AZ1366: An inhibitor of tankyrase and the canonical Wnt pathway that limits the persistence of non-small cell lung cancer cells following EGFR inhibition

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Translational Relevance

Non-small cell lung cancer (NSCLC) is the most common form of cancer worldwide. Despite progress in the treatment of NSCLC driven by alterations in signaling through the EGFR gene, the prognosis for patients with these mutations generally remains poor and new therapeutic strategies are urgently needed. Recent efforts have identified the canonical Wnt/β-catenin pathway as a means of persistence for EGFR-driven NSCLCs treated with EGFR-inhibitors. Here, we show that co-treatment of these cancers with an EGFR-inhibitor and AZ1366, a novel tankyrase inhibitor which effectively reduces signaling through the Wnt/β-catenin pathway, induces tumor cell senescence, reduces tumor growth and increases survival in a subset of cell lines that can undergo Wnt pathway modulation. These data strongly support further evaluation of tankyrase inhibitors as a co-treatment strategy for EGFR-driven NSCLC.
Tankyrase inhibition limits persistence of EGFR-driven NSCLC

Abstract

Purpose: The emergence of EGFR-inhibitors such as gefitinib, erlotinib and osimertinib has provided novel treatment opportunities in EGFR-driven non-small cell lung cancer (NSCLC). However, most patients with EGFR-driven cancers treated with these inhibitors eventually relapse. Recent efforts have identified the canonical Wnt pathway as a mechanism of protection from EGFR-inhibition and that inhibiting tankyrase, a key player in this pathway, is a potential therapeutic strategy for the treatment of EGFR-driven tumors.

Experimental Design: We performed a preclinical evaluation of tankyrase inhibitor AZ1366 in combination with multiple EGFR-inhibitors across NSCLC lines, characterizing its anti-tumor activity, impingement on canonical Wnt signaling and effects on gene expression. We performed pharmacokinetic (PK) and pharmacodynamic (PD) profiling of AZ1366 in mice and evaluated its therapeutic activity in an orthotopic NSCLC model.

Results: In combination with EGFR-inhibitors, AZ1366 synergistically suppressed proliferation of multiple NSCLC lines and amplified global transcriptional changes brought about by EGFR-inhibition. Its ability to work synergistically with EGFR inhibition coincided with its ability to modulate the canonical Wnt pathway. PK and PD profiling of AZ1366-treated orthotopic tumors demonstrated clinically-relevant serum drug levels and intratumoral target inhibition. Finally, co-administration of an EGFR inhibitor and AZ1366 provided better tumor control and improved survival for Wnt-responsive lung cancers in an orthotopic mouse model.

Conclusions: Tankyrase inhibition is a potent route of tumor control in EGFR-dependent NSCLC with confirmed dependence on canonical Wnt signaling. These data strongly support further research.
evaluation of tankyrase inhibition as a co-treatment strategy with EGFR inhibition in an identifiable subset of EGFR-driven NSCLC.
Introduction

Lung cancer is the most commonly diagnosed cancer and leading cause of cancer death worldwide (1). More than half of new diagnoses occur in late stage disease which carries a poor prognosis, and despite some advances in treatment regimens, median overall survival remains less than 12 months (2). The preceding decade has been witness to a revolution in treatment strategies with the increased use of targeted therapies in many cancers, including non-small cell lung cancer (NSCLC), which represents 80-85% of lung cancer cases. Clinical studies have demonstrated that small molecule-based targeting of the epidermal growth factor receptor (EGFR) delivers improved responses and prolonged progression-free survival (PFS) over conventional chemotherapy for patients with sensitizing EGFR mutations (3-5). Radiological imaging indicates that with these therapies, most tumor shrinkage occurs within weeks of treatment initiation, but that only a fraction of cells are eliminated despite the fact that all have the EGFR mutation, and thus $10^{11}$-$10^{12}$ cells persist after therapy (6). While the mechanisms of mutation-based resistance are becoming increasingly clear (7,8) (and in the case of secondary kinase mutations, can now be targeted by third-generation EGFR-inhibitors(9,10)), the innate mechanisms that allow a fraction of EGFR-mutant cells to persist in the presence of EGFR inhibitors are poorly understood. Elucidation of how these cells persist should facilitate the development of rational combinations therapies with improved outcomes.

Dysregulation of the Wnt/$\beta$-catenin pathway has emerged as a recurrent theme in various cancers. 90% of sporadic colorectal cancers exhibit aberrant Wnt signaling activity (11), loss-of-function mutations in $AXIN$ have been found in hepatocellular carcinoma (12,13), and $\beta$-catenin mutations have been described in ovarian adenocarcinomas (14), medulloblastoma (15), and thyroid tumors (16). Although mutations in the canonical Wnt pathway are uncommon in
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NSCLC (17,18), altered expression of various Wnt pathway components and β-catenin have been associated with a poor prognosis (19,20). Because of its involvement in a multitude of developmental processes and maintenance of adult tissue homeostasis, efforts to inhibit the Wnt/β-catenin pathway have been met with toxicity and narrow therapeutic windows (21). A number of agents to target this pathway have entered clinical trials, but to our knowledge, none have yet been approved.

The central feature of canonical Wnt pathway control is the regulated proteolysis of the downstream effector β-catenin by the β-catenin destruction complex, which includes adenomatous polyposis coli (APC), GSK3B, and Axin-1 (22). Axin-1 is considered the limiting component for β-catenin degradation, and is itself PARsylated by two members of the poly(ADP-ribose) polymerase superfamily, tankyrase-1 and tankyrase-2 (23). Recent work highlighting the role of the tankyrases in the control of canonical WNT signaling has fueled interest in the development of inhibitors to target this enzyme (24). Numerous studies have shown that inhibition of tankyrase can induce cell killing in Wnt-dependent models of colorectal cancer, and the growing body of knowledge on the importance of the Wnt pathway and β-catenin in multiple cancers has stimulated several directed discovery efforts for tankyrase inhibitors (25-28).

Previously, we defined tankyrase as a mechanism of inherent NSCLC cell persistence in the face of EGFR-inhibition (29). Here we have developed a therapeutic strategy to leverage this knowledge, defining and characterizing a combination therapy targeting EGFR and tankyrase for EGFR mutant NSCLC. We demonstrate that combining EGFR inhibitors with AZ1366, a novel small-molecule inhibitor of tankyrase1 and 2, represses growth and proliferation of NSCLC lines with dependence on signaling through the canonical Wnt pathway. We show that
AZ1366 amplifies the global transcriptional changes brought about by EGFR inhibition, and that its actions within the canonical Wnt pathway are necessary to bring about its synergistic effects. Furthermore, combined inhibition of both EGFR and tankyrase represses tumor growth and provides a significant survival advantage in mice harboring orthotopic tumors over EGFR inhibition alone. Our data suggest tankyrase inhibition as a potential route of combinatorial therapy in EGFR-dependent NSCLC with confirmed dependence on canonical Wnt signaling.
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Materials and Methods

Cell lines

293FT cells and the NSCLC lines H1650 and HCC827 were obtained from the University of Colorado Cancer Center Tissue Culture Shared Resource within the past 3 years. HCC4011 was purchased from ATCC (Manassas, VA, USA) in 2012. PC9 and HCC4006 were provided by Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, USA) in 2013 and 2006, respectively. H3255 was provided by Drs. Bruce Johnson and Pasi Janne (Dana-Farber Cancer Institute, Boston, USA) in 2006. PC9T790M was provided by Dr. Lynn Heasley (University of Colorado, Denver, USA) in 2013. H3122 was provided by Dr. Robert Doebele (University of Colorado, Denver, USA) in 2016. All cell lines were authenticated by the authors within the 6 months prior to submission by short tandem repeat (STR) analysis. All NSCLC lines were cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (Sigma, St Louis, USA) at 37°C in a humidified 5% CO2 incubator. 293FT cells were cultured in IMDM supplemented with 10% FBS.

Pharmacological agents

Gefitinib, osimertinib (AZD9291) and AZ1366 were provided by AstraZeneca. Erlotinib was purchased from Tocris pharmaceuticals. Alectinib was provided by Dr. Robert Doebele. Each of these compounds was resuspended in DMSO at 10 mM, and subsequently diluted in culture media for further studies. Wnt3a (R&D Biosystems) was resuspended in sterile PBS for a stock concentration of 200 μg/ml.

Orthotopic mouse lung cancer model

Athymic nude mice (8-12 week old females) were obtained from an in-house breeding colony, maintained by the veterinary staff at the University of Colorado Anschutz Medical Campus.
Mice were anesthetized using isoflurane and positioned laterally such that their flank was accessible. A small skin incision was made over the ribcage to visualize the lung. Cultured, luciferase-tagged NSCLC cells (1x10^6) were injected directly into the lung using a 28-gauge needle, inserted to a depth of 3 mm. Tumor growth was assessed every 14 days using a Living Image IVIS system. Once a mouse’s tumor reached a threshold of 5x10^8 p/s, the mouse was randomized into a treatment group. For the HCC4006 and H1650 lines the treatment groups were as follows: Vehicle (Methocel E4M), gefitinib (6.25 mg/kg), AZ1366 (either 25 or 50 mg/kg) or a combination of gefitinib and either dose of AZ1366. For the PC9T790M line, mice were treated with either vehicle (Methocel E4M), osimertinib (6.25 mg/kg), AZ1366 (25 mg/kg) or a combination of osimertinib and AZ1366. All drug combinations were delivered by oral gavage daily (5/7 days). Tumor growth was assessed every 14 days by IVIS imaging and mice were weighed bi-weekly. The event resulting in euthanasia was reaching moribund criteria, as evaluated by the veterinary staff. Mice were maintained in accordance with institutional guidelines, and all experimental procedures were approved by the University of Colorado Institutional Animal Care and Use Committee.

Statistical analysis

All error shown is standard deviation and is based on biological replicates. Unless otherwise indicated, one-way ANOVA with Tukey post-test was used to compute p-values. We analyzed survival of mice in the orthotopic model by the Kaplan-Meier method and compared the curves using a log-rank (Mantel-Cox) test. P-value designations for all figures: * - <0.05, ** - <0.01, *** - <.001, **** - <.0001. Results of clonogenic assays were analyzed for synergistic, additive or antagonistic effects using the combination index (CI) method developed by Chou and Talalay (30). The quantified CI values were determined using CompuSyn software (ComboSyn Inc.).

See Supplementary Information for a description of additional methods.
Results

Combined tankyrase inhibition via AZ1366 and EGFR inhibition synergistically suppresses NSCLC proliferation.

We have previously shown that genetic and pharmacological inhibition of tankyrase-1 increases susceptibility to EGFR inhibition (29). A recent high throughput proteomics screen identified the pyrimidinone nicotinamide mimetic, AZ1366, as a potent and selective inhibitor of TNSK1/2 with good bioavailability in mouse and rat and activity within the canonical Wnt pathway in DLD-1 cells (AZ1366 = Compound 9) (28). To confirm that AZ1366 effectively inhibits tankyrase and has activity within the canonical Wnt pathway in lung cancer cells, we treated HCC4006 cells with various concentrations of AZ1366. AZ1366 stabilized Axin-1 in a dose-dependent manner (Figure 1A), and provided a corresponding decrease in the mRNA level of multiple β-catenin dependent targets, including Survivin, c-Myc and Axin2, albeit with different dose-dependencies (Figure 1B). Stabilization of Axin-1 has been established as a reliable proxy to indicate inhibition of tankyrase (23). To test the effects of dual tankyrase and EGFR blockade in vitro, we treated HCC4006 cells with the EGFR inhibitor gefitinib, AZ1366, or a combination of both. By 48 hours, gefitinib markedly suppressed phosphorylation of EGFR and downstream ERK, and treatment with AZ1366 resulted in stabilization of Axin-1. As expected, the combination of gefitinib and AZ1366 suppressed EGFR and ERK phosphorylation, and stabilized Axin-1 (Figure 1C). After 7 days (3 days of drug exposure followed by 4 days of growth in the absence of drugs), the anti-proliferative effects of gefitinib treatment were augmented with AZ1366 in a dose-dependent manner. Similar patterns of synergy were seen for the combination of AZ1366 with erlotinib and osimertinib (Figures 1D and 1E). Through analysis of cell-cycle changes induced by treatments, we show that the primary effect of AZ1366 on gefitinib-treated cells is an increase in the fraction of cells in G0/G1 and a decrease in cells in the S/M or G2 phases, demonstrating the effectiveness of AZ1366 as an amplifier of EGFR-inhibitor action.
Tankyrase inhibition limits persistence of EGFR-driven NSCLC (Supplemental Figure 2F). Interestingly, when combined with alectinib in EML4-ALK-driven H3122 cells, AZ1366 was able to achieve a moderate degree of synergy (Supplemental Figure 3E). These results demonstrate that tankyrase inhibition may be a useful co-treatment strategy in numerous tyrosine kinase driven lung cancers.

AZ1366 amplifies global transcriptional changes brought about by treatment with gefitinib

To evaluate transcriptional changes brought about by treatment with this combination of drugs, we treated HCC4006 cells with gefitinib, AZ1366 or a combination of the two for 24 hours (which precedes any alteration in viability) and performed RNA-seq and subsequent transcriptional analysis. Quality control analyses indicate that there were no systematic outliers in quadruplicate samples generated for any of the 4 treatment conditions. Additionally, using unsupervised clustering methods, all samples cluster by treatment (Supplemental Figure 1A-C). Hierarchical clustering demonstrates that a significant proportion of differentially expressed genes are being coordinately up and down regulated in the combination treatment and that most of the combination effect is additive for the two single agents. The comparison between vehicle and combination treatment shows that a significant proportion of differentially expressed genes are being up (red arrow) and down- (blue arrow) regulated (Supplemental Figure 2A). Most of the significant transcriptional changes observed in the samples treated with the drug combination were driven by gefitinib, which had 262 up-regulated genes, and 125 down-regulated genes. However, while there was a relatively small number of significant transcriptional changes observed in the AZ1366-treated samples (18 up, 30 down), the addition of AZ1366 delivered a large amplifying effect to the combination treated samples, which had 467 significantly up-regulated and 865 down-regulated genes (Supplemental Figure 2B).
To evaluate the effects of treatment on pathway modulation, we performed BROAD and MetaCore Gene Set Enrichment Analysis (GSEA) on the FPKM gene values for each of the treatment conditions vs. vehicle. BROAD GSEA revealed that combination treatment had significant effects on signaling through the EGFR pathway, interferon response pathways, and cell cycle (Supplemental Figure 1D). While most changes in gene expression were driven by gefitinib, there is clear evidence of amplification of these changes with addition of AZ1366. As shown in Supplemental Figure 2C, while EGFR inhibition had the expected impact on genes known to be regulated downstream of EGFR (including MYC targets and cell cycle genes), we observed a consistent enhancement of these effects across these gene sets, for both up (EGFR UP) and down (EGFR DN) regulated genes. GSEA-categorized Wnt-regulated gene signatures were statistically poorly supported in the AZ1366 treated cells (FDR q-value > 0.05); however, the signatures are anti-coordinated with treatment (down signatures in traditionally Wnt up-regulated genes, and up signatures in Wnt down-regulated genes; Supplemental Figure 1E). Indeed, individual examination of prototypical Wnt-regulated transcripts Axin-2, Birc5, CCND1 and Myc indicates that AZ1366 and gefitinib coordinately downregulate Wnt gene expression, which is further decreased in the combination treated cells (Supplemental Figure 2D). Furthermore, when changes are queried against the Molecular Signature Database (MSigDB) Wnt hallmark gene set, which contains genes up-regulated by activation of WNT signaling through accumulation of β-catenin, it is evident that AZ1366 treatment generally results in the down-regulation of Wnt-dependent genes, and that perturbations in these genes observed in the combination treatment are driven largely by AZ1366 (Figure 1F).

Coadministration of EGFR and tankyrase inhibitors exerts antiproliferative effects through inhibition of the canonical Wnt pathway.

To evaluate the effect of AZ1366 on gefitinib treatment across NSCLC lines, we treated 6 EGFR-mutant lines with various doses of both AZ1366 and gefitinib. After 72 hours of treatment...
and 5 days of clonogenic outgrowth, we observed variable synergy between the two drugs; 3 of the 6 lines (HCC4006, H3255, H1650 displayed a high degree of synergy, HCC827 and HCC4011 had a low degree of synergy, and PC9 showed no additive or synergistic effect (Figure 2A). The presence of synergy did not correlate with sensitivity to EGFR inhibition (e.g. HCC4006 and PC9, with divergent degrees of synergy, have roughly equivalent IC$_{50}$ for gefitinib). The combinatorial index values for the drug combination are shown in Figure 2B. We sought to determine whether the source of the differences between the synergistic and non-synergistic lines was due to inherent differences in canonical Wnt pathway activity for different cell lines. Stimulation with exogenous Wnt3a ligand (considered a prototypical canonical Wnt ligand (31)) brought about an increase in mRNA levels for the β-catenin dependent target genes Survivin, c-Myc and Axin-2 in HCC4006, which was abrogated by co-treatment with AZ1366. In contrast, there was no effect of Wnt3a or AZ1366 treatment on transcript levels in PC9 (Figure 2C). At the protein level, treatment with AZ1366 led to stabilization of Axin-1 in both HCC4006 and PC9, but Wnt3a resulted in an increase in Birc5 only in HCC4006, which is again reversed by AZ1366 treatment (Figure 2D).

**Loss of Axin-1 is sufficient to abrogate the synergistic effects of AZ1366**

To further validate that AZ1366 is working through the canonical Wnt pathway and that these direct effects on Axin stabilization are sufficient for its synergistic activity, we used shRNA to knockdown Axin-1 in two synergistic lines, HCC4006 and H1650, and assessed whether the loss of Axin-1 resulted in a loss of synergy with gefitinib. RT-PCR and Western blots were used to confirm sufficient knockdown of Axin-1 (Supplemental Figures 3A and 3B). In both lines tested, HCC4006 and H1650, the synergistic effects on cell proliferation of AZ1366 were lost with knockdown of Axin-1 (Figure 3A). Additionally, we performed a competition assay in HCC4006 cells for which GFP-labeled non-targeting shRNA cells were cultured with non-GFP, Axin-1 knockdown cells (see Supplemental Figure 3C for schematic). The increase in the ratio
of non-GFP/GFP cells with time indicates that treatment with AZ1366 had a larger growth inhibitory effect on GFP-bearing wild type cells (Figure 3B), which was augmented when cells were co-treated with AZ1366 and gefitinib. Interestingly, analyses of cell expansion post-gefitinib treatment revealed that the loss of Axin-1 reduced the growth inhibitory effects of gefitinib itself in H1650 and H3255 (2 of the 3 synergistic lines tested) (Figure 3C). Given that gefitinib treatment also reduces the expression of the β-catenin target Birc5 (Figure 2D), this reduction in gefitinib activity could be due to the increase in β-catenin targets brought about by the reduction in available Axin-1 (Figure 3D).

The ability of tankyrase inhibitor AZ1366 to synergize with EGFR inhibition coincides with its ability to modulate the canonical Wnt pathway.

In order to evaluate whether the ability of AZ1366 to synergize with EGFR inhibition coincides with its ability to modulate the canonical Wnt pathway downstream of Axin-1, we transfected the 7TFP construct as described in (32) into a synergistic (HCC4006) and a non-synergistic (PC9) cell line. This reporter construct induces expression of luciferase upon stimulation of the canonical Wnt pathway. Using this reporter, we show a dose-dependent increase in luciferase expression with Wnt3a treatment at significantly lower doses in HCC4006 than PC9 (Figure 4A), indicating that the HCC4006 line exhibits greater sensitivity to Wnt pathway stimulation than does PC9. When we combined Wnt pathway stimulation with gefitinib and AZ1366 treatment using 7TFP, we observed that in HCC4006 cells the combinatorial efficacy of gefitinib/AZ1366 on cell proliferation was preserved under Wnt stimulation (Figure 4B). AZ1366 did not impact gefitinib efficacy in PC9 cells, either with or without Wnt3a stimulation (data not shown). In HCC4006 cells, treatment with AZ1366 reduced β-catenin-dependent transcription (as indicated by luciferase activity) both with or without concomitant gefitinib treatment, which was even more apparent following stimulation with Wnt3a. In contrast, no significant changes in Wnt-dependent
transcription were observed in PC9 cells (Figure 4C). Treatment of HCC4006 and PC9 with XAV939, a different tankyrase inhibitor, in combination with gefitinib yielded effects on cell proliferation similar to those of AZ1366, with synergy observed in the HCC4006, but not the PC9 line (Supplemental Figure 3D).

Co-administration of an EGFR inhibitor and AZ1366 provided increased tumor control and improved survival in mice harboring Wnt-responsive tumors. We evaluated the combination of EGFR inhibitors gefitinib and osimertinib with AZ1366 in an orthotopic mouse model. Luciferase-expressing HCC4006, H1650 and PC9 cells harboring the T790M mutation (which makes them resistant to first generation inhibitors, heretofore called PC9T790M) cells were injected unilaterally into the right lung of Nu/Nu mice, and tumor burden was assessed bi-weekly by IVIS after administration of luciferin. Mice implanted with the gefitinib sensitive lines (HCC4006, H1650) were treated with gefitinib and AZ1366, while mice bearing the PC9T790M line were treated with osimertinib and AZ1366. We operated under a "threshold-model" whereby tumors were allowed to reach a pre-determined size before the mouse harboring that tumor was randomized and distributed into a treatment group. There were no significant differences in either the average or distribution of starting luminescence between treatment groups (Supplemental Figure 4A). Once assigned to a treatment group, mice were treated once daily (5/7 days) by oral gavage with Methocel E4M (vehicle), 3.125 mg/kg gefitinib, 25 or 50 mg/kg AZ1366, or a combination thereof (HCC4006 and H1650 lines), or 3.125 mg/kg of osimertinib, 25 mg/kg AZ1366 or a combination thereof (PC9T790M line). In proliferation assays, the addition of AZ1366 to osimertinib or gefitinib had similar synergistic and non-synergistic effects in HCC4006 and PC9T790M cells, respectively (Supplemental Figure 4C). Mice were monitored for changes in weight and tumor burden bi-weekly, and for moribund criteria by the CU Anschutz Veterinary staff. There were no significant differences in weight loss or final weight at death between treatment groups for any cell line (data not shown).
Interestingly, in each of the three iterations of the experiment, EGFR tyrosine kinase inhibitor (TKI) treatment (or treatment with the combination of the TKI and AZ1366) did not result in a significant decrease in tumor burden, but instead a slowing of tumor growth. Treatment efficacy was compared by calculating the tumor doubling time for the tumors present in each mouse. Tumor burden at end of life was divided by tumor burden at the start of treatment, which was then compared to the number of days over which the mouse was monitored to calculate a growth rate per day and subsequent doubling time. There were no significant differences between the tumor burden at death among the treatment groups for any of the cell lines (Supplemental Figure 4B). For the HCC4006 and H1650 tumors, the addition of AZ1366 to gefitinib treatment increased efficacy above that achieved by gefitinib alone (representative mice shown in Figure 5A, all data shown in Figure 5B. Given the highly divergent variance between the treatment groups (Bartlett’s test p-value for all three cell lines <.00001), the p-values shown represent the results of a Mann-Whitney U test (33). The median survival achieved by adding AZ1366 to treatment with an EGFR inhibitor alone increased from 92 to 152 and 59 to 168 days for HCC4006 and H1650, respectively (Figure 5C) Consistent with in vitro results, there was no difference in treatment efficacy between osimertinib and the combination treatment of osimertinib and AZ1366 for the PC9T790M tumors (Figure 5B).

Serum pharmacokinetic parameters were assessed from drug concentration profiles following a single dose of gefitinib, AZ1366 or the combination thereof at 4, 24, 72 or 168 hours. Serum concentrations of both gefitinib and AZ1366 fell throughout the time course and were undetectable at 168 hours after administration. There was no significant change in serum gefitinib concentration brought about by the coadministration of AZ1366. The addition of gefitinib to AZ1366 appeared to increase the serum concentration of AZ1366 at the earliest timepoint assessed, but this difference was not sustained at later timepoints (Figure 6A).
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Intratumoral pharmacodynamics parameters were assessed after single-dose treatment of gefitinib, AZ1366 or the combination at 24, 72 or 168 hours. pERK was assessed as an indicator of EGFR inhibition (and thus gefitinib activity), and Axin-1 stabilization as an indicator of AZ1366 activity. By 24 hours, there was a detectable and consistent decrease in the presence of pERK in the gefitinib and combination treated tumors that was sustained through 72 hours, and lost by 168 hours. Axin-1 was consistently stabilized by single-agent treatment with AZ1366 by 24 hours, which was also sustained through 72 hours and lost by 168 hours post-treatment. Interestingly, the combination of gefitinib and AZ1366 achieved a lower degree of Axin-1 stabilization at 24 and 72 hours than treated with AZ1366 alone, albeit the stabilization achieved was above that seen in the vehicle or gefitinib treated samples (Figure 6B).

To assess the role of co-administration of EGFR inhibitors with AZ1366 on survival, mice were monitored in a blinded manner by the veterinary staff for moribund criteria. Upon meeting these criteria, mice were euthanized. We found no significant impact of single-agent AZ1366 treatment on animal survival in any of the three cell lines tested, at either the 25 or 50 mg/kg dose. In two of the three lines evaluated (HCC4006 and PC9), EGFR inhibition provided the expected increase in survival over vehicle treatment. Importantly, in the Wnt-responsive HCC4006 and H1650 lines, co-administration of 25 mg/kg of AZ1366 with gefitinib provided a significant survival advantage over treatment with gefitinib alone. In the Wnt-non-responsive PC9T790M line, there was no effect of the addition of AZ1366 to AZD9291 on mouse survival (Figure 5D). Of note, co-administration of 50 mg/kg AZ1366 did not lead to a similar survival advantage in either the HCC4006 or H1650 cell lines (this dose was not tested in PC9T790M), which may indicate a narrow therapeutic window for this drug combination (Supplemental Figure 4D).
Discussion

The now widespread use of EGFR TKIs in EGFR-sensitive NSCLC has inevitably led to great effort to address mechanisms of resistance. While significant insight into mutations acquired post-therapy has been achieved (34), there remains a lack of understanding of the signaling pathways that contribute to the fact that in the face of treatment, a population of EGFR-expressing cells persists. It has recently been suggested that this persistent subpopulation can actually promote the subsequent development of acquired resistance mutations (35); thus, targeting the mechanisms of persistence with rational combination therapies at the outset of therapy will likely produce better long term outcomes. Previously, we defined tankyrase as mechanism of escape from targeted inhibition of EGFR in EGFR-sensitive NSCLC (29). In this report, we demonstrate the efficacy of a novel tankyrase inhibitor, AZ1366, in combination with multiple EGFR inhibitors in an orthotopic model of NSCLC. Importantly, we demonstrate that inhibiting the actions of tankyrase within the canonical Wnt pathway contributes to its observed synergism and that the addition of AZ1366 to gefitinib significantly amplifies the effects of EGFR inhibition on overall transcription. Furthermore, our characterization of dual tankyrase/EGFR inhibition as variably synergistic between NSCLC lines, and the correlation of that synergism with canonical Wnt pathway modulation suggest β-catenin transcriptional targets as a possible biomarker for patients whose tumors are most likely to respond to a combination of tankyrase and EGFR inhibitors.

Several recent studies have suggested that dysregulation of Wnt/β-catenin signaling is important in NSCLC, however, the complicated protein-protein interaction domains in the β-catenin destruction and transcriptional complexes make it difficult to target directly with small molecules (36). The discovery of tankyrases as activating enzymes of Wnt/β-catenin signaling...
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has led to efforts to design and characterize tankyrase inhibitors for use in multiple cancer types (27,37-39). However, inhibition of tankyrase alone is not sufficient to fully suppress Wnt signaling, and results in only partial or negligible tumor suppression, even in tumors with significant dependence on β-catenin (37,40). Focus has thus shifted to establishing the utility of tankyrase inhibition in combination with other therapies, and efficacy of tankyrase inhibitors in combination with PI3K/AKT inhibitors (41,42), MEK inhibitors (37) (43), EGFR inhibitors (29), and ionizing radiation(44) has been suggested.

Multiple reports have characterized first generation tankyrase inhibitors XAV-939 and G007-LK but have indicated that the potential for clinical use of these compounds may be low due to narrow therapeutic windows and poor pharmacokinetics (37,45). Thus characterization of new inhibitors as they develop will be important to bring tankyrase inhibitors into clinical use. We report here on a novel tankyrase inhibitor that possesses synergistic activity with multiple generations of FDA-approved EGFR inhibitors across a panel of NSCLC lines and in an orthotopic model. We note that the lack of benefit in the orthotopic model at the higher dose of AZ1366 tested may indicate a narrow therapeutic window for this tankyrase inhibitor as well, but this observation will require follow-up in future pre-clinical work. Our findings show that AZ1366 exhibited some degree of synergism in most of the cell lines tested and suggest that in EGFR-dependent cancers, the Wnt pathway is important more often than not. This is consistent with recent reports that β-catenin contributes to lung tumor development induced by mutant EGFR and that acquisition of β-catenin mutations can occur together with the emergence of kinase-domain escape mutations after treatment with first-generation inhibitors (46) (3,47). Ultimately, our study adds to a growing understanding of the canonical Wnt pathway as an essential player in the development of resistance to EGFR inhibition and suggests β-catenin modulation as a means to prolong the clinical efficacy of EGFR inhibitors in an identifiable subset of patients.
While tankyrases are key regulators of canonical Wnt signaling, they are known to play other roles in cellular processes, including the regulation of telomere length (48), the polarization of the mitotic spindle (49), and activation of the YAP/HIPPO pathway (50). While our report provides evidence that the stabilization of Axin-1 is essential for the anti-tumor effects of tankyrase inhibition in combination with EGFR inhibition, its synergistic activity and the significant amplification of the transcriptional changes brought about by EGFR inhibition may not be entirely explained by its actions within the Wnt pathway. Indeed, we observed significant changes to the transcriptional profile of cells treated with AZ1366 that were unrelated to canonical Wnt signaling, including dysregulation of genes involved in the cell cycle, direct changes to Axl, and EGFR expression itself, although some of these changes could be downstream of canonical Wnt target genes like MYC and CCND1. While most studies have characterized the combinatorial efficacy of tankyrase inhibitors with other therapies as being Wnt-dependent, it has been reported that synergy of tankyrase inhibition in combination with MEK inhibitors occurs independent of β-catenin, likely through a feedback loop involving FGFR2 (43). Further studies are needed to elucidate the importance of the Wnt-independent changes we observed in AZ1366 treated cells.

In summary, our work has characterized a novel tankyrase inhibitor and demonstrated its synergistic activity with three FDA-approved EGFR inhibitors. We have shown in vivo efficacy of these combinations in multiple NSCLC lines and intratumoral inhibition of the drug targets. Importantly, we established that inhibiting the role of tankyrase in the canonical Wnt signaling is essential to this synergy. Our correlation of synergy with canonical Wnt pathway modulation suggests β-catenin transcriptional targets as a possible biomarker for patients whose tumors are most likely to respond to a combination of tankyrase and EGFR inhibitors.
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Tankyrase inhibition limits persistence of EGFR-driven NSCLC


Figure legends

Figure 1. Effect of gefitinib and AZ1366 co-treatment on Axin-1 stabilization, downstream β-catenin dependent targets, cell viability and cell cycle markers. A) Western analyses were performed on HCC4006 cells treated continuously for 48 hours with the indicated dose of AZ1366. B) RT-PCR performed on HCC4006 cells treated for 24 hours with the indicated doses of AZ1366. Results and statistics are representative of 3 independent experiments. Values were first normalized to a GAPDH internal control, then compared to a DMSO experimental control. C) Western analyses were performed on HCC4006 cells treated for 24 hours with DMSO (Veh), 90 nM Gefitinib (Gef), 100 nM AZ1366 (1366) or a combination thereof (Combo). D) Flow-cytometry-based proliferation assay using propidium iodide as a live dead stain assessing the number of live cells after 3 days of treatment and 4 days of outgrowth with the indicated combination of an EGFR TKI and AZ1366. Clonogenic assay Combinatorial Index values are indicated in Figure 1E. F) Heat map of MSigDB HALLMARK_WNT genes. Heat maps in C and E were all normalized by gene using raw FPKM values and constructed in the Broad Institute's GENE-E mapping program. Abbreviations: “V”=DMSO vehicle, “G”=gefitinib, “13”=AZ1366, “C”=Combo.

Figure 2. Differential effects of AZ1366 and Wnt-responsiveness in various cell lines. A) Clonogenic assays were performed with various doses of gefitinib and AZ1366 treatment for 72 hours, followed by 5 days of clonogenic outgrowth. Each dose was repeated in triplicate (HCC4006, H1650, H3255, HCC827) or quintuplicate (PC9, H4011). Data shown is representative of at least 2 independent experiments. Numbers on the Y axis indicate the calculated number of colonies. B) CI values for AZ1366 + gefitinib combinations shown in Figure 3A. Gefitinib doses are indicated as follows: “low” = 1 nM in H3255; 10 nM in H1650, HCC4006, HCC827, PC9; 30 nM in HCC4011; “med” = 3 nM in H3255; 30 nM in H1650, HCC4006, HCC827, PC9; 90 nM in HCC4011; “high” = 10 nM in H3255; 90 nM in H1650,
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HCC4006, HCC827, PC9; 2700 nM in HCC4011. Shading is indicated as follows: white = CI value of <0.67, light grey = CI value of between 0.67 and 1.0, dark grey = CI value >1.0 C) RT-PCR performed on HCC4006 and PC9 cells after 48 hours of stimulation with exogenous Wnt3a ligand (25 ng/ml), AZ1366 (100 nM) or a combination of Wnt3a and AZ1366. Values were internally normalized to GAPDH, then normalized to DMSO treatment for each cell line. Three independent replicates for each treatment condition were performed. D) HCC4006 and PC9 cells were treated for 48 hours with the indicated dose of Wnt3a ligand, and/or AZ1366 (100 nM). Western blots were performed on flash frozen cell lysates. Images are representative of three independent experiments.

**Figure 3. Axin-1 is required for the ability of AZ1366 to synergize with gefitinib in NSCLC lines.** A) HCC4006 and H1650 cells harboring either a non-targeting shRNA (scramble) or Axin-1 shRNA (Axin1-1 KD and Axin1-2KD) were treated with DMSO vehicle, 100 nM gefitinib and either 0, 20, or 100 nM AZ1366 (increasing dose of AZ1366 indicated by triangles under graphs). Cell numbers were determined by flow cytometry after 3 days of treatment. B) GFP-tagged HCC4006 cells were cultured in an initial 1:1 ratio with cells harboring an Axin-1 shRNA construct. The ratio of GFP to non-GFP cells was evaluated at 1, 2, 3 and 7 days after treatment initiation. C) HCC4006, H1650 and H3255 cells with either a non-targeting shRNA (scramble) or shRNA targeting Axin-1 were treated with either DMSO vehicle or 100 nM gefitinib for 3 days, then allowed to grow out for 4 days. Statistics shown compare the ratio of cells in gefitinib to vehicle for scramble relative to each of the Axin-1 knockdowns. D) Axin-1, c-Myc and Survivin were evaluated by Western blot in HCC4006 Axin-1 knockdown cells cultured for 72 hours.

**Figure 4. AZ1366 inhibits basal and Wnt3a-activated β-catenin-dependent transcription in HCCC4006 but not PC9 cells.** A) 7TFP Wnt reporter construct-transfected cells were treated with Wnt3a ligand at the indicated doses for 72 hours. Luciferase activity in cell lysates was
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measured in three independent replicates. B) Flow-based proliferation assay. Cells were cultured with drug for 72 hours, followed by a 4-day period with drugs. C) 7FTP Wnt reporter construct-transfected HCC4006 or PC9 cells were treated with 90 nM Gefitinib, 25 ng/ml recombinant Wnt3a, 100 nM AZ1366, or a combination thereof. Luciferase activity of cell lysate was measured after 72 hours of continuous treatment in three independent replicates.

Figure 5. In vivo treatment efficacy of AZ1366 in combination with various EGFR inhibitors. A) Mice representative of the changes in tumor burden observed for treatment with gefitinib and gefitinib + AZ1366; mice shown were implanted with HCC4006. Numbers on mice indicate normalized tumor burden. B) Comparison of tumor growth rate (as indicated by tumor doubling time) between treatment groups. Calculation was performed as follows: Doubling rate = 2/(# days alive/(Tumor burden at death/Tumor burden at start of treatment). Mantel-Cox comparison of survival between gefitinib and gefitinib+AZ1366 groups is shown. C and D) Median survival in days (C) and a survival curve for the mice in each treatment group for each cell line evaluated. Doses indicated are 6.125 mg/kg for gefitinib, 3.125 mg/kg for osimertinib, 25 mg/kg AZ1366.

Figure 6. Serum pharmacokinetics and tumor pharmacodynamics for gefitinib and AZ1366 A) Serum pharmacokinetics for mice treated with gefitinib and gefitinib + AZ1366. Blood and serum isolated from mice at 4, 24, 72 and 168 hours after a single dose of drug was given by oral gavage. B) Tumor pharmacodynamics as determined by SALLY western blotting for Axin-1 and pERK. The numbers on the left side of the figure indicate the SALLY ladder standards. Abbreviations: “V”=DMSO vehicle, “G”=gefitinib, “13”=AZ1366, “C”=Combo.
FIGURE 1

A

AXIN-1

β-actin

C

D

Survivin

E

Cl Values

Gefitinib

AZ1366 (nM)

10 nM 30 90

Gefitinib

AZ1366 (nM)

10 nM 30 90

Erlotinib

10 nM 30 90

Osimertinib

5 nM 10 50

F

Cell cycle
HCC4006  PC9  HCC827  HCC4011

Gef

AZ1366

H3255  H1650  HCC4006  HCC4011  HCC827  PC9

low

10 nM 0.67 1.06 2.99 >10 2.34 >10

30 0.46 0.66 0.79 1.11 0.49 >10

90 0.48 0.62 0.81 1.68 0.67 >10

med

10 0.70 0.95 0.76 0.57 0.67 >10

30 0.61 0.32 0.41 0.22 0.53 >10

90 0.27 0.27 0.48 0.22 0.53 >10

high

10 0.66 0.64 0.56 0.51 1.12 >10

30 0.31 0.25 0.28 0.32 0.91 >10

90 0.25 0.12 0.22 0.30 0.84 >10

Survivin

C-myc

Axin-2

HCC4006

Axin-1

Survivin

β-actin

β-actin
**FIGURE 3**

**A**

![Graphs showing effects of scramble, Axin1-1 KD, and Axin1-2 KD on HCC4006 and H1650 cell lines.](image)

**B**

![Graphs showing effects of Vehicle, Gefitinib, AZ1366, and Combo treatments on % non-GFP population over days for various cell lines.](image)

**C**

![Bar charts showing number of live cells for HCC4006, H1650, and H3255 cell lines under different conditions.](image)

**D**

![Images of western blot analysis showing expression of Axin-1, Survivin, C-Myc, and β-actin proteins for different cell lines.](image)
FIGURE 5

A) Median Survival (days)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCC4006</th>
<th>H1650</th>
<th>PC9T790M</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>41</td>
<td>55</td>
<td>52</td>
</tr>
<tr>
<td>EGFR TKI</td>
<td>92</td>
<td>59</td>
<td>110</td>
</tr>
<tr>
<td>AZ1366</td>
<td>29</td>
<td>41</td>
<td>60</td>
</tr>
<tr>
<td>Combination</td>
<td>152</td>
<td>168</td>
<td>117</td>
</tr>
</tbody>
</table>

B) Calculated Doubling Time (days)

C) % surviving

D) % surviving
FIGURE 6

A

![Graph showing ng/ml gefitinib and AZ1366 concentrations over time for Gef and Gef+AZ1366 treatments.](image)

B

![Western blot images for Axin-1, pERK, and GAPDH at 24h, 72h, and 168h.](image)
AZ1366: An inhibitor of tankyrase and the canonical Wnt pathway that limits the persistence of non-small cell lung cancer cells following EGFR inhibition

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