Onvansertib in Combination With Chemotherapy and Bevacizumab in Second-Line Treatment of *KRAS*-Mutant Metastatic Colorectal Cancer: A Single-Arm, Phase II Trial

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ABSTRACT

PURPOSE This phase II study evaluated the efficacy and tolerability of onvansertib, a polo-like kinase 1 (PLK1) inhibitor, in combination with fluorouracil, leucovorin, and irinotecan (FOLFIRI) + bevacizumab for the second-line treatment of *KRAS*-mutant metastatic colorectal cancer (mCRC).

PATIENTS AND METHODS This multicenter, open-label, single-arm study enrolled patients with *KRAS*-mutated mCRC previously treated with oxaliplatin and fluorouracil with or without bevacizumab. Patients received onvansertib (15 mg/m² once daily on days 1-5 and 15-19 of a 28-day cycle) and FOLFIRI + bevacizumab (days 1 and 15). The primary end point was the objective response rate (ORR), and secondary endpoints included progression-free survival (PFS), duration of response (DOR), and tolerability. Translational and preclinical studies were conducted in *KRAS*-mutant CRC.

RESULTS Among the 53 patients treated, the confirmed ORR was 26.4% (95% CI, 15.3 to 40.3). The median DOR was 11.7 months (95% CI, 9.4 to not reached). Grade 3/4 adverse events were reported in 62% of patients. A post hoc analysis revealed that patients with no prior bevacizumab treatment had a significantly higher ORR and longer PFS compared with patients with prior bevacizumab treatment: ORR of 76.9% versus 10.0% (odds ratio of 30.0, $P < .001$) and median PFS of 14.9 months versus 6.6 months (hazard ratio of 0.16, $P < .001$). Our translational findings support that prior bevacizumab exposure contributes to onvansertib resistance. Preclinically, we showed that onvansertib inhibited the hypoxia pathway and exhibited robust antitumor activity in combination with bevacizumab through the inhibition of angiogenesis.

CONCLUSION Onvansertib in combination with FOLFIRI + bevacizumab showed significant activity in the second-line treatment of patients with *KRAS*-mutant mCRC, particularly in patients with no prior bevacizumab treatment. These findings led to the evaluation of the combination in the first-line setting (ClinicalTrails.gov identifier: NCT06106308).

ACCOMPANYING CONTENT

Appendix

Data Sharing Statement

Protocol

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INTRODUCTION

The mainstay of management for patients with metastatic colorectal cancer (mCRC) harboring oncogenic *KRAS* mutations has not significantly changed in 20 years. Cytotoxic chemotherapy with fluoropyrimidines combined with either oxaliplatin or irinotecan forms the backbone of treatment. The addition of antiangiogenic therapy, notably bevacizumab—a monoclonal antibody that targets vascular endothelial growth factor A—enhances chemotherapy efficacy in both first- and second-line treatments.[1-3] However, the benefit of combining chemotherapy and antiangiogenic agents in the second-line setting remains limited, with an objective response rate (ORR) around 15% and a median progression-free survival (PFS) of 6-7 months.[3-5]

Developing therapies targeting mutant KRAS (≈45% of patients with CRC)[6] has been challenging. Inhibitors of

CONTEXT

Key Objective

This phase II study evaluated the tolerability and efficacy of the polo-like kinase 1 (PLK1) inhibitor onvansertib in combination with chemotherapy and bevacizumab as second-line treatment for *KRAS*-mutant metastatic colorectal cancer (mCRC).

Knowledge Generated

The combination therapy was well tolerated, achieving an objective response rate (ORR) of 26.4%, with post hoc analyses revealing significantly greater clinical benefit in bevacizumab-naïve patients, who had an ORR of 76.9% compared with 10.0% in those previously exposed to bevacizumab. Translational studies suggested synergy between onvansertib and bevacizumab, whereas prior bevacizumab exposure appeared to contribute to treatment resistance.

Relevance *(A.H. Ko)*

The addition of a PLK 1 inhibitor to chemotherapy/bevacizumab exhibits very promising activity in patients with previously treated but bevacizumab-naive *KRAS*-mutant mCRC. Further data from larger randomized clinical trials will help determine whether this may prove to be a useful strategy for this patient population in need of mechanistically novel therapeutic agents.*

*Relevance statement written by *JCO* Associate Editor Andrew H. Ko, MD, FASCO.

$KRAS^{G12C}$, a relatively rare mutation in CRC (approximately 3% of patients),[6] have shown modest single-agent activity,[7-9] prompting the exploration of combination therapies.[10-12] Most patients with mCRC with *KRAS* mutations still lack effective targeted therapies, underscoring the urgent need for innovative treatments.

Polo-like kinase 1 (PLK1) has emerged as a promising target. The serine/threonine kinase PLK1 regulates the cell cycle by controlling mitotic entry and progression.[13] Onvansertib, a selective PLK1 inhibitor, has shown potent antitumor activity in CRC preclinical models, both as a single agent and in combination with irinotecan.[14,15] Additionally, synthetic lethality between PLK1 inhibition and *KRAS* mutation has been observed in CRC models, enhancing the therapeutic potential for *KRAS*-mutated patients.[15,16] We recently reported that onvansertib combined with fluorouracil, leucovorin, and irinotecan (FOLFIRI) and bevacizumab was safe in patients with *KRAS*-mutant mCRC,[15] prompting further evaluation in phase II trials.

In our phase II study, we assessed the antitumor activity and tolerability of onvansertib at 15 mg/m^2 in combination with FOLFIRI and bevacizumab for the second-line treatment of patients with *KRAS*-mutant mCRC. The observation of differential clinical benefit, dependent on prior bevacizumab exposure, prompted comprehensive translational analyses to understand the interplay between onvansertib and bevacizumab exposure. Here, we report the prospective trial and subsequent translational analyses.

PATIENTS AND METHODS

Study Design and Patients

This study (ClinicalTrials.gov identifier: NCT03829410) was an open-label, single-arm phase Ib/II study conducted at seven sites in the United States, evaluating the efficacy and tolerability of onvansertib in combination with FOLFIRI and bevacizumab in second-line treatment of *KRAS*-mutant mCRC. The phase I portion, previously reported, described detailed eligibility criteria.[15] Briefly, patients age ≥18 years with histologically confirmed metastatic and unresectable CRC and a *KRAS* mutation in exons 2, 3, or 4 detected in the primary tumor or the metastasis by a Clinical Laboratory Improvement Amendments–certified laboratory were eligible. Patients were required to have received ≥6 weeks of oxaliplatin and fluoropyrimidine, with or without bevacizumab, and to have either progressed within 6 months of treatment or shown intolerance to oxaliplatin. Eastern Cooperative Oncology Group performance status of 0 or 1 and adequate organ function were required. Key exclusion criteria included microsatellite instability-high/mismatch repair deficiency, BRAF V600 mutation, more than one prior chemotherapy regimen for metastatic disease, and untreated brain metastasis.

The study protocol was approved by each participating site's institutional review board and/or independent ethics committee and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All participants provided written informed consent.

TABLE 1. Baseline Demographics and Disease Characteristics

Characteristic	Patients (n = 53)
Median age (range), years	54 (34-83)
Sex, No. (%)	
Male	27 (50.9)
Female	26 (49.1)
Race, No. (%)	
White	44 (83.0)
Other	9 (17.0)
ECOG PS, No. (%)	
0	29 (54.7)
1	24 (45.3)
Primary tumor site, No. (%)	
Left colon or rectum	34 (64.1)
Right colon	18 (34.0)
Unknown	1 (1.9)
Metastatic disease at diagnosis, No. (%)	
Yes	44 (83.0)
No	9 (17.0)
Liver metastasis, No. (%)	
Yes	39 (73.6)
No	14 (26.4)
Metastatic lesions, No. (%)	
Single	3 (5.7)
Multiple	50 (94.3)
Metastatic organs, No. (%)	
Single	14 (26.4)
Multiple	39 (73.6)
Received prior bevacizumab, No. (%)	
Yes	40 (75.5)
No	13 (24.5)

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status.

Treatment

Onvansertib was administrated orally at the recommended phase 2 dose (RP2D) of 15 mg/m² once daily on days 1-5 and 15-19 of a 28-day cycle in combination with FOLFIRI (irinotecan 180 mg/m² intravenous (IV); leucovorin 400 mg/m² IV; fluorouracil (FU) 400 mg/m² IV bolus; FU 2,400 mg/m² continuous IV infusion for 46 hours) and bevacizumab (5 mg/kg IV) administered once daily on days 1 and 15 until disease progression, death, unacceptable toxicity, or withdrawal from the study. The elimination of the FU bolus and leucovorin was allowed at the investigator's discretion.

End Points

The primary end point of the phase II study was the investigator-assessed ORR according to RECIST version 1.1 (v1.1) in patients who received at least one cycle (28 days) of treatment. Secondary efficacy end points included disease control rate (percentage of patients with best overall response being complete response [CR], partial response [PR] or stable disease [SD]), PFS (time from treatment initiation to progression or death, whichever occurs first), duration of response (DOR, time from first response [CR or PR] to progression or death, whichever occurs first), and reduction in *KRAS*-mutant allelic burden in liquid biopsies.

Assessments

Tumor response was assessed using computed tomography or magnetic resonance imaging per RECIST v.1.1, at screening and during treatment at every other cycle until end of treatment. For patients who discontinued study treatment for reasons other than progressive disease, follow-up information was collected until the patient started new therapy, underwent a procedure, experienced progressive disease, or died, for up to 1 year.

Safety assessments in patients who received at least one treatment dose included physical examinations, laboratory test results, electrocardiograms, and monitoring of adverse events (AEs) graded by the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0.

Blood samples were collected at baseline and after completion of cycle 1 to analyze *KRAS*-mutant circulating tumor DNA (ctDNA) as described previously.[15] For patients with available archival tumor tissue, the presence of *KRAS* mutations was tested using the Tempus xT assay (Tempus Labs Inc, Chicago, IL).

Translational and Preclinical Studies

Tempus Real-World Patient Sample Selection and Analysis

Biopsies from patients with *KRAS*-mutant mCRC were selected from the Tempus real-world database for gene expression analysis. Selected biopsies were from patients treated in first-line therapy with oxaliplatin with or without bevacizumab. Differential expression analysis and gene set enrichment analysis (GSEA) were performed as described in Appendix 1 (online only).

Preclinical Studies

In vitro and in vivo studies were performed using the human *KRAS*-mutant CRC cell lines HCT116 (*KRAS* G13D), SW620 (*KRAS* G12V), LoVo (*KRAS* G13D), and DLD-1 (*KRAS* G13D) as described in Appendix 1.

Statistical Analyses

This study used a binomial superiority one-sample test with a null hypothesis set at an ORR of 15% and an alternative hypothesis at an ORR of 30%. The sample size was estimated at approximately 80 patients to obtain 85% power with a type I error of 2.5%. Patient characteristics and AEs were

TABLE 2. Summary of Treatment-Related Adverse Events Reported in ≥10% of Patients

	No. of Patients With the Event,[a] No. (%)				
Adverse Event	Any Grade	Grade 1	Grade 2	Grade 3	Grade 4
Any adverse events	53 (100)	51 (96.2)	47 (88.7)	33 (62.3)	4 (7.5)[b]
Fatigue	39 (73.6)	24 (45.3)	12 (22.6)	3 (5.7)	0
Neutropenia	38 (71.7)	1 (1.9)	15 (28.3)	19 (35.8)	3 (5.7)
Nausea	33 (62.3)	21 (39.6)	8 (15.1)	4 (7.5)	0
Diarrhea	28 (52.8)	14 (26.4)	10 (18.9)	4 (7.5)	0
Stomatitis	24 (45.3)	14 (26.4)	7 (13.2)	3 (5.7)	0
Alopecia	17 (32.1)	13 (24.5)	4 (7.5)	0	0
Leukopenia	17 (32.1)	4 (7.5)	10 (18.9)	3 (5.7)	0
Vomiting	16 (30.2)	8 (15.1)	5 (9.4)	3 (5.7)	0
Anemia	15 (28.3)	13 (24.5)	1 (1.9)	1 (1.9)	0
Thrombocytopenia	15 (28.3)	12 (22.6)	3 (5.7)	0	0
Constipation	11 (20.8)	10 (18.9)	1 (1.9)	0	0
Dehydration	10 (18.9)	3 (5.7)	4 (7.5)	3 (5.7)	0
Hypertension	10 (18.9)	2 (3.8)	3 (5.7)	5 (9.4)	0
Abdominal pain	8 (15.1)	6 (11.3)	1 (1.9)	1 (1.9)	0
Decreased appetite	8 (15.1)	4 (7.5)	4 (7.5)	0	0
Epistaxis	8 (15.1)	8 (15.1)	0	0	0
Palmar-plantar erythrodysesthesia syndrome	7 (13.2)	5 (9.4)	2 (3.8)	0	0
Aspartate aminotransferase increased	6 (11.3)	4 (7.5)	2 (3.8)	0	0
Lymphopenia	6 (11.3)	0	6 (11.3)	0	0

[a]If a patient experienced an adverse event multiple times, the maximum grade is counted.
[b]Grade 4 colonic perforation was reported in one patient.

summarized using descriptive statistics. PFS and DOR were estimated using the Kaplan-Meier method with 95% CI. Post hoc univariable and multivariable analyses were used to determine the impact of baseline characteristics on ORR by a logistic regression model and on PFS by the Cox proportional hazards regression model. Variables with *P* values <.05 in univariable analyses were set as covariates in the multivariable analyses.

One-way analysis of variance with the Dunnet multiple comparison test was used for analyses of preclinical studies; *P* values <.05 were considered statistically significant.

Analyses were performed using R version 4.2.3 and GraphPad Prism version 10.0.02.

RESULTS

Patients

Between December 2019 and October 2023, 53 patients were treated with onvansertib at 15 mg/m^2, including five patients treated in phase Ib (Fig 1).[15] Patient baseline demographics and disease characteristics are summarized in Table 1. As of January 29, 2024, all patients had completed their on-treatment visits and follow-up period, and the median follow-up duration was 6.4 months (range, 1.6-30.3). The reasons for discontinuing treatment were disease progression (n = 32, 60.4%), pursued curative surgery (n = 10, 18.9%), patient choice (n = 6, 11.3%), and AEs (n = 4, 7.5%). One patient (1.9%) enrolled in an expanded access program to continue receiving onvansertib following the study's closure.

KRAS Mutations

The frequency of *KRAS* mutations in our study aligned with the expected distribution for this patient population[6] (Appendix Table A1). The presence of a *KRAS* mutation was confirmed for all patients through molecular testing of plasma ctDNA and/or archival tumor tissue (Appendix Table A2).

Clinical Efficacy

All patients treated with onvansertib RP2D completed at least one cycle of treatment and were included in the efficacy analysis (Fig 1). The investigator-assessed ORR was 26.4% (95% CI, 15.3 to 40.3), including one CR and 13 PR, and all responses were confirmed (Appendix Fig A1). Seven (50%) of the responders achieved PR at their first disease evaluation scan (two cycles of treatment). The median time to achieve a response was 86 days. Thirty-nine (73.6%) patients had a decrease in target lesion size

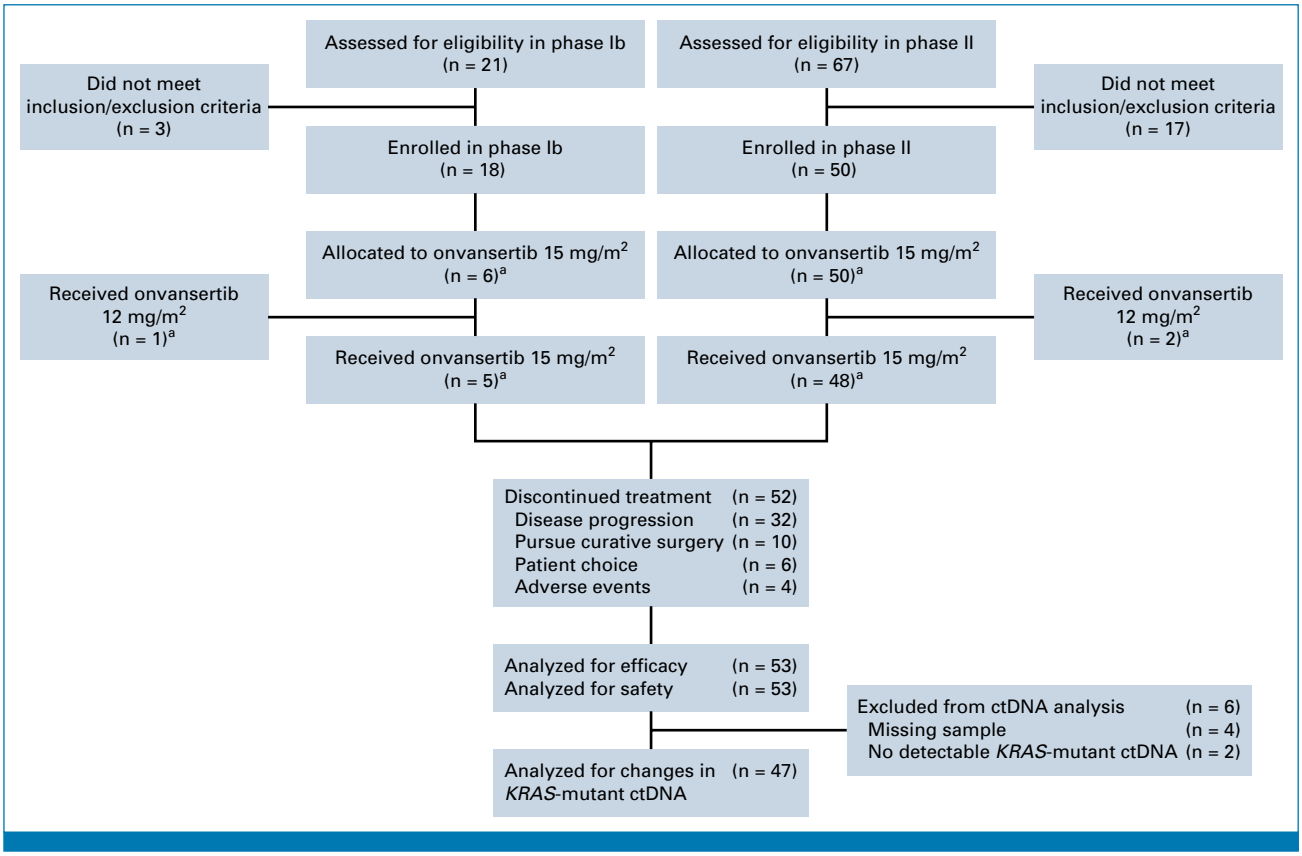


FIG 1. Flow diagram of the phase Ib and phase II. [a]Once daily on days 1-5 and 15-19 of a 28-day cycle. ctDNA, circulating tumor DNA; Onv, onvansertib.

from baseline, and the disease control rate, including CR, PR, and SD, was 92.5% (49/53 patients). The median DOR was 11.7 months (95% CI, 9.4 to not reached), and the median PFS was 8.4 months (95% CI, 6.0 to 14.8; Appendix Fig A1).

ctDNA

We previously described that a ≥90% decrease in *KRAS*-mutant ctDNA after one cycle of treatment was associated with clinical benefit to onvansertib + FOLFIRI and bevacizumab.[15] Here, patients achieving CR/PR (n = 12) had a significantly greater decrease in *KRAS*-mutant ctDNA compared with those with SD or PD (n = 35, Appendix Fig A2). Twenty patients had a ≥90% decrease in *KRAS*-mutant ctDNA and, when compared with those with <90% reduction (n = 27), exhibited a significantly higher ORR of 55.0% (95% CI, 31.5 to 76.9) versus 3.7% (95% CI, 0.1 to 19.0) and a significantly longer median PFS of 12.6 months (95% CI, 10.0 to not reached) versus 5.8 (95% CI, 3.9 to 9.2; Appendix Fig A2).

Safety

Treatment-emergent adverse events (TEAEs) of any grade were reported in all patients with the most common being fatigue (73.6%), neutropenia (71.7%), nausea (62.3%), diarrhea (52.8%), and stomatitis (45.3%; Table 2). Grade 3 and 4 TEAEs were reported in 33 (62.3%) and four (7.5%) patients, respectively. The grade 4 TEAEs were neutropenia in three patients (5.7%) and colonic perforation in one patient (1.9%). The most common grade 3 TEAEs were neutropenia in 19 patients (35.8%), hypertension in five patients (9.4%), and diarrhea and nausea in four patients (7.5%) each. No neutropenic fever was reported. Four patients discontinued treatment because of an AE, but none were related to onvansertib.

Association Between Prior Bevacizumab Treatment and Clinical Benefit

In a post hoc analysis, we evaluated the association between clinical benefit and baseline demographics or disease characteristics. Univariable analyses for radiographic response and PFS indicated superior clinical benefit for patients who had not received bevacizumab as part of their first-line treatment (bevacizumab-naïve) and for patients without metastatic disease at the time of diagnosis (Table 3). Multivariable analyses were carried out with these two characteristics to adjust for potential confounding effects on treatment outcomes. Only the absence of prior bevacizumab treatment remained independently associated with clinical benefit (Table 3). Bevacizumab-naïve patients (n = 13) had an ORR of 76.9% (95% CI, 46.2 to 95.0) and a median PFS of

TABLE 3. Univariable and Multivariable Analyses for Radiographic Response and PFS

	Univariable Analyses				Multivariable Analyses			
	Radiographic Response		PFS		Radiographic Response		PFS	
Parameter	OR (95% CI)	*P*	HR (95% CI)	*P*	OR (95% CI)	*P*	HR (95% CI)	*P*
Age, years								
>70 *v* ≤70	0.92 (0.04 to 7.97)	.946	1.64 (0.49 to 5.44)	.449				
Sex								
Male *v* female	0.64 (0.18 to 2.19)	.480	0.80 (0.40 to 1.60)	.532				
Race								
Other *v* White	2.72 (0.58 to 12.3)	.196	0.86 (0.35 to 2.11)	.745				
ECOG PS								
1 *v* 0	0.38 (0.09 to 1.35)	.137	1.08 (0.55 to 2.13)	.827				
Primary tumor site								
Right colon/other *v* left colon/rectum	0.99 (0.26 to 3.48)	.990	2.07 (0.98 to 4.37)	.065				
Metastatic disease at diagnosis								
Yes *v* no	**0.11 (0.02 to 0.51)**	**.005**	**3.28 (1.00 to 10.8)**	**.022**	0.59 (0.07 to 6.16)	.636	0.82 (0.19 to 3.60)	.795
Liver metastasis								
Yes *v* no	0.86 (0.23 to 3.69)	.832	1.53 (0.70 to 3.34)	.269				
Metastatic lesions								
Multiple *v* single	0.70 (0.06 to 15.9)	.785	2.00 (0.27 to 14.8)	.446				
Metastatic organs								
Multiple *v* single	0.54 (0.14 to 2.13)	.367	1.69 (0.73 to 3.92)	.200				
Received prior bevacizumab								
No *v* yes	**30.0 (6.5 to 187.6)**	**<.001**	**0.16 (0.05 to 0.51)**	**<.001**	**23.7 (4.2 to 201.0)**	**<.001**	**0.14 (0.03 to 0.61)**	**.002**

NOTE. Bold indicates statistically significant association between the variable of interest and the outcome.
Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; OR, odds ratio; PFS, progression-free survival.

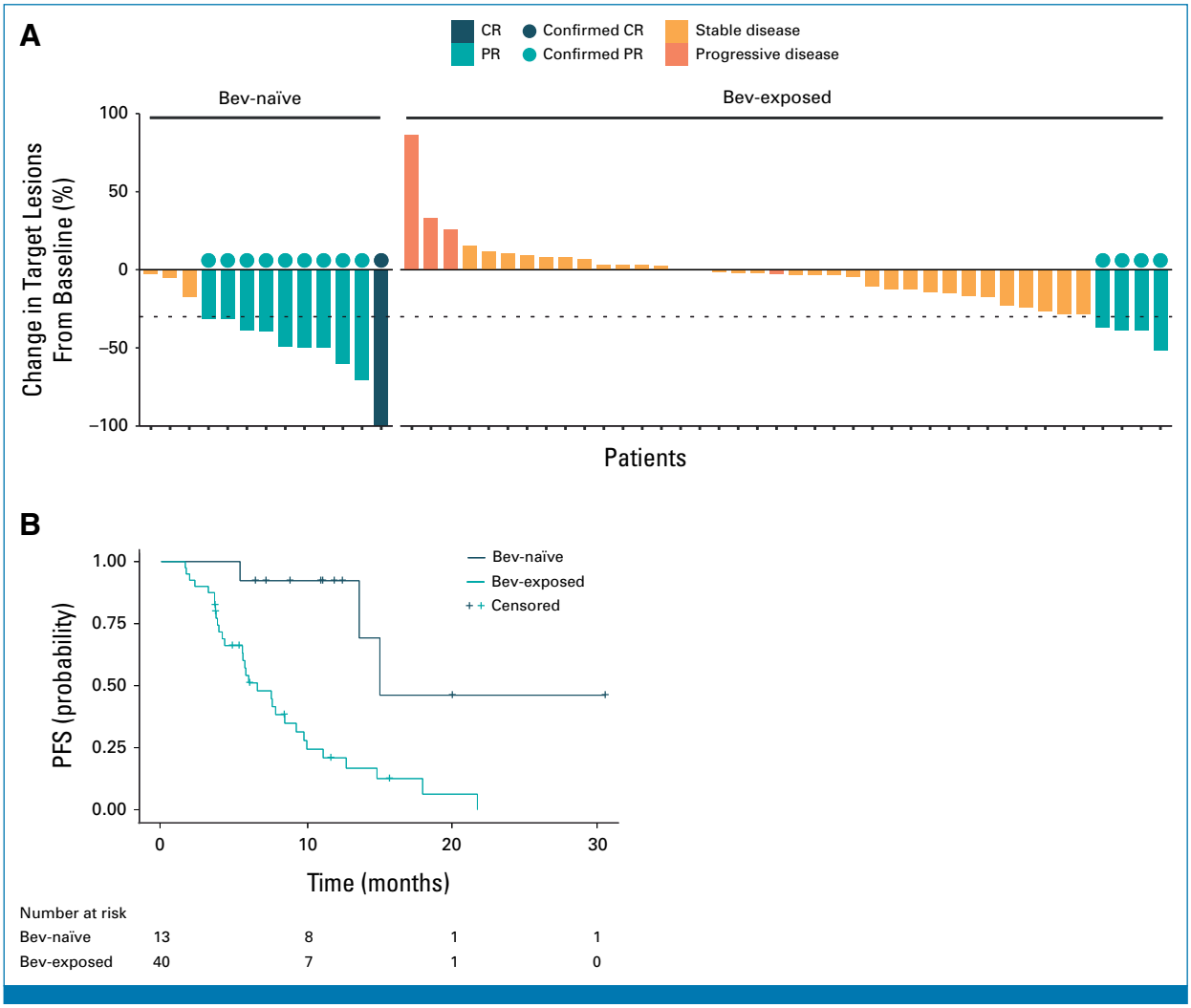


FIG 2. Antitumor activity according to prior bevacizumab treatment. (A) Best percentage change from baseline in target lesions. The dashed line at −30% change represents the RECIST v1.1 cutoff to define PR. Confirmed responses are indicated. (B) Kaplan-Meier curve of the PFS. Bev, bevacizumab, CR, complete response; PFS, progression-free survival; PR, partial response.

14.9 months (95% CI, 13.5 to not reached), compared with an ORR of 10.0% (95% CI, 2.8 to 23.7) and a median PFS of 6.6 months (95% CI, 5.6 to 9.8) for patients who received bevacizumab in first line (bevacizumab exposed, n = 40; Fig 2). The study was ended early based on the result of this subgroup analysis, showing significantly higher clinical benefit in bevacizumab-naïve patients.

Transcriptomic Changes Associated With Bevacizumab Treatment

We next investigated potential resistance mechanisms arising from bevacizumab treatment. We leveraged the Tempus real-world database and compared tumor transcriptomes obtained from *KRAS*-mutant mCRC patients after first-line treatment with either oxaliplatin (bevacizumab-naïve, n = 71) or oxaliplatin + bevacizumab (bevacizumab exposed, n = 64), as depicted in Figure 3A. Patient baseline characteristics were well balanced between these two cohorts (Appendix Table A3). Using GSEA on the MSigDB Oncogenic Signature and Hallmarks of Cancer gene sets,[17] we identified pathways upregulated in bevacizumab-exposed tumors (Fig 3B). These included pathways associated with bevacizumab resistance, such as the hallmark hypoxia and the oncogenic signature related to the angiogenic factor placental growth factor (PGF).[18-21] Furthermore, this analysis revealed upregulation of the hallmarks G2M checkpoint and mitotic spindle in bevacizumab-exposed tumors, two pathways linked to PLK1's functions in mitosis,[13] and potential contributors of onvansertib resistance. Collectively, these data suggest that transcriptomic changes after bevacizumab exposure might drive resistance to both bevacizumab and onvansertib.

Onvansertib and Bevacizumab Antitumor Activity in *KRAS*-Mutant CRC Xenografts

We evaluated the antitumor activity of onvansertib and bevacizumab in three *KRAS*-mutant CRC xenograft models. The combination demonstrated robust antitumor activity

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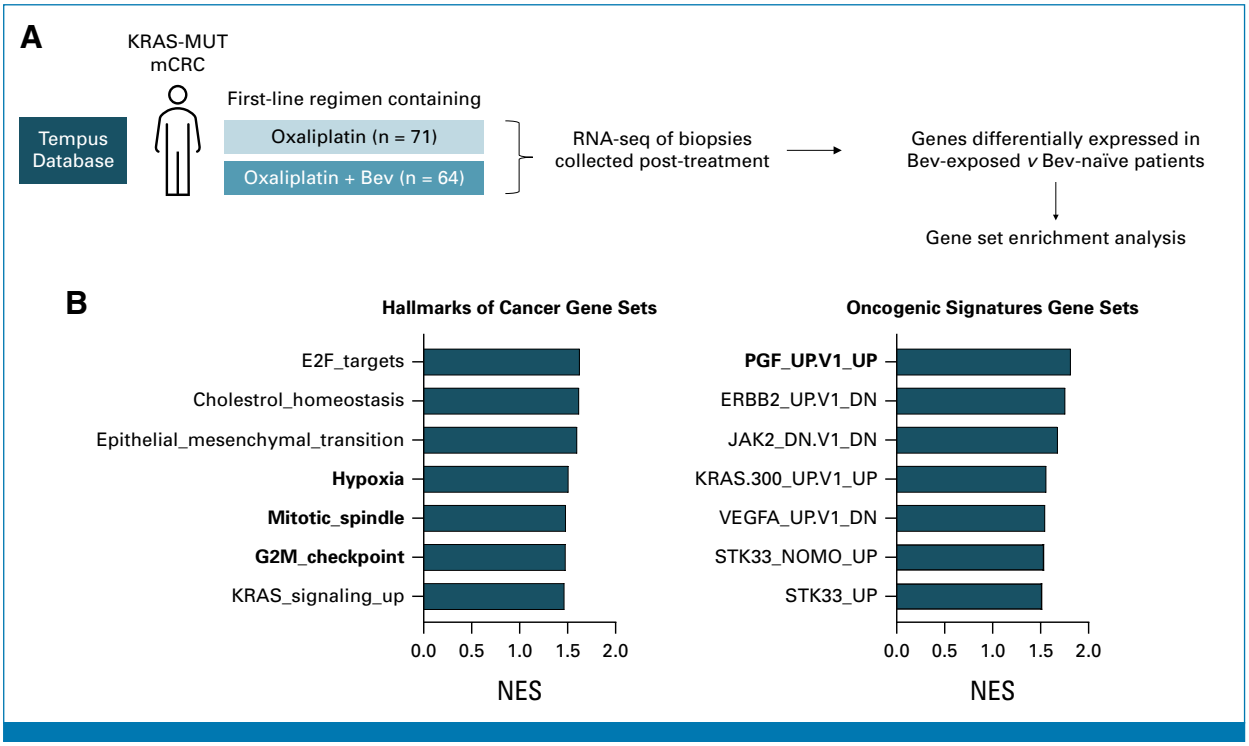


FIG 3. Transcriptomic changes associated with bevacizumab treatment. (A) Schematic representation of analysis workflow. (B) Gene set enrichment analysis of differentially expressed genes in patients treated with oxaliplatin + bevacizumab (bevacizumab-exposed) compared with oxaliplatin (bevacizumab-naïve). Bar plots show NES of pathways significantly upregulated in bevacizumab-exposed patients (FDR $P < .05$). Pathways in bold indicate pathways potentially involved in onvansertib and bevacizumab resistance. Bev, bevacizumab; FDR, false discovery rate; mCRC, metastatic colorectal cancer; NES, normalized enrichment scores.

across all three xenografts and superior inhibition of tumor growth compared with individual treatments (Fig 4A). Tumor examination revealed reduced vascularization with onvansertib or bevacizumab single agents and even greater reduction with the combination therapy (Fig 4B, Appendix Fig A3). These findings were corroborated through quantification of blood vessels using the endothelial marker CD31 (Fig 4C). Furthermore, significant reduction in tumor cell proliferation and increase in apoptosis were observed with the combination (Appendix Fig A3).

PLK1 Regulates the Hypoxia Pathway in *KRAS*-Mutant CRC Cells

We further explored PLK1's role in angiogenesis. In tumors, hypoxia results in the stabilization of the hypoxia-inducible factor 1α (HIF1α), which triggers the expression of genes promoting angiogenesis, metabolic changes, and cell survival.[22] Notably, onvansertib treatment led to a dose-dependent reduction in HIF1α protein expression in four *KRAS*-mutant CRC cell lines subjected to hypoxia (Fig 4D). These data corroborate recent findings showing that PLK1 contributes to HIF1α stability in hypoxia.[23] Transcriptomic profiling of HCT116 and SW620 cells exposed to hypoxia revealed that onvansertib significantly downregulated the hypoxia pathway and the closely associated metabolic pathway glycolysis in both cell lines (Fig 4E). In the four cell lines, onvansertib inhibited the hypoxia pathway by preventing the induction of genes involved in angiogenesis, metabolic reprogramming, and cell survival (Appendix Fig A4). Similarly, depletion of PLK1 by siRNAs in HCT116 and SW620 cells subjected to hypoxia led to a marked decrease in both HIF1α protein and the mRNA expression of hypoxia-related genes (Appendix Fig A4).

DISCUSSION

To our knowledge, this is the first study to evaluate the combination of a PLK1 inhibitor with standard-of-care systemic therapy in second-line treatment of patients with *KRAS*-mutant mCRC. The regimen of onvansertib + FOLFIRI and bevacizumab resulted in a confirmed ORR of 26.4%. Although our study fell short on meeting its primary end point of a 30% ORR, we unexpectedly identified a bevacizumab-naïve subpopulation that achieved an ORR of 76.9%. On the basis of these exceptional outcomes, this study was discontinued prematurely to focus on the clinical development of onvansertib for bevacizumab-naïve patients. This decision was supported by preclinical studies demonstrating onvansertib's impact on the hypoxia pathway and its combined effect with bevacizumab. Translational

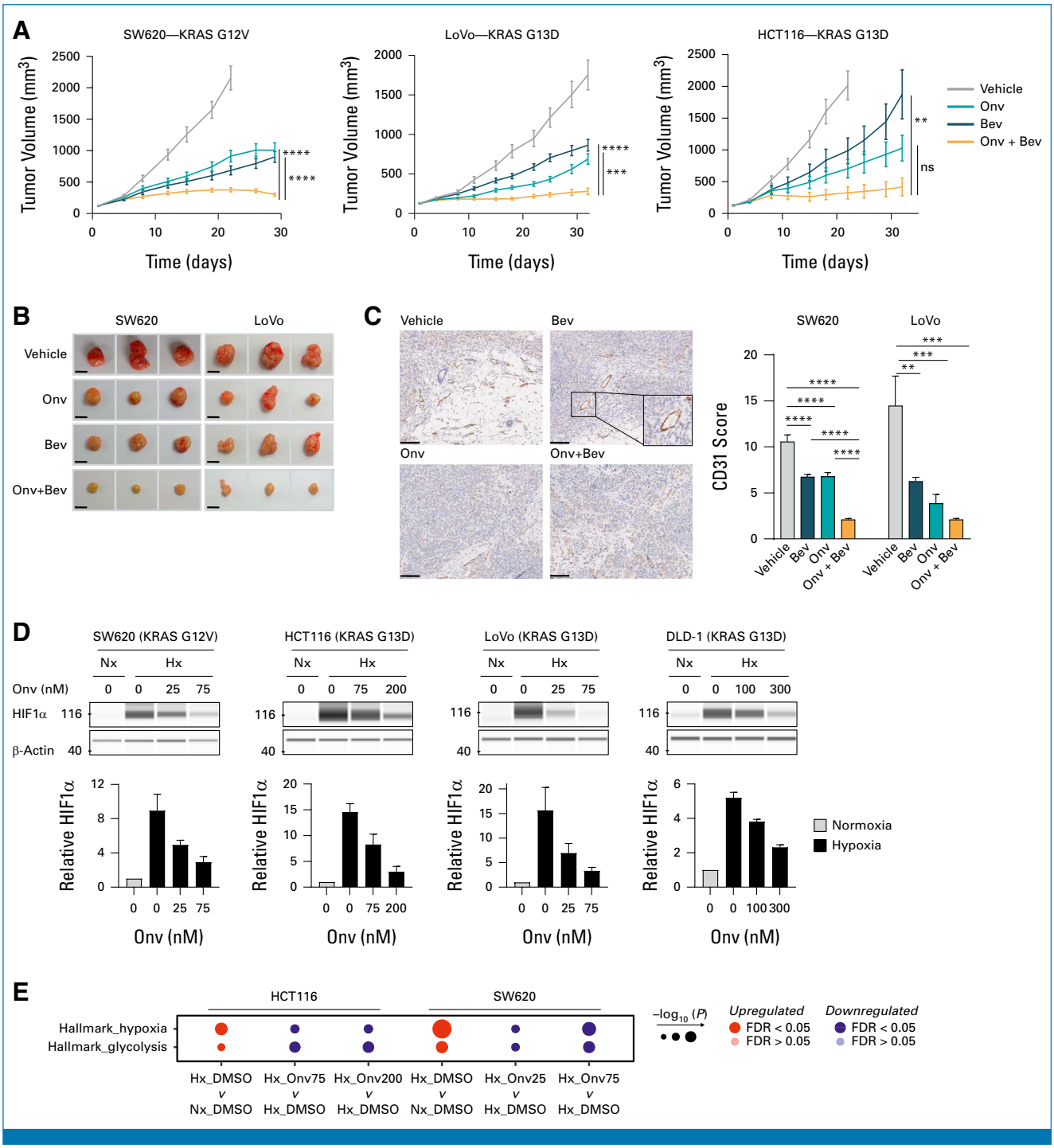


FIG 4. Antitumor activity of onvansertib and bevacizumab in *KRAS*-mutant CRC xenografts and regulation of the hypoxia pathway by PLK1. (A-C) Tumor-bearing mice were treated with vehicle, onvansertib, and/or bevacizumab for 30-33 days. (A) Tumor growth curves. (B) SW620 and LoVo tumor photographs, scale bar: 10 mm. (C) Representative immunohistochemical images of SW620 tumors stained with anti-CD31 antibody (LoVo tumors are shown in Appendix Fig A3), scale bar: 100 μm. CD31+ blood vessels were quantified; graphs represent the mean score ± SEM for each treatment group (n = 6/group). Statistical analyses were performed using one-way ANOVA. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. (D, E) *KRAS*-mutant CRC cell lines were treated with DMSO or onvansertib at the indicated doses for 20 hours and then exposed to hypoxia for 4 hours. (D) Representative Simple Western images of HIF1α and β-actin and quantification of HIF1α expression normalized to β-actin and normoxia control sample. Graphs represent mean ± SEM of ≥3 independent experiments. (E) Dot plot showing hallmarks of cancer hypoxia and glycolysis gene set enrichment analyses from comparisons of the indicated groups. The size of each dot corresponds to significance ($-\log_{10}(P)$). ANOVA, analysis of variance; Bev, bevacizumab; FDR, false discovery rate; HIF1α, hypoxia-inducible factor 1α; Hx, hypoxia; Nx, normoxia; Onv, onvansertib; PLK1, polo-like kinase 1.

studies indicated that bevacizumab-treated tumors develop resistance mechanisms, likely impeding the synergy between onvansertib and bevacizumab (graphical summary provided in Appendix Fig A5).

Combining chemotherapy and bevacizumab, the most commonly used second-line therapy in mCRC, has limited efficacy, with an ORR ranging between 5% and 23% and a median PFS of 6-7 months.[2,3,24,25] Additionally, the presence of a *KRAS* mutation is associated with poorer outcomes.[26,27] In comparison, our study showed encouraging efficacy with an ORR of 26.4% and median PFS of 8.4 months. Notably, all responses were confirmed and durable, with a median DOR of 11.7 months. The ORR of bevacizumab-naïve patients was 76.9%, and their median PFS was 14.9 months, substantially exceeding any historical outcomes. By contrast, bevacizumab-exposed patients had an ORR of 10.0% and median PFS of 6.6 months, aligning with the expected outcomes for standard of care. Second-line regimens such as FOLFOX + bevacizumab or FOLFIRI + aflibercept evaluated in bevacizumab-naïve patients with mCRC resulted in an ORR of approximately 20% and a median PFS of around 7 months,[2,4] highlighting that the exceptional efficacy observed in our study in bevacizumab-naïve patients is unique to onvansertib.

Hypoxia activates adaptive cellular pathways through stabilization of the HIF transcription factors, promoting tumor survival, angiogenesis, and metastasis, thereby contributing to treatment resistance. Our preclinical studies demonstrated that onvansertib inhibits the hypoxia pathway by downregulating HIF1α protein, likely contributing to reduced tumor vascularization and robust antitumor activity of onvansertib and bevacizumab. Although dual inhibition of angiogenesis has the potential to enhance antitumor effects and delay resistance, combining bevacizumab with other antiangiogenic agents like sunitinib has proven challenging because of high toxicities.[28,29] By contrast, the combination of onvansertib with FOLFIRI and bevacizumab was well tolerated, with no increase in vascular toxicities beyond those expected from bevacizumab alone (eg, hypertension, bleeding, clotting),[30] supporting the hypothesis that onvansertib's antiangiogenic activity is mediated through tumor cell-specific pathways, sparing normal vasculature. Onvansertib also downregulated glycolysis-related genes disrupting another critical survival pathway in cancer cells. Glycolysis is essential for supporting energy needs, biosynthetic processes, and adaption to the hypoxic microenvironment in tumor cells, especially those with oncogenic *KRAS* mutations.[31] By targeting the hypoxia pathway, onvansertib may inhibit tumor growth through dual mechanisms: It collaborates with bevacizumab to block angiogenesis and inhibits crucial tumor survival pathways such as glycolysis, offering a promising two-hit therapeutic strategy against *KRAS*-mutant CRC. Additional investigations are warranted to determine whether this strategy is unique to mCRC tumors harboring *KRAS* mutations.

Resistance to targeted therapies can involve the activation of compensatory signaling pathways. For instance, adaptive resistance to $KRAS^{G12C}$ inhibitors can occur via EGFR-mediated reactivation of the mitogen-activated protein kinase pathway and combining EGFR and $KRAS^{G12C}$ inhibitors has shown to enhance antitumor activity.[10-12,32] Our clinical data suggest that first-line treatment with bevacizumab results in resistance to onvansertib. Real-world data analysis indicated that bevacizumab-exposed tumors from patients with *KRAS*-mutant mCRC exhibited an upregulation of pathways related to PLK1 functions in mitosis, potentially contributing to onvansertib resistance. Further validations using independent data sets and experimental testing are warranted to corroborate this hypothesis.

There are several limitations to our clinical study to consider. First, this was a single-arm study with no control group. Second, the study was terminated before reaching its estimated sample size, resulting in reduced statistical power. Finally, the increased clinical benefit observed in bevacizumab-naïve patients was based on a post hoc analysis of a small subset of patients, underscoring the need for further validation through larger and randomized controlled trials.

In conclusion, our study demonstrates the efficacy and tolerability of onvansertib + FOLFIRI and bevacizumab in second-line treatment of patients with *KRAS*-mutant mCRC. Patients with no prior exposure to bevacizumab showed marked increased efficacy with an ORR of 76.9% and a median PFS of 14.9 months, underscoring the unique sensitivity of this population to onvansertib. This exceptional response, alongside our translational studies showing onvansertib's effect on the hypoxia pathway and angiogenesis, supports shifting onvansertib development to the frontline setting, where all patients are bevacizumab naïve. CRDF-004 (ClinicalTrails.gov identifier: NCT06106308) is an open-label, multicenter, randomized phase II study, currently enrolling to evaluate the efficacy and tolerability of first-line onvansertib + chemotherapy + bevacizumab versus chemotherapy + bevacizumab in patients with *RAS*-mutant mCRC.

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Onvansertib in Combination With Chemotherapy and Bevacizumab in Second-Line Treatment of *KRAS*-Mutant Metastatic Colorectal Cancer: A Single-Arm, Phase II Trial

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APPENDIX 1. METHODS

Tempus Real-World Patient Sample Selection and Analysis

Biopsies were obtained within a time frame of 6 weeks to 1 year after initiating first-line treatment and prior starting second-line therapy. Differential expression analysis was performed using a Wilcoxon rank-sum test and a linear model controlling for tumor purity. Genes were ranked by the product of the Log10 transformed *P* value and fold change. Differential expression results were then used for gene set enrichment analysis (GSEA) using the fgsea package.[33,34] Gene sets were selected from the Molecular Signatures Database (MSigDB)[17] including MSigDB hallmark and oncogenic signature gene sets.

Cell Culture and Transfection

Human *KRAS*-mutant colorectal cancer cell lines HCT116, SW620, LoVo, and DLD-1 (ATCC, Manassas, VA) were cultured in RPMI (Cat# 30-2001, ATCC) supplemented with 10% FBS (Cat#16000069, ThermoFisher Scientific, Waltham, MA) and 1× penicillin-streptomycin solution (Cat#30-2300, ATCC). The cells were incubated at 37°C in a humidified chamber containing 5% CO_2 and 20% O_2. Hypoxic conditions were achieved by culturing cells in Ruskinn-Baker Invivo$_2$ 400 workstation (SP0530) at 1% O_2 and 5% CO_2. The cell lines were confirmed negative for Mycoplasma using MycoAlert Mycoplasma Detection kit (Cat#LT07-318, Lonza, Basel, Switzerland).

For gene silencing experiments, cells were transiently transfected with 50 nM of SMARTpool siRNA containing a mixture of four siRNAs (ON-TARGETplus siRNA, Horizon Discovery, Cambridge, UK) targeting *PLK1* (Cat#L-003290-00-0005), using Lipofectamine RNAiMAX transfection reagent (Cat#13778075, ThermoFisher Scientific) following the manufacturer's protocol. The ON-TARGETplus nontargeting siRNA pool (Cat#D-001810-10-05) was used as the control.

Protein Analysis

Cells lysates were prepared in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 25 mM β-glycerophosphate, 150 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 1 mM Na4P2O7, 10% glycerol, 1% IGEPAL CA-630) supplemented with protease inhibitor cocktail (Cat#P8340, Millipore Sigma) and phosphatase inhibitor cocktail (Cat#P5726, Millipore Sigma, Burlington, MA). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Cat#23227, ThermoFisher Scientific) according to the manufacturer's instructions. Protein lysates were analyzed by size-based capillary electrophoresis using the Jess Simple Western instrument (Bio-Techne, Minneapolis, MN) as per manufacturer's instructions and described previously.[35] The following primary and secondary antibodies were used: PLK1 (Cat#4513, dilution 1:200, Cell Signaling Technology, Danvers, MA), hypoxia-inducible factor 1α (Cat#ab179483, dilution 1:100, Abcam, Cambridge, UK), β-Actin-HRP (Cat#sc-47778, dilution 1:500; Santa Cruz Biotechnology, Dallas, TX), and anti-rabbit secondary HRP (Cat#042-206, dilution 1:1, Bio-Techne). Data analysis was performed using Compass software for Simple Western.

RNA Extraction

Cells were lysed in 700 μL of Trizol LS Reagent (Cat#10296028, ThermoFisher Scientific) and total RNA was isolated using the PureLink RNA Mini Kit (Cat#12183025, ThermoFisher Scientific) per the manufacturer's instructions.

Real-Time Quantitative Polymerase Chain Reaction

The concentration and purity of RNA were determined using a NanoDrop 2000C spectrophotometer (ThermoFisher Scientific). The reverse transcription of RNA was performed using Superscript IV VILO Master Mix (Cat#11766050, ThermoFisher Scientific). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in triplicates with Primetime gene expression master mix (Cat#1055771, Integrated DNA Technologies, Coralville, IA) on a CFX384 thermocycler (Bio-Rad, Hercules, CA). Primers were purchased from Integrated DNA Technologies, and primer reference numbers are provided in Appendix Table A4. The housekeeping gene *RPLP0* was used to normalize expression. Relative expression values were calculated using the comparative cycle threshold method (ΔΔCt).

RNA-Seq Library Generation, High-Throughput Sequencing, and Analysis

For RNA-sequencing, biological triplicate samples were analyzed. Total RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and RNA integrity was checked with the TapeStation 4200 (Agilent Technologies, Santa Clara, CA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina per the manufacturer's instructions (Cat#:E7775, New England Biolabs, Ipswich, MA). Briefly, mRNAs were initially enriched with Oligo d(T) beads. Enriched mRNAs were fragmented for 15 minutes at 94°C. First strand and second strand cDNA were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by PCR with limited cycles. The sequencing library was validated on the Agilent TapeStation and quantified by using Qubit 2.0 Fluorometer and by quantitative PCR (KAPA Biosystems, Wilmington, MA).

The sequencing libraries were multiplexed and clustered onto a flow cell on the Illumina NovaSeq instrument according to manufacturer's instructions. The samples were sequenced using a 2 × 150 bp Paired End configuration. Image analysis and base calling were conducted by the NovaSeq Control Software. Raw sequence data (.bcl files) generated from Illumina NovaSeq was converted into fastq files and demultiplexed using Illumina bcl2fastq software (version 2.20). One mismatch was allowed for index sequence identification.

Gene expression was quantified using Salmon v1.9.0 with GRCh38 (GENCODE release 44).[36] Differential gene expression was assessed using DESeq2 (version 1.42.1).[37] Log_2 fold-changes (LFC) were shrunken using the ashr algorithm[38] and *P* values were adjusted using independent hypothesis weighting against the baseMean counts and the Benjamini-Hochberg method.[39,40]

For each comparison (hypoxia DMSO *v* normoxia DMSO and hypoxia onvansertib *v* hypoxia DMSO), genes were ranked by their LFC and GSEA carried out against the Hallmarks of Cancer gene set collection from MSigDB (version 2023) using fGSEA (version 1.28.0).[17,34]

Genes that were differentially regulated in response to both exposure to hypoxic conditions and treatment with onvansertib in both cell lines were selected. The variance transformed (rlog) gene counts for these genes in each cell and contrast were z-scaled and clustered, using complete linkage and the Euclidean distance, and plotted as a heatmap using the ComplexHeatmap R package (version 2.18.0).[41]

In Vivo Studies

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Crown Bioscience before being carried out. The care and use of animals were conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care. Female Balb/c nude mice (6-9 weeks, GemPharmatech Co. Ltd, Nanjing, China) were inoculated subcutaneously with HCT116, SW620, or LoVo cells. Once tumors reached 100-200 mm^3, mice were randomly assigned and treated with vehicle, onvansertib (oral, 45 mg/kg, once daily), bevacizumab (intraperitoneally, 5 mg/kg for SW620 and HCT116, 1 mg/kg for LoVo, twice a week) or the combination. Onvansertib (provided by Cardiff Oncology) was prepared as described before,[15] and bevacizumab (HY-P9906, MedChemExpress, Monmouth Junction, NJ) was diluted in saline. Tumor volumes and body weights were measured twice a week. Tumors collected at the end of this study were subjected to gross morphology and histology analyses.

Histopathology Analyses

Tumor samples were collected at the end of this study and fixed for 24 hours in 10% neutral buffered formalin for immunohistochemical (IHC) and hematoxylin and eosin (H&E) staining. BrdU (2 mg/mice, Cat#HY-15910, MedChemExpress) was administered to the mice 24 hours before tumor sample collection. Fixed tumor tissues were paraffin-embedded, sectioned, and placed on positively charged glass slides. Sections were deparaffinized and stained with anti-CD31 (Cat#ab28364, dilution 1:50, Abcam) or anti-BrdU (Cat#ab6326, dilution 1:600, Abcam) antibodies for 1 hour at 37°C using a Leica Bond III autostainer with Leica detection kit (Cat#DS9800, Leica Biosystems). Brightfield whole slide imaging was performed using Aperio AT2 scanner (Leica Biosystems, Nussloch, Germany).

The digital images were examined by a board-certified pathologist to quantify apoptotic cells and CD31-positive vessels. For each sample, tumor cells exhibiting apoptotic features were manually counted across five randomly selected fields of view at 50 μm magnification. CD31-positive vessels were identified as round/oval CD31-positive endothelial layer within the vascular wall and counted in five random fields per tumor at 100 μm magnification. The number of BrdU-positive tumor cells and the intensity of the stain were quantified using Halo AI classifier (Indica Labs, version 3.6). Stain intensity was scored as negative, weak, moderate, or strong, and an H-score was calculated for each sample as follows: [(0 × % negative cells) + (1 × % weak positive cells) + (2 × % moderate positive cells) + (3 × % strong positive cells)].

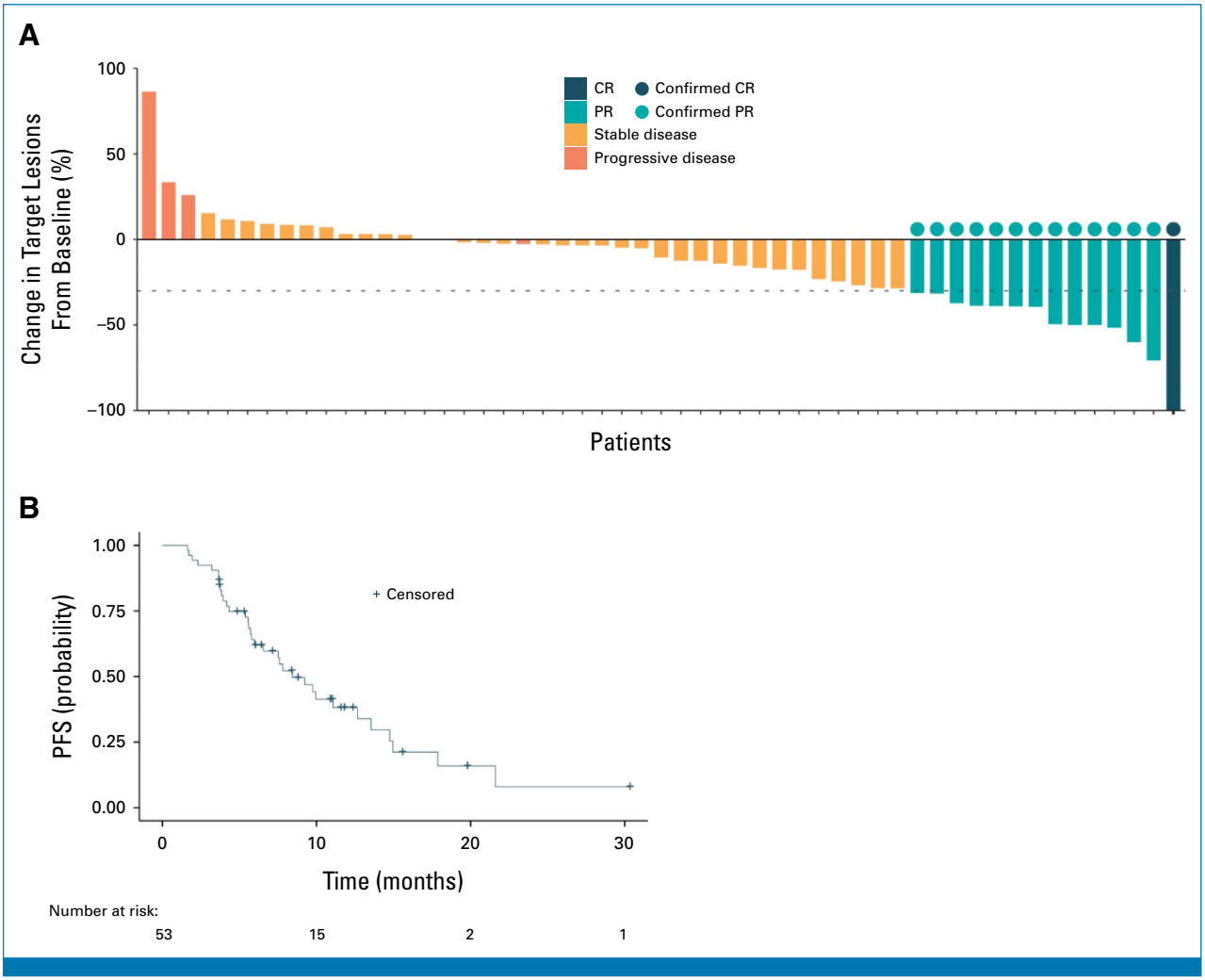


FIG A1. Antitumor activity (n = 53). (A) Best percentage change from baseline in target lesions. The dashed line at −30% change represents the RECIST v1.1 cutoff to define PR. Confirmed responses are indicated. (B) Kaplan-Meier curve of the PFS. CR, complete response; PFS, progression-free survival; PR, partial response.

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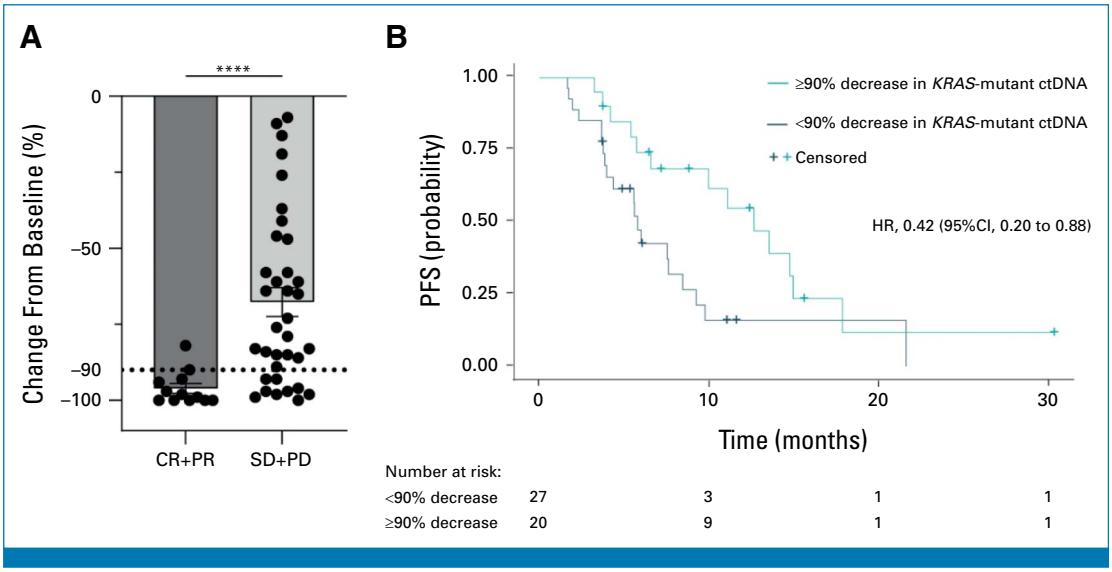


FIG A2. Association between changes in *KRAS*-mutant ctDNA and clinical benefit. (A) Percentage change in *KRAS*-mutant ctDNA from baseline after one cycle of treatment according to best response. Data analyzed by unpaired *t*-test with Welch correction, **** corresponds to $P < .0001$. (B) PFS of patients who exhibited a ≥90% decrease in *KRAS*-mutant ctDNA after 1 cycle of treatment and in patients with less than 90% decrease. CR, complete response; ctDNA, circulating tumor DNA; HR, hazard ratio; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease.

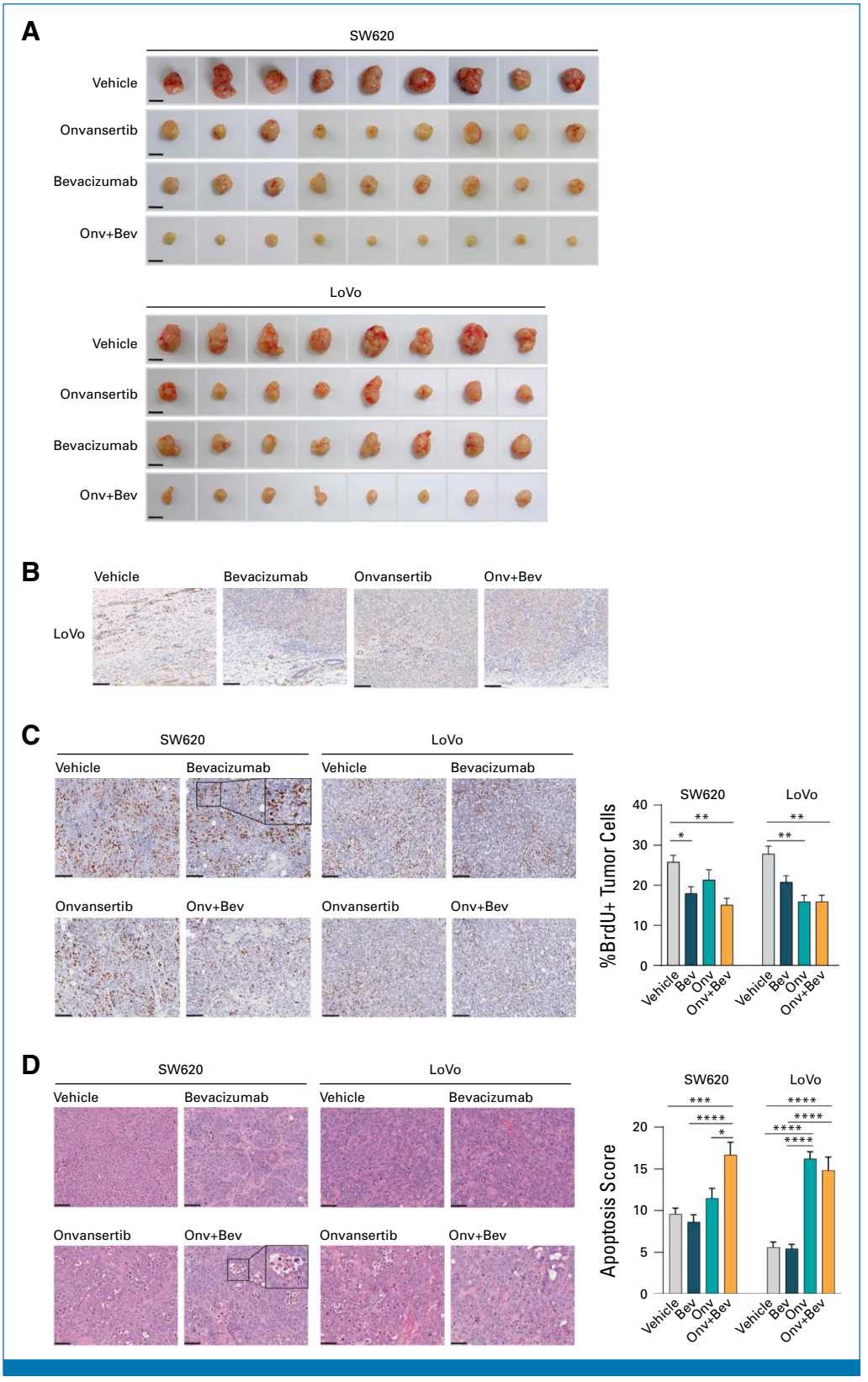


FIG A3. Antitumor activity of Onv and Bev in *KRAS*-mutant CRC xenografts, related to Figure 4. SW620 and LoVo tumors after treatment with vehicle, Onv, and/or Bev for 30-33 days. (A) SW620 and LoVo tumor photographs, scale bar 10 mm. (B) Representative immunohistochemical images of LoVo tumors stained with anti-CD31 antibody, scale bar 100 μm. (C) Representative immunohistochemical images of SW620 and LoVo tumors stained with anti-BrdU antibody, scale bar 100 μm. Graph represents the % of BrdU+ proliferating tumor cells (mean ± SEM) for each treatment group (n = 6/group). (continued on following page)

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FIG A3. (Continued). (D) Representative H&E images of SW620 and LoVo tumors, scale bar 50 μm. Tumor cells with apoptotic morphology were scored; graph represents the score (mean ± SEM) for each treatment group (SW620: n = 9/group, LoVo: n = 6/group). Statistical analyses were performed using one-way ANOVA. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. ANOVA, analysis of variance; Bev, bevacizumab; H&E, hematoxylin and eosin; Onv, onvansertib.

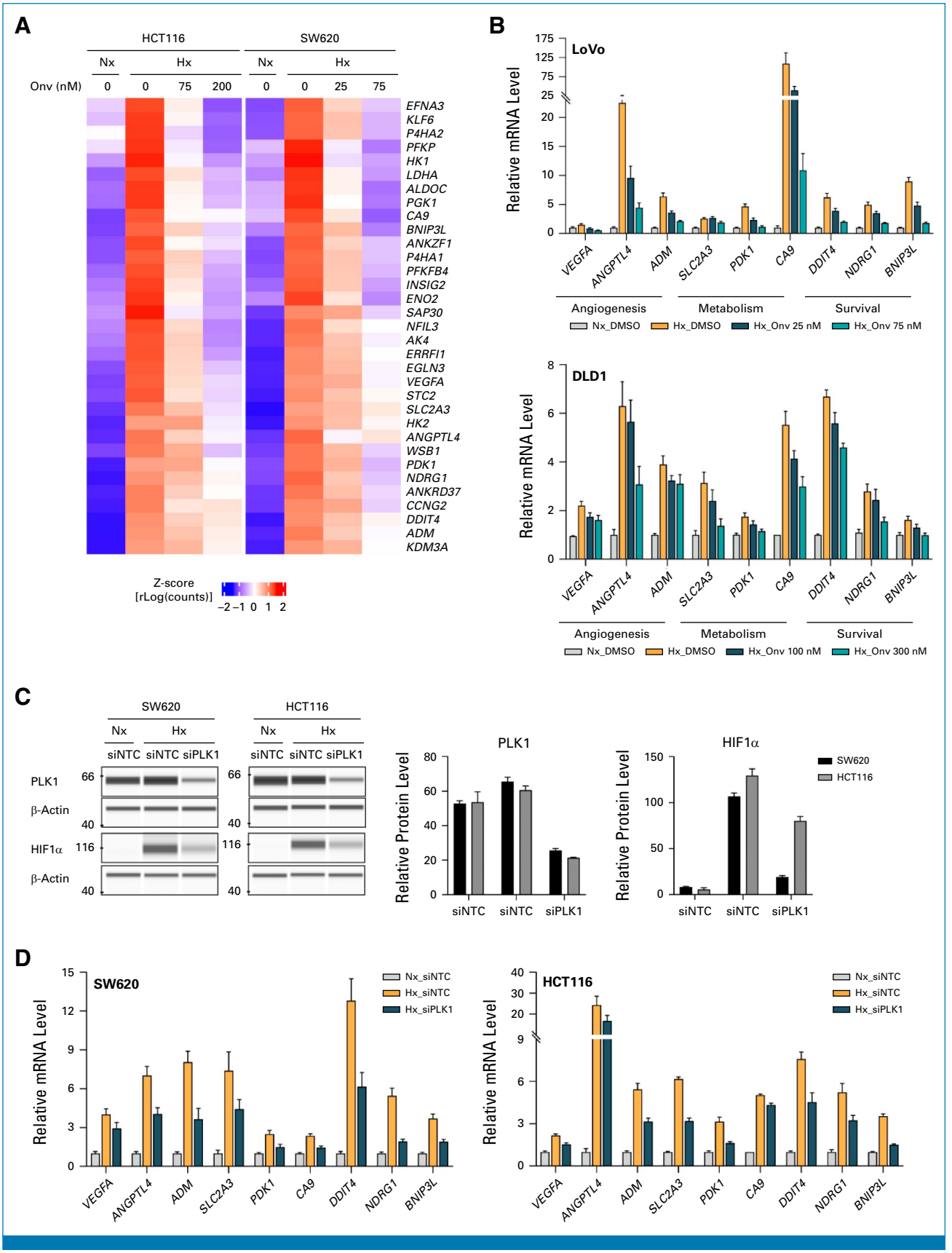


FIG A4. PLK1 regulates the hypoxia pathway, related to Figure 4. (A, B) *KRAS*-mutant CRC cells were treated with DMSO or Onv at the indicated doses for 20 hours and then exposed to hypoxia or kept in normoxia for 4 hours. (A) Heatmap of hypoxia-related genes significantly regulated by Onv in HCT116 and SW620 cells based on the RNA-seq analysis. (B) Expression (continued on following page)

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FIG A4. (Continued). of hypoxia-related genes in LoVo and DLD-1 cells, assessed by RT-qPCR and normalized to the housekeeping gene *RPLP0*. Bar graphs represent expression relative to Normoxia_DMSO sample. (C, D) SW620 and HCT116 cells were transfected with nontargeting control siRNA (siNTC) or *PLK1* targeting siRNA (siPLK1) for 20 hours and then exposed to hypoxia for 4 hours. (C) Left: Simple Western images of PLK1, HIF1α, and β-actin. Right: HIF1α and PLK1 protein expression normalized to β-actin. (D) Expression of hypoxia-related genes assessed by RT-qPCR and normalized to the housekeeping gene *RPLP0*. Bar graphs represent expression relative to Normoxia_siNTC. (B-D) Data are shown as mean ± SEM of at least three independent biological replicates. HIF1α, hypoxia-inducible factor 1α; Hx, hypoxia; Nx, normoxia; Onv, onvansertib; PLK1, polo-like kinase 1; RT-qPCR, real-time quantitative polymerase chain reaction.

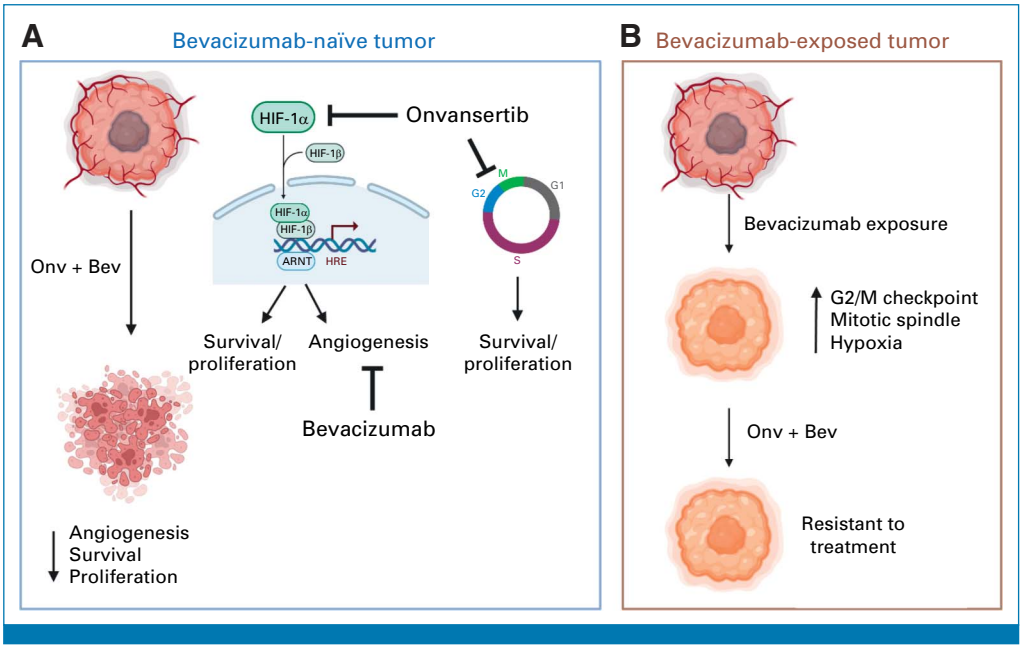


FIG A5. Graphical summary of the proposed mechanisms of Onv and Bev combination therapy in Bev-naïve and Bev-exposed tumors. (A) In Bev-naïve tumors, the combination of Onv and Bev effectively inhibits tumor cell survival, proliferation, and angiogenesis through the indicated mechanisms. (B) In Bev-exposed tumors, Bev exposure leads to upregulation of mitotic and hypoxia pathways resulting in resistance to both Onv and Bev. Figure was created with BioRender.com. Bev, bevacizumab; HIF1α, hypoxia-inducible factor 1α; Onv, onvansertib.

TABLE A1. Frequency of *KRAS* Mutations

KRAS Mutation	Patients, No.	%
G12D	16	30
G12V	10	19
G12A	6	11
G13D	6	11
G12C	4	8
A146T	3	6
Q61H	3	6
G12S	2	4
K117N	2	4
G12R	1	2
Total	53	100

TABLE A2. List of *KRAS* Mutations Detected in Liquid and Tumor Biopsies

Patient	*KRAS* Mutation Detected in Liquid Biopsy	*KRAS* Mutation Detected in Archival Tissue
1	G12A	G12A
2	G12D	NA
3	A146T	NA
4	G12D	G12D
5	G12V	NA
6	G13D	NA
7	G12D	NA
8	G12A	NA
9	G12D	NA
10	G12D	G12D
11	G12D	G12D
12	G12A	G12A
13	G12D	NA
14	Not detected	Q61H
15	K117N	K117N
16	Q61H	NA
17	A146T	NA
18	G12A	G12A
19	Not detected	Q61H
20	G12V	G12V
21	G12V	NA
22	G12D	G12D
23	G12D	NA
24	G12A	G12A
25	G12C	G12C
26	G13D	G13D
27	G12S	G12S
28	G13D	Not detected
29	G12D	G12D
30	G13D	NA
31	G12C	NA
32	G12D	NA
33	G12V	NA
34	G12D	G12D
35	G12R	G12R
36	G12D	G12D
37	G12S	NA
38	G12V	G12V
39	G12A	G12A
40	G12C	G12C
41	G12V	NA
42	K117N	K117N
43	G12V	G12V
44	G12D	G12D
45	A146T	NA
46	G12V	G12V
47	G13D	G13D
48	G12V	G12V
49	G12A	G12A
50	G12D	G12D
51	G13D	G13D
52	G12V	G12V
53	G12D	G12D

Abbreviation: NA, no data available.

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TABLE A3. Patient Demographics and Baseline Characteristics of the Tempus Cohorts

Characteristic	Bev-Naïve	Bev-Exposed	*P*
Patients, No.	71	64	
Mean age at biopsy collection, years	55.6	55.0	.774
Sex, No. (%)			1.000
Female	36 (50.7)	33 (51.6)	
Male	35 (49.3)	31 (48.4)	
Ethnicity, No. (%)			.062
White	30 (42.3)	32 (50.0)	
Other	19 (26.8)	6 (9.4)	
Unknown	22 (31.0)	26 (40.6)	
ECOG PS, No. (%)			.218
0	28 (39.4)	20 (31.3)	
1	29 (40.8)	28 (43.8)	
>1	5 (7.0)	2 (3.1)	
Unknown	9 (12.7)	14 (21.9)	
Biopsy site, No. (%)			.992
Liver	26 (36.6)	25 (39.1)	
Lower GI	23 (32.4)	20 (31.2)	
Pelvic and upper body soft tissue	10 (14.1)	9 (14.1)	
Other	12 (16.9)	10 (15.6)	
Metastatic disease at diagnosis, No. (%)			.157
Yes	53 (74.6)	39 (60.9)	
No	14 (19.7)	22 (34.4)	
Unknown	4 (5.6)	3 (4.7)	
KRAS mutation			.665
G12D	21 (30)	19 (30)	
G12V	15 (21)	14 (22)	
G13D	11 (15)	6 (9)	
G12C	8 (11)	5 (8)	
Other	16 (23)	20 (31)	

Note: *P* values are based on χ^2 test for categorical variables and *t* test for continuous variables.
Abbreviations: Bev, bevacizumab; ECOG PS, Eastern Cooperative Oncology Group performance status.

TABLE A4. List of Primers Used for Real-Time Quantitative Polymerase Chain Reaction

Gene	Assay ID
ADM	Hs.PT.56a.25211580.g
VEGFA	Hs.PT.58.1149801
ANGPTL4	Hs.PT.58.25480012
CA9	Hs.PT.56a.2192889
PDK1	Hs.PT.58.19794808
SLC2A3	Hs.PT.58.39033300
DDIT4	Hs.PT.58.38843854.g
BNIP3L	Hs.PT.56a.1103799
NDRG1	Hs.PT.58.4224283
RPLP0	Hs.PT.39a.22214824