TTK Inhibitors as a Targeted Therapy for CTNNB1 (β-catenin) Mutant Cancers

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Abstract

The spindle assembly checkpoint kinase TTK (Mps1) is a key regulator of chromosome segregation and is the subject of novel targeted therapy approaches by small-molecule inhibitors. Although the first TTK inhibitors have entered phase I dose escalating studies in combination with taxane chemotherapy, a patient stratification strategy is still missing. With the aim to identify a genomic biomarker to predict the response of tumor cells to TTK inhibitor therapy, we profiled a set of preclinical and clinical TTK inhibitors from different chemical series on a panel of 66 genetically characterized cell lines derived from different tumor types (Oncolines). Cell lines harboring activating mutations in the CTNNB1 gene, encoding the Wnt pathway signaling regulator β-catenin, were on average up to five times more sensitive to TTK inhibitors than cell lines wild-type for CTNNB1. The association of CTNNB1-mutant status and increased cancer cell line sensitivity to TTK inhibition was confirmed with isogenic cell line pairs harboring either mutant or wild-type CTNNB1. Treatment of a xenograft model of a CTNNB1-mutant cell line with the TTK inhibitor NTRC 0066-0 resulted in complete inhibition of tumor growth. Mutations in CTNNB1 occur at relatively high frequency in endometrial cancer and hepatocellular carcinoma, which are known to express high TTK levels. We propose mutant CTNNB1 as a prognostic drug response biomarker, enabling the selection of patients most likely to respond to TTK inhibitor therapy in proof-of-concept clinical trials.

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Introduction

Targeted therapies exploit unique features of tumor cells, permitting cancer specificity, while having limited effects on healthy cells. Many targeted therapies are genotype-selective, enabling the selection of patients most likely to respond by the detection of specific genomic alterations in tumors, such as gene amplifications, translocations, or point mutations. Well-known examples are the use of growth factor receptor kinase inhibitors in breast cancer patients with amplification of the HER2 gene (1), and inhibitors of a mutant form of the serine/threonine kinase B-Raf (2). The dual-specificity protein kinase TTK, commonly referred to as Mps1, is a component of the spindle assembly checkpoint, a surveillance mechanism that ensures the fidelity of chromosome segregation (3). Inhibition of TTK kinase activity with small-molecule kinase inhibitors leads to chromosome segregation errors by allowing mitotic exit in the presence of unattached kinetochores (4–6). After several rounds of cell division, the accumulation of chromosome segregation errors results in cancer cell death by apoptosis (7, 8). Several TTK inhibitors have been shown to reduce the growth of xenografts of human cancer cell lines from diverse tumor tissue origin in mice (5, 7–13). In an immunocompetent mouse model of triple-negative breast cancer (TNBC; ref. 8), and in patient-derived xenograft models (12), TTK inhibitors increased the efficacy of taxane chemotherapy.

Because of its role in proper chromosome segregation, TTK inhibition also kills nontransformed proliferating cells (6, 14), and concerns have been raised whether sufficient therapeutic window will be achievable in the clinic (11). However, we showed that mice could undergo repeated treatments with the selective small-molecule inhibitor NTRC 0066-0 over a period of more than 150 days in combination with docetaxel, resulting in tumor remission and without signs of toxicity (8). Four TTK inhibitors are currently evaluated in phase I dose escalating studies, some in combination with paclitaxel (refs. 12, 13; http://www.clinicaltrials.gov; clinical studies NCT02138812, NCT02366949, NCT02792465; EudraCT no.: 2014-002023-10).

Despite these advances, development of a patient stratification strategy would greatly help the applicability of TTK inhibitors in the clinic. However, unlike mutant BRAF in metastatic melanoma (2), there is no relationship between mutated or activated TTK and malignancy status known. Furthermore, whereas TTK is highly expressed in several cancer types, the relationship between expression level and severity of disease is complex and contradictive (14). For example, high TTK expression correlates with poor prognosis in hepatocellular carcinoma (HCC; ref. 15) and Her2-positive breast cancer (14), while low TTK expression correlates with poor patient outcome in triple-negative states (14). In the past, sensitivity to TTK inhibition has been associated with mutations in TP53 or PTEN (16, 17), but these results have not been further confirmed (7, 13).

To enable the selection of patients most likely to respond to TTK inhibitor therapy, we profiled a set of ten preclinical and clinical inhibitors on a panel of 66 genetically characterized cancer cell lines derived from different tumor tissues (Oncolines; refs. 18,
Drug sensitivity was related to the mutation status of 114 cancer genes in an unbiased way. Mutant CTNNB1, coding for the Wnt signaling pathway regulator β-catenin, was identified as a unique prognostic drug response biomarker for TTK inhibitors. Experiments with isogenic cell line pairs and a mouse xenograft model confirmed that mutant CTNNB1 associates with sensitivity to TTK inhibitor therapy.

Materials and Methods

TKK inhibitors

All TTK inhibitors were synthesized in-house according to published procedures (5, 20–24), except Mps1-IN-1 (6) and TC-Mps1-12 (10), which were purchased from Tocris. Compounds were stored as solids at room temperature and dissolved in dimethyl sulfoxide before experiments.

Kinase assays

Binding affinity (K_d) for TTK was determined by surface plasmon resonance (8). Selectivity over Aurora A, Aurora C, and Polo-like kinase 1 (PLK1) was determined in enzyme assays performed at 100 μM ATP using full-length enzymes from Carna and Lance time-resolved fluorescence (PerkinElmer). The inhibitor constant (K_i) was calculated from the half-maximum inhibitory concentration (IC_{50}) with the Cheng–Prusoff equation.

Cancer cell lines

Cancer cell lines were obtained from the ATCC from 2011 to 2014 and cultured in ATCC-recommended media. All experiments were carried out within nine passages of the original vials from ATCC who authenticated all cell lines by short tandem repeat analysis. The mutation status of all cell lines was obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC) version 75 (http://cancer.sanger.ac.uk/cosmic; ref. 25) and was confirmed by DNA sequence analysis and found to be identical as reported in the COSMIC database version 75 (http://cancer.sanger.ac.uk/cosmic; ref. 25) and was found to be identical as reported in the COSMIC database version 75 (http://cancer.sanger.ac.uk/cosmic; ref. 25).

Mutations in Cancer cell lines, from the Cancer Cell Line Encyclopedia (CCLE; ref. 28). Subsequently, Pearson correlations were calculated between the expression levels of each individual gene and 10 log(IC_{50})s. The list was sorted from a high correlation and is less sensitive to outliers because it does not assume normal data distribution.

Gene set analysis

Gene expression profiles were downloaded for 57 of the 66 Oncolines cell lines, from the Cancer Cell Line Encyclopedia (CCLE; ref. 28). Subsequently, Pearson correlations were calculated between the expression levels of each individual gene and 10 log(IC_{50})s. The list was sorted from a high correlation between expression and sensitivity, to anticorrelation. This sorted list was used as input for Gene Set Analysis (GSA) with the package piano in R (27, 29). Gene sets were obtained from the MSigDB database (software.broadinstitute.org/gsea/msigdb). To limit the number of tested hypotheses, we restricted GSA to Wnt/β-catenin signaling-related sets from the Protein Interaction Database (PID) and Chemical and Genetical Perturbations (CGP) collections.

In vivo studies

Intervention studies in mouse xenograft models of the human β-catenin-mutant lung carcinoma cell line A-427 and the TNBC cell line MDA-MB-231 were performed at Charles River Laboratories. Experiments were conducted in accordance to protocols approved by the Charles River Discovery Services Animal Care and Use Committee. The animal care and use program at Charles River Discovery is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).
A427 cells were implanted subcutaneously into nude mice on day 0. Once the tumors reached a volume of 150 mm³, mice were treated with vehicle or 20 mg/kg NTRC 0066-0 administrated orally every other day (Q2D, n = 10 mice per treatment group). Tumor growth was monitored using caliper measurement three times weekly. The study was terminated when tumor size of the vehicle-treated mice reached a maximum size of 750 mm³. MDA-MB-231 cells were implanted orthotopically into nude mice on day 0. Once the tumors reached a volume of 200 mm³, mice were treated with vehicle or 20 mg/kg NTRC 0066-0 orally every other day (n = 10 mice per treatment group). Tumor growth was monitored using caliper measurement three times weekly. The study was terminated when tumor size of the vehicle-treated mice reached a maximum size of 1,500 mm³.

Statistical comparisons between compound-treated (NTRC 0066-0) and nontreated (vehicle) were analyzed by t test (unpaired, two-tailed) using GraphPad Prism StatView software (Abacus Concepts, Inc.). Relative inhibition of tumor growth (TGI) was calculated from the relative tumor volume (RTV) as follows: TGI = 100 x 1 – (TVtreated / TVcontrol). Where TVi is the average tumor volume at the end of the study, and TVc is the average tumor volume at the initiation of treatment (13).

Results
Activating mutations in CTNNB1 correlate with cellular response to TTK inhibitors

We previously described the biological characterization of NTRC 0066-0, a subnanomolar potent inhibitor of TTK in enzyme activity and binding assays (8). The compound inhibited the proliferation of a wide variety of cancer cell lines with a potency similar to that of classic cytotoxic agents, and was efficacious in mouse cancer models, without toxicity (8). NTRC 0066-0 only kills proliferating cells, but there is no relationship between cell doubling time and half maximal inhibitory potency (IC50) in cell proliferation assays (8, 30). Because cell line profiling is one of the best ways to discover if certain genomic alterations confer drug sensitivity (28), we extended the number of cancer cell lines examined from 45 to 66, and related differences in cell lines to the presence or absence of mutations in 23 known cancer genes (18). Cell lines were ranked on potency in a waterfall plot (Fig. 1A). Differences in inhibitory potency were related to cancer gene mutation status by ANOVA, and displayed in a volcano plot (Fig. 1B). This revealed that NTRC 0066-0 preferentially inhibits the proliferation of cell lines harboring a mutant copy of the CTNNB1 gene, encoding the Wnt pathway regulator β-catenin (31).

Mutant CTNNB1 is represented by four cell lines in the panel (Table 1): the lung cancer cell line A427, the colon carcinoma cell line HCT116, and the colorectal adenocarcinoma cell lines LS 174T and SW48. These are indicated in red in the waterfall plot (Fig. 1A). The difference in sensitivity between CTNNB1 wild-type and cell lines mutant for CTNNB1, expressed as a differences in −10logIC50 (ΔpIC50), was −0.49 and the difference in median values was −0.44 (Supplementary Tables S1A–S1C). This means that CTNNB1-mutant cell lines are on average three times more sensitive to NTRC 0066-0 than cell lines that are wild-type for this gene. All four cell lines contain point mutations or deletions in serine or threonine residues encoded by a cancer hotspot region in exon 3 of the CTNNB1 gene (Table 1). Mutations in these regions impair the phosphorylation by upstream kinases that mark β-catenin for proteosomal degradation (32). Accumulated β-catenin transfers to the nucleus where it activates the transcription of Wnt-dependent genes, such as MYC (31).

To determine whether mutant CTNNB1 is a common marker of increased tumor cell line sensitivity to TTK inhibitors, we further profiled a set of well-characterized TTK inhibitors based on different chemical scaffolds on the cell panel (Supplementary Fig. S1; Supplementary Table S1A). Compounds 4 and 5 are derivatives of NTRC 0066-0 with increased binding affinity for TTK (33). Mps-BAY2b (Bayer A.G.; refs. 7, 22) is a structural analogue of BAY 1217389 and based on a different chemical backbone than BAY 1161909 (refs. 12, 20–21; Supplementary Fig. S1). NMS-P715 (5) is a structural analogue of the clinical compound of Servier, S81694 (34). Te-Mps-1-12 (10), Mps-1-IN-1-6 (6), and MPI-0479605 (9, 23) are research tool compounds.

The TTK selectivity of the different inhibitors has been demonstrated by profiling experiments on large kinase assay panels (5–10, 12). To compare them head-to-head, we further tested their activity in enzyme assays for Aurora A, Aurora C, and Polo-like kinase 1 (PLK1; Supplementary Table S1D). Although some inhibitors show cross-reactivity with Aurora A and PLK1, all compounds showed at least 100 times selectivity over Aurora A, Aurora C, and PLK1 (Supplementary Table S1D). Moreover, cellular IC50, especially for CTNNB1-mutant cell lines, are lower than the biochemical IC50 for the off-targets, indicating that these play a negligible role in the antiproliferative effects of the inhibitors.

All TTK inhibitors preferentially inhibited CTNNB1-mutant cell lines in the ANOVA (Fig. 1C; Supplementary Fig. S2). Compound 5 showed the strongest targeting of 6 times (Supplementary Table S1B). The significance of the differences was confirmed in a Wilcoxon rank sum test, which is less sensitive to outliers (Supplementary Table S1C). Because the mutant cell lines are mostly from colon cancer origin, we compared the pIC50 in mutant cell lines with wild-type colon cancer lines specifically. This confirmed the preferential sensitivity of CTNNB1-mutant lines to TTK inhibitors (Supplementary Tables S1E and S1F). To test if the effect was related to cell doubling time, we defined a subset of wild-type cell lines showing comparable or faster doubling rates than mutants (19), and used this set as comparator (Supplementary Table S1G). Also in this case, differences between mutant and wild-type lines remained significant. In fact, the cell line that was most sensitive to TTK inhibitors, A427, has a doubling time that is only average across the cell line panel (Supplementary Table S1H).

To determine the uniqueness of the association between TTK inhibitor sensitivity and mutant CTNNB1, we next related sensitivity to a broader set of, in total, 114 cancer genes that occur as variants in at least three of the 66 cell lines (ref. 19; Supplementary Table S1I). For NTRC 0066-0, this revealed one novel gene, in which mutation associated with sensitivity, that is, ARHGAP95, encoding Rho GTase activating protein (ΔpIC50 = −0.48; Padjusted = 0.13, mutated in four cell lines; Supplementary Table S1J). Mutation in this gene also related with sensitivity to other TTK inhibitors (Supplementary Table S1K). Notably, cell lines that are mutant for APC, encoding another regulator of the Wnt pathway, were also slightly more sensitive to NTRC 0066-0 (ΔpIC50 = −0.18), but the correlation was not statistically significant (Padjusted = 0.53, 11 cell lines; Supplementary Table S1I). However, when...
CTNNB1 and APC-mutant cell lines are analyzed as one group, sensitivity differences are significant (F_pooled = 0.11, 14 cell lines). The same trend was seen for the other TTK inhibitors (Supplementary Table S1K). Only NMS-P715, which has relatively low cellular potency, showed significant preferential targeting of APC alone, in addition to CTNNB1 (Supplementary Fig. S2; Supplementary Table S1K).

It should be noted that, like others (7, 12, 13), we did not find a relationship between TP53 and PTEN mutations and TTK inhibitor sensitivity, as reported in early studies (16, 17). Furthermore, in a genomic biomarker analysis of 122 anticancer agents (19), we only found MEK inhibitors that preferentially targeted CTNNB1, as previously reported (18), indicating that the observed CTNNB1 targeting is not caused by the selection of cell lines in the panel, but a result of compound properties, namely TTK inhibition.

To investigate the influence of assay conditions and cell panel composition, we reanalyzed the profiling results reported by other groups working on TTK inhibitors (refs. 12, 34, 35; Supplementary Table S2A). All three panels include HCT116 and LS 174T, and two include SW48. Two panels included a cell line with a CTNNB1 exon 3 hotspot mutant that was not included in the Oncolines cell panel. Reanalyzing the IC_{50}s in these studies with a Wilcoxon rank sum test revealed significant associations of cancer cell line sensitivity and mutant CTNNB1 for the clinical compound BAY 1217389 (12) and for the preclinical compound CCT271850 (ref. 35; Supplementary Table S2B). Reanalyzing the data for NMS-P715 (34) and BAY 1161909 (12) moreover revealed a trend for TTK inhibitor sensitivity of ten TTK inhibitors for CTNNB1-mutant cell lines in comparison with 122 anticancer agents profiled on the same panel (19).

To further scrutinize the relation between CTNNB1 mutation and sensitivity to TTK inhibitors, we acquired two additional cell lines from the ATCC containing CTNNB1 exon 3 hotspot mutations, and that were not part of the Oncolines panel. These are the duodenum carcinoma line HuTu 80 and the ovary carcinoma line TOV-112D (Table 1). As control, we also acquired TOV-21G, an ovary carcinoma line with a non-hotspot CTNNB1 mutation. This was originally isolated in the same laboratory as TOV-112D, but from a different patient (Table 1). All TTK inhibitors were tested in proliferation assays in the three lines (Fig. 1D; Supplementary Table S3A). A Wilcoxon rank sum test across all inhibitors demonstrated that the CTNNB1-mutant cell lines HuTu 80 and TOV-112D were more sensitive than wild-type cell lines (P = 0.01 and P = 0.005, respectively), whereas the control cell line TOV-21G showed no difference in sensitivity (P = 0.87). This further validates mutations in the exon 3 hot spot region as a prognostic biomarker for TTK inhibitor sensitivity.
Validation of mutant CTNNB1 as a marker of TTK inhibitor sensitivity using isogenic cell lines

To determine whether mutant CTNNB1 was sufficient to confer increased sensitivity to TTK inhibitors, proliferation assays were performed with a pair of isogenic cell lines derived from the colon carcinoma cell line HCT116 (26). Parental HCT116 cells harbor a deletion of three base pairs in one copy of the CTNNB1 gene, resulting in deletion of the regulatory serine residue at position 45 of β-catenin (Table 1). The genotype of the cell line for CTNNB1 is referred to as ΔS45/+.

Isogenic cells lacking the mutated CTNNB1 gene copy (−/+), were less sensitive to TTK inhibitors than parental HCT116 cells (Fig. 2A), which are heterozygous for mutant CTNNB1 (ΔS45/+). Difference in sensitivity, expressed as a shift in pIC50 (ΔpIC50), was approximately one-half log unit for NTRC 0066-0 (ΔpIC50 0.43). In addition, the maximum effect at the highest compound concentration was decreased by 21% in the cell line lacking the mutated CTNNB1 gene copy (Fig. 2A). In contrast, isogenic cells lacking the wild-type gene copy of CTNNB1 (ΔS45/−) showed similar sensitivity to TTK inhibitors as parental HCT116 cells (Fig. 2A). The doubling times of the HCT116 (ΔS45/+), parental), (ΔS45/−) and (−/+), respectively, which is consistent with the lower ability of the (−/+) variant to form colonies, as reported earlier (26).

The other TTK inhibitors also showed less activity in proliferation assays with the isogenic cell line lacking the mutant CTNNB1 gene copy (Fig. 2B and C; Supplementary Fig. S4; Supplementary Table S3B). The differences in sensitivity were of similar magnitude as those between wild-type and mutant lines in the 66 cell line panel, with compound 5 showing the strongest targeting. There the ΔIC50 between parental (ΔS45/+), and the isogenic (−/+), cell line was 0.58 (Supplementary Table S3B).

To study the mechanism of the interaction between mutant CTNNB1 and TTK inhibition, we studied the effect of NTRC 0066-0 on cytoplasm-to-nuclear translocation of β-catenin and expression of the Wnt pathway target gene Axin2 by Q-PCR in the HCT116 parental and the isogenic (−/+), cell line, and the lung carcinoma cell line A427, which is homozygous mutant for CTNNB1 (Table 1). Activation of the Wnt pathway, as determined by Axin2 gene expression, correlated with CTNNB1-mutant status, but there was no direct effect of TTK inhibition (Supplementary Fig. S3A), indicating that TTK inhibitors do not directly interfere with the Wnt pathway.

Validation using functional assay data and GSA

To further explore the mechanism of the association between CTNNB1 mutation and sensitivity to TTK inhibition, we determined β-catenin pathway activity in sensitive cell lines from the Oncolines panel. First, we ascertained by Western blot analysis and qPCR that the CTNNB1-mutant cell lines in the Oncolines panel contain nuclear β-catenin and express Axin2. This was indeed the case, as already had been described in the literature (Supplementary Fig. S3B; refs. 26, 32, 36). Second, to independently study if TTK inhibitor sensitivity is linked to β-catenin transcriptional activity, we used a literature dataset of 85 cancer cell lines labeled “active” or “inactive” in a β-catenin/TCF4 reporter assay (37). Of this dataset, six cell lines with validated reporter assay activity are also part of the Oncolines cell panel (A-549, COLO 205, DLD-1, SW48, and SW480), as well as seven “inactive” cell lines (AspC-1, BxPC-3, MIA Paca-2, NCI-H82, OVCA-3, RKO, and TT). Comparison of inhibitor responses between these two groups indicates that the cell lines active in the β-catenin/TCF4 reporter assay are also significantly more sensitive to TTK inhibitors (P < 0.05). Student t test and Wilcoxon test, Supplementary Table S2C. Thus, these literature data validate the biomarker and suggest that TTK inhibitor sensitivity is linked to β-catenin transcriptional activity.

As markers for drug sensitivity can also be found from mRNA expression profiles (29) we next used GSA on the cell panel data. We generated a list of genes of which high expression correlates with sensitivity to NTRC 0066-0, using gene expression information from 57 cell lines. Because many more lines than the CTNNB1 mutants are analyzed, GSA provides an orthogonal analysis of the biomarker’s mechanism. We investigated if genes related to Wnt/β-catenin signaling were significantly

![Figure 2](https://www.aacrjournals.org/MolCancerTher/article-pdf/16/11/2613/541402/mct-2017-0342.pdf)

**Figure 2.**

Validation of mutant CTNNB1 as drug sensitivity biomarker for TTK inhibitors using isogenic cell lines. A, Representative dose–response curves of NTRC 0066-0 in proliferation assays with parental HCT116 cells (ΔS45/+), red) and an isogenic cell line lacking the mutated CTNNB1 gene copy (−/+), green) or the wild-type gene copy (ΔS45/−), blue) cell line. NTRC 0066-0 was used to determine IC50 values for each cell line. B and C, Box plot showing distribution of relative potencies (ΔpIC50) of TTK inhibitors in proliferation assays in the three isogenic cell lines. Every dot represents 1 of the 10 TTK reference inhibitors. For data in B and C, averages of three independent experiments were used. ΔpIC50 differences compared with the average over the three isogenic variants are shown. Maximum effect is defined as the difference between top and bottom of dose–response curves. a Wilcoxon rank sum test on the combined inhibitor data shows that TTK inhibitors have significantly more potent ΔIC50 and higher maximum effects in both ΔS45/+ and ΔS45/− cells compared with nonmutant (+/+; P < 0.001).
inhibitors have been positioned as potential new drugs in combination with taxane chemotherapy in TNBC (8, 12). Our unbiased gene set analysis (GSA) corroborates that TTK inhibitor–sensitive cell lines have an active β-catenin pathway.

**In vivo efficacy and potential therapeutic relevance in human cancers**

We have previously shown that 20 mg/kg NTRC 0066-0 given orally every other day reduces tumor growth in a mouse xenograft model of the human TNBC cell line MDA-MB-231 by 61% (8). To investigate whether TTK inhibitor therapy is more efficacious in the context of mutated CTNNB1, we attempted to set up an in vivo model using HCT116 and its isogenic variants. However, this was hampered by the appearance of ulcerations in xenografts of the untreated HCT 116 parental cell line, preventing further studies for ethical reasons. As an alternative, we investigated whether the superior in vitro sensitivity of the cell line A427 also translates to an in vivo situation. An in vivo efficacy study was therefore carried out with a xenograft model of the lung carcinoma cell line A427, which is homozygous mutant for the CTNNB1 gene (36). In this model, NTRC 0066-0 reduced tumor growth by 99% (Fig. 3A). In a parallel study, NTRC 0066-0 reduced tumor growth in the MDA-MB-231 model by 70% (Fig. 3B). At the doses used, there was no significant effect on body weight of the mice in both models (Fig. 3C), in agreement with our previous study (8).

**Frequency of CTNNB1 mutations in patients**

Our studies suggest that TTK inhibitors may be in particular effective in cancers characterized by mutations in the CTNNB1 gene. To determine the potential relevance for targeted therapy, we analyzed The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) for the occurrence of CTNNB1 mutations in different indications. CTNNB1 gene mutations were filtered for mutations corresponding to the four regulatory phosphorylation sites in β-catenin encoded by exon 3. These mutations are frequently detected in tumor samples from endometrial cancer and hepatocellular carcinoma (HCC; Table 2), which both have a high incidence. Whereas endometrial cancer is often treated with surgery, there is a high medical need of more effective therapies for HCC. Adrenal cancer and medulloblastoma are cancers with relatively low incidence and relatively high expression (>10%) of mutant CTNNB1 (Table 2). Whereas mutations in CTNNB1 are detected at a much lower frequency in prostate and lung, both cancers have a high incidence (Table 2).

**Discussion**

Inhibition of the spindle assembly checkpoint kinase TTK is a relatively new, and still experimental therapeutic approach to selectively target proliferating cancer cells. Supported by positive results from efficacy studies in preclinical models (8, 12, 14), TTK inhibitors have been positioned as potential new drugs in combination with taxane chemotherapy in TNBC (8, 12). Our unbiased cell panel screen shows that TTK inhibitors are indeed very effective in TNBC cell lines (8, 30), but do not confirm earlier results of TP53 or PTEN targeting (16, 17). Instead, our unbiased cell panel profiling indicates that TTK inhibitors may be more effective in cancers characterized by activating mutations in the CTNNB1 gene.

The distribution of CTNNB1-mutant cell lines in the waterfall plot (Fig. 1A) indicates that mutant CTNNB1 is not the sole determinant of TTK inhibitor sensitivity. Recently, we showed by time-lapse image analysis of six cancer cell lines from the 66 cancer cell line panel, that chromosomally stable aneuploid cell lines are relatively more sensitive to TTK inhibition than...
intrinsiclly chromosomally unstable cell lines (30). Treatment of sensitive cell lines with TTK inhibitors increased the percentage of cells undergoing mis-segregations, which was already maximal in resistant cell lines. This suggests that TTK-sensitive lines are more dependent on an active SAC (30). In that study, we used the near-diploid cell line HCT116 that is chromosomally stable and CTNNB1 mutant. Two other chromosomally stable cell lines studied, LoVo and A-172 (30), are wild-type for CTNNB1. Interestingly, the CTNNB1-mutant lines SW48 and LS 174T are also near-diploid and chromosomally stable, whereas A-427 is near-triploid and chromosomally stable (40, 41). Thus, the CTNNB1-mutant lines have a similar stable karyotype as found earlier in relatively sensitive cells (30). The mechanistic link between CTNNB1 mutation and sensitivity to TTK inhibition is probably related to β-catenin signaling, as indicated by the results from the functional assays and gene set analysis. Whereas β-catenin has a well-known role in cell adhesion and regulation of gene expression (31), β-catenin also contributes to mitosis in the establishment of a bipolar mitotic spindle (42). Activation of Wnt signaling by CTNNB1 or APC mutations has been reported to increase chromosomal instability and cause aneuploidy (43–45). TTK, on the other hand, has been shown to protect aneuploid cells from mitotic catastrophe (4, 42). Interestingly, both β-catenin signaling and mitosis are mediated by FOXM1, which appeared from the GSA (38, 39). Like TTK expression, β-catenin phosphorylation status fluctuates with the cell cycle (47). β-catenin is maximally active during G1–S and disappears at G2–M under the influence of its transcriptional product conductin/Axin2 (47). This is probably because maintaining Wnt-dependent transcription during mitosis is toxic to cells (47). TTK inhibitors could disrupt the cycling process because they reduce time in mitosis by overriding the SAC (8, 30), leaving β-catenin–mutant cells no time to critically downregulate Wnt signaling during mitosis (Fig. 4).

On the basis of these data, we hypothesize that the synthetic lethal effect between TTK inhibition and CTNNB1 mutation arises because deregulated β-catenin signaling in mutant cells makes them more sensitive to mitotic catastrophe. Cells that survive the mutation therefore possess a chromosomally stable karyotype maintained by a strong mitotic checkpoint activation through FOXM1 and cell-cycle control through conductin/Axin2. As a result, CTNNB1-mutant cells particularly depend on the SAC and thereby TTK, making them vulnerable to TTK inhibitors (Fig. 4).

The reason why CTNNB1 mutation shows more significant association than APC mutation could lie in the fact that β-catenin engages in a direct feedback regulation with conductin/Axin2 (47) and FOXM1 (38), whereas APC can also interfere with mitosis through a β-catenin–independent process called Wnt/STOP (48). The mechanistic basis of the association between activated β-catenin and cell response to TTK inhibitors however requires further study. Although our cell panel profiling results do not confirm earlier work on TP53 or PTEN targeting of TTK inhibitors, they do confirm that TTK inhibitors are more active in TNBC cell lines than in cell lines from other cancer types. For instance, the average IC50 of NTRC 0066-0 on four TNBC cell lines [BT-20, BT-349, Hs 578T, MDA-MB-231] is 49 nmol/L, whereas the average IC50 for all other cell lines in the panel is 99 nmol/L (and 123 nmol/L for all non-TNBC breast cancer lines). Although CTNNB1 mutations are rare in TNBC, multiple studies have confirmed that the Wnt/catenin pathway is often activated in TNBC, and is indicative for prognosis (49, 50). A potential pathway activator could be the Wnt ligand Wnt10b, which is involved in mammary development and plays an important role in development of TNBC tumors (52). Therefore, the preferential targeting of TNBC lines and CTNNB1-mutant cell lines by TTK inhibitors might be related.

We propose that mutations in sequences that regulate the degradation of β-catenin can be used as prognostic drug response biomarkers to select patient populations that are most likely to respond to TTK inhibitor therapy (53), and suggest new potential applications for TTK inhibitors, such as endometrial cancer and HCC. Notably, high TTK expression correlates with poor prognosis in HCC (15). Our study provides an additional argument to pursue the development of TTK inhibitor therapy for HCC and other diseases driven or dependent on activated β-catenin. During the course of our analysis, we discovered however that almost no CTNNB1 hotspot mutant cell lines are available from these cancers. The only exception is SNU-398, a liver line that expresses Hepatitis B viral sequences. Thus, there is an urgent need for more CTNNB1-mutant cell line models for liver and endometrial cancers.

Interestingly, β-catenin signaling has also been implicated in intrinsic resistance against immunotherapy in melanoma by regulation of T-cell infiltration (54). In a recent article, Mason and colleagues (13) showed that TTK inhibitor therapy in combination with anti-programmed cell death 1 (PD-1) antibody can induce tumor regression in a syngeneic mouse model of human cancer. One possible explanation of this response, raised by the authors, is the induction of genomic instability and apoptotic cell
death by TTK inhibition, which may promote tumor immunity. Alternatively, or in addition, as suggested by our work, TTK inhibition may directly regulate T-cell response by affecting β-catenin–regulated immune response.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: G.J.R. Zaman, J.A.D.M. de Roos, R.C. Buijsman, J.C.M. Uitdehaag
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