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Supplemental Information

Paradox-Breaking RAF Inhibitors that Also Target SRC Are Effective in Drug-Resistant BRAF Mutant Melanoma

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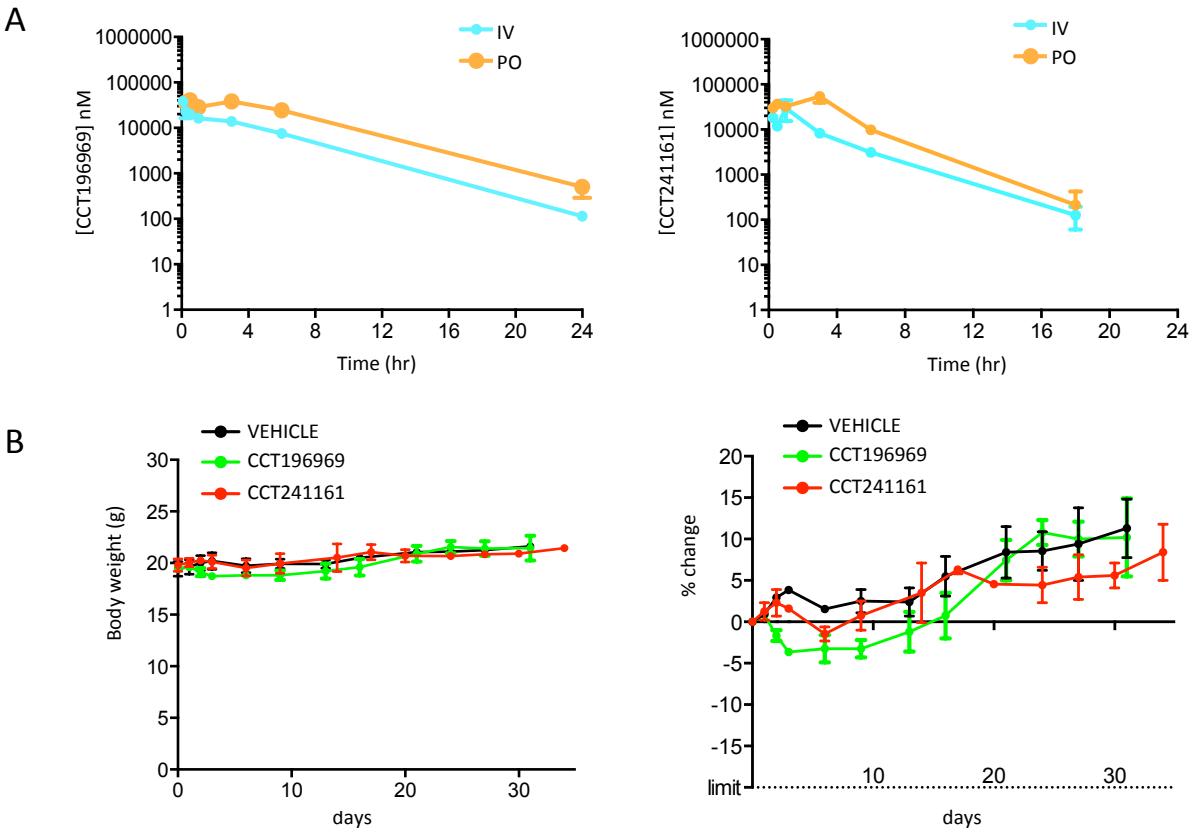


Figure S1, related to Figure 1.

A. Pharmacokinetic studies performed in Balb/c mice for CCT196969 and CCT241161. Plasma levels at time point 24 hr (for CCT196969) or 14 hr (for CCT241161) show concentration of ~1 µM when administered by oral gavage PO=oral administration (10 mg/kg/day); IV=intravenous administration (2 mg/kg/day).

B. Body weights of mice treated with vehicle, CCT196969 and CCT241161 by oral gavage at 30 mg/kg/day for 4 days. Body weight (g) was monitored daily for the first 4 days then twice a week. Bars represent SEM.

Table S1, related to Figure 1. Pharmacokinetic parameters for CCT196969 and CCT241161 in plasma following oral and intravenous dosing.

Plasma - CCT196969	Tmax (hr)	Cmax (nM)	t1/2lz (hr)	AUClast (nM.hr)	F%
IV	0.083	38118	3.0	154467	
PO	0.5	40503	3.3	416286	54%

Plasma - CCT241161	Tmax (hr)	Cmax (nM)	t1/2lz (hr)	AUClast (nM.hr)	F%
IV	1	29776	2.8	95020	
PO	3	54223	2.5	274772	58%

*(IV= Intravenous administration, PO=Oral administration, Cmax= maximum concentration, Tmax= time of maximum concentration, AUC(0-t)= area under the concentration time curve, F% =oral bioavailability and T½λz= elimination half life).

Table S2, related to Figure 1. CCT241161 and CCT196969 plasma levels in NSG and nude mice post-treatment.

CCT196969 plasma levels at end of therapy 1 hr post-last dose		
	Plasma	Plasma
	NSG mice (nM)	nude mice (nM)
Mouse 1	20838	35344
Mouse 2	29922	112554
Mouse 3	27704	27092
Mouse 4	10642	30050
Mouse 5	28142	50848
Mouse 6	26052	62818
Mouse 7	42148	
Mouse 8	19052	
Mouse 9	19534	
Mouse 10	25420	
Average (nM)	24945	53118

CCT241161 plasma levels at end of therapy 1 hr post-last dose		
	Plasma	Plasma
	NSG mice (nM)	nude mice (nM)
Mouse 1	53922	70022
Mouse 2	49800	94314
Mouse 3	48390	44068
Mouse 4	28044	75390
Mouse 5	47682	65960
Mouse 6	38810	66766
Mouse 7	53466	
Mouse 8	81140	
Mouse 9	36062	
Average (nM)	48591	69420

Table S3, related to Figure 1. CCT241161 and CCT196969 tumor levels in NSG and nude mice post-treatment

CCT196969 tumor levels at end of therapy 1 hr post-last dose		
	Tumor	Tumor
	NSG mice (nM)	nude mice (nM)
Mouse 1	6073	6259
Mouse 2	3295	14434
Mouse 3	No sample	3475
Mouse 4	2521	7632
Mouse 5	4081	6041
Mouse 6	17652	8600
Mouse 7	15090	
Mouse 8	7652	
Mouse 9	5803	
Mouse 10	7181	
Average (nM)	7705	7740

CCT241161 tumor levels at end of therapy 1 hr post-last dose		
	Tumor	Tumor
	NSG mice (nM)	nude mice (nM)
Mouse 1	10964	8101
Mouse 2	16568	6168
Mouse 3	8881	6160
Mouse 4	3930	7066
Mouse 5	8001	4994
Mouse 6	6989	6725
Mouse 7	9985	
Mouse 8	11672	
Mouse 9	13072	
Average (nM)	10007	6536

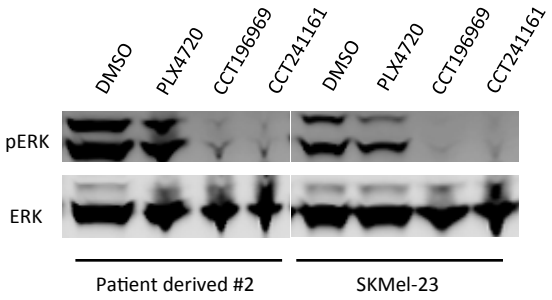


Figure S2, related to Figure 2.
Phospho-ERK (pERK) and ERK2 levels in patient #2 cells or SK-Mel 23 cells treated for 4 hr with DMSO (D), PLX4720 1 µM, CCT196969 1 µM or CCT241161 1 µM.

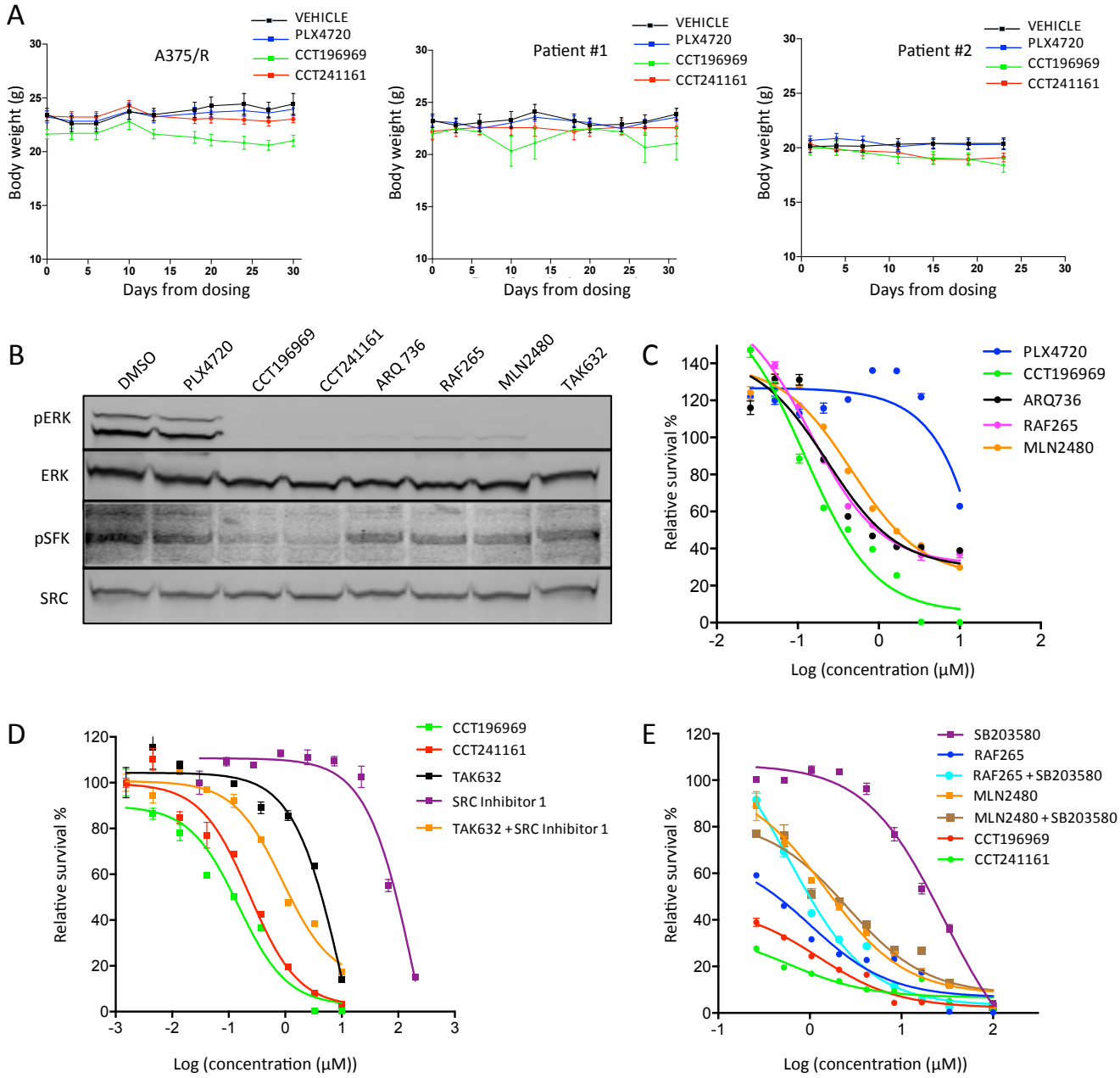


Figure S3, related to Figure 3.

A. Body weight curves of nude and NGS mice treated with vehicle, PLX4720, CCT196969 and CCT241161 by oral gavage. Body weight (g) was monitored twice a week during the period of treatment

B. Phospho-ERK (pERK), ERK2, pSFK and SRC in patient #2 cells treated for 4 hr with DMSO (D), PLX4720 1 μM, CCT196969 0.5 μM, CCT241161 0.5 μM, ARQ736 0.5 μM, RAF265 0.5 μM, MLN2480 0.5 μM or TAK632 0.5 μM.

C. Cell proliferation assay (CellTiter Glo) on patient #2 cells treated with PLX4720, CCT196969, ARQ736, RAF265, MLN2480.

D. Cell proliferation assay (CellTiter Glo) on patient #2 cells treated with SRC Inhibitor 1, TAK632 (alone or in combination with 40 μM SRC Inhibitor 1), CCT196969 or CCT241161.

E. Cell proliferation assay (CellTiter Glo) on patient #2 cells treated with p38 inhibitor SB203580, RAF265 (alone or in combination with 10 μM SB203580), MLN 2480 (alone or in combination with 10 μM SB203580), CCT196969 or CCT241161.

Bars represent SEM.

Table S4, related to Figure 3. Clinical history of patients studied herein.

Patient	BRAF mutation status	Diagnosis	Treatment	Months on treatment	Best response
#1	BRAFV600E	Metastatic melanoma stage III	naïve (no treatment)	N/A	N/A
#2	BRAFV600E	Metastatic melanoma stage IV	vemurafenib	3	PR
#3	BRAFV600E	Metastatic melanoma stage IV	vemurafenib	15	CR
#4	BRAFV600E	Metastatic melanoma stage IV	vemurafenib	5	PR
#5	BRAFV600E	Metastatic melanoma stage IV	vemurafenib	2	PD
#13	BRAFV600E	Metastatic melanoma stage IV	Dabrafenib + Trametinib	5	PR

Table S5, related to Figure 3. RPPA analysis of phosphorylated:total protein ratios in compound treated cells following normalization to DMSO.

	PLX4720 1 µM					CCT241161 1 µM					CCT196969 1 µM				
Analyte Phospho:total Ratio	P1	P2	P3	mean	Std Dev.	P1	P2	P3	mean	Std dev.	P1	P2	P3	mean	Std Dev.
p70 S6 Kinase P Thr421,Ser424/total	1.14	1.15	1.04	1.11	0.06	1.07	1.23	1.10	1.14	0.09	0.95	1.03	1.14	1.04	0.10
Tuberin P S1387/total	1.42	0.92	1.21	1.18	0.25	1.23	0.75	1.16	1.05	0.26	1.59	0.99	1.08	1.22	0.32
PDK-1 P Ser241/total	1.02	1.18	1.10	1.10	0.08	1.02	1.06	1.05	1.05	0.02	0.91	1.08	0.99	0.99	0.09
Rb P Ser780/total	0.81	0.89	0.99	0.90	0.09	0.96	1.04	1.11	1.04	0.08	1.08	0.94	1.01	1.01	0.07
EGFR P Tyr1173/total	0.87	0.91	0.65	0.81	0.14	1.09	0.99	0.66	0.91	0.23	1.10	1.10	0.78	0.99	0.18
FAK1 P Y397/total	0.54	0.74	0.93	0.74	0.19	1.09	0.57	0.92	0.86	0.27	0.28	0.24	0.47	0.33	0.12
Tsc-2 (Tuberin) P Thr1462/total	1.16	1.09	0.91	1.06	0.13	0.95	0.89	0.65	0.83	0.16	1.06	0.91	0.54	0.84	0.26
c-Jun P Ser73/total	0.54	1.10	0.62	0.75	0.30	0.80	1.09	0.47	0.79	0.31	0.69	1.07	0.41	0.72	0.33
Rb P Ser807,Ser811/total	0.74	0.85	0.81	0.80	0.06	0.70	0.98	0.60	0.76	0.20	0.59	0.91	0.47	0.66	0.23
Metp/total	1.05	0.91	1.03	1.00	0.08	0.80	0.68	0.78	0.75	0.06	0.70	0.73	0.79	0.74	0.04
S6 Ribosomal protein p Ser240,Ser244/total	0.55	1.23	0.81	0.86	0.34	0.40	1.03	0.74	0.72	0.32	0.22	0.68	0.36	0.42	0.23
MTOR P 2448/total	0.89	0.82	0.60	0.77	0.15	0.78	0.92	0.46	0.72	0.24	0.69	0.99	0.57	0.75	0.21
p70 S6 Kinase P Thr389/total	0.84	1.20	0.86	0.97	0.20	0.58	1.06	0.49	0.71	0.31	0.43	0.69	0.29	0.47	0.20
ErbB-3/Her3/EGFR P Tyr1289/total	1.36	1.00	0.81	1.06	0.28	0.73	0.73	0.53	0.66	0.11	0.83	0.84	0.60	0.76	0.13
ErbB-2/Her2/EGFR P Tyr1248/Tyr1173/total	1.60	0.79	1.30	1.23	0.41	0.98	0.30	0.65	0.64	0.34	0.89	0.31	0.52	0.57	0.29
p90 S6 kinase (Rsk1-3) P Thr359,Ser363/total	0.76	0.99	0.41	0.72	0.29	0.62	0.95	0.32	0.63	0.31	0.49	0.79	0.37	0.55	0.22
p53 P Ser15/total	1.16	0.97	0.58	0.90	0.30	0.90	0.34	0.64	0.62	0.28	1.26	0.76	-0.04	0.66	0.65
Stat5 p Tyr695/total	0.67	0.87	0.92	0.82	0.13	0.64	0.71	0.48	0.61	0.12	0.61	0.66	0.46	0.58	0.11
S6 Ribosomal protein P Ser235,Ser236/total	0.37	1.19	0.49	0.68	0.44	0.20	1.22	0.40	0.60	0.54	0.12	0.69	0.13	0.31	0.32
GSK-3-alpha/beta P Ser21/Ser9/total	0.84	1.47	0.89	1.07	0.35	0.67	0.69	0.43	0.60	0.14	0.44	0.52	0.37	0.45	0.08

SAPK/JNK P Thr182,Tyr185/total	0.83	0.75	0.71	0.77	0.06	0.64	0.60	0.46	0.57	0.10	0.56	0.61	0.45	0.54	0.08
GSK-3-beta P Ser9/total	0.84	1.00	0.91	0.92	0.08	0.61	0.62	0.41	0.55	0.12	0.40	0.39	0.29	0.36	0.06
p38 MAPK PThr180,Tyr182/total	0.78	0.90	0.54	0.74	0.19	0.57	0.70	0.28	0.52	0.22	0.61	0.83	0.48	0.64	0.18
Met P Tyr1234/total	0.64	0.53	0.89	0.69	0.19	0.24	0.46	0.67	0.46	0.22	0.38	0.59	0.62	0.53	0.13
Mek-phosphoser217/221/total	0.59	0.99	0.63	0.73	0.22	0.29	0.39	0.37	0.35	0.05	0.28	0.38	0.50	0.39	0.11
Akt P Ser473/total	0.50	0.86	0.79	0.72	0.19	0.43	0.35	0.27	0.35	0.08	0.26	0.16	0.20	0.20	0.05
Src (family) P Tyr416/total	0.88	1.01	0.86	0.91	0.08	0.24	0.23	0.26	0.24	0.01	0.27	0.29	0.37	0.31	0.05
p44/42 MAPK (ERK1/2) P Thr202/Thr185,Tyr204/Tyr187/total	0.41	0.76	0.46	0.54	0.19	0.05	0.09	0.04	0.06	0.03	0.06	0.08	0.03	0.06	0.02
IRS-1 P S636/639/total	3.77	0.78	-0.18	1.45	2.06	-4.50	0.92	0.42	-1.05	3.00	3.27	0.81	26.31	10.13	14.07

(P1: cell line clone A from patient #1; P2: cell line clone B from patient #1; P3: cell line derived from patient #2).

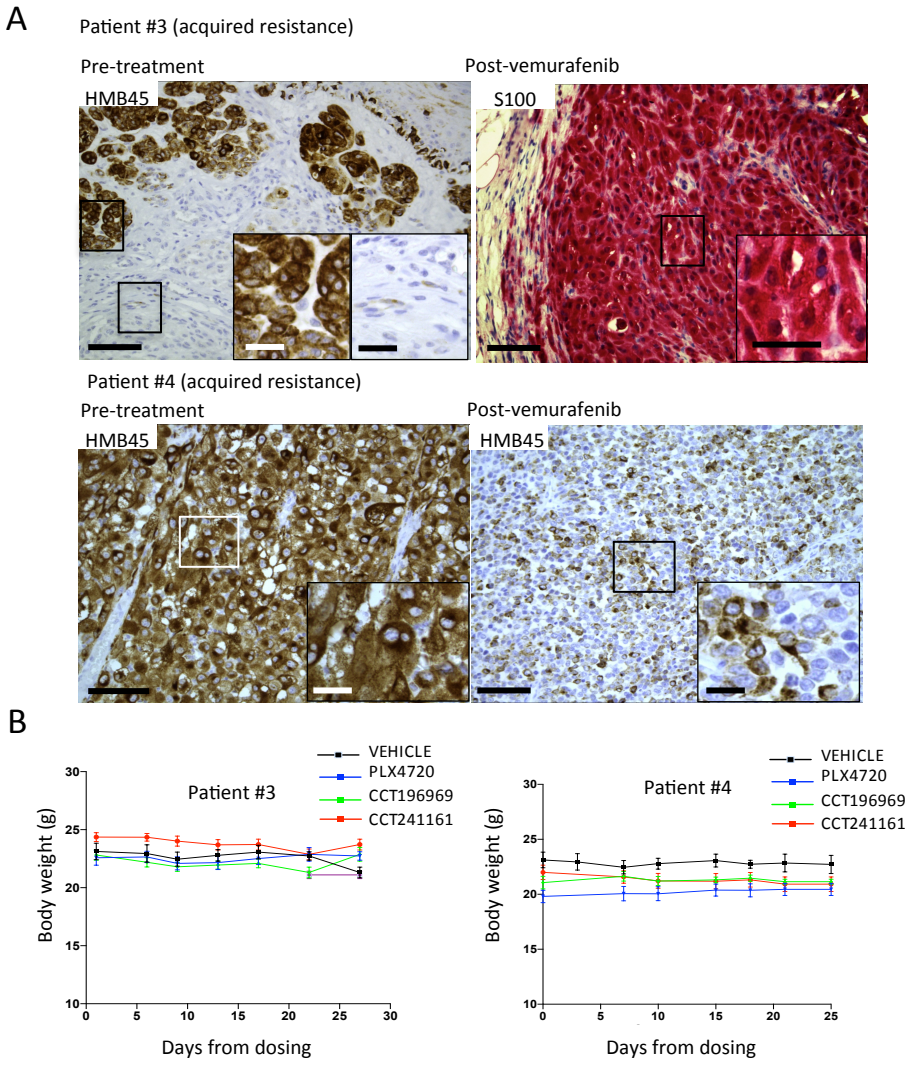


Figure S4, related to Figure 4.

A. HMB45/MelanA or S100 in pre- and post-vemurafenib tumors from patient #3 and #4. Scale bars: 100 µm, inset 30 µm.

B. Body weight curves of NGS mice treated with vehicle, PLX4720, CCT196969 and CCT241161 by oral gavage. Body weight (g) was monitored twice a week during the period of treatment. No significant difference in body weight was observed with any of the treatments.

Bars represent SEM.

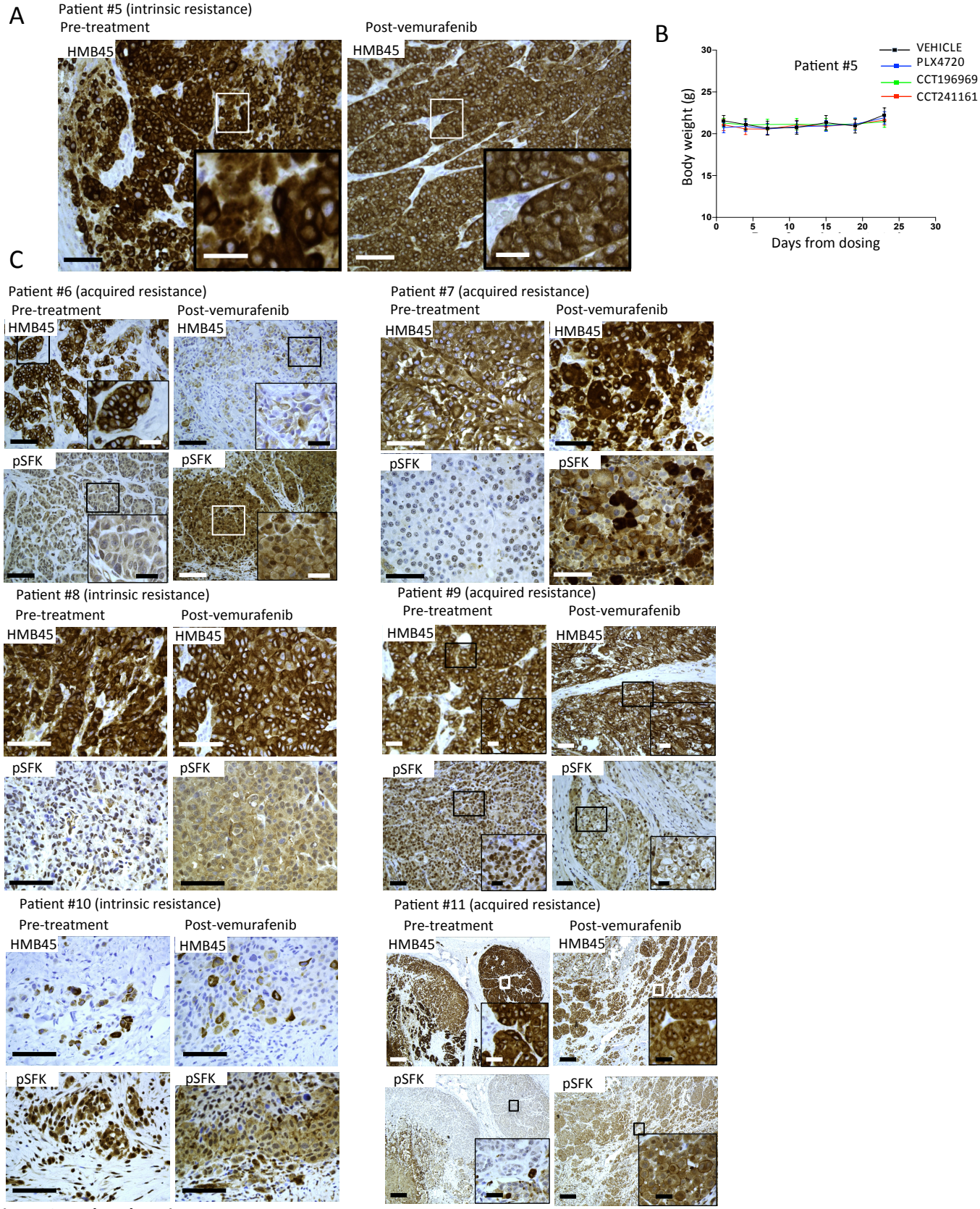


Figure S5, related to Figure 5.

A. HMB45/MelanA or S100 in pre- and post-vemurafenib tumors from patient #5. Scale bars: 100 µm, inset 30 µm.

B. Body weight curves of NGS mice treated with vehicle, PLX4720, CCT196969 and CCT241161 by oral gavage. Body weight (g) was monitored twice a week during the period of treatment. No significant difference in body weight was observed with any of the treatments. Bars represent SEM.

C. HMB45/MelanA and pSFK in pre- and post-vemurafenib tumors from patient #6, #7, #8, #9, #10 and #11. Scale bars: 100 µm, inset 30 µm. Patient 11 scale bars: 300 µm, inset 25 µm

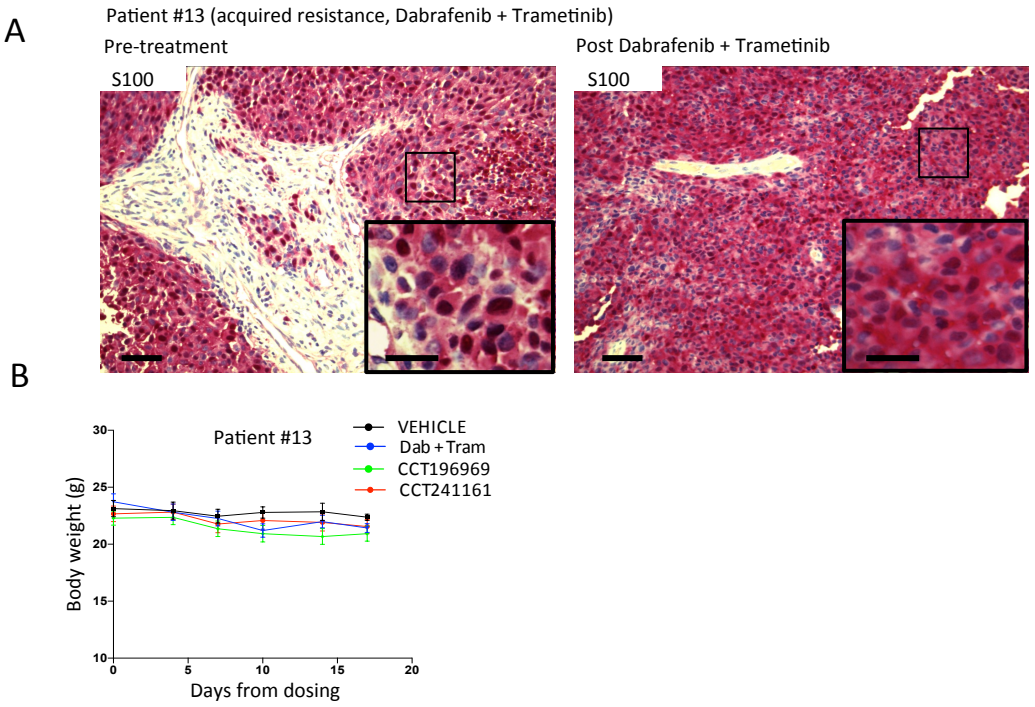


Figure S6, related to Figure 6.

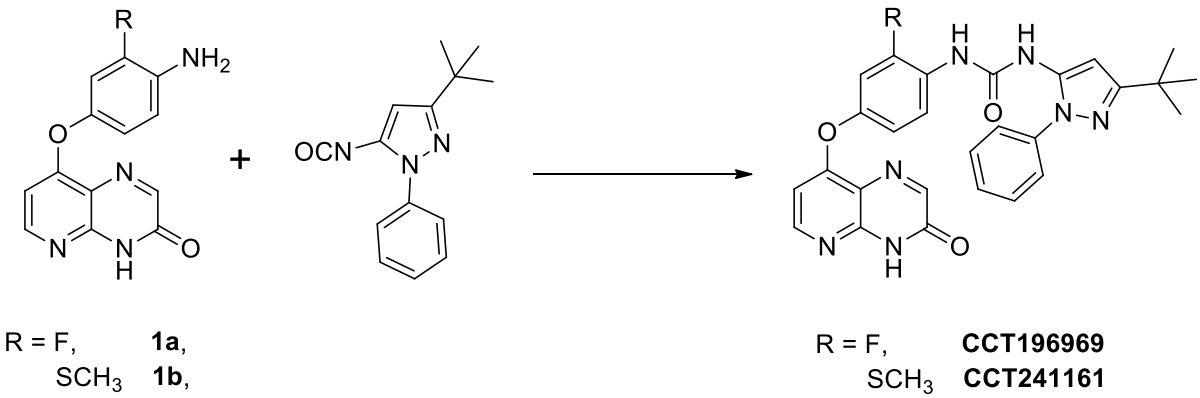
A. S100 in pre- and post-vemurafenib tumors from patient #13. Scale bars: 100 µm, inset 30 µm.

B. Body weight curves of NGS mice treated with vehicle, dabrafenib and trametinib, CCT196969 and CCT241161 by oral gavage. Body weight (g) was monitored twice a week during the period of treatment. No significant difference in body weight was observed with any of the treatments. Bars represent SEM.

Supplemental Experimental Procedures

Experimental chemistry. All starting materials, reagents and solvents for reactions were reagent grade and used as purchased. Chromatography solvents were HPLC grade and were used without further purification. LC-MS analyses were performed on a Micromass LCT/Water's Alliance 2795 HPLC system using 5 µm Atlantis C18, 50 mm × 2.1 mm columns at 22°C with the following solvent system: Aqueous: water + 0.1% formic acid; Organic: 0.1% formic + acetonitrile, at a flow rate of 1 mL/min. Method A: gradient starting with 100% aqueous to 100% organic in 2.5 minutes at room temperature and a flow rate of 0.6 mL/min or method B gradient starting with 100% aqueous to 100% organic in 5 minutes at 40°C (column temperature) at a flow rate of 0.6 mL/min. UV detection was at 215 nm and ionisation was positive or negative ion electrospray. The molecular weight scan range was 50-1000. Samples were injected at 3 µL on a partial loop fill. NMR spectra were recorded in DMSO-d_6 on a Bruker Advance 500 MHz spectrometer. Chemical shifts (δ) are given in ppm and are referenced to residual, not fully deuterated solvent signal (*i.e.* DMSO-d_5). Coupling constants (*J*) are given in Hz. Accurate Mass Measurement was performed with a Waters Micromass LCT Premier orthogonal acceleration Time-of-Flight Mass Spectrometer 4GHz TDC with LockSpray™ enable mass measurements of 5 ppm or better for m/z of 400 or greater and 2 mDa or better for m/z of 400 or less. Calibration reference: Wpos_150208.cal or Wneg_150208a.cal. MassLynx v4.1 SCN 633 was the operating software using the in-built elemental composition to report data. Minimum 10 scans combined across a MS peak. The purity of the final compounds was determined by HPLC and ^{1}H-NMR as described above and is higher than 95%.

CCT241161 and CCT196969 were synthesised starting from the amine intermediates **1a** and **1b** which were previously reported (Aalto et al., 2001) (see Scheme 1).



Scheme 1

Synthesis of 1-(3-*tert*-butyl-1-phenyl-1H-pyrazol-5-yl)-3-(2-fluoro-4-(3-oxo-3,4-dihydropyrido [3,2-b]pyrazin-8-yloxy)phenyl)urea (CCT196969)

A solution of 8-(4-amino-3-fluorophenoxy)pyrido[2,3-b]pyrazin-3(4H)-one **1a** (45 mg, 165mmol) in dry THF (10 mL) under argon was treated with 3-tert-butyl-5-isocyanato-1-phenyl-1H-pyrazole (1.2 eq; and as reported (Suijkerbuijk et al.)]) and the pale yellow solution stirred at room temperature. After 2h, the solution was concentrated to dryness under vacuum, dissolved in 30 mL DCM and washed with citric acid (2 x 20 mL). The organic layer was dried ($MgSO_4$), concentrated to 10 mL and 50 mL hexane added. The desired compound which precipitated as a cream coloured solid was filtered and dried. Yield: 50 mg (60%). ^{1}H-NMR (DMSO-d_6), δ (ppm), J (Hz): 1.28 ppm (s, 9H, *tert*- Bu), 6.40 (s, 1H, H_{pyz}), 6.66 (d, J=5.6 Hz, 1H, H_{Py}), 7.04 (m, 1H, H_{arom}), 7.29 (m, 1H, H_{arom}), 7.42 (m, 1H, H_{arom}), 7.53-7.55 (m, 4H, H_{arom}), 8.16-8.17 (m, 2H, H_{arom}), 8.37 (d, J=5.6 Hz, 1H, H_{Py}), 8.83 (s, 1H, NH), 8.98 (s, 1H, NH), 12.90 (br s, 1H, NH); ^{13}C-NMR (DMSO-d_6), δ (ppm), J (Hz): 30.2, 32.0, 95.1, 106.5, 108.5 (d, J_{FC}=22 Hz), 116.4 (d, J_{FC}=3 Hz), 118.4, 121.8, 124.4, 124.9 (d, J_{FC}=12 Hz), 127.4, 129.3, 136.9, 138.4, 145.5, 148.6 (d, J_{FC}=10 Hz), 151.2, 151.3, 152.2,

152.3 (d, J_{FC}=245 Hz), 153.3, 156.4, 160.5, 160.8, 171.2; ^{19}F NMR (470 MHz, DMSO-d_6): δ= -125.2 ppm; LC-MS (*m/z*): 514.2 (M+H, 100), rt=4.93 min; HRMS (5.95 min): *m/z* calcd. for $C_{27}H_{25}FN_7O_3$ [M+H$^+$]: 514.19974; found: 514.19964.

Synthesis of 1-(3-*tert*-butyl-1-phenyl-1H-pyrazol-5-yl)-3-(2-(methylthio)-4-(3-oxo-3,4-dihydropyrido[2,3-b]pyrazin-8-yloxy)phenyl)urea (CCT241161)

A similar method was used by reacting 8-(4-amino-3-(methylthio)phenoxy)pyrido[3,2-b]pyrazin-3(4H)-one, **1b**, (100 mg, 333 mmol) and 3-*tert*-butyl-5-isocyanato-1-phenyl-1H-pyrazole (2 eq) in 600 mL DMSO for 19.5 hrs. at room temperature. The reaction mixture was diluted with water, the precipitate was recovered by filtration and washed with acetonitrile to afford the title compound (175mg, 97%) as a white powder.

^{1}H-NMR ($CDCl_3$-d6), δ (ppm), J (Hz): 1.28 (s, 9H, *tert*- Bu), 2.43 (s, 3H, CH_3), 6.36 (s, 1H, H_{pyz}), 6.60 (d, 1H, $H_{Py,5}$, J=5.6 Hz), 7.03 (dd, 1H, H_{arom}, J=8.8 Hz, J=2.7 Hz), 7.21 (d, 1H, H_{arom}, J=2.7 Hz), 7.39-7.43 (m, 1H, H_{arom}), 7.53-7.54 (m, 4H, H_{arom}), 7.77 (d, 1H, H_{arom}, J=8.8 Hz), 8.18 (s, 1H, H_{arom}), 8.35 (d, 1H, $H_{Py,}$ J=5.6 Hz), 8.37 (s, 1H, NH), 8.98 (s, 1H, NH), 12.89 (s, 1H, NH). ^{13}C-NMR (DMSO-d6), δ (ppm), J (Hz): 15.3, 30.0(3), 31.9, 96.2, 106.1, 117.7, 118.2, 119.3, 123.9(2), 124.3, 127.0, 129.1(2), 131.8, 133.7, 136.8, 138.5, 145.3, 149.9, 150.8, 152.0(2), 156.3, 160.6, 160.7. LC-MS (*m/z*): 542 (M+H, 100), rt=2.60min. HRMS (EI): *m/z* (M+H, 100) calcd for $C_{28}H_{27}N_7O_3S$: 542.1968; found: 542.1968.

Pharmacokinetics studies. The test compounds, CCT1976969 and CCT241161, were suspended in DMSO:water (1:19 v:v) for the oral dosing and DMSO:Tween:Saline (10:1:89 v:v:v) for the intravenous dosing at 0.2 ml per 20 g body weight. Eighteen female mice (Balb/c) were dosed via oral gavage at a concentration of 10 mg/kg/day and twenty one female mice (Balb/c) were dosed intravenously at a concentrations of 2 mg/kg/day. Treatment was a single dose by oral gavage or intravenous injection.

Three mice were terminally exsanguinated (cardiac puncture under isoflurane anaesthesia) at 5 minutes (IV only), 15 minutes, 30 minutes, 1, 3, 6 and 18 hr post dose. Heparinised blood was centrifuged at 1000 g for 3 minutes at room temperature and the resultant plasma was separated and placed in liquid nitrogen until transferred to -80°C.

Following protein precipitation the samples were centrifuged for 30 minutes in a refrigerated centrifuge (4°C) at 2800 rpm. The supernatant was analysed by Liquid Chromatography Mass Spectrometry (LC-MS/MS) for the CCT196969 and CCT241161 plasma concentrations. Non-compartmental analysis was performed on plasma concentration data by computer software WinNonlin v5.3.

Single dose tolerability investigations with vehicle or CCT196969 were assessed at 20 mg/kg/day or 40 mg/kg/day by Aurigon Toxicoop, Hungary in CD-1 mice (2/group) in accordance with GLP principles. Test compound was formulated in 5% DMSO in water and administered by oral gavage. The vehicle and test compounds were made up and administered within 90 minutes of preparation at a standard dosage volume of 10 mL/kg, individual doses being calculated on the basis of individual body weight.

Repeat dose oral toxicity investigations with CCT196969 at 20 mg/kg qd or vehicle were assessed for 24 days in CD-1 mice (8/group). CCT1976969 or CCT241161 or vehicle were assessed at 30 mg/kg qd for 4 days in CD-1 mice (2/group) and followed for >30 days. Further independent investigations (Aurigon Toxicoop, Hungary) of repeat dose studies assessed on the higher dose of 25 mg/kg qd for 19 days in CD-1 mice (5/group) with CCT196969 in accordance with GLP principles. Test compound was administered, formulated and dosed as above, by oral gavage.