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Tankyrase inhibition sensitizes melanoma to PD-1 immune checkpoint blockade in syngeneic mouse models

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The development of immune checkpoint inhibitors represents a major breakthrough in cancer therapy. Nevertheless, a substantial number of patients fail to respond to checkpoint pathway blockade. Evidence for WNT/β-catenin signaling-mediated immune evasion is found in a subset of cancers including melanoma. Currently, there are no therapeutic strategies available for targeting WNT/β-catenin signaling. Here we show that a specific small-molecule tankyrase inhibitor, G007-LK, decreases WNT/β-catenin and YAP signaling in the syngeneic murine B16-F10 and Clone M-3 melanoma models and sensitizes the tumors to anti-PD-1 immune checkpoint therapy. Mechanistically, we demonstrate that the synergistic effect of tankyrase and checkpoint inhibitor treatment is dependent on loss of β-catenin in the tumor cells, anti-PD-1-stimulated infiltration of T cells into the tumor and induction of an IFNγ- and CD8+ T cell-mediated anti-tumor immune response. Our study uncovers a combinatorial therapeutical strategy using tankyrase inhibition to overcome β-catenin-mediated resistance to immune checkpoint blockade in melanoma.

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Cancer immunotherapy is undergoing rapid advances. Treatment of patients using immune checkpoint inhibitors, such as antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) and programmed death-ligand 1 (PD-L1) that enhance T cell-mediated immune responses against cancer, is considered a major breakthrough.

However, many cancer patients, including 40–65% of melanoma patients, do not respond to checkpoint inhibitor treatment and the underlying mechanisms are not well understood. Resistance mechanisms are currently being mapped, and cellular signaling pathways and components, such as epidermal growth factor receptor (EGFR), phosphoinositide-3-kinase (PI3K)/AKT, serine/threonine kinase (AKT), vascular endothelial growth factor (VEGF) and B-Raf proto-oncogene, serine/threonine kinase (BRAF)/V600E, as well as Wingless-type mammary tumor virus integration site (WNT)/β-catenin signaling, emerge as promising targets for therapeutic intervention.

WNT/β-catenin signaling can play a central regulatory role in immune cell homeostasis, development and function as well as in peritumoral T cell activation, differentiation and tumor-immune cell interplay. β-catenin is the key transcriptional regulator of WNT/β-catenin signaling. β-catenin-induced immune evasion is found in 13% of all tumors and 42% of cutaneous melanoma.

A recent study, using genetically engineered murine melanoma models, revealed that tumors expressing a dominant stable form of β-catenin showed negligible T-cell infiltration and were resistant to checkpoint blockade therapy. In these β-catenin-positive tumors, production of CCL4 or increased tumor infiltration by CD8+ T cells was not G007-LK-mediated enhanced release of the BATF3-lineage dendritic cells (DC) to the tumor microenvironment and finally defective host priming of antigen-specific T cells. In contrast, in melanoma tumors with low levels of β-catenin, DCs and CD8+ T cells migrated into the tumor and the tumor-killing activities of CD8+ T cells could be unleashed or enhanced by the use of checkpoint inhibitors. Hence, interventions that reduce WNT/β-catenin signaling may have the potential to broaden the anti-tumor spectrum of checkpoint inhibitors.

Although dysregulation of WNT signaling is a hallmark characteristic in a major fraction of cancers, anti-cancer therapy that targets this pathway is currently not available in clinical practice. Target identification and characterization of the small-molecular WNT/β-catenin signaling inhibitor XAV939 revealed that telomeric repeat factor (TRF1)-interacting ankyrin-related ADP-ribosyl polymerases 1 and 2 (tankyrase 1 and 2, TNKS1/2) are key and druggable regulatory enzymes in the signaling pathway. TNKS1/2 catalyze the post-translational modification poly(ADP-ribosyl)ation. AXIN1 and AXIN2 proteins are the main rate-limiting structural proteins that together with adenomatous polyposis coli (APC) control the formation of the β-catenin degradosome, which also contains the β-catenin-targeting kinase glycogen synthase kinase 3 beta (GSK3β). TNKS1/2 poly(ADP-ribosyl)ate AXIN proteins to earmark them for degradation by the ubiquitin-proteasomal system. Inhibition of TNKS1/2 can therefore lead to stabilization of AXIN proteins and hence the degradosomes. This, in turn, enhances the phosphorylation and degradation of the central transcriptional regulator β-catenin and inhibits WNT/β-catenin signaling.

TNKS1/2 catalytic activity does not only regulate the stability of AXIN proteins, but also interferes with additional biological mechanisms and cell signaling pathways including telomere homeostasis, mitotic spindle formation, vesicle transport, and energy metabolism, as well as AKT/PI3K, AMPK and Hippo signaling. In Hippo signaling, tankyrase inhibitor-mediated stabilization of angiomotin (AMOT) proteins shifts the subcellular location of the transcription cofactors yes associated protein 1 (YAP) and tfafazzin (TAZ), leading to a reduction of oncogenic YAP signaling. Recent reports show that YAP signaling may support immune evasion in cancer and melanoma by inducing PD-L1 expression, whereas others show that enhanced YAP signaling, due to loss of the regulating kinases large tumor suppressor kinase 1 and 2 (LATS1/2), may promote an anti-cancer immune response.

Checkpoint inhibitor treatment, including blockade of the PD-1 receptor, has shown limited efficacy in the murine B16-F10 melanoma model, despite strong expression of the ligand PD-L1 on the tumor cells; a feature attributed to low tumor infiltration by effector CD8+ T cells. G007-LK is a potent preclinical stage tankyrase inhibitor with a high selectivity towards tankyrase 1 and 2 and a favorable pharmacokinetic profile in mice with an oral bioavailability of 76% and a t1/2 of 2.6 hours in female mice.

Here, we describe G007-LK-mediated blockade of both WNT/β-catenin and YAP signaling in B16-F10 cells in vitro and in vivo. We show that two murine melanoma models display resistance to monotherapy with either anti-PD-1 or G007-LK. A synergistic anti-tumor effect was observed upon combined anti-PD-1/G007-LK treatment. We show that the mechanistic basis for the synergy was not G007-LK-mediated enhanced release of the BATF3-lineage CD-attracting chemokine CCL4 or increased tumor infiltration by CD8+ T cells. Instead, we find that alterations in T cell infiltration are mainly orchestrated by anti-PD-1 treatment alone. Next, we provide evidence that combined anti-PD-1/G007-LK treatment of B16-F10 tumors is effectuated by G007-LK-induced loss of β-catenin in the tumor cells and induction of an interferon-γ (IFNγ) and CD8+ T cell-dependent anti-tumor immune response. Finally, upon RNA sequencing of G007-LK-treated human melanoma cell lines and B16-F10 cells, we reveal a transcriptional response profile for a cell line subpopulation displaying high relative baseline YAP signaling activity and predisposition for reduced MITF expression upon tankyrase inhibition.

**Results**

**G007-LK inhibits WNT/β-catenin and YAP signaling.** Tankyrase inhibition can inhibit proliferation and viability in a subset of cancer cell lines in vitro. When the anti-proliferative effect of G007-LK on cultured B16-F10 mouse melanoma cell line was monitored, only a limited cell growth reduction was observed (Supplementary Fig. 1a, b). Efficacy of G007-LK treatment on WNT/β-catenin and YAP signaling in B16-F10 cells was then explored in vitro and in vivo.

In cell culture, G007-LK-treated B16-F10 cells displayed stabilization of TNKS1/2 and AXIN1 proteins (Fig. 1a, Supplementary Fig. 2a and Supplementary Fig. 27), as well as formation of cytoplasmic TNKS1/2-containing puncta (Supplementary Fig. 3), indicating the formation and accumulation of β-catenin degradosomes.

Moreover, in WNT3a-induced B16-F10 cells, G007-LK treatment reduced the level of nuclear and cytoplasmic β-catenin protein, transcription of the WNT/β-catenin signaling target genes, such as Axin2 and transcription factor 7 (Tcf7) and also luciferase-based WNT/β-catenin signaling reporter activity (Fig. 1a–c and Supplementary Figs. 2b, c, 4a, c, 5b, 27 and Supplementary Table 1a, b). No distinct effect of G007-LK on WNT/β-catenin signaling was observed in neither B16-F10 cells without WNT3a stimulation nor in β-catenin knockout cells (B16-F10Cntnb1K0), indicating low endogenous pathway activity in vitro (Fig. 1a–c, Supplementary Figs. 4a, 5a–e and...
Supplementary Table 1a, b). Treatment with G007-LK was unable to counteract luciferase-based WNT signaling reporter activity rescued by overexpression of N-terminal mutated β-catenin in B16-F10<sub>Ctnnb1</sub>KO cells (Supplementary Fig. 5f). These results suggest that tankyrerase inhibition only induces turn-over of wild-type β-catenin containing intact GSK3β phosphorylation sites.

G007-LK treatment also stabilized angiomotin like 1 (AMOTL1) and angiomotin like 2 (AMOTL2) proteins and decreased transcription of YAP signaling target genes, such as cellular communication network factor 1 (Ccn1, previously named Cyr61), cellular communication network factor 1 (Ccn2, previously named Ctgf), Amotl2 and YAP signaling luciferase reporter activity (Supplementary Figs. 4b, 6a–c, 28 and Supplementary Table 1a,b). The nuclear YAP protein level, instead of being reduced upon tankyrerase inhibition as previously reported<sup>27,38</sup>, actually increased in both B16-F10 and HEK293 cells upon G007-LK treatment (Supplementary Fig. 6a, d and 28). Confocal imaging further revealed that G007-LK treatment induced the aggregation of puncta, predominantly in the cytoplasma, with not only colocalized AMOTL1-YAP and AMOTL2-YAP but also AMOTL1-TNKS1/2 and AMOTL2-TNKS1/2 (Supplementary Fig. 7a, b).

Next, C57BL/6 N mice with established B16-F10 tumors were treated with G007-LK for four days. This treatment destabilized TNKS1/2 and stabilized AXIN1 protein levels, similar to previous reports<sup>23</sup>, and decreased β-catenin protein levels as well transcription of WNT/β-catenin target genes in the tumors (Fig. 2a, b and Supplementary Figs. 8 and 29). In parallel, AMOTL2 protein was stabilized and transcription of the YAP signaling target genes Ccn1, Ccn2, and Amotl2 were reduced in the tumors (Supplementary Figs. 9a–c and 29).

These results show that tankyrase inhibitor treatment using G007-LK can attenuate WNT/β-catenin signaling and YAP signaling target gene expression in B16-F10 cells in vitro and in vivo.

Synergistic tankyrase and PD-1 inhibition treatment effect. To test whether tankyrase inhibition can counteract resistance to immune checkpoint blockade, B16-F10 tumors were established subcutaneously in C57BL/6 N mice. Neither monotherapy with G007-LK, PD-L1 nor PD-1-blocking antibodies reduced tumor size. However, combined anti-PD-1/G007-LK treatment, but not the anti-PD-L1/G007-LK combination, reduced tumor volume and weight (Fig. 3a and Supplementary Fig. 10a–e). In addition, the combined anti-PD-1/G007-LK treatment also reduced WNT/β-catenin signaling and tumor volume of murine Clone M-3<sup>Z1</sup> melanoma in immunocompetent DBA/2 N mice (Fig. 3b, Supplementary Figs. 11a, c–f and 29). No signs of toxicity, intestinal injury (Supplementary Fig. 10g) or change in body weights were observed in any of the mouse experiments (Supplementary Figs. 10f and 11b). To examine longer-term efficacy of combined anti-PD-1/G007-LK treatment, B16-F10-bearing C57BL/6 N mice were followed until the entire control group reached the endpoint criterion. In the three surviving anti-PD-1/G007-LK-treated mice (18.5%) (Fig. 3c and Supplementary Fig. 12a–d), histopathological evaluation of immunostained tumor sections detected no viable tumor cells. Instead, the tumor implant site was infiltrated by macrophages loaded with melanin, presumably derived from B16-F10 cells (Fig. 3d).

In summary, the tested murine melanoma models are resistant to single-agent anti-PD-1 or G007-LK treatment. In contrast, a synergistic anti-tumor effect and eradication of a subset of the tumors was observed upon combined anti-PD-1/G007-LK treatment.

Tankyrase inhibition alters intratumoral cytokine composition. We next pursued the mechanistic basis for the observed synergy of anti-PD-1 and G007-LK treatment. Previous work in a genetically modified mouse melanoma model have indicated that decreased WNT/β-catenin signaling in the tumor cells promoted adaptive immune responses within the tumor by enhanced secretion of the cytokine CCL4<sup>21</sup>. The subsequent chemotaxis of dendritic cells to the tumor site was reported to support infiltration and activation of tumor-reactive CD8<sup>+</sup> T cells<sup>41</sup>. To identify alterations in cytokine secretion upon treatment, conditioned supernatants from matrigel-embedded B16-F10 tumors<sup>59</sup>...
were screened using multiplex immunoassays while cell cultures were analyzed using ELISA assays. Few alterations were detectable in anti-PD-1-treated tumors, however, G007-LK treatment was associated with increased levels of three cytokines and decreased levels of five cytokines (>30% difference compared to control) (Fig. 4a and Supplementary Fig. 13). Similar changes in cytokine secretion were apparent upon anti-PD-1/G007-LK treatment (Fig. 4a and Supplementary Fig. 13). Notably, G007-LK treatment reduced CCL4 levels in B16-F10 tumors (Fig. 4b) but no reduction was detected in cultured B16-F10 cells (Fig. 4c). Ccl4 transcript was not inversely correlated to its previously described negative regulator activating transcription factor 3 (Atf3) in either wild-type B16-F10 cells or B16-F10Ctnnb1KO cells when compared to wild-type cells (Fig. 4d, e).

In conclusion, the results suggest that G007-LK treatment, but not anti-PD-1 treatment, mainly alters the intratumoral cytokine composition. The herein observed anti-PD-1/G007-LK-induced anti-tumor effect cannot be attributed to enhanced CCL4 secretion.

The treatment effect depends on β-catenin in tumor, IFNγ, and CD8+ T cells. A knockout of β-catenin in B16-F10 cells can serve as a model to recapitulate G007-LK-mediated blockade of WNT signaling. To evaluate β-catenin-mediated immune evasion in the B16-F10 syngeneic mouse melanoma model, β-catenin was knocked out in B16-F10 cells (B16-F10Ctnnb1KO, Supplementary Fig. 5a, b) and one of the cell lines, B16-F10Ctnnb1KO1, was used to establish subcutaneous tumors in C57BL/6 N mice. Compared to vehicle control mice, anti-PD-1-treated mice displayed reduction in tumor size, indicating loss of anti-PD-1 resistance in β-catenin-deficient tumors (Fig. 5a and Supplementary Fig. 14a–d). The result suggests that the synergistic anti-PD-1/G007-LK treatment effect seen in wild-type B16-F10 tumors (Fig. 3a) is, to a considerable part, attributed to G007-LK-induced reduction of β-catenin levels in the melanoma cells of the tumors.

To evaluate if the observed effects of anti-PD-1/G007-LK treatment are mediated by an adaptive immune response, B16-F10 challenge experiments were repeated in recombinase-deficient (Rag2−/−) mice, which lack functional T and B cells, but possess functionally intact natural killer (NK) cells. No significant effect of anti-PD-1/G007-LK treatment was observed in such mice (Fig. 5b and Supplementary Fig. 15a, b). Although, a contributory role of NK cells and the innate immune system cannot be entirely excluded, the result indicates that the anti-PD-1/G007-LK treatment effect is orchestrated by an adaptive immune response.

Next, we further evaluated the adaptive immune response by performing selective elimination of either CD8+ T cells or IFNγ. Antibody-mediated CD8+ T cell depletion abrogated the anti-tumor effect of anti-PD-1/G007-LK treatment (Fig. 5c and Supplementary Fig. 16a, b). Similarly, neutralization of IFNγ resulted in increased tumor growth comparable to anti-PD-1/G007-LK-treated mice (Fig. 5c and Supplementary Fig. 16a, b). In summary, these results confirm a role of CD8+ T cells and IFNγ as mediators of the synergistic effect of anti-PD-1/G007-LK treatment.

To assess immune cell infiltration upon treatment, we next performed flow cytometry analysis using tumors of comparable size collected on day 7–17 (Supplementary Figs. 17a and 18a, b). No increase in tumor leukocyte abundance was observed in any of the treatment groups when compared to the control group (Supplementary Fig. 17b). An increase in total T cell and CD8+ T cell infiltration was seen in both the anti-PD-1 and anti-PD-1/G007-LK groups, whereas CD4+ T cells were similarly increased across all treatment groups (Fig. 5d and Supplementary Fig. 17c). No differences in the abundance of CD45+ or CD8+ T cells expressing the memory marker CD44 were detected (Supplementary Fig. 17d). Treg cells constituted approximately 1% of infiltrating CD45+ cells in all groups (Supplementary Fig. 17e). Myeloid DCs were decreased in the anti-PD-1 and anti-PD-1/G007-LK-treated groups while CD103+ DCs were equally present in all treatment groups (Supplementary Fig. 17f). Lymphoid DCs, myeloid-derived suppressor cells (M-MDSC) and neutrophils were present at comparable levels across all treatment groups (Supplementary Fig. 17g).

Subsequently, infiltration of CD8+ T cells in tumors sections was scored using treated wild-type and β-catenin knock-out B16-F10 tumors. Only anti-PD-1 and anti-PD-1/G007-LK-treated B16-F10Ctnnb1KO tumors showed an increase in CD8+ T cell infiltration when compared with controls (Fig. 5e and Supplementary Fig. 19). The result suggests that only PD-1 inhibition, and not loss of β-catenin in the tumor cells, contributes to increased chemotaxis of CD8+ T cells.

To evaluate direct effects of G007-LK on T cell effector functions, we assayed the in vitro activation of MHC class I (H2-Kb)-restricted and ovalbumin-specific CD8+ T cells. The presence of G007-LK moderately enhanced T cell proliferation...
following cognate interaction with antigen-presenting cells, but did not affect proliferation following polyclonal activation by immobilized anti-CD3/anti-CD28 antibodies (Supplementary Fig. 20a). The presence of G007-LK did not affect the expression of T cell activation markers (CD26L, CD44, CD25, and CD69) or secretion of interleukin 2 (IL2) or IFNγ (Supplementary Fig. 20b–d). The addition of G007-LK induced a slight increase in intracellular granzyme B expression in CD8+ T cells following cognate or polyclonal activation, possibly indicating increased effector function (Supplementary Fig. 20e).

In summary, combined anti-PD-1/G007-LK treatment of B16-F10 tumors is dependent on tankyrase inhibitor-mediated loss of β-catenin in the tumor and induces an IFNγ and CD8+ T cell-dependent growth-inhibitory effect. Changes in myeloid DC and T cell infiltration are likely attributable to anti-PD-1 treatment, but do not alone seem to cause anti-tumor activity.

RNA sequencing reveals a subpopulation transcriptional response profile. The efficiency of tankyrase inhibitor-mediated inhibition of WNT/β-catenin and YAP signaling is known to be cell type and context-dependent. The beneficial synergistic anti-PD-1/G007-LK cell treatment effect seen on immunosurveillance in B16-F10 tumors may therefore vary between melanomas based on differences in cell signaling pathway activities and genetic background. In addition, the here utilized B16-F10 murine melanoma model, lacking the BRAFV600E mutation41,42, only partially recapitulates the genetic features of human melanoma. Thus, murine B16-F10 cells and a panel of 18 human melanoma cell lines, were exposed to G007-LK treatment followed by RNA sequencing and bioinformatic analyses.

In the untreated group, no clear correlation was found between baseline transcription of WNT/β-catenin signaling target genes (Supplementary Fig. 21a) or the mutation load (Supplementary Fig. 3) Dual inhibition of tankyrase and PD-1 confers synergistic anti-tumor efficacy in mouse melanoma. a B16-F10 tumor (s.c.) end volume upon anti-PD-1/G007-LK treatment (−83% when compared to control) in C57BL/6 N mice treated from day 10 through 21. Control diet (n = 10), G007-LK diet (n = 10), anti-PD-1 (n = 11), and anti-PD-1/G007-LK (n = 8). Mann-Whitney rank sum tests are indicated by †(P < 0.05) and ‡(P < 0.01). For a, b Mean values are indicated by grey lines. Absence of depicted statistical comparisons indicates lack of statistical significance. b Clone M-3Z1 tumor (s.c.) end volume reduction upon anti-PD-1/G007-LK treatment (−53% when compared to control) in DBA/2 N mice treated from day 8–18. Control diet (n = 8), G007-LK diet (n = 8), anti-PD-1 (n = 11), and anti-PD-1/G007-LK (n = 10). Two-tailed t-test is indicated by *(P < 0.05). c Kaplan-Meier plot showing survival of B16-F10-recipient (s.c.) C57BL/6 N mice treated with control (n = 19, blue) or anti-PD-1/G007-LK (n = 16, black) from day 10 to 38. Difference between the two graphs: One-tailed log rank (Mantel-Cox) test, P = 0.087, hazard ratio = 1.75, 95% CI: 0.78–3.93. d Representative images from H&E (left and middle panels) and F4/80 and Hoechst-stained (in green and blue, respectively, right panels) tumors from control (n = 5, upper panels) and tumor implant sites of anti-PD-1/G007-LK-treated surviving mice (n = 2, lower panels). Macrophages laden with melanin are highlighted with arrows. Scale bars: H&E = 100 µm (original magnification × 100) and H&E higher magnification and F4/80 Hoechst = 50 µm (original magnification × 400).
Whitney rank sum test is indicated by third quartiles and maximum and minimum whiskers. One representative experiment of two repeated assays with three replicates is shown. Data from minimum three independent experiments with three replicates each are shown. Two-tailed RT-qPCR analyses of (KO1 and 2) treated with G007-LK (1 µM) were predisposed for decreased MITF expression (Fig. 6b and Supplementary Fig. 23a, b and Supplementary Table 2). Notably, in B16-F10 tumors, transcription of Mitf was moderately reduced upon G007-LK treatment (Fig. 6c). Attenuated WNT/β-catenin and/or YAP signaling activity was observed in nearly all samples upon G007-LK treatment (Fig. 6d). These changes in signaling pathway activities did not correlate with changes in MITF expression, nor act as a predictive marker for MITF regulation (Fig. 6e and Supplementary Figs. 24b–e and 25a, b). In conclusion, YAPhigh is a marker for a melanoma subgroup that includes B16-F10 (Fig. 6e) and tracks with tankyrase inhibitor-induced reduction in MITF expression (Supplementary Fig. 26 and Supplementary Table 3).

Discussion

Here we show, for the first time to our knowledge, proof-of-concept results for a previously unreported therapeutic strategy using tankyrase inhibitor-mediated blockade of WNT/β-catenin signaling to counteract β-catenin-supported immune evasion and resistance to checkpoint inhibition in syngeneic murine melanoma models.

The G007-LK treatment effect could be recapitulated in vivo by knockout of β-catenin in B16-F10 tumor cells. Although not further supported by a rescue experiment re-introducing N-terminal mutant β-catenin, the result suggests that loss of WNT/β-catenin signaling in tumor cells is sufficient to trigger a gain of susceptibility and a synergetic relationship to checkpoint inhibition. Nevertheless, we cannot exclude the possibilities that...
tankyrase inhibition impacts additional biological mechanisms in the tumor cells, in the tumor microenvironment, systemically or on immune cell subpopulations that can contribute to the treatment effect. In addition to its effect on WNT/β-catenin signaling, G007-LK treatment stabilized AMOT proteins and reduced YAP signaling-mediated gene expression in B16-F10 cell culture and tumors. An absence of reduced nuclear YAP and TAZ protein levels, as also seen in HEK293 cells, along with the here observed aggregation of colocalized YAP, AMOT proteins and TNKS1/2-containing puncta, is inconsistent with previous reports indicating that further evaluation of tankyrase inhibitor-induced interference with YAP signaling is necessary.

Our results provide evidence that the anti-tumor effect of combined tankyrase and checkpoint inhibitor treatment against B16-F10 tumors is dependent on both IFNγ and CD8+ T cells and occurs, at least in part, through direct suppression of WNT/β-catenin signaling within the tumor cells. The precise mechanistic basis of this synergistic effect remains to be investigated. Previous work performed in genetically modified models indicate that lowered WNT/β-catenin signaling resulted in increased intratumoral CCL4 cytokine release, which lead to enhanced influx of CD103+/BATF3+ pDCs and subsequent activation of CD8+ T cell anti-tumor activity. Our results indicate that G007-LK treatment indeed could change the intratumoral cytokine composition; however, we found no evidence for increased CCL4 cytokine levels. In addition, assessment of the intratumoral immune response following G007-LK monotherapy did not yield significant quantitative alterations in T cell or antigen-presenting cell (APC) subsets in treated mice. While it cannot be excluded that qualitative or more subtle quantitative changes in particular tumor-specific CD8+ T cell subsets might be responsible for the observed therapeutic effects, an increase in overall CD8+ T cell infiltration was not observed. Only a moderate enhancement of proliferation and granzyme B expression was observed in G007-LK-treated MHC class I (H2-Kb-) restricted and ovalbumin-specific CD8+ T cells, which could indicate enhanced effector function. In depth follow-up studies in the framework of tankyrase inhibition are required, including assessment of the effects of alterations in individual cytokine and chemokine levels on the T cell response.

The use of model systems with defined and...
traceable anti-tumor CD8+ T cell specificities would also be of value in determining the effects of tankyrase inhibition on the kinetics and qualitative characteristics of the ensuing immune response.

The RNA sequencing of 18 tankyrase inhibitor-treated human melanoma cell lines and B16-F10 cells shows that tankyrase inhibition context-dependently can influence WNT/β-catenin and YAP signaling, not only in murine B16-F10 cells, but importantly also in a subset of 18 human melanoma cell lines. The results indicate that tankyrase inhibition may have a potential for treatment of a subset of patients with the human disease in combination with checkpoint inhibition. Analysis of the RNA sequencing data revealed a subgroup-specific transcriptional response profile. Upon tankyrase inhibition, the subgroup displaying elevated baseline YAP signaling activity was susceptible to reduced MITF expression. Presently, neither the meeting points between MITF, its regulation by YAP/TEA domain transcription factor (TEAD), activator protein 1 (AP-1)
and tankyrase, nor the function in immune regulation and control of susceptibility to checkpoint-inhibitor therapy are well characterized\(^\text{46-51}\). Further molecular and functional evaluations are required for establishing a precise set of rules for context-dependent treatment efficiency using combined tankyrase and checkpoint inhibitor treatment in human melanoma. Translation of the combinatorial therapeutic strategy, using tankyrase inhibition to counteract β-catenin-induced resistance to immune checkpoint blockade, should also be evaluated to test treatment efficacy against other cancers\(^\text{57-59}\).

Tankyrase inhibitors have been suspected to cause intestinal toxicity\(^\text{46-58}\) and bone loss\(^\text{52-53}\) in certain mouse models. In this study, and similar to previous reports\(^\text{28-29,36-53}\), we observed no signs of toxicity, intestinal injury or body weight changes in G007-LK-treated mice. G007-LK is a preclinical stage tankyrase inhibitor.

Methods

**Cell culture.** The mouse melanoma cell line B16-F10 (ATCC \(^\text{CRL-6475}\)) and human HEK293 cells (ATCC \(^\text{CRL-1573}\)) were obtained from the American Type Culture Collection. Clone M-3–2 (ProQnase) cells were derived from Clone M-3 cells (ATCC \(^\text{CCL-53.1}\)) after implantation in DBA/2 NCi mice (DBA/2N, Charles River). The cell cultures were generally kept below 20 passages (~10 weeks) and the melanoma cells were grown in RPMI-1640 medium (R8758, Sigma Aldrich) while HEK293 cells were grown in DMEM medium (D6429, both Sigma Aldrich). The cell culture medium was supplemented with 1% penicillin-streptomycin (P4333, Sigma Aldrich) and 5% fetal bovine serum (FBS, 10270-106, Gibco) and grown at 37 °C in humidified incubators with 5% CO\(_2\). The cells were routinely monitored (upon thawing and monthly) for mycoplasma using MycoAlert mycoplasma detection kit (Lonza). B16-F10 cells were authenticated by short tandem repeat profiling and subsequent analysis confirming murine C57BL/6 origin (Leibniz-Institute DSMZ). General protocol for treatment of cultured B16-F10 cells: Cells were seeded one day before treatment to reach ~20% confluence for 72 or 24 h treatments, respectively. The cell culture medium was changed for medium containing 0.01% DMSO (D8418, Sigma Aldrich), 1 µM or 10-cm dishes: 16.5 µg luciferase reporter and 3 µg Renilla luciferase. For over-expression assays using 6-well plates: 2.2 µg luciferase reporter, 0.1 µg del-β-catenin, DA β-catenin or pCI-neo (empty vector, Promega) and 0.2 µg Renilla luciferase reporter for assays using FuGENE \(\text{HD}\) (Promega). On day 1, B16-F10 cells were seeded to reach 50-60% confluence on day 2 for co-transfections (for reporter assays using 10-cm dishes: 16.5 µg luciferase reporter and 3 µg Renilla luciferase. For over-expression assays using 6-well plates: 2.2 µg luciferase reporter, 0.1 µg del-β-catenin, DA β-catenin or pCI-neo (empty vector, Promega) and 0.2 µg Renilla luciferase reporter for assays using FuGENE \(\text{HD}\) (Promega). On day 3, the cells were trypsinized and seeded in 96-well plates and treatment was added on day 4.

**Luciferase reporter assays.** The following luciferase reporter vectors were used: Wild-type (WT) β-catenin signaling pathway reporter with 7X TCF binding sites: St-Luc, gift from V. Korinek, SFF-Luc (negative control reporter with mutated TCF binding sites: SuperTOPflash-luciferase, gift from V. Korinek), 8xG/TIC- luciferase (Hippo and TAP signaling pathway reporter: 34615, Addgene, provided by Dr. Stefano Piccolo), del-β-catenin or with deleted-del-β-catenin, gift from R. Kemler) or point mutated (dominant active [DA] β-catenin [S33, 37, 41, 45 A], gift from R. Kemler) N-terminal domain containing GSK3β phosphorylation sites and Renilla luciferase (pRL-TK, Promega). On day 1, B16-F10 cells were seeded to reach 50-60% confluence on day 2 for co-transfections (for reporter assays using 10-cm dishes: 16.5 µg luciferase reporter and 3 µg Renilla luciferase. For over-expression assays using 6-well plates: 2.2 µg luciferase reporter, 0.1 µg del-β-catenin, DA β-catenin or pCI-neo (empty vector, Promega) and 0.2 µg Renilla luciferase reporter for assays using FuGENE \(\text{HD}\) (Promega). On day 3, the cells were trypsinized and seeded in 96-well plates and treatment was added on day 4.

**RNA isolation and real-time qRT-PCR.** Total RNA was isolated from cell lines and tumor samples using GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma Aldrich). The RNA concentration was measured using Nanodrop 2000c spectrophotometer (Thermo Scientific). cDNA was synthesized from the purified RNA using SuperScript™ II VLO cdNA Synthesis Kit (Invitrogen). Real-time qRT-PCR was performed using SYBR® Green PCR Mastermix (Thermo Fisher Scientific). Primer pairs were designed using Primer3 tool (Table S1). 

**Western blot analysis.** Treated cells were washed in PBS and lysed in NP40 Cell lysis buffer (Invitrogen) containing protease inhibitors. The nuclei were pelleted and separated from the cytoplasmic/cell membrane supernatant fractions. RIPA lysis buffer (89091, Thermo Fisher Scientific) containing phosphatase (4906845001) and protease inhibitors (496316001, both Sigma Aldrich) was added to the nuclei followed by sonication (Bioruptor®Plus, Diagenode). Tumor samples were prepared using TissueRuptor II (9002755, Qiagen), RIPA buffer and sonication. Protein concentrations were measured using Pierce™ BCA Protein Assay Kit ( Pierce BCA Protein Assay Kit). The protein samples were separated by SDS PAGE (Invitrogen) and immunoblotted (Immobilon-P PVDF Membrane, Millipore) using the following primary antibodies: Tankyrase-1/2 (TNKS1/2, H-350, sc-8337, Santa Cruz Biotechnology), AXIN1 (C7B2, 3323, Cell Signaling Technology), non-phospho (active) β-catenin (D15A1, 8814, Cell Signaling Technology), β-catenin (610153, 1:500, BD Biosciences), YAP (sc-101199, Santa Cruz Biotechnology), TAZ (HPA007415, Sigma Aldrich), AMOT (sc-166924, Santa Cruz Biotechnology), AMOTLI1 (PA5-42267, Thermo Fisher Scientific), AMOTL2 (PA5-78770, Thermo Fisher Scientific), GSK3β (12456, Cell Signaling Technology), phosphorylated GSK3β (Serine [Ser] 9) (8933, Cell Signaling Technology), GAPDH (D60, Santa Cruz Biotechnology), β-tubulin III (T2200, Sigma Aldrich), actin (A2066, Sigma Aldrich), and lamin B1 (ab16048, Abcam) were added as loading controls. Primary antibodies were visualized with HRP-conjugated secondary antibodies (mouse anti-rabbit IgG, sc-2357, Santa Cruz Biotechnology, monkey anti-rabbit IgG, 711-035-152, Jackson Immunoresearch) and enhanced with chemiluminescent substrate (ECL™ Prime Western Blotting Detection Reagent, RP2236, GE Healthcare) and ChemiDoc Touch Imaging System (Bio-Rad). Band quantifications (band of interest versus loading control) was performed using Image Lab Software 5.2.1 (Bio-Rad).

**CRISPR/Cas9-based knockout.** DNA sequences targeting exon 4 of mouse ctcnbb (β-catenin associated protein), 5′-Ctnnb1 _gRNA1: 5′-GATTAACTACTGGATAGCC-3′. The gRNA sequence was inserted into the pSpCas9(BB)-2A-GFP vector (PX458, 48138, Addgene) for transfection into tumor cells using polyethylenimine (40872, Sigma Aldrich). After 24 h, GFP-expressing cells were single-cell sorted (BD FACSAria II, BD Biosciences). Genomic DNA was isolated from isolated clones, the relevant gene fragment was amplified by PCR (Forward primer: 5′-GTCCTCGAGCTAGCCGTCAGAG-3′; Reverse primer: 5′-ACATCA CTTGTTATCTTGCCTCCT-3′) and screened by Sanger sequencing for non-sense mutations in Ctnnb1. Additional verification of gene knockout was performed by immunofluorescence staining (primary antibody β-catenin (Mm00443610_m1), 1:500, Bio-Rad) and secondary antibody goat ant-mouse (go Alexa 488 A28175, 1:500, Thermo Fisher Scientific)) and western blot analysis (β-catenin, 1:500).

**Immunofluorescence, SIM and confocal microscopy.** Cells grown on coverslips pre-coated with poly-L-lysine (sc-286689, Santa Cruz Biotechnology) were fixed in 4% paraformaldehyde (P6148, Sigma Aldrich) for 15 min at room temperature and permeabilized with 0.1% Triton-X100/PBS (T8787, Sigma Aldrich, 15 min at room temperature, followed by 1 hour (at room temperature) SIM incubation at 4°C (for confocal images) incubations with primary and secondary (antibodies 1 h at room temperature) diluted in PBS with 4% bovine serum albumin. Nuclear counterstaining was performed with DAPI (D9542, Sigma Aldrich, 1 µg/mL, 5 min at room temperature) and coverslips were mounted in ProLong Diamond Antifade Mountant (Invitrogen, Thermo Fisher Scientific). The following primary antibodies were used: β-catenin (610153, 1:500, BD Biosciences), Tumor昌-1/2 (H-350, sc-8337, 1:500, Santa Cruz Biotechnology [for SIM imaging]), Tumor昌-1/2 (E10, sc-365897, Nature).
immersion objective, images were acquired using Zen software (Zeiss). The confocal microscope using standard rendered from all Z planes.

For experiments using B16-F10 cells in C57BL/6 N mice: Intraperitoneal injections of anti-PD-1 or anti-PD-L1 were administered on day 10, 13, 17 and every 4 days from day 21 until the end for the survival analysis. For experiments in C57BL/6 N mice: On day 11, 48 tumor-bearing animals (primary tumors reaching 20–100 mm³) were randomized into four groups (n = 12), each treated with (i), (ii), (iii), or (v). On day 25, mice were euthanized, and the tumors were dissected, weighed and volumes re-measured with caliper. Three mice from each group received antibody treatment. For ethical reasons, due to skin ulcerations in the tumor area.

Tumor growth assay using B16-F10 cells in C57BL/6 N mice: On day 11, 48 tumor-bearing animals (primary tumors reaching 20–100 mm³) were randomized into four groups (n = 12), each treated with (i), (ii), (iii), or (v). On day 25, mice were euthanized, and the tumors were dissected, weighed and volumes re-measured with caliper. Three mice from each group received antibody treatment. For ethical reasons, due to skin ulcerations in the tumor area.

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matrigel mixture) in 6–8 weeks old female C57BL/6 mice (Taconic). On day 6, animals were distributed into four groups and treatments (i) (n = 3), (ii) (n = 6), (iii) (n = 6) and (iv) (n = 6) were administered intraperitoneally and IFNγ quantification in culture supernatants was determined using ELISA Max Deluxe kits (431004 and 430804, Biolegend). For flow cytometric analysis of surface markers on APC/SIINFEKL or ConA-activated CD8+ T cells, the cells were pooled from 8–12 wells from the setup described previously and the following antibodies were used: Anti-CD26L (1705-09-L, Southern biotech), anti-CD103 (11-1301, Southern Biotech) or anti-CD25 (17-0251-82, Biolegend), anti-CD44 (103049, Biolegend), and anti-CD3e (35-0031, TONBO Biosciences). For intracellular staining, the cells were incubated for 4 h in protein transport inhibitor before permeabilization and fixation, according to the manufacturer instructions (Fix/Perm Kit for Flow with GolgiStop, 17-57415, Biolegend) and then stained with anti-gramy-B (12-8898-82, Biolegend). All antibodies were used at a working concentration of 2 µg/mL. The samples were assayed using the Attune NxT flow cytometer (Thermo Fisher Scientific) and FlowJo software (BD Biosciences).

**Tumor flow cytometry analysis.** Primary tumor from treatment groups (i), (ii), and (iii) was collected and processed for flow cytometry analysis to determine the presence of subpopulations of T cells and myeloid-derived suppressor cells (carried out by ProQinase39). For analysis of T cells and myeloid-derived suppressor cells, animals were treated for 7–17 days to obtain similarly distributed primary tumor volumes ranging from 80–240 mm³ (Supplementary Fig. 17a). The animal experiment was approved by local animal experiment authorities (Norwegian Food Safety Authority, Norway) and in compliance with FELASA guidelines and the EC Directive 2010/63/EU. For Bio-Plex Pro Mouse Chemokine Panel 33-plex analysis, cell supernatants from treated cells and conditioned media were included. Variants found in RNAseq data were cross-referenced with COSMIC (v82) was used as input. Due to the variable coverage in RNAseq data, additional SNVs found in an external unpublished gene panel sequencing experiment for the same cell lines, were included. Variants found in both datasets were annotated using ANNOVAR (2017-07-17)69. The data analysis was performed by the Bioinformatics Core Facility (Oulu University Hospital, Norway). DEGs analysis, including log2-fold change and adjusted p-values, were uploaded into Ingenuity Pathway Analysis (IPA) version 10.0 (Qiagen). The DEGs analysis data were analyzed using the core analysis function with the Ingenuity Knowledge Base (genes only) reference set and direct relationships, with no filters set for node types, data sources, confidence, species, tissues and cell lines and mutations. For the IPA core analyses, the log2-fold and or adjusted p-value cutoffs are specified in the figure legends.

**Statistics and reproducibility.** No sample size calculation was performed. Sample sizes for both in vivo and in vitro experiments were determined based on experience, pilots and preliminary experiments as well as what was reported in the literature. Samples sizes for each experiment and numbers of independent repeats are indicated for each figure legend. All in vitro experiments included at least 2 independent biological replicates, except for Fig. 4a, b (2±3) and Fig. 5e (2±3). For all in vivo assays, all attempts at replication were successful through repeated experiments (two or more replications). Sigma Plot 12.5 (Systat Software Inc.) was used to perform statistical tests: Student’s t-test for comparisons with homogenous variances (Shapiro-Wilk test, P > 0.05) and Mann-Whitney rank sum tests for comparisons where the normality assumption was violated (Shapiro-Wilk test, P < 0.05). GrapPad Prism 7 was used for Kaplan-Meier estimations and statistical analysis. Single outlier detections
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