-BINDING PROTEIN-COUPLED RECEPTOR 5 (LGR5), a marker for colon crypt stem cells (19), and the differentiation marker CYTOKERATIN 20 (KRT20). In COLO320DM, G007-LK induced molecular characteristics of differentiation by reducing LGR5 and increasing KRT20 expression (Fig. 2C). BKM120 did not affect expression of either marker, while erlotinib enhanced LGR5 and reduced KRT20 expression, supposedly moving the colorectal cancer cells toward a more stem-like character. G007-LK counteracted the effect of erlotinib, increasing the expression of KRT20 and moderating LGR5 expression.

In HCT-15 cells, G007-LK strongly induced KRT20 and reduced LGR5 expression, while both BKM120 and erlotinib induced expression of both markers (Fig. 2D). G007-LK attenuated the BKM120 and erlotinib induced LGR5 induction and massively increased KRT20 levels (243.5-fold with G007-LK/BKM120/erlotinib treatment).

In conclusion, G007-LK most effectively induced molecular characteristics of differentiation in both cell lines, while erlotinib...
AKT signaling pathways (21, 22), and are required for the G1 phase transition (23, 24). Induced CMYC levels may also inhibit expression of CYCLIN-DEPENDENT KINASE 1A (CDKN1A) and 1B (CDKN1B) genes (22), which are negative regulators of cell-cycle progression from G1- to S-phase through inhibition of CCND1 expression (25).

In COLO320DM, neither G007-LK nor BKM120 alone significantly changed the number of Annexin V-positive (apoptotic) cells, but when combined, apoptosis was significantly induced compared with control (Fig. 3A). Apoptosis could not be related to nuclear levels of β-Catenin and FOXO3a (Supplementary Fig. S13; ref. 16). Single erlotinib treatment significantly induced apoptosis, which was further increased in combination with BKM120, but not with G007-LK. Combined BKM120/erlotinib treatment most potently induced apoptosis (>3.5-fold), an effect that was attenuated by G007-LK.

In COLO320DM, G007-LK treatment significantly increased the number of G1-phase cells (Fig. 3B; Supplementary Fig. S14), indicative of cell-cycle arrest and consistent with moderate growth inhibition. This was supported by reduced expression of CCND1 and CMYC, and increased expression of CDKN1A and CDKN1B (Fig. 3C; Supplementary Fig. S14). The tendency to more G1-phase cells with BKM120 and erlotinib became statistically relevant when each was combined with G007-LK. G1-phase increase was accompanied by fewer S-phase cells, reduced expression of CMYC and CCND1, and increased expression of CDKN1A and CDKN1B. Combining G007-LK/BKM120/erlotinib significantly enhanced numbers of cells in G1-phase. Also, the differential effect on CCND1, CMYC, CDKN1A, and CDKN1B expression was most prominent with G007-LK/BKM120/erlotinib treatment. Protein levels of CCND1 and CMYC reflected transcript expression (Fig. 3D).

In HCT-15 cells, apoptosis was not significantly changed with any combinations of inhibitor treatments, although G007-LK treatment showed a tendency toward more apoptosis (Fig. 3E). G007-LK treatment did not affect cell-cycle distribution (Fig. 3F; Supplementary Fig. S14), reflecting the lack of growth reduction. Erlotinib significantly increased the number of cells in the G1-phase compared with control when combined with either G007-LK or BKM120, reflected by a significantly increased CDKN1A and CDKN1B expression (Fig. 3G; Supplementary Fig. S14). The impact of inhibitors on CMYC and CCND1 expression was more complex. Both CMYC and CCND1 transcription was increased by both BKM120 and erlotinib treatment, while G007-LK reduced their expression both alone and in combination with BKM120 and/or erlotinib. The protein levels of CCND1 and CMYC reflected well their transcript levels (Fig. 3H).

P53 is a tumor suppressor known to be involved in many cancer-relevant functions, including growth arrest, apoptosis, and colony formation (26), presumably through interactions with CMYC (27, 28). Both COLO320DM and HCT-15 cells carry TP53 mutations (Supplementary Fig. S1), which compromise their ability to induce G1 arrest through enhanced CDKN1A expression. Inhibitor treatments, in particular when combined, significantly increased TP53 expression in COLO320DM (Supplementary Fig. S14). However, this was not reflected by P53 protein levels which remained unaltered (Fig. 3D).

In HCT-15 cells, TP53 expression levels were significantly increased by both single and combined BKM120 and erlotinib treatment and attenuated by G007-LK (Supplementary Fig. S14).
Inhibitor-mediated changes in proliferation and colony-forming ability were reflected by changes in cell-cycle distribution and apoptosis through an altered balance between cell-cycle genes. Fold induction of apoptotic (Annexin V+) cells after incubation with inhibitors as indicated in COLO320DM (A) and HCT-15 (E) cells compared with DMSO control treatment. Changes in percentage of cells in G1 phase of the cell cycle upon inhibitor treatments in COLO320DM (B) and HCT-15 (F) cells. Expression levels of CCND1, and CDKN1A upon incubation with inhibitors as indicated in COLO320DM (C) and HCT-15 (G) cells. Gene expression is normalized to internal GAPDH levels. Data represent mean relative expression values (±SD) of 3 independent experiments, each with 3 technical replicates. Representative regulations in CCND1, CMYC, and P53 protein levels are shown in COLO320DM (D) and HCT-15 (H). *, P < 0.05 by two-tailed t test; **, P < 0.01 by one-tailed t test. Complete statistical analysis is found in Supplementary Statistics.

TP53 expression levels were well reflected by P53 protein levels (Fig. 3H).

An esiRNA-mediated knockdown of TP53 did not change growth response to inhibitors in COLO3230DM cells. In contrast, HCT-15 cells showed a statistically relevant enhancement in their response to combined G007-LK/BKM120 and G007-LK/erlotinib treatment with TP53 knockdown (Supplementary Fig. S15).

Taken together, in COLO320DM cells, combined inhibitor treatments reduced proliferation and colony formation accompanied by a combination of reduction in CMYC and CCND1 protein levels, enhanced expression of CDKN1A and CDKN1B cell-cycle regulators, and induction of apoptosis. In HCT-15 cells, the colony-forming ability was reflected by changes in CCND1 and CMYC protein levels, while proliferation was reflected by changes in CMYC, and restrained by upregulated P53.

**Inhibitor effects on WNT signaling pathway components**

Next, we investigated how inhibitor treatments affected their respective biotargets, and the WNT/β-catenin and AKT/mTOR signaling pathways. G007-LK exposure may either stabilize or destabilize its primary biotarget TNKS1/2 in a contextual way that is currently poorly understood (10, 11). In COLO320DM and HCT-15, protein levels of TNKS1/2 were stabilized upon G007-LK treatment (Fig. 4A and B). Furthermore, AXIN1 and AXIN2, which are structural proteins in the WNT/β-Catenin destruction complex, were stabilized. BKM120 selectively stabilized AXIN1 (although less powerful than G007-LK) in COLO320DM and HCT-15, while it did not affect AXIN2. Erlotinib did not stabilize either of the two AXIN proteins. AXIN2 stabilization was maximal by single G007-LK treatment, while both BKM120 and erlotinib reduced G007-LK–mediated AXIN2 stabilization.
β-CATENIN is the key transmitter of the canonical WNT signaling pathway, and its stability is regulated by the destruction complex (29). Active GSK3β targets β-CATENIN for ubiquitination and degradation by the proteasome by N-terminal phosphorylation of β-CATENIN (at serine 33/37 and threonine 41). GSK3β also phosphorylates AXIN1/2, enhancing its binding to β-CATENIN and further stabilizing the β-CATENIN destruction complex (30, 31). The activity of GSK3β is regulated by several pathways, including AKT signaling (32), which inactivates GSK3β through phosphorylation of serine 9 (pGSK3β(S9)). In COLO320DM, both G007-LK and BKM120 reduced the level of inactive pGSK3β(S9) (Fig. 4C). However, only G007-LK treatment, which also stabilized AXIN2, reduction of pGSK3β(S9) led to increased N-terminal phosphorylation (S33/S37/T41) of β-CATENIN and reduced levels of both total and non-phospho(activ)β-CATENIN (ABC). Reduction of pGSK3β(S9) by BKM120 (without AXIN2 stabilization) did not increase N-terminal phosphorylation of β-CATENIN. This is in line with reports showing that GSK3β activation through AKT inactivation does not affect canonical WNT signaling (33). Under all conditions, the levels of pβ-CATENIN(S33/S37/T41) corresponded inversely with the levels of total β-CATENIN, indicating that the pβ-CATE- NIN(S33/S37/T41) was targeted for degradation. Intriguingly, upon combining G007-LK/BKM120/erlotinib treatment, N-terminal phosphorylation of β-CATENIN was massively increased, accompanied by a strong reduction of both total, ABC, C-terminal phosphorylated β-CATENIN, and nuclear β-CATENIN (Fig. 4C, Supplementary Fig. S16). This effect was seen despite reduced AXIN2/1 stabilization.

In HCT-15 cells, neither G007-LK, BKM120, nor erlotinib single treatment affected pGSK3β(S9) levels (Fig. 4D), although we observed a mild increase of pβ-CATENIN(S33/S37/T41) levels by G007-LK, which, despite massive stabilization of AXIN1/2, was not reflected by either the total, ABC, C-terminal phosphorylated, or nuclear levels of β-CATENIN (Fig. 4D; Supplementary Fig. S16). Only when G007-LK was combined with BKM120 or erlotinib, levels of pGSK3β(S9) were moderately reduced, pβ-CATENIN(S33/S37/T41) increased, and the total levels of β-CATENIN were reduced. Hence, in HCT-15 cells, BKM120 and erlotinib acted as enabling factors for the destabilization of β-CATENIN by G007-LK. In conclusion, inhibition of TNKS by G007-LK alone activated GSK3β and reduced the level of β-CATENIN in COLO320DM, while in HCT-15 cells, a combination of inhibitors was needed.

Nuclear β-CATENIN is a direct regulator of AXIN2 transcription in a negative feedback loop (34, 35). Nuclear levels of β-CATENIN should therefore positively correlate to AXIN2 transcription. In both COLO320DM and HCT-15 cells, nuclear levels of ABC correlated with total levels of ABC under all conditions (Fig. 4C and D). In COLO320DM cells, reduced AXIN2 expression correlated well with G007-LK-mediated reduction of nuclear ABC (Fig. 4C and E). Interestingly, also in HCT-15 cells, G007-LK led to a reduction of AXIN2 transcripts, despite the absence of altered nuclear ABC levels (Fig. 4D and F). In HCT-15 cells, the levels of ABC were therefore not predictive for AXIN2 transcription. Previous studies have shown that nuclear levels of AXIN2 negatively regulate WNT/β-CATENIN signaling by binding to the β-CATENIN/TCF complex (36).

In COLO320DM, nuclear AXIN2 levels correlated positively with total levels of AXIN2, while negatively with nuclear ABC (Fig. 4A and C). Hence, COLO320DM cells exhibited a G007-LK

![Figure 4](image-url)
Inhibitor effects on AKT/mTOR and EGFR signaling pathway components

Inhibition of PI3K signaling by BKM120 should prevent downstream activation of AKT (8). In both COLO320DM and HCT-15 cells, BKM120 significantly reduced activated AKT (pAKT(S473)), while total AKT levels were unaffected (Fig. 5A and B). Interestingly, in COLO320DM cells, but not in HCT-15 also, G007-LK moderately reduced pAKT(S473). Oppositely, erlotinib enhanced pAKT(S473) levels in both cell lines. This was unexpected as PI3K signaling is downstream of EGFR. In both cell lines, combined G007-LK/BKM120/erlotinib treatment reduced pAKT(S473) levels most effectively.

mTOR is a downstream target of AKT that affects mRNA translation through regulation of the S6 RIBOSOMAL PROTEIN (S6RP). In COLO320DM, all inhibitors reduced the active form of S6RP (pS6RP(S240/244)), and the active form of its regulator P70S6K (pP70S6K(T389); Fig. 5C). P70S6K activity was further inhibited by combined inhibitor treatments, in particular by G007-LK/BKM120/erlotinib treatment. As G007-LK affected both β-CATENIN and AKT/mTOR signaling in COLO320DM, the reduced proliferation and colony formation by this inhibitor is likely to be impacted by the combined alteration of both pathways. In contrast, BKM120 led to an inhibition of the AKT/mTOR signaling pathway only, while erlotinib enhanced AKT signaling and reduced mTOR activity. Inhibitor combinations reliably reduced both pathways.

In HCT-15 cells, BKM120 and erlotinib reduced active P70S6K and S6RP (Fig. 5D). When combined, their effect on pS6RP(S240/244) was highly potentiated and reflected the significant reduction in cell proliferation and colony formation. G007-LK treatment, although clearly stabilizing AXIN1/2, did not affect the levels of either protein. Hence, TNKS inhibition appeared to be uncoupled from impacting both the AKT/mTOR and WNT/β-catenin signaling in HCT-15 cells. Despite enhanced AKT activation with erlotinib in both cell lines, both active P70S6K and S6RP were reduced. This suggests that AKT and mTOR were differentially regulated with erlotinib treatment as compared with G007-LK and BKM120 treatment, where both AKT and P70S6K/S6RP activity were equally regulated.

Erlotinib affects EGFR activity by preventing receptor dimerization, reliably affecting phosphorylation of tyrosine 1068 (pEGFR(1068); ref. 37). Because of low expression of EGFR in COLO320DM (38, 39), only weak bands of pEGFR(1068) were obtained even with high protein concentrations (Fig. 5E, bottom). As expected, pEGFR(1068) was reduced with erlotinib, while it was unaffected by G007-LK, and enhanced by BKM120. In HCT-15 cells, pEGFR(1068) was strongly reduced by erlotinib treatment, while both G007-LK and BKM120 induced pEGFR(1068) protein level (Fig. 5F), which was further increased when the two were combined. Increased pEGFR(1068) was efficiently attenuated by erlotinib treatment. In conclusion, combined G007-LK/BKM120/erlotinib treatment reduced expression of hallmark proteins of both β-CATENIN and AKT/mTOR signaling pathways in both COLO320DM and HCT-15 cells, and EGFR activity in HCT-15 cells.

GSK3β is a protein functionally linking AKT and WNT signaling and has been implied in the TNKS/AXIN effect on RAS signaling (32, 40). As we observed that TNKS inhibition affected both

Figure 5.

Effect of G007-LK, BKM120, and erlotinib treatment on AKT/mTOR and EGFR signaling pathway components. Protein levels of total and active AKT (phospho-serine 473 AKT; P-AKT) in COLO320DM (A) and HCT-15 (B) cells. Protein levels of total and active P70S6K (phospho-tyrosine389-P70S6K; P-P70S6K) and total and active S6RP (phospho-serine240/244-S6RP; P-S6RP) in COLO320DM (C) and HCT-15 (D) cells. Protein levels of total and active EGFR (phospho-tyrosine 1068 EGFR; P-EGFR) protein levels in COLO320DM (E) and HCT-15 (F) cells, and total RAS in COLO320DM (G) and HCT-15 (H) cells. Fifteen to 20 µg protein was probed with the indicated antibodies, and 40 µg for P-EGFR in the bottom panel in E. Data represent one experiment of at least two.
WNT/β-Catenin and AKT/mTOR signaling in COLO320DM but not in HCT-15 cells, we tested whether the inhibitor impact on RAS expression could be indicative for the differential response of both cell lines. Activated GSK3β has been shown to destabilize both β-Catenin and RAS in colorectal cancer cells (40), and RAS is an upstream regulator of AKT (41). However, G007-LK at the tested dose did not affect RAS protein level neither in COLO320DM nor in HCT-15 cells (Fig. 5G and H). There were no detectable changes in RAS levels upon BKM120 or erlotinib treatment in mutant HCT-15 cells, BKM120 slightly and erlotinib substantially reduced RAS levels in all treatment combinations, and most substantially upon G007-LK/BKM120/erlotinib treatment.

**Inhibitor impacts on TNKS/β-Catenin complex formation**

Axin1/2 together with GSK3β, APC, and CK1ε are components of the β-Catenin destruction complex (42, 43). As β-Catenin was reduced by TNKS inhibition (G007-LK) in COLO320DM, but not in HCT-15 cells, we investigated by immunofluorescence whether TNKS and β-Catenin colocalized upon inhibitor treatments in both cell lines.

In COLO320DM, β-Catenin was localized predominantly cytoplasmic and nuclear with control treatment. Following G007-LK treatment, β-Catenin localization was reduced in the nucleus and colocalized with TNKS containing puncta in the cytoplasm (Fig. 6A; Supplementary Fig. S17). Colocalization of TNKS and β-Catenin was observed in all treatments containing G007-LK despite attenuated Axin1/2 levels by BKM120 and erlotinib treatment (Fig. 4A). No TNKS puncta were observed upon BKM120 and/or erlotinib treatment.

In HCT-15 cells, both TNKS and β-Catenin were predominantly found at the cell membrane upon control treatment. G007-LK induced TNKS-containing puncta, but β-Catenin was not colocalized in these puncta (Fig. 6B; Supplementary Fig. S17). Although G007-LK induced N-terminal β-Catenin phosphorylation and reduced β-Catenin levels in combination with BKM120 or erlotinib, in HCT-15 cells, β-Catenin degradation appears to be independent from a complex containing TNKS.

**Combined inhibitor treatment exerts additive or synergistic effects on colorectal cancer xenograft growth reduction**

To explore the possible additive effects of combined inhibitor treatments against tumor growth in vivo, xenografts were established using COLO320DM-Luc2, HCT-15-Luc2, and HCT-15 cells in CB17SCID mice. Mice carrying the xenografts were treated with moderate doses of the compounds [G007-LK (10 mg/kg), BKM120 (3 mg/kg), and erlotinib (15 mg/kg)], produced a more significant tumor reduction. At elevated drug doses [G007-LK (50 mg/kg), BKM120 (15 mg/kg), and erlotinib (30 mg/kg)] produced a more significant tumor size reduction. Combined G007-LK/BKM120/erlotinib treatment (39% reduction, Fig. 7A; Supplementary Figs. S16 and S17). Treatment with erlotinib in combination with G007-LK or BKM120 resulted in 39% or 25% tumor reduction, respectively, a change without statistical relevance compared with a combined G007-LK/BKM120/erlotinib treatment (39% reduction, Fig. 7A; Supplementary Figs. S16 and S17). Treatment at elevated drug doses [G007-LK (50 mg/kg), BKM120 (15 mg/kg), and erlotinib (30 mg/kg)] produced a more significant tumor size reduction. At these doses, single-agent treatment with G007-LK, BKM120, and erlotinib resulted in 78%, 75%, and 55% tumor size reduction, respectively, while combined G007-LK/BKM120 treatment enhanced the effect of single treatments to an 86% reduction, and more pronounced upon G007-LK/BKM120/erlotinib treatment (91% reduction, Fig. 7A; Supplementary Figs. S18 and S19). Molecular analysis of tumor protein extracts reflects in vitro analysis (Supplementary Fig. S20).
In HCT-15 xenografts, single-agent treatments caused only trends at moderate doses compared with control (G007-LK; 3%, BKM120; 18%, erlotinib; 21%). Only when erlotinib was combined with a G007-LK/BKM120 treatment, a more robust reduction on tumor growth was observed (34% against 16% reduction; Fig. 7B; Supplementary Figs. S18 and S19). At elevated drug doses, single treatment with G007-LK, BKM120, and erlotinib resulted in 3%, 76%, and 8% tumor size reduction, respectively. Combined G007-LK/BKM120/erlotinib treatment resulted in a striking 94% tumor size reduction compared with the control, effectively halting tumor growth (Fig. 7B; Supplementary Figs. S18 and S19).

Mouse body weights (Supplementary Fig. S21) indicated overall tolerability of the treatment regimes, including to the increased dose G007-LK/BKM120/erlotinib treatment.

Discussion
In this study, we have investigated a combinatorial therapeutic approach combining TNKS/WNT, PI3K, and EGFR inhibition to explore treatment strategies for colorectal cancer. Although both PI3K and EGFR inhibitors are in a clinical stage (6–9), TNKS inhibitors are still at a preclinical exploratory stage due to a limited efficacy of WNT inhibitors in current preclinical colorectal cancer models (11, 12) and to intestinal cytotoxicity attributed to WNT pathway inhibition (12, 14). In addition, the contextual background of colorectal cancer cells required to render WNT inhibitors efficacious remains poorly understood, although the length of the APC protein is proposed to play an important role (44).

Our study revealed that G007-LK exerts a dual effect on b-CATENIN and AKT/mTOR activity in the TNKS inhibitor–sensitive (affecting proliferation) COLO320DM and SW403 cell lines, but not in the inhibitor-insensitive HCT-15 and SW480 cells. In HCT-15 cells, G007-LK rather initiated EGFR feedback activation. Contextual differences, such as the mutational background of cells, may be important for enabling or blocking this dual pathway response.

In both COLO320DM and HCT-15 cells, erlotinib enhanced activation of AKT, while reducing the activity of both EGFR and mTOR effectors. Similar effects have been observed with EGFR...
Inhibition in non–small cell lung cancer, where AKT activation was induced either through activation of STAT3 (45) or ERK-2 (46). Enhanced AKT activity could also be induced by reduced SRP (mTOR) activity, which may relieve the TORC1 feedback regulation and enhance the interaction between PI3K and IRS proteins (47).

In KRAS-mutant HCT-15 cells, G007-LK neither affected the levels of ABC nor proliferation, despite the classical stabilization of both TNKS and AXIN1/2. A possible explanation may be the subcellular distributions of β-CATENIN and TNKS. In Drosophila, active RAS signaling counteracts AXIN-mediated degradation of Armadillo (the Drosophila β-CATENIN) by recruiting the destruction complex to the cell membrane (48). In accordance, dual inhibition of TNKS and MEK has reduced cell proliferation in vitro than combined TNKS/MEK inhibition in some colorectal cancers, including KRAS wild-type COLO320DM. Hence, combined TNKS/PI3K/EGFR inhibition may be a potent treatment option that is independent of a KRAS-mutant background.

Although both KRAS mutations and the degree of APC truncation could affect the sensitivity to TNKS inhibition (12, 44), we observed that combined BMK120/erlotinib treatment sensitized HCT-15 cells to G007-LK treatment, reduced ABC, proliferation, and the colony-forming ability, possibly because erlotinib attenuated the induced EGFR feedback activation mediated by G007-LK and BMK120, accompanied by a reduction in RAS protein levels. Combined G007-LK/BMK120/erlotinib therefore most potently reduced both WNT, AKT, mTOR, EGFR, and RAS signaling in both COLO320DM and HCT-15 cells.

TNKS inhibition has shown to induce puncta in colorectal cancer cells containing functional components of the β-CATENIN destruction complex, including TNKS, β-CATENIN, and AXIN1/2 (43). In our study, TNKS inhibition alone induced stabilization of both AXIN1 and AXIN2. However, when combined with BMK120 and erlotinib, AXIN1/2 stabilization was reduced to the level of control with G007-LK/BMK120/erlotinib treatment. Despite reduced AXIN1/2 stabilization, TNKS-containing puncta were formed in the presence of G007-LK in all treatment regimes. β-CATENIN colocalized with these puncta only in COLO320DM but not in HCT-15 cells. Notwithstanding, G007-LK/BMK120/erlotinib treatment strongly reduced ABC in both cell lines. We therefore assume that AXIN1/2 stabilization is not required for TNKS puncta formation, and neither correlates with degradation of β-CATENIN.

Our study shows that combined TNKS/PI3K/EGFR inhibition, mutually affecting in a context-dependent manner components of the WNT/β-CATENIN, AKT/mTOR, EGFR, and RAS signaling pathways, may provide a treatment strategy for colorectal cancer cells with divergent mutational backgrounds. The TNKS/PI3K/EGFR inhibitor combination also led to a significant reduction of tumor size in vivo without affecting animal body weight. Although possible adverse effect of this combination will have to be further explored, TNKS/PI3K/EGFR inhibition may provide a versatile combination expanding the possibilities of TNKS/MEK inhibition in KRAS-mutant colorectal cancer tumors.

Disclosure of Potential Conflicts of Interest
J. Waaler has ownership interest (including patents) in G007-LK. S. Krauss has ownership interest (including patents) in G007-LK. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: N.T. Solberg, J. Waaler, S. Krauss
Development of methodology: N.T. Solberg, J. Waaler, P.A. Olsen
Acquisition of data (provided animals, provided facilities, etc.): N.T. Solberg, J. Waaler, J. Mygland
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.T. Solberg, J. Waaler, P.A. Olsen, K. Lund
Writing, review, and/or revision of the manuscript: N.T. Solberg, J. Waaler, K. Lund, L. Mygland, P.A. Olsen, S. Krauss
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.T. Solberg, K. Lund, P.A. Olsen, S. Krauss
Study supervision: N.T. Solberg, S. Krauss

Acknowledgments
The work was supported by grants given to Stefan Krauss by the Norwegian Cancer Society contract 2327614, 4561000, and 5803958, the Research Council of Norway contract 226290/030, and the Norwegian Health Region South East contract 2013012. We would like to acknowledge René Beyer and the IOI Oleo GmbH for the Mygplol 810N drug carrier solution and Jennifer Dombiński for technical support during initial xenograft experiments.

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 July 10, 2017; revised September 30, 2017; accepted November 10, 2017; published OnlineFirst December 8, 2017.

References


TANKYRASE Inhibition Enhances the Antiproliferative Effect of PI3K and EGFR Inhibition, Mutually Affecting β-CATENIN and AKT Signaling in Colorectal Cancer

Nina T. Solberg, Jo Waaler, Kaja Lund, et al.


Updated version

Supplementary Material

Cited articles

This article cites 48 articles, 19 of which you can access for free at:
http://mcr.aacrjournals.org/content/16/3/543.full#ref-list-1

Citing articles

This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/16/3/543.full#related-urls

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, use this link http://mcr.aacrjournals.org/content/16/3/543.

Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.