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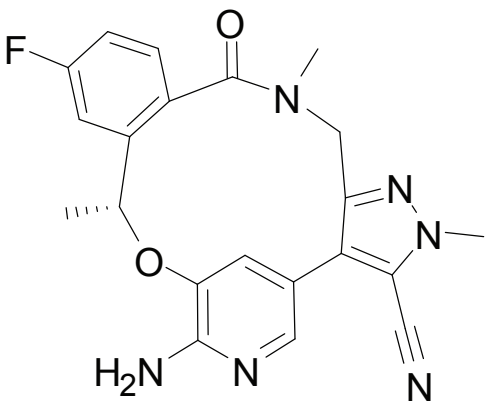

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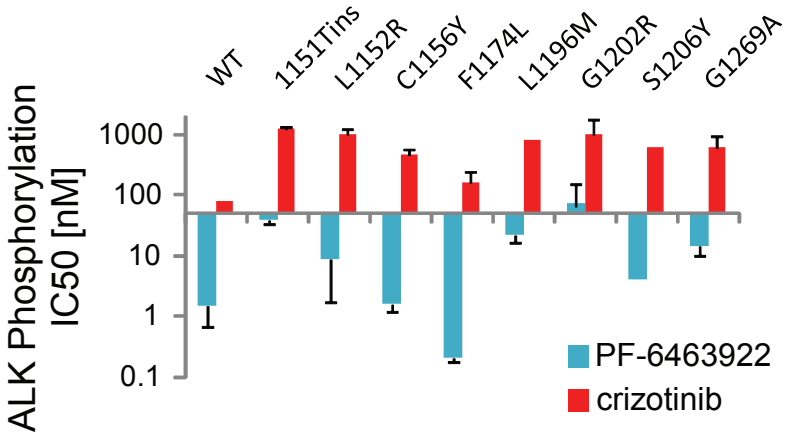


Figure S1: Related to Figure 1.

(A) Chemical structure of PF-06463922: (10R)-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-(metheno)pyrazolo[4,3h][2,5,11] benzoxadiazacyclotetradecine-3-carbonitrile.

(B) IC50 values of crizotinib and PF-06463922 for the inhibition ALK autophosphorylation in engineered NIH3T3 cells expressing the indicated ALK fusion mutant. Values are presented as mean ± SD (n=3).

Table S1 related to Figure 1.

PF-06463922 inhibitory potency against WT and ALK resistant mutants in cell assays

IC_{50} < 100 nM IC_{50} ≥ 100 < 200nM IC_{50} ≥ 200nM	[a]Cellular ALK Phosphorylation Mean [c]IC_{50} (nM)				[b]Cell Proliferation Mean [c]IC_{50} (nM)			
Target/Cell Line	PF-6463922	Xalkori	ceritinib	CH-5424802	PF-6463922	Xalkori	ceritinib	CH-5424802
EML4-ALK v1/ H3122 (endogenous) NIH3T3 (engineered) BaF3 (engineered)	1.9 1.5 3.6	82 80 90	102 NA 41	24 62 24	2.4 NA 2.7	81 NA 88	30 NA 25	27 NA 39
EML4-ALK v3a/b/ H2228(endogenous) NIH3T3 (engineered)	1.3 1.0	118 50	NA NA	NA NA	4.1 NA	60 NA	NA NA	13 NA
EML4-ALK^{L1196M}/ H3122 (engineered) NIH3T3 (engineered) BaF3 (engineered)	15 21 43	535 843 1154	142 NA 70	200 250 113	30 NA 28	973 NA 420	NA NA 26	119 NA 79
EML4-ALK^{G1269A}/ SNU2535 H3122 (engineered) NIH3T3 (engineered) BaF3 (engineered)	19 14 15 80	507 504 605 689	195 137 NA 134	173 64 NA 112	45 20 NA 36	3845 623 NA 466	96 NA NA 21	161 NA NA 73
EML4-ALK^{G1202R}/ NIH3T3 (engineered) BaF3 (engineered)	77 113	1003 562	>1000 549	>1000 362	NA 80	NA 560	NA 309	NA 595
EML4-ALK^{I1151Tins}/ NIH3T3 (engineered) BaF3 (engineered))	38 50	1268 902	1066 296	NA 126	NA 57	NA 783	NA 200	NA 172
EML4-ALK^{L1152R}/ NIH3T3 (engineered)	9	1026	NA	NA	NA	NA	NA	NA
EML4-ALK^{S1206Y}/ NIH3T3 (engineered) BaF3 (engineered)	4.2 3.2	626 152	NA 60	NA 29	NA 5.9	NA 140	NA 26	NA 33
EML4-ALK^{C1156Y}/ NIH3T3 (engineered) BaF3 (engineered)	1.6 15	478 406	NA 177	NA 21	NA 6.7	NA 166	NA 50	NA 30
EML4-ALK^{F1174L}/ NIH3T3 (engineered) BaF3 (engineered)	0.2 4.0	165 150	NA 161	NA 26	NA 4.8	NA 106	NA 40	NA 28
NPM-ALK/ Kapas299(endogenous)	1.2	38	NA	NA	3	62	NA	27
KIF5B-ALK v1 NIH3T3 (engineered)	0.5	29	NA	NA	NA	NA	NA	NA

[a]: The ALK-tyrosine 1604 phosphorylation in cell lysates was determined utilizing a commercially available PathScan® Phospho-ALK Chemiluminescent Sandwich ELISA Kit (Cell Signaling Technologies).
[b]: Cell proliferation was determined by utilizing a commercially available CellTiter Glow assay kit (Promega).
[d]: IC_{50} values were calculated by concentration-response curve fitting using a four-parameter analytical method.

Definitions: NA = not available. Mean values are derived from n = 2-101.

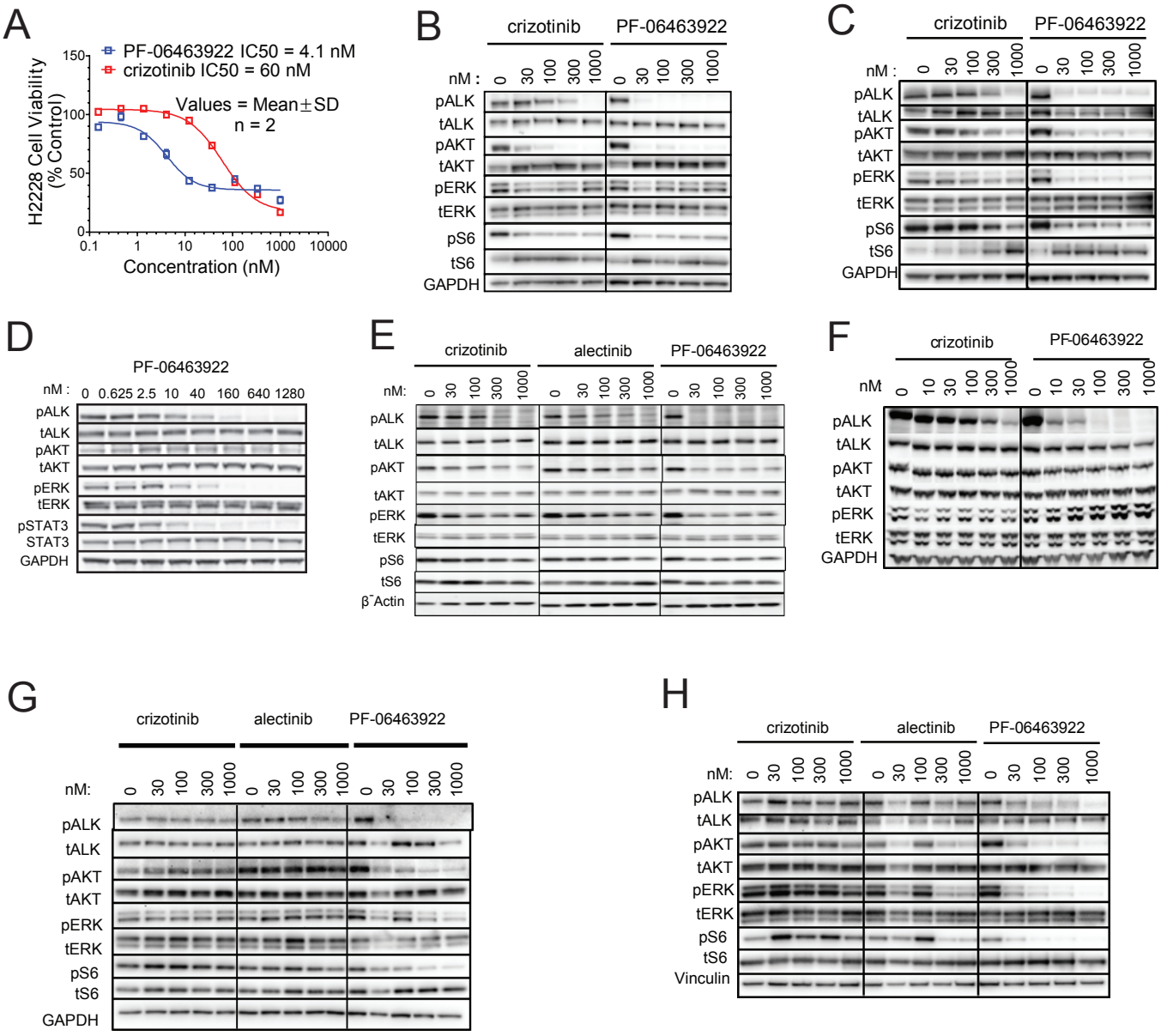


Figure S2. Related to Figure 2.

Comparison of PF-06463922 and other clinically available ALK inhibitors. (A) Cell viability assay of H2228 cells treated with the indicated doses of crizotinib or PF-06463922 for 72 hours. (B) Inhibition of ALK fusion mediated signal transduction analysis in H2228 cells treated with the indicated doses of crizotinib or PF-06463922 for 6 hours. (C) Inhibition of ALK fusion mediated signal transduction analysis in H3122 EML4-ALK^{WT} cells treated with the indicated doses of crizotinib or PF-06463922 for 6 hours (cf. Fig. 2A). (D**)** Inhibition of ALK fusion mediated signal transduction analysis in H3122 EML4-ALK^{L1196M} cells treated with the indicated doses of crizotinib or PF-06463922 for 3 hours (cf. Fig. 2B). (E**)** Inhibition of ALK fusion mediated signal transduction analysis in H3122 EML4-ALK^{V1280L} cells treated with the indicated doses of crizotinib, alectinib or PF-06463922 for 6 hours (cf. Fig. 2F). (F) Inhibition of ALK fusion mediated signal transduction analysis in SNU2535 (EML4-ALK^{G1269A}) cells treated with the indicated doses of crizotinib or PF-06463922 for 3 hours (cf. Fig. 2G). (G) Inhibition of ALK fusion mediated signal transduction analysis in MGH021-5 (SQSTM1-ALK^{G1202R}) cells treated with the indicated doses of crizotinib, alectinib or PF-06463922 for 6 hours (cf. Fig. 2H). (H) Inhibition of ALK fusion mediated signal transduction analysis in MGH056-1 (EML4-ALK^{I1171T}) cells treated with the indicated doses of crizotinib, alectinib or PF-06463922 for 6 hours (cf. Fig. 2I). Undesired conditions were removed but all images belong to the same original images.

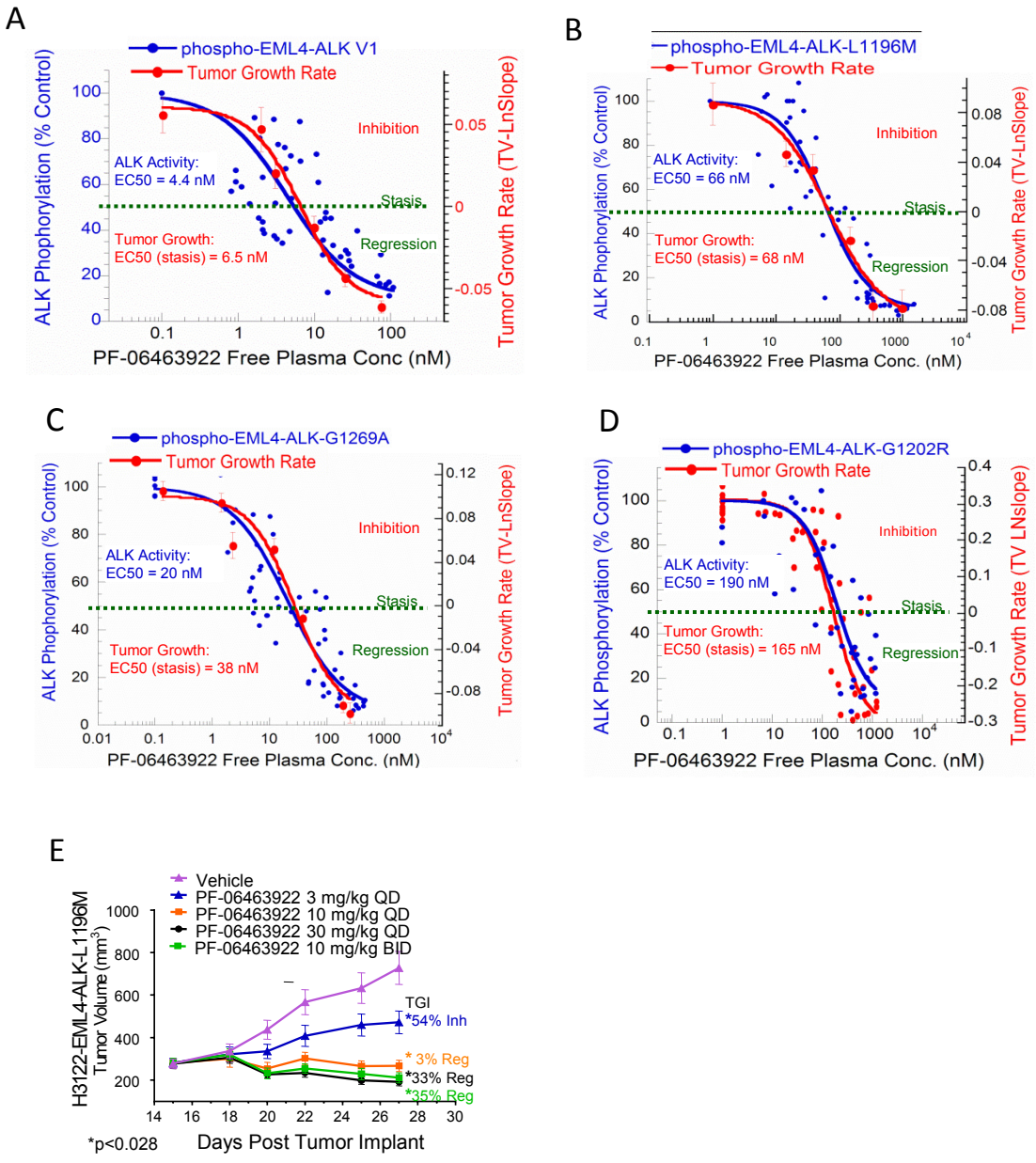


Figure S3 related to Figure 3: PF-06463922 antitumor efficacy in ALK fusion- driven subcutaneous tumor xenograft models in mice. (A-D) Hill plots showing relationships between PF-06463922 free plasma concentration to ALK target inhibition or tumor growth rate in H3122 model expressing endogenous EML4-ALK^{WT} (A), in H3122 model expressing engineered EML4-ALK^{L1196M} (B), in H3122 model expressing engineered human EML4-ALK^{G1269A} (C) and in 3T3 model expressing engineered human EML4-ALK^{G1202R} (D) (cf. Fig. 3A-D). (E) Comparison in antitumor efficacies of PF-06463922 QD vs. BID oral dosing schedules in the H3122-EML4-ALK^{L1196M} model. Tumor growth, ALK phosphorylation and plasma concentration values are presented as mean ± SEM (n=8-12).

Table S2 related to Figure 3.

Antitumor Efficacy of PF-06463922 in ALK Fusion Positive Tumor Models *In Vivo*

TumorModel/ Study #	Initial [a]TV (mm^3)	Route & Schedule &Dose (mg/kg/day)	PK Parameters [b](nM free)			[c]% pALK Target Inhibition (hr)							Antitumor Efficacy			
			Cmax	Cmin	Cave	0.5	1	3	7	8	24	[d]Emax	[e]%TGI (Day)	[f]% Regression (Day)	Emax	[g]P Value Treated vs control
H3122-EML4-ALK-L1196M	280	PO,QD, 3	521		102	61	74	73	57	NA	41	NA	54% (d13)	NA	<	0.02833
	280	PO,QD, 10	1636		508	81	90	57	92	NA	47	NA	NA	3% (d13)	<	<0.00001
	280	PO,QD, 30	3193		1728	93	95	91	97	NA	68	NA	NA	33% (d13)	=	<0.00001
	280	PO,BID, 10	787		395	NA	88	84	71	89	30	NA	NA	35% (d13)	=	<0.00001
H3122-EML4-ALK-L1196M	310	SC Infus, 0.5	15	15	15	6.2						<	49% (d13)	NA	<	0.00342
	310	SC Infus, 1.5	38	38	38	27						<	84% (d13)	NA	<	<0.00001
	310	SC Infus, 5	150	150	150	60						<	NA	30% (d13)	<	<0.00001
	310	SC Infus, 15	346	346	346	89						=	NA	57% (d13)	=	<0.00001
	310	SC Infus, 40	961	961	961	94						=	NA	57% (d13)	=	<0.00001
H3122-EML4-ALK-G1269A	220	SC Infus, 0.2	3.2	3.2	3.2	9						<	9% (d12)	NA	<	0.33445
	220	SC Infus, 0.6	5.2	5.2	5.2	59						<	59% (d12)	NA	<	0.00069
	220	SC Infus, 2	23	23	23	41						<	50% (d12)	NA	<	0.00546
	220	SC Infus, 6	65	65	65	49						<	96% (d12	NA	<	<0.00001
	220	SC Infus, 20	272	272	272	86						=	NA	52% (d12)	=	<0.00001
	220	SC Infus, 25	366	366	366	87						=	NA	59% (d12)	=	<0.00001
NIH3T3-EML4-ALK-G1202R	340	SC Infus, 0.75	28	28	28	14						<	0% (d6)	NA	<	0.05952
	340	SC Infus, 2.5	109	109	109	8						<	71 (d6)	NA	<	0.00070
	340	SC Infus, 7.5	388	388	388	49						<	NA	34% (d6)	<	<0.00001
	340	SC Infus, 20	624	624	624	80						=	NA	76% (d6)	=	<0.00001
	340	SC Infus, 25	725	725	725	82						=	NA	77% (d6)	=	<0.00001
H3122 (EML4-ALK V1)	280	SC Infus, 0.06	2.0	2.0	2.0	45						<	0% (d13)	NA	<	0.01368
	280	SC Infus, 0.2	3.1	3.1	3.1	43						<	49% (d13)	NA	<	0.00019
	280	SC Infus, 0.6	10	10	10	46						<	NA	6% (d13)	<	<0.00001
	280	SC Infus, 1.5	25	25	25	70						<	NA	45% (d13)	<	<0.00001
	280	SC Infus, 3	75	75	75	83						=	NA	59% (d13)	=	<0.00001

a: TV (tumor volume) was measured by electronic caliper.
b: Plasma drug concentrations were measured by LCMS, and the fraction of unbound (free) concentrations were used.
c: pALK levels in tumors were measured by ELISA assay using the tumor protein extracts.
d: Emax = maximum effect.
e: %TGI (tumor growth inhibition) was calculated as: 100 * (tumor $volume_{final}$ – tumor $volume_{initial}$ for the treated group)/ (tumor $volume_{final}$ – tumor $volume_{initial}$ for vehicle group)
f: % tumor regression was calculated as: 1 – (tumor $volume_{final}$/ tumor $volume_{initial}$)
g: p values were derived by ANOVA analysis.

Table S3 related to Figure 3.

PF-06463922 Hill equation parameters in *in vivo* tumor xenograft studies in mice.

Tumor Model/ Study type	PF-06463922 dosing Route	Hill equation parameters	
		[a]ALK phosphorylation	[b]Tumor growth rate[60]
H3122-EML4-ALK^{L1196M} /[a]SC tumor growth	[c]SC mini-pump infusion	fixed Emax = 100, fixed Baseline = 6, Slop = -1.3 ± 0.13, IC_{50} = 66 ± 6.3 nM, R^2 = 0.89.	fixed E_{max} = 0.09, fixed Baseline = -0.09, Slop = -1.02 ± 0.16, IC_{50} = 68 ± 12 nM, R^2 = 0.98.
NIH3T3-EML4-ALK^{G1202R} /SC tumor growth	SC mini-pump infusion	fixed Emax = 100, fixed Baseline = 8, Slop = -1.35 ± 0.29, IC_{50} =190 ± 34 nM, R^2 = 0.67.	fixed E_{max} = 0.31, fixed Baseline = -0.31, Slop = -1.44 ± 0.23, IC_{50} = 165 ± 20 nM, R^2 = 0.82.
H3122-EML4-ALK^{G1269A} /SC tumor growth	SC mini-pump infusion	fixed Emax = 100, fixed Baseline = 5, Slop = -0.9 ± 0.11, IC_{50} = 20 ± 3.0, R^2 = 0.79.	fixed E_{max} = 0.09, fixed Baseline = -0.11, Slop = -1.05 ± 0.22, IC_{50} = 38 ± 8.8 nM, R^2 = 0.96.
H3122 (EML4-ALK V1)/SC tumor growth	SC mini-pump infusion	fixed Emax = 100, fixed Baseline = 10, fixed Slop = -1, IC_{50} = 4.4 ± 0.65, R^2 = 0.62.	fixed E_{max} = 0.06, fixed Baseline = -0.06, Slop = -1.35 ± 0.18, IC_{50} = 6.5 ± 0.75 nM, R^2 =0.98.

[a:] ALK phosphorylation in tumors were measured by ELISA assay.
[b:] Tumor growth rate was determined as described by Jackson, B. C. 1992.
[c:] SC = subcutaneous.

Table S4 related to Figure 3.

PF-06463922 efficacious concentrations derived from PKPD studies in ALK-fusion positive tumor models in mice

Model/ Regimen	[a]ALK phosphorylation [b]Ceff (free nM)				Antitumor Efficacy Ceff (free nM)		
	[c]EC_{50}	EC_{65}	EC_{90}	[d]Emax	Tumor Stasis (100% [e]TGI)	Tumor 30% [f]regression	Emax (52-86% regression)
H3122-EML4 -ALK^{L1196M} SC tumor/infusion	66	106	356	NA	68	126	346
3T3-EML4-ALK^{G1202R} SC tumor/infusion	190	285	682	NA	165	254	624
H3122-EML4-ALK^{G1269A} SC tumor/infusion	20	40	232	272	38	68	272
H3122 (EML4-ALK V1) SC infusion	4.4	8	39	NA	6.5	10	25-75
H3122 (EML4-ALK V1) brain tumor/infusion	NA	NA	NA	157	NA	NA	126

[a:] ALK phosphorylation in tumors was determined by ELISA assay using the tumor protein extracts.
[B:] Ceff = Efficacious concentration. Unbound (free) plasma drug concentrations were used.
[c:] EC_{50} = 50% of Effective concentrations.
[d:] Emax = maximal efficacy observed.
[e:] TGI = tumor growth inhibition
[f:] % tumor regression was calculated as: 1 – (tumor $volume_{final}$/ tumor $volume_{initial}$)

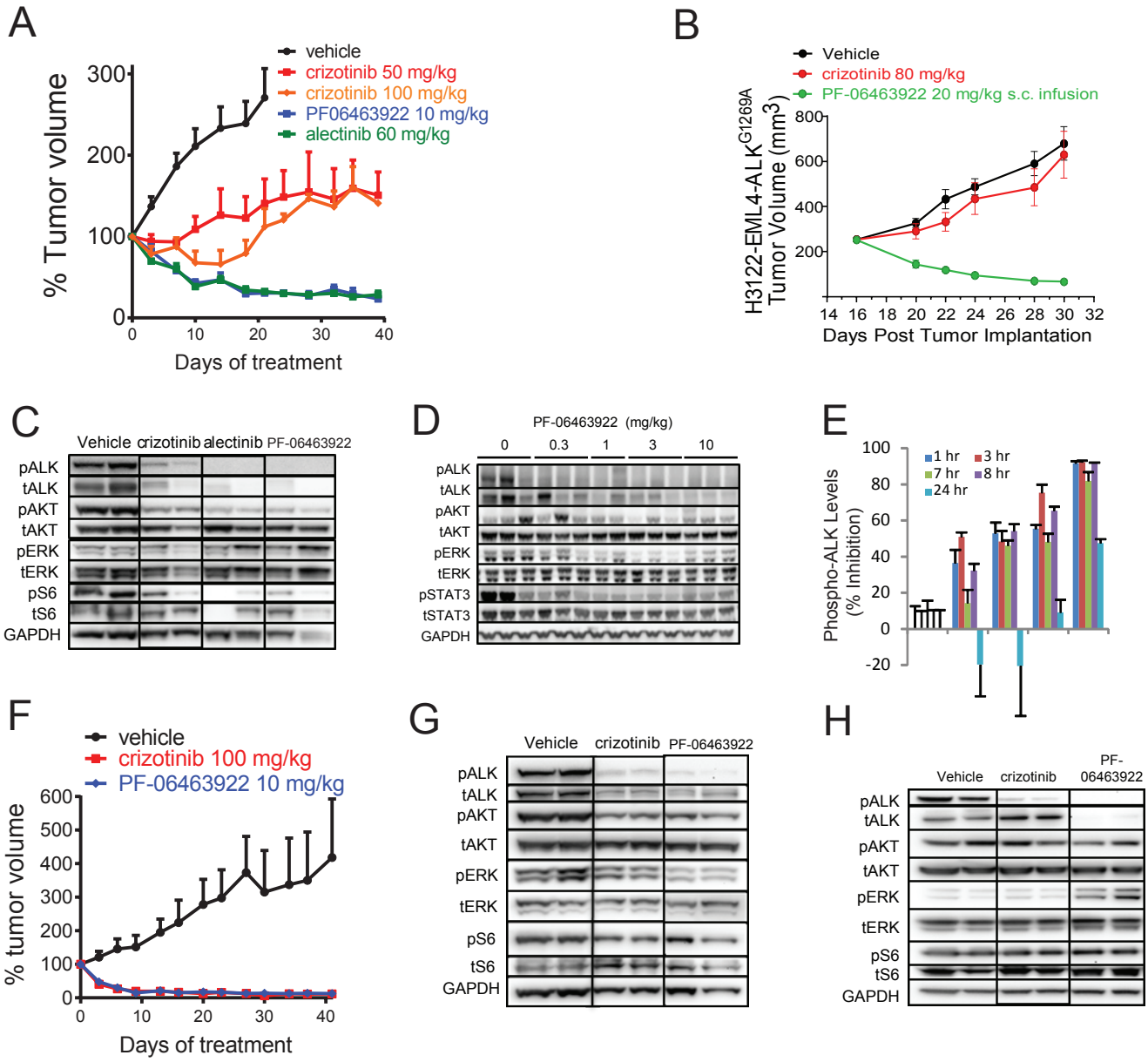
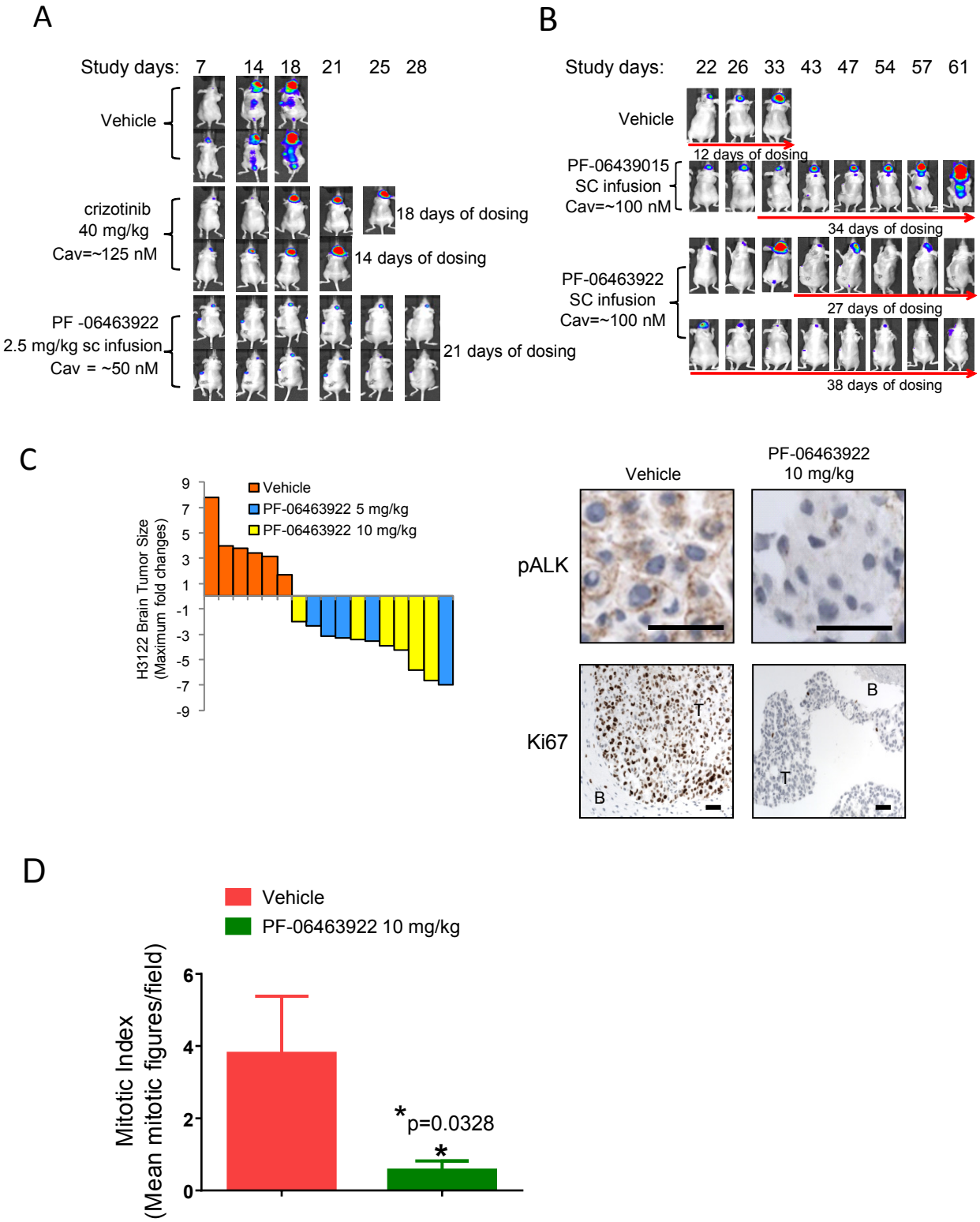


Figure S4 related to Figure 4.

PF-06463922 antitumor efficacy in ALK fusion driven subcutaneous tumor xenograft models in mice. (A) Subcutaneous tumor growth of H3122 cell line treated with crizotinib 50 mg/kg and 100 mg/kg QD or alectinib 60 mg/kg QD or PF-06463922 10 mg/kg BID for 40 days. Values represent mean + SEM (n=5-10) (B) Subcutaneous tumor growth of H3122 EML4-ALK^{G1269A} tumor model treated with crizotinib 75 mg/kg oral BID or PF-06463922 20 mg/kg pump infusion for 30 days. Values represent mean ± SEM (n=12). (C) Pharmacodynamic analysis of H3122-EML4-ALK^{WT} subcutaneous tumors treated for 3 days and collected 3 hours after last treatment. (D) Pharmacodynamic analysis of H3122-EML4-ALK^{L1196M} subcutaneous tumors treated for 3 days and collected 3 hours after last treatment (cf. Fig. 4A). (E) phospho-ALK quantification by ELISA assay on H3122-EML4-ALK^{L1196M} subcutaneous tumors treated for 3 days and collected at different time points after last treatment (cf. Fig. S5E). Values = Mean ± SEM (n=7-9). (F) Subcutaneous tumor growth of EML4-ALKWT MGH006 naive patient derived cell line treated with crizotinib 100 mg/kg QD or PF-06463922 10 mg/kg BID for 40 days. Values represent mean + SEM (n=6). (G) Pharmacodynamic analysis of MGH006 subcutaneous tumors treated for 3 days and collected 3 hours after last treatment. (H) Pharmacodynamic analysis of MGH051 subcutaneous tumors treated for 3 days and collected 3 hours after last treatment (cf. Fig. 4B).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Cav=~125 nM
18 days of dosing
14 days of dosing
PF -06463922
2.5 mg/kg sc infusion
Cav = ~50 nM
21 days of dosing
B
Study days: 22 26 33 43 47 54 57 61
Vehicle
12 days of dosing
PF-06439015
SC infusion
Cav=~100 nM
34 days of dosing
PF-06463922
SC infusion
Cav=~100 nM
27 days of dosing
38 days of dosing
C
H3122 Brain Tumor Size
(Maximum fold changes)
Vehicle
PF-06463922 5 mg/kg
PF-06463922 10 mg/kg
Vehicle
PF-06463922
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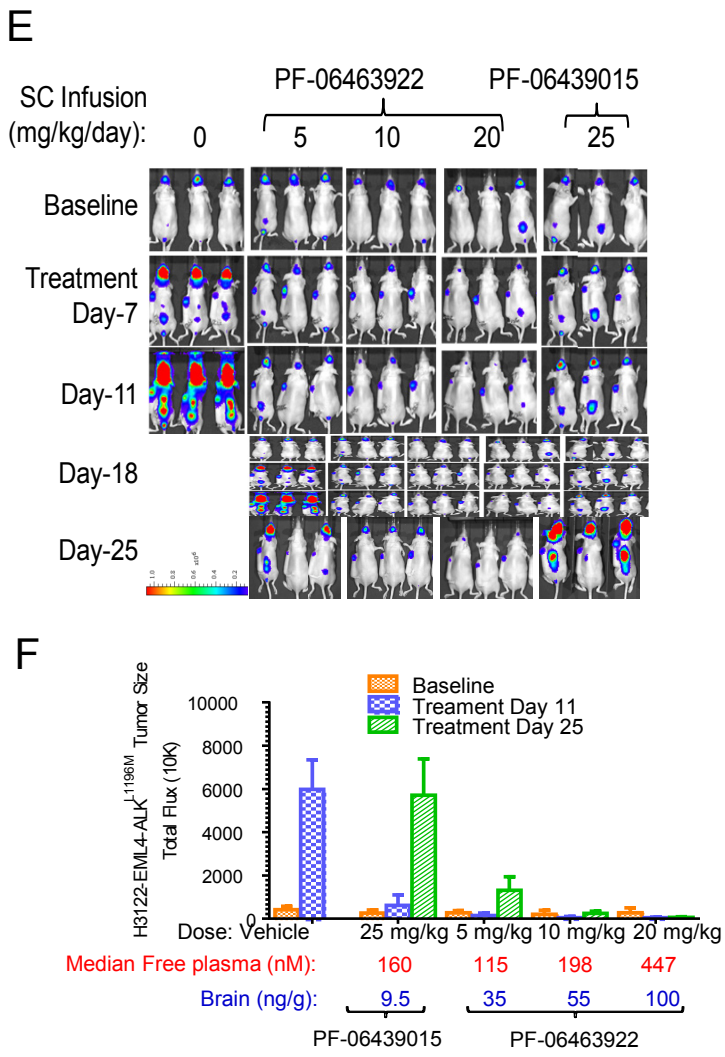

Figure S5. Related to Figure 5.

PF-06463922 antitumor efficacy in ALK fusion-driven brain intracranial tumor models in mice. (A) Representative IVIS images of mice H3122 EML4-ALK^{WT} brain intracranial tumors. Animals treated with crizotinib or PF-06463922 as indicated (cf. Fig. 5A-D). Cav (average plasma drug concentration) are mentioned. (B) Representative IVIS images of mice bearing H3122 EML4-ALK^{WT} brain intracranial tumors. Animals were treated with either PF-06463922 (6-12 mg/kg) or the related non-brain penetrant compound PF-06439015 (36 mg/kg). (C) Fold changes in individual tumor size following PF-06463922 treatment in H3122 EML4-ALK^{WT} intracranial brain xenograft study and representative images of IHC stained for phospho-ALK or Ki67 in the H3122 intracranial brain tumors in the groups indicated. Scale bars in top two panels represent 25 μm and scale bars in bottom two panels represent 50 μm. (D) Decrease in mitotic index following PF-06463922 treatment (cf. Fig. 5A-D). Values are shown as mean ± SEM (n=4-5) (E) Representative IVIS images of mice bearing H3122 EML4-ALK^{L1196M} brain intracranial tumors. Animals were treated with either PF-06463922 or the related non-brain penetrant compound PF-06439015 as indicated. (F) Fold changes in individual tumor size following PF-06463922 treatment in H3122 EML4-ALK^{L1196M} intracranial brain xenograft study. Plasma free and brain concentration of PF-06463922 are indicated for both compounds. Tumor volumes, mitotic index and PF-06463922 concentration values are presented as mean ± SEM (n=4-6).

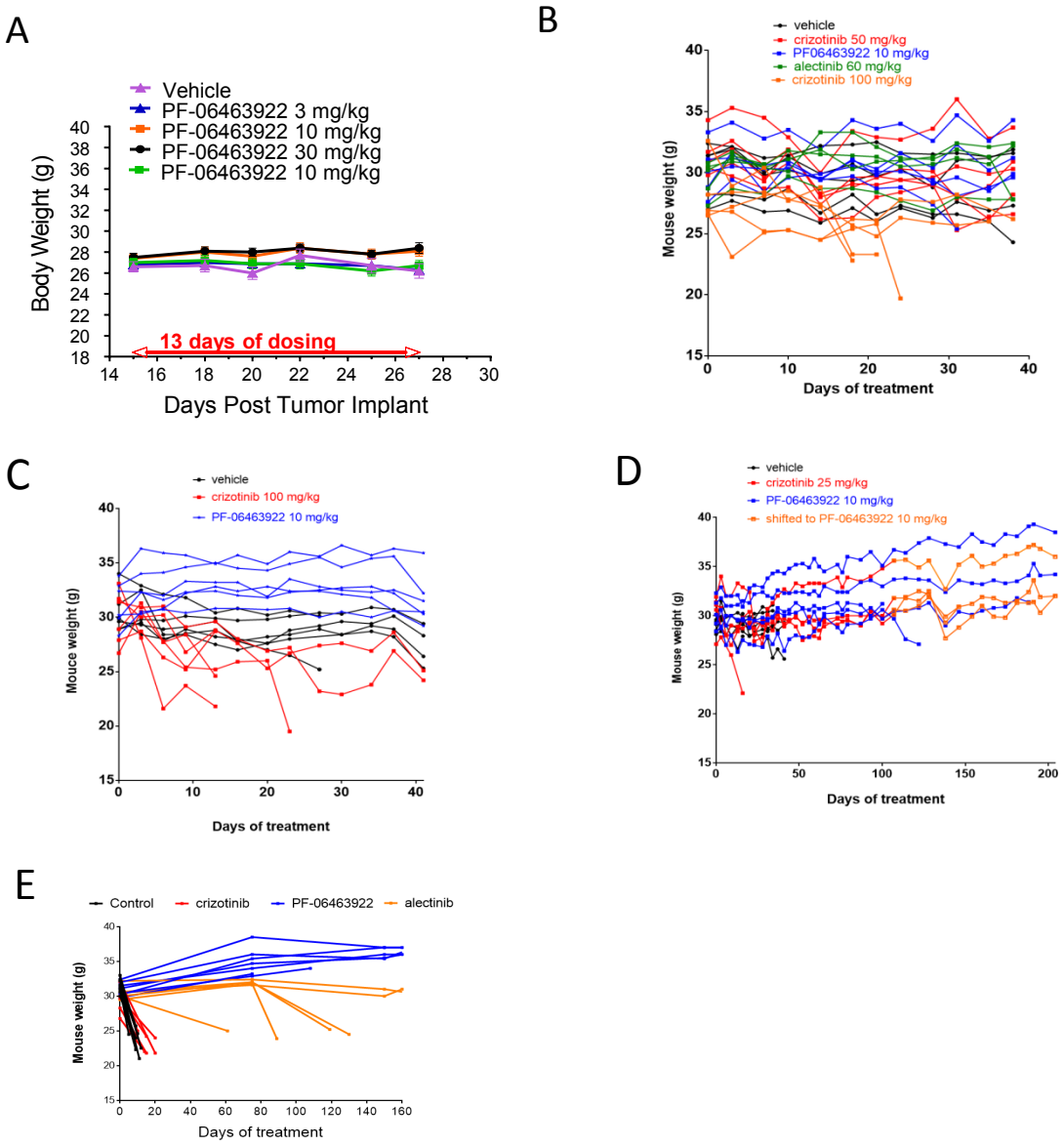

Figure S6 related to Figure 6.

Mouse body weight changes during compound treatment. (A) Mean mouse body weight in subcutaneous tumor growth study of H3122 EML4-ALK^{L1196M} cell line treated with PF-06463922 QD or BID oral dosing from 3 to 10 mg/kg (cf. Fig. S5A). Values are shown as mean ± SEM (n=15). (B) Individual mouse body weights in subcutaneous tumor growth study of H3122 EML4-ALK^{WT} cell line treated with crizotinib 50 mg/kg and 100 mg/kg QD or alectinib 60 mg/kg QD or PF-06463922 10 mg/kg BID for 40 days (cf. Fig. S5B). (C) Individual mouse body weights in subcutaneous tumor growth study of EML4-ALK^{WT} MGH006 naive patient derived cell line treated with crizotinib 100 mg/kg QD or PF-06463922 10 mg/kg BID for 40 days (cf. Fig. S5H). (D**)** Individual mouse body weights in long term subcutaneous tumor growth study of EML4-ALK^{WT} MGH051 crizotinib resistant patient derived cell line treated with crizotinib 25 mg/kg QD or PF-06463922 10 mg/kg BID. The mice treated with crizotinib were shifted to PF-06463922 after 107 days of treatment (cf. Fig. 4B). (E) Individual mouse body weights in brain intracranial tumor growth study of H3122 EML4-ALK^{WT} cell line treated with crizotinib 100 mg/kg QD or alectinib 60 mg/kg QD or PF-06463922 10 mg/kg BID for 150 days (cf. Fig. 5B).

Supplemental Experimental Procedures

IVIS imaging

The IVIS Lumina II imaging system (Xenogen Corporation, Alameda, CA) was used to monitor growth of brain tumors expressing luciferase. Mice received an intraperitoneal (i.p.) injection of d-luciferin (150 mg/kg, stock solution 15 mg/mL in sterile PBS, Goldbio, St. Louis, MO). After 10 min, isoflurane-anesthetized animals were placed in the imaging chamber. The bioluminescent images were acquired and data were analyzed based on total photon flux emission (photons) using Living Image software.

Immunohistochemistry (IHC) Staining and IHC scoring

Following standard tissue processing, xenograft tumor specimens were formalin-fixed, paraffin-embedded and sectioned at a thickness of 4-5 μm onto positively charged microscope slides. Deparaffinization, rehydration, antigen retrieval, and immunohistochemical staining techniques were performed using the fully automated BOND MAX instrument or BOND-III Staining System instrument (Leica Microsystems, Wetzlar, Germany). Heat induced epitope retrieval was performed with either Leica Bond Epitope Retrieval Solution 1 or 2. Nonspecific immunolabeling was reduced with Cyto Q Background Buster (Innovex Biosciences, Richmond, California, United States) or Protein Block either from Dako (DakoCytomation , Glostrup, Denmark) or Spring Bioscience (Pleasanton, California, Unite States). The specific primary antibody was detected using the Leica Bond Polymer Refine Detection system. Prior to immunolabeling development with 3,3'diaminobenzidine tetrahydrochloride (DAB), endogenous peroxidase activity was blocked with hydrogen peroxide. All tumor sections were counterstained with hematoxylin, dehydrated, cleared, and mounted before pathological evaluation. Positive and negative control slides for each marker were evaluated to ensure the specificity of the staining. All samples were evaluated for cellular staining patterns (compared to controls) to ensure that consistent staining was present and to establish the range of the staining frequency/distribution and intensity. Any discrepancies noted in staining consistency may prompt additional or repeat staining prior to evaluation. Once the consistency of staining was confirmed, the staining frequency/distribution and staining intensity (if notably variable between samples) were tabulated for all samples; one or more additional slide reviews may be done at the conclusion of the initial evaluation to confirm patterns observed on the initial evaluation. Mean values and the range of staining scores were evaluated between groups and notable differences and/or patterns were compared between samples from vehicle (or untreated) and treated animals and between individual treatment groups.

Supplemental References

Jackson, B. C. (1992). The theoretical foundations of cancer chemotherapy introduced by computer models. Academic Press, Inc., San Diego, CA, USA.