Supplementary Methods

CB-17SC female mice (Taconic Farms, Germantown NY), were used to propagate subcutaneously implanted kidney/rhabdoid tumors, sarcomas (Ewing, osteosarcoma, rhabdomyosarcoma), and neuroblastoma (1). Mice bearing subcutaneous tumors each received drug when tumors reached between 200 mm^3 and 500 mm^3. Mice were randomized to groups of 10. All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee (IACUC) of the appropriate consortium member.

A tumor *event* was defined as a quadrupling of tumor volume from the day of treatment initiation, where tumor volume was estimated from caliper measurements as $(4/3) \times \pi \times ((\textit{length} + \textit{width}) / 4)^3$. Tumor volumes were measured weekly. The exact time-to-event was estimated by interpolating between the measurements directly preceding and following the event, assuming log-linear growth. Differences in event-free survival (EFS) between experimental groups were tested using the exact log-rank test or the Gehan-Wilcoxon test. Relative tumor volume (RTV) was defined for each mouse as the ratio of its current tumor volume divided by baseline tumor volume. Comparisons between treatments groups of the minimum attained RTV were performed using the Wilcoxon rank sum test.

The objective response categories of progressive disease (PD, which is subdivided among treated mice into PD without and with growth delay, PD1 and PD2 respectively), stable disease (SD), partial response (PR), complete response (CR), and maintained complete response (MCR), are defined below.

- **PD:** <50% tumor regression throughout study and >25% tumor growth at end of study
  - **PD1:** PD and the mouse's time-to-event ≤ 200% the Kaplan-Meier (KM) median time-to-event in control group
  - **PD2:** PD and the mouse's time-to-event > 200% of the KM median time-to-event in control group
- **SD:** <50% tumor regression throughout study and ≤25% tumor growth at end of study
- **PR:** ≥50% tumor regression at any point during study, but measurable tumor throughout study period
- **CR:** Disappearance of measurable tumor mass during the study period
- **MCR:** No measurable tumor mass for at least 3 consecutive weekly readings at any time after treatment has been completed

Overall group response was determined by the median response among evaluable mice. Each individual mouse was assigned a score from 0 to 10 based on their response (PD1 = 0, PD2 = 2, SD = 4, PR = 6, CR = 8, and MCR = 10) with the median for each group determining overall response. If the median score was halfway between a response categories, the objective response was assigned to the lower response category (e.g. a median response score of 9 is scored CR).

Mice with possible treatment-related death (i.e. drug toxicity), mice with failed engraftment, and mice which unexpectedly died for reasons unrelated to treatment were excluded from statistical analyses of time-to-event, minimum tumor volume, and objective response.

Reference

1. Houghton PJ, Morton CL, Tucker C, Payne D, Favours E, Cole C, et al. The Pediatric Preclinical Testing Program: description of models and early testing results. Pediatr Blood Cancer. 2007;49(7):928-40.

Supplementary Table S1. *In vitro* activity of prexasertib against PPTC cell lines.

Cell Line	Histology	rIC_{50} (nM)*	Panel rIC_{50}/Line rIC_{50}	In/Out%^
Karpas-299	anaplastic large-cell lymphoma	0.9	3.4	-99
CCRF-CEM (1)	acute lymphoblastic leukemia	4.2	0.8	-99
CCRF-CEM (2)	acute lymphoblastic leukemia	3	1.1	-100
COG-LL-317	acute lymphoblastic leukemia	2.6	1.3	-100
MOLT-4	acute lymphoblastic leukemia	3.4	0.9	-100
NALM-6	acute lymphoblastic leukemia	5.1	0.6	-100
RS4;11	acute lymphoblastic leukemia	4.5	0.7	-99
Kasumi-1	acute myeloid leukemia	3.5	0.9	-92
SJCRH30	alveolar rhabdomyosarcoma	1.4	2.3	-96
Rh41	alveolar rhabdomyosarcoma	8.8	0.4	-85
RD	embryonal rhabdomyosarcoma	22	0.1	8
Rh18	embryonal rhabdomyosarcoma	2.9	1.1	-75
CHLA-10	Ewing's sarcoma	1.7	1.9	-88
CHLA-258	Ewing's sarcoma	2.4	1.4	-74
CHLA-9	Ewing's sarcoma	1.6	2.0	-64
TC-71	Ewing's sarcoma	1.6	2.0	-100
SJ-GBM2	glioblastoma multiforme	2.6	1.2	-84
BT-12	atypical teratoid/rhabdoid tumor	6.6	0.5	13
CHLA-266	malignant rhabdoid tumor	15	0.2	25
CHLA-136	neuroblastoma	0.9	3.4	-55
CHLA-90	neuroblastoma	3.7	0.9	-71
NB-1643	neuroblastoma	4.8	0.7	-90
NB-EBc1	neuroblastoma	1.7	1.8	-94
Ramos-RA1	non-Hodgkin's lymphoma	6.5	0.5	-95
Median		3.2	1.0	-91
Minimum		0.9	0.1	-100
Maximum		22	3.4	25

*rIC_{50}: relative IC_{50}

^relationship between number of input cells (in) and number of cells following 96h of treatment (out)

Supplementary Table S2. Summary of high content imaging results.

Cell Line	Endpoint	EC_{50} (nM)*	Min. %responders**	Max. %responders
A673	γH2AX	4.7	4.1	33
	cl. caspase 3	6.4	3.7	17
	pATM S1981	4.7	3.1	47
	pDNA-PKcs S2056	5.1	3.3	32
MG-63	γH2AX	18	<1.0	69
	cl. caspase 3	21	1.8	9
	pATM S1981	11	3.3	67
	pDNA-PKcs S2056	10	9.1	88
SJCRH30	γH2AX	3.9	<1.0	38
	cl. caspase 3	4.5	1.5	23
	pATM S1981	4.4	<1.0	64
	pDNA-PKcs S2056	2.8	7.3	79
Rh41	γH2AX	6.6	2.0	36
	cl. caspase 3	16	1.6	16
	pATM S1981	8.1	1.6	45
	pDNA-PKcs S2056	7.4	1.4	40
RD	γH2AX	7.0	3.5	45
	cl. caspase 3	12	3.6	19
	pATM S1981	9.6	2.5	48
	pDNA-PKcs S2056	6.9	2.5	53

*Relative EC_{50} values were calculated for each cell line and endpoint.

**%responders = % of cells positive for specific endpoint

Supplementary Table S3. Summarized results of *in vivo* experiments evaluating prexasertib monotherapy in mouse models of pediatric tumors.

Model	Xenograft type	Tumor type	*n* /arm	Chemotherapy		Analysis day	Chemotherapy	Prexasertib
				Agent	Dose/Schedule		% T/C or regression on treatment analysis day^	
SJCRH30	CDX	alveolar rhabdomyosarcoma	6	Doxorubicin	5 mg/kg, Q7Dx4	41	48.8	**-66.8**
ST162	PDX	alveolar rhabdomyosarcoma	4	Doxorubicin	5 mg/kg, Q7Dx4	39	**21.8**	**-92.8**
CTG-0926	PDX	DSRCT*	5	Doxorubicin	5 mg/kg, Q7Dx4	28	53.1	**-76.3**
CTG-1213	PDX	embryonal rhabdomyosarcoma	5	Actinomycin D	0.25 mg/kg, Q21Dx2	29	63.3	**-22.6**
CTG-1116	PDX	embryonal rhabdomyosarcoma	4	Doxorubicin	5 mg/kg, Q7Dx4	17	72.9	**8.1**
RD	CDX	embryonal rhabdomyosarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	104	**19.0**	31.1
CTG-0142	PDX	Ewing's sarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	32	55.7	78.5
CTG-0785	PDX	Ewing's sarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	14	75.7	68.4
CTG-0816	PDX	Ewing's sarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	46	**18.0**	52.3
CTG-0994	PDX	Ewing's sarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	21	104.4	**-56.8**
RD-ES	CDX	Ewing's sarcoma	8	Doxorubicin	2 mg/kg, Q7Dx1	25	130.7	60.9
				Cyclophosphamide	100 mg/kg, Q7Dx3		**-60.7**	
CTG-1072	PDX	hepatoblastoma	5	Cisplatin	5 mg/kg, Q7Dx4	28	154.5	171.0
A-204	CDX	malignant rhabdoid tumor	6	Doxorubicin	5 mg/kg, Q7Dx4	52	**37.5**	50.6
CTG-0241	PDX	osteosarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	32	115.9	62.8
CTG-0242	PDX	osteosarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	39	**5.2**	**9.1**
CTG-0243	PDX	osteosarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	16	263.3	46.0
CTG-1064	PDX	osteosarcoma	5	Doxorubicin	5 mg/kg, Q7Dx4	33	181.6	37.9
Y79	CDX	retinoblastoma	6	Doxorubicin	5 mg/kg, Q7Dx4	44	**48.5**	**37.1**
Rh10	CDX	alveolar rhabdomyosarcoma	10	-	-	21	-	**-58.2**
EW-5	CDX	Ewing's sarcoma	10	-	-	14	-	107.7
KT-12	CDX	malignant rhabdoid tumor	10	-	-	21	-	**45.9**
KT-13	CDX	malignant rhabdoid tumor	10	-	-	14	-	10.6
RBD-1	CDX	malignant rhabdoid tumor	10	-	-	21	-	**-90.5**
COG-N-421x	CDX	neuroblastoma	10	-	-	28	-	**6.4**
COG-N-453x	CDX	neuroblastoma	10	-	-	23	-	**-86.1**
COG-N-Felix-x	CDX	neuroblastoma	10	-	-	10	-	**-50.0**
NB-1643-x	CDX	neuroblastoma	10	-	-	19	-	**1.8**
NB-EBc1-x	CDX	neuroblastoma	10/5[†]	-	-	7	-	**-72.5**
NB-SD-x	CDX	neuroblastoma	10	-	-	27	-	**-40.7**
OS31	CDX	osteosarcoma	10	-	-	21	-	**64.5**
OS33	CDX	osteosarcoma	10	-	-	14	-	**48.7**

*desmoplastic small round cell tumor [†]*n* = 10 vehicle, 5 prexasertib-treated **^ : *p* < 0.05**

Supplementary Table S4. Summarized results of *in vivo* combination studies of prexasertib plus chemotherapy in pediatric cancer mouse models.

Model	Xenograft type	Tumor type	*n* /arm	Chemotherapy		Prexasertib dose Schedule	Analysis day	Chemotherapy	Prexasertib	Prexa + Chemo	Combination effect^
				Agent	Dose, Schedule			% ΔT/C or regression on analysis day			
SJCRH30	CDX	aRMS	4	Doxorubicin	5 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	39	**60.0**	**-62.0**	**-94.9***‡	**Greater than additive**
SJCRH30	CDX	aRMS	5	Cyclophosphamide	100 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	46	**33.5**	**0.10**	**-91.4***‡	**Greater than additive**
				Irinotecan	2.5 mg/kg, [QDx5,restx14]x2			**12.2**		**-85.8***‡	**Additive**
Rh41	CDX	aRMS	4	Cyclophosphamide	100 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	88	42.9	**-36.8**	**-71.5***‡	**Additive**
				Irinotecan	2.5 mg/kg, [QDx5,restx14]x2			**-13.4**		**-86.2***‡	**Additive**
CTG-1458	PDX	DSRCT	5	Cyclophosphamide	100 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	35	**41.9**	**-90.5**	**-91.7**‡	No effect
RD	CDX	eRMS	5	Doxorubicin	5 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	76	**21.2**	**4.00**	**0.50**‡	Less than additive
A673	CDX	Ewing's sarcoma	5	Irinotecan	2.5 mg/kg, [QDx5,restx16]x2	10 mg/kg [BIDx3D, restx4D]x4	28	60.6	**38.8**	**-66.3***‡	**Greater than additive**
COG-N-Felix-x	CDX	neuroblastoma	10	Irinotecan	2.5 mg/kg, [QDx5]	10 mg/kg [BIDxD1,3,5]x3	14	**0.50**	**-4.10**	**-85.0***‡	**Additive**
NB-EBc1-x	CDX	neuroblastoma	10	Irinotecan	2.5 mg/kg, [QDx5]	10 mg/kg [BIDxD1,3,5]x3	14	**-10.2**	**2.60**	**-94.3***‡	**Additive**
CCSARC005	PDX	osteosarcoma	5	Cisplatin	4 mg/kg, Q7Dx3	10 mg/kg (8 mg/kg in combo) [BIDx3D, restx4D]x4	62	**63.1**	**63.7**	**-23.0***‡	**Greater than additive**

aRMS: alveolar rhabdomyosarcoma; DSRCT: desmoplastic small round cell tumor; eRMS: embryonal rhabdomyosarcoma

***p* < 0.05 compared to vehicle**

* *p* < 0.05 compared to prexasertib alone

‡ *p* < 0.05 compared to chemotherapy alone

^BLISS independence method

Supplementary Table S5. Summary of results from *n* of 1 Ewing's sarcoma PDX studies.

Model	Irinotecan	Prexasertib	Prexa + Irinotecan/TMZ
	Best ΔT/C or regression (%)		
CTG-0143	-100.0	32.0	-100.0
CTG-1126	20.9	78.4	16.0
CTG-1413	-100.0	4.27	106.0
CTG-1651	-93.8		7.63
CTG-1663	13.3	53.8	
CTG-2003	8.28	54.7	23.5
CTG-2113	57.4	73.0	26.6

Supplementary Table S6. Summary of *in vivo* combination results in PPTC models.

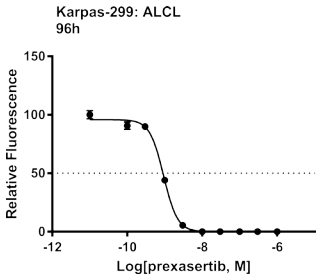
Model	Tumor type	Prexasertib 10 mg/kg	Prexasertib 4 mg/kg	Irinotecan 2.5 mg/kg	Irinotecan (2.5 mg/kg) + prexasertib (4 mg/kg)	Combination v prexasertib (10 mg/kg)	Combination v irinotecan (2.5 mg/kg)
NB-SD-x*	neuroblastoma	44.9 / 2.5^	19.1 / 1.1	33.5 / 1.9	34.9 / 2.0	<0.001	0.857
		CR	PD1	CR	CR		
NB-EBc1-x	neuroblastoma	25.3 / 6.8	11.8 / 3.2	17.9 / 4.8	32.9 / 8.8	**0.011**	**<0.001**
		PR	PD2	PR	PR		
NB-1643-x*	neuroblastoma	22.7 / 2.4	13.8 / 1.5	22.0 / 2.3	25.0 / 2.6	0.121	0.919
		PR	PD1	PR	CR		
COG-N-Felix-x	neuroblastoma	31.4 / 6.4	10.4 / 2.1	24.3 / 4.9	26.8 / 5.5	0.004	0.065
		PR	PD2	PR	PR		
COG-N-453x*	neuroblastoma	36.8 / 2.6	22.9 / 1.6	35.1 / 2.4	30.4 / 2.1	0.013	0.09
		CR	PR	CR	CR		
COG-N-421x*	neuroblastoma	33.3 / 2.0	19.5 / 1.2	31.3 / 1.9	31.0 / 1.9	0.07	0.994
		PR	PD1	PR	PR		
EW-5	Ewing's sarcoma	6.9 / 1.0	6.3 / 1.0	21.8 / 3.3	22.3 / 3.4	**<0.001**	0.189
		PD1	PD1	PD2	PD2		
Rh10	alveolar rhabdomyosarcoma	53.3 / 1.8	27.5 / 0.9	67.6 / 2.2	53.8 / 1.8	0.653	0.041
		PR	PD1	CR	CR		
Rh41	alveolar rhabdomyosarcoma	27.7 / 2.1	17.0 / 1.3	31.1 / 2.4	39.5 / 3.0	0.202	0.029
		PD2	PD1	PR	PR		
RBD-1	malignant rhabdoid tumor	77.4 / 4.5	39.0 / 2.3	27.4 / 1.6	43.8 / 2.5	<0.001	**<0.001**
		MCR	PD2	SD	PR		
KT-12	malignant rhabdoid tumor	37.6 / 1.3	31.4 / 1.1	44.5 / 1.6	45.5 / 1.6	0.241	0.87
		PD1	PD1	PD1	PD1		
OS31	osteosarcoma	20.6 / 1.2	18.3 / 1.1	25.0 / 1.5	33.0 / 2.0	**<0.001**	**<0.001**
		PD1	PD1	PD1	PD1		
OS33	osteosarcoma	21.3 / 1.5	18.2 / 1.3	30.1 / 2.2	38.5 / 2.8	**<0.001**	**<0.001**
		PD1	PD1	PD2	PD2		

*: *MYCN*- amplified xenograft lines.

^Data presented as "median time to event" / "ratio of median time to event for treated and control groups" (See Supplementary Materials for a more detailed explanation).

Red: significant differences in time to event between the single agent compared to the control.

Gray shading: significantly greater treatment effect for combination compared to single agent comparator at its optimal dose.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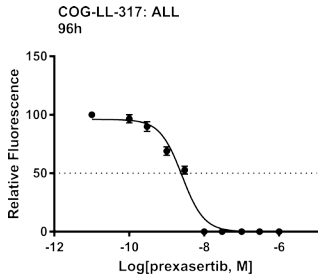
Log[prexasertib, M]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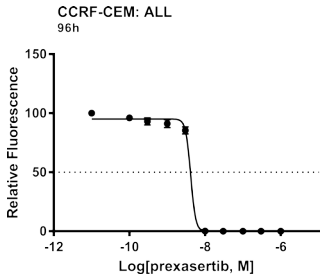
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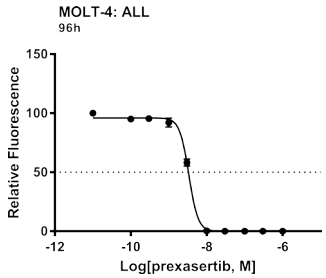
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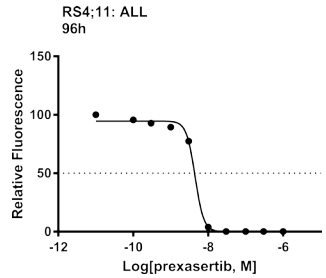
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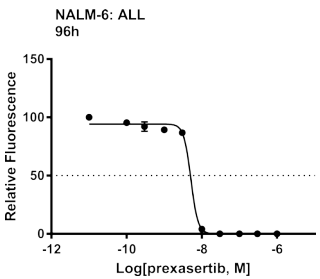
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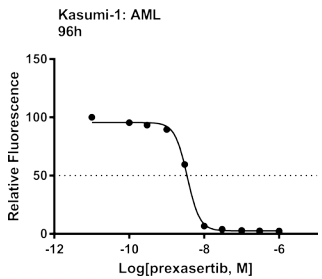
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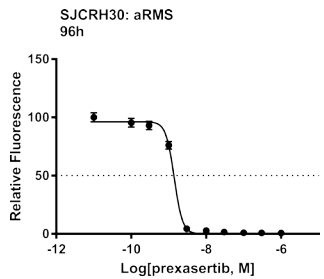
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Relative Fluorescence
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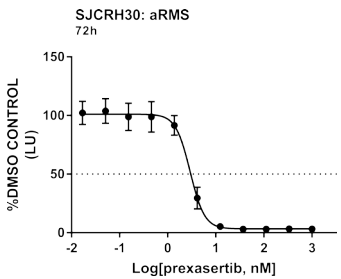
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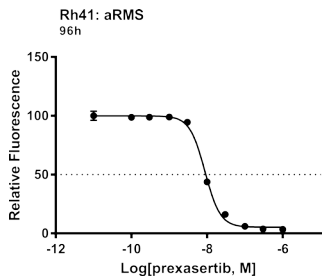
Rh41: aRMS
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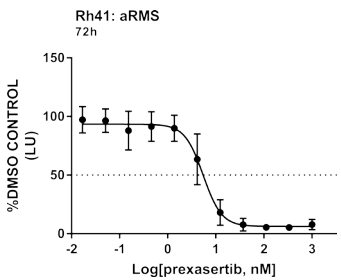
Relative Fluorescence
Log[prexasertib, M]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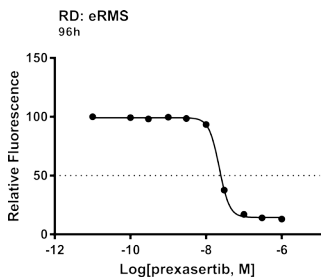
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Relative Fluorescence
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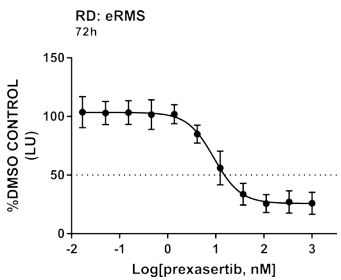
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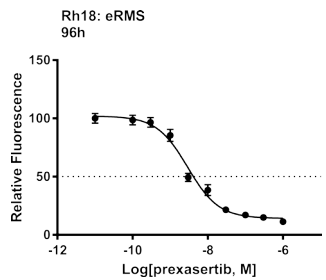
Rh18: eRMS
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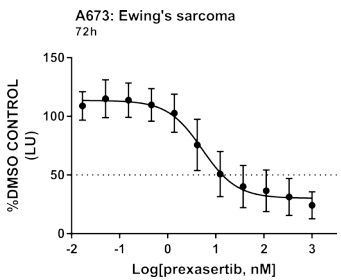
A673: Ewing's sarcoma
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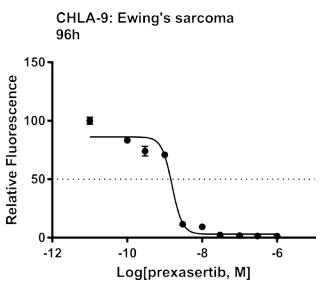
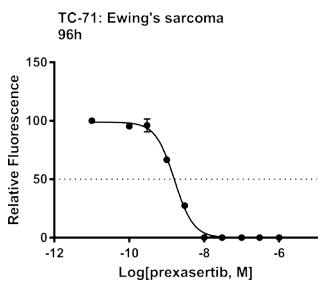
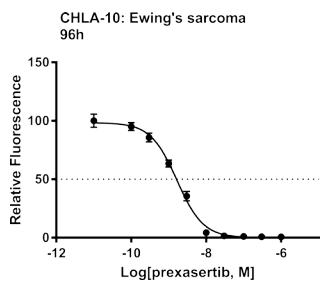
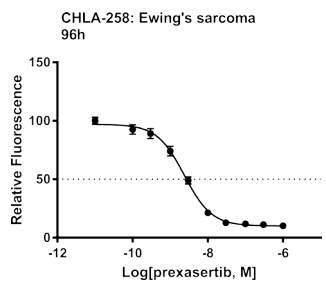
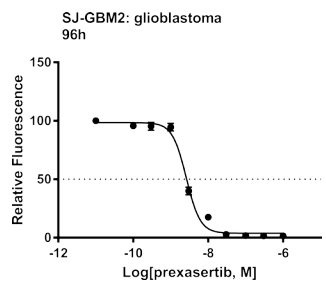
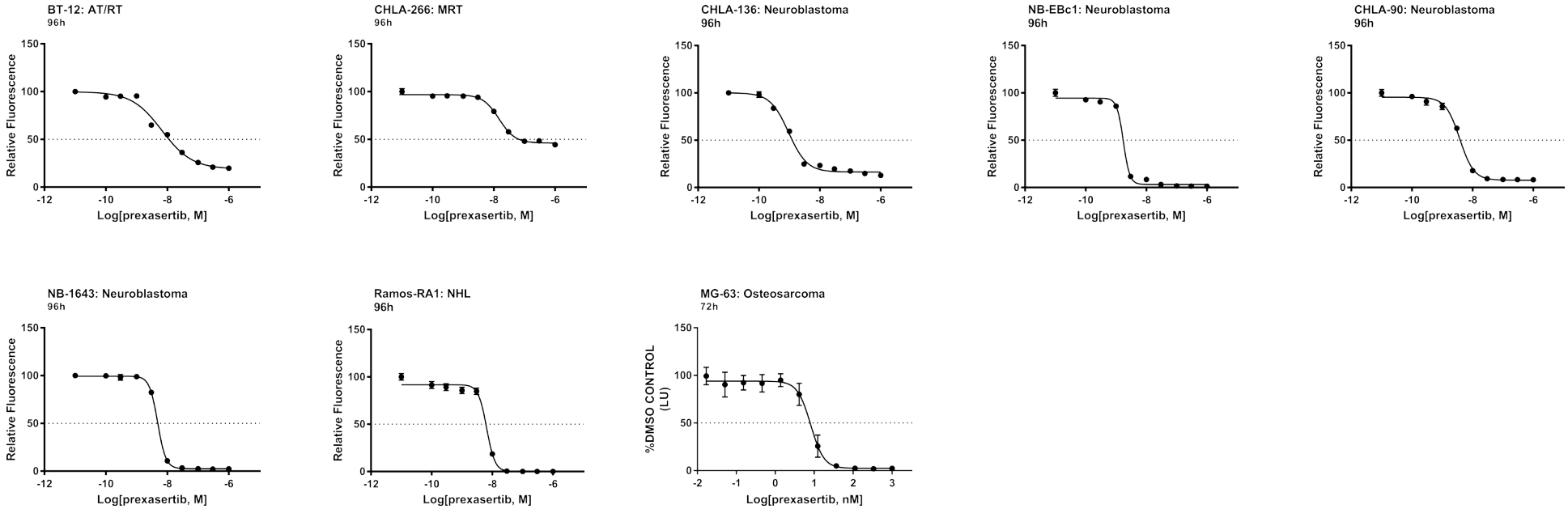

CHLA-9: Ewing's sarcoma
96h
Relative Fluorescence
Log[prexasertib, M]


TC-71: Ewing's sarcoma
96h
Relative Fluorescence
Log[prexasertib, M]

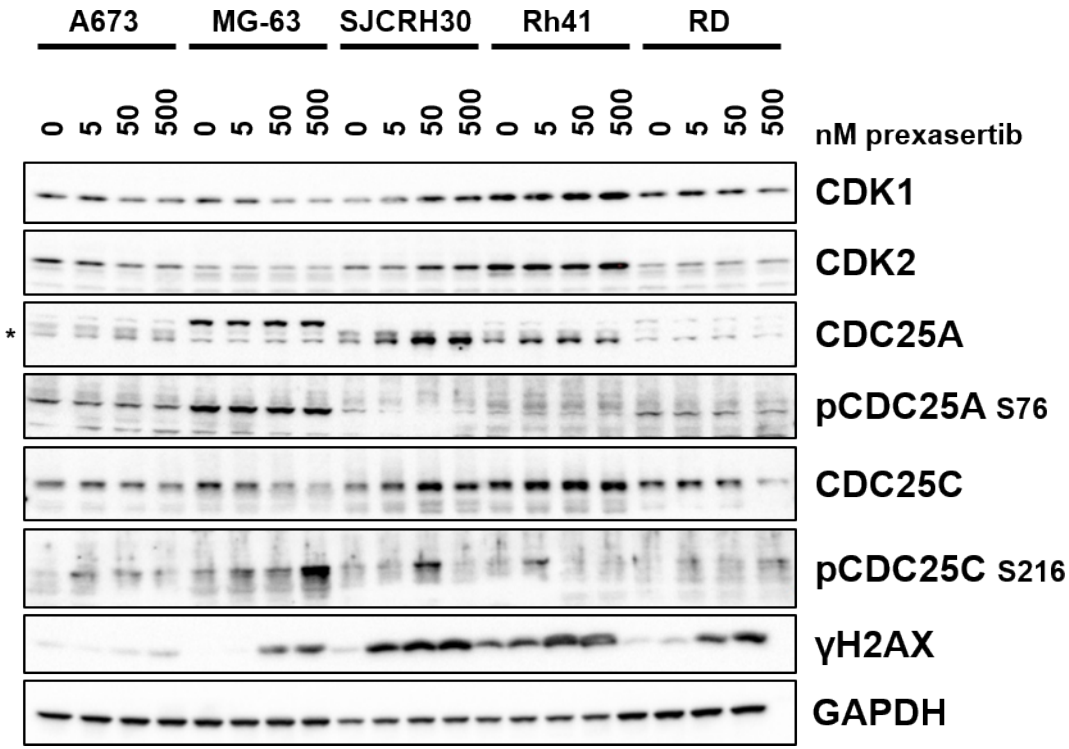

CHLA-10: Ewing's sarcoma
96h
Relative Fluorescence
Log[prexasertib, M]


CHLA-258: Ewing's sarcoma
96h
Relative Fluorescence
Log[prexasertib, M]

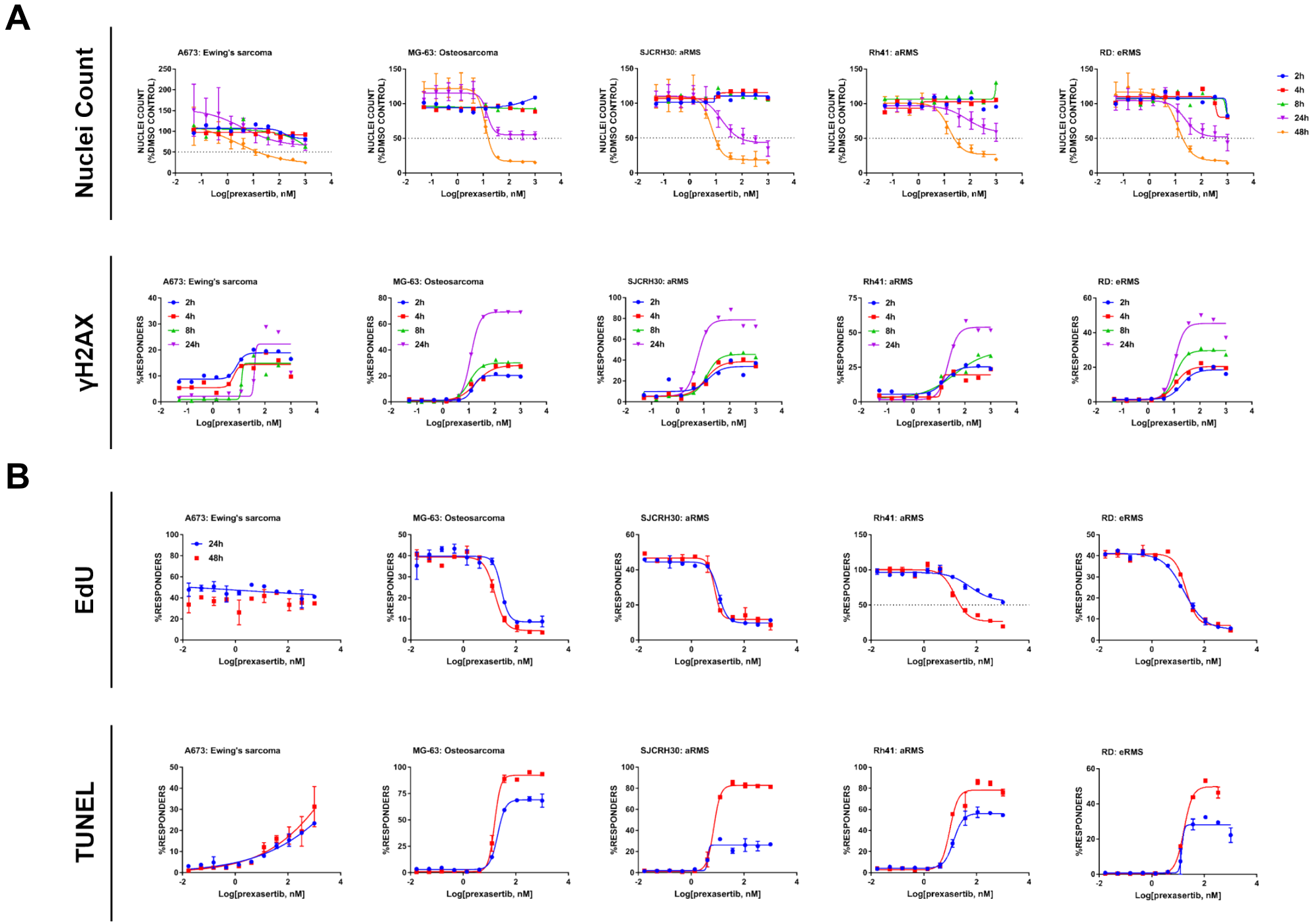

SJ-GBM2: glioblastoma
96h
Relative Fluorescence
Log[prexasertib, M]



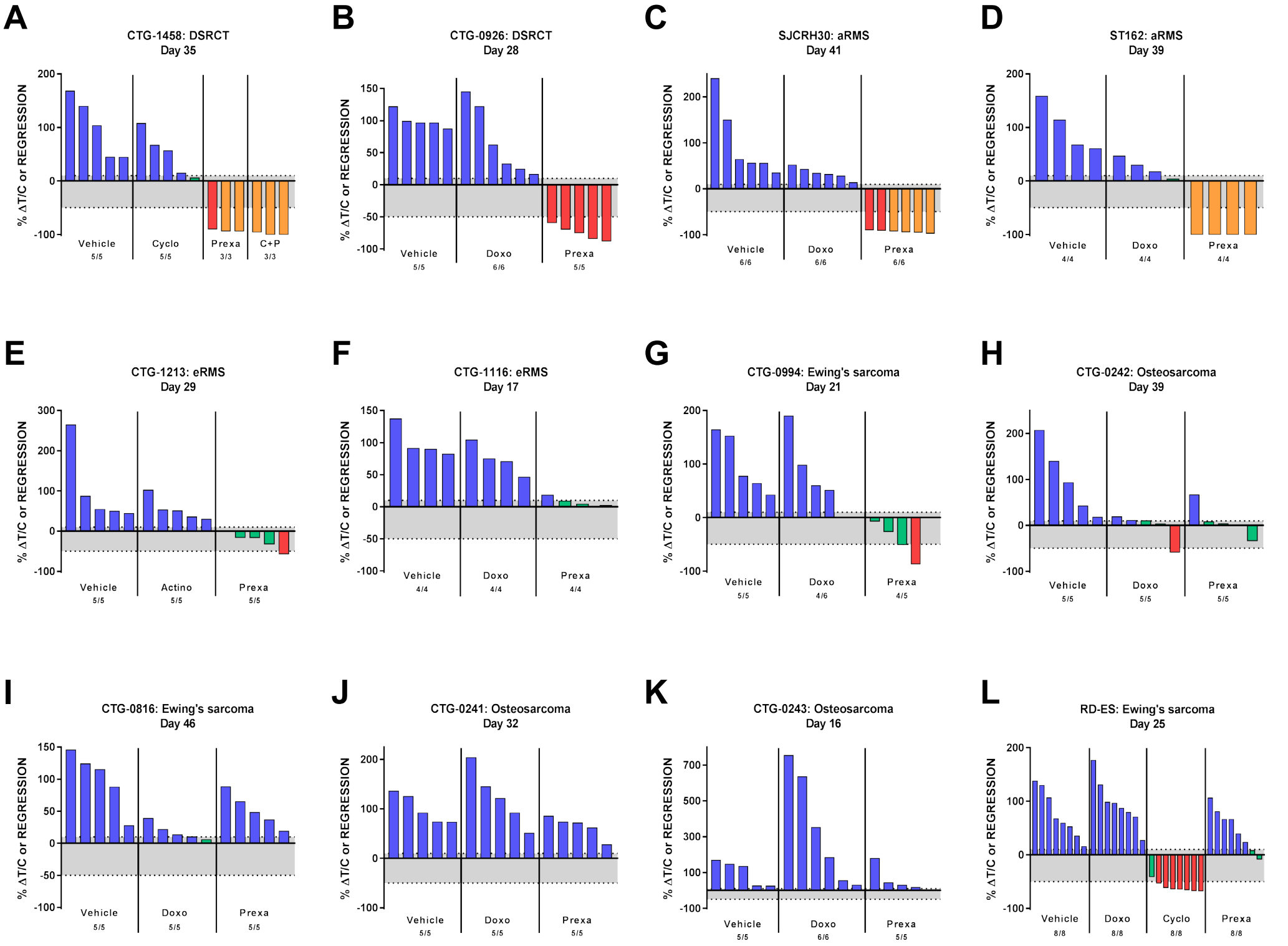
Supplementary Figure S1. Prexasertib reduces cell proliferation in a panel of pediatric bone and soft tissue tumor cell lines. Pediatric cancer cell lines were evaluated after 72h or 96h of prexasertib treatment by CellTiter Glo or digital image fluorescence microscopy, respectively, and EC_{50} values were calculated (reported in Table 1). Data are plotted as the average ± SEM of triplicate experiments.

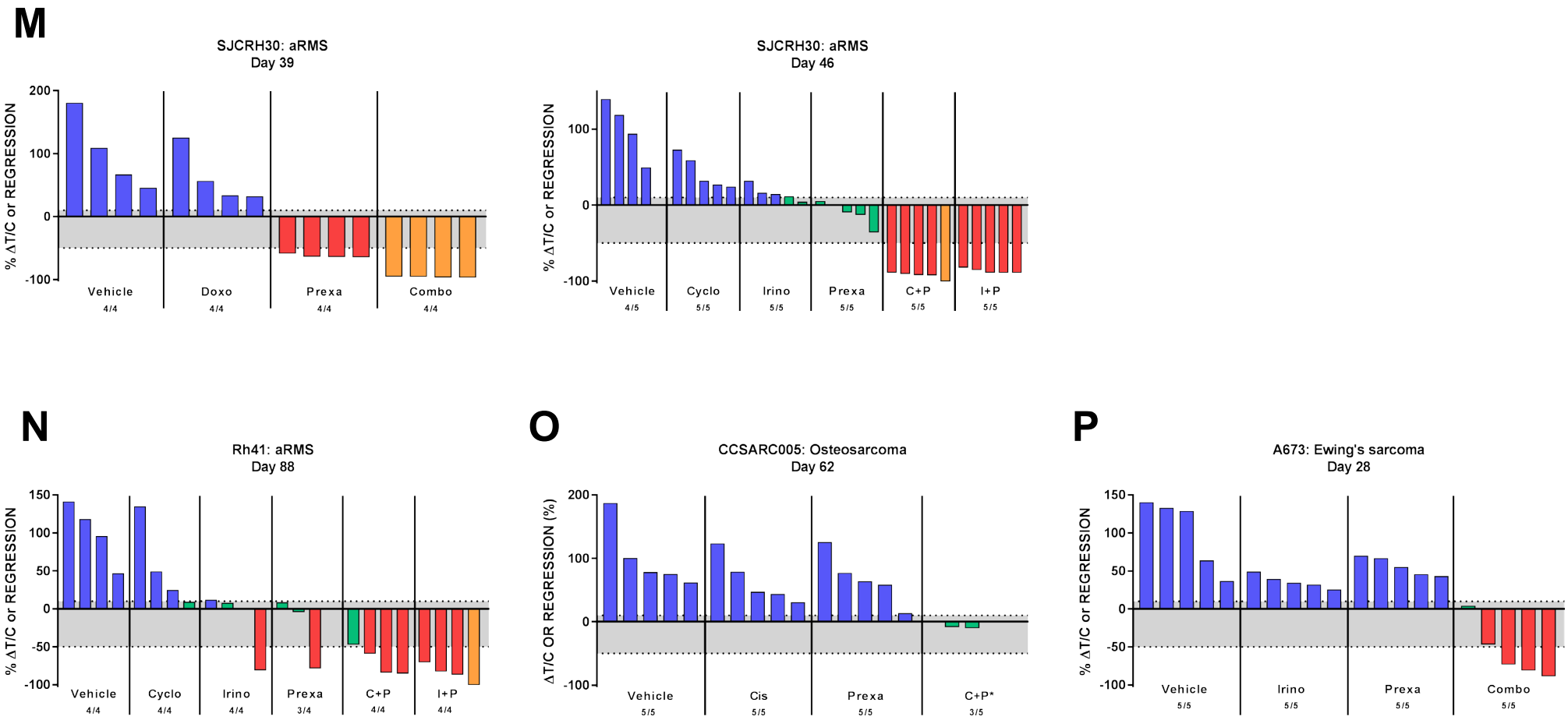


Supplementary Figure S2. CHK1 inhibition by prexasertib has limited influence on CDC25A/C and CDK1/2 expression. Pediatric sarcoma cell lines were treated with the indicated concentration of prexasertib for 24h. Whole cell lysates were probed for the indicated total and phosphorylated proteins. The asterisk next to the CDC25A panel marks the correct band size (59 kDa).

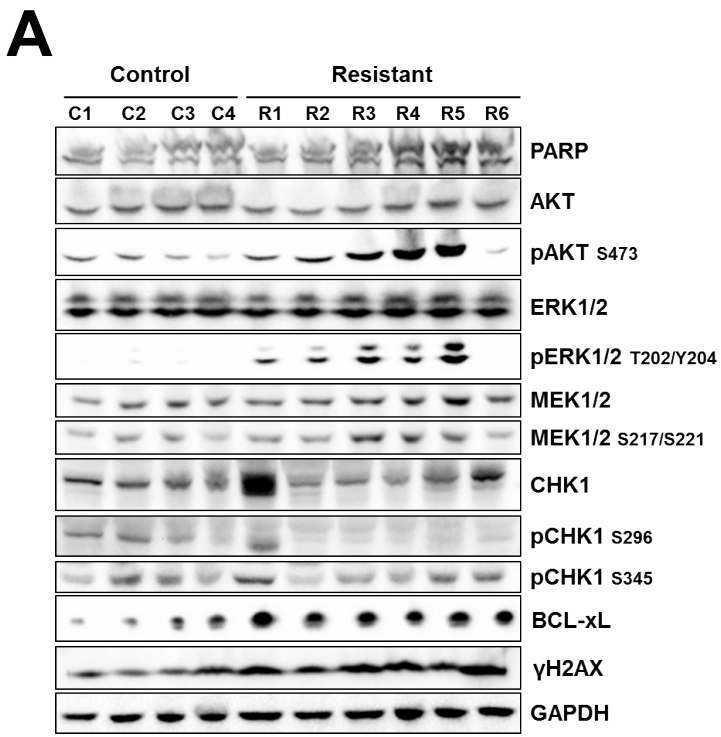


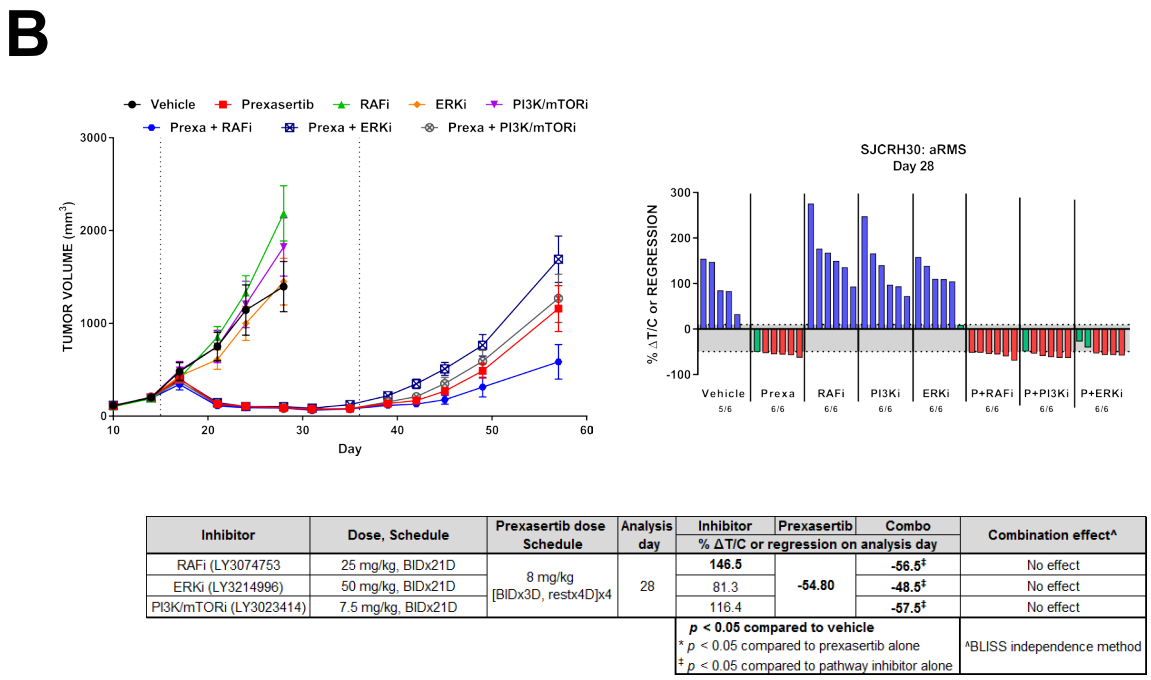
Supplementary Figure S3. Prexasertib reduces DNA synthesis and increases DNA damage and cell death in pediatric sarcoma cell lines. Pediatric sarcoma cell lines were treated with range of concentrations of prexasertib and evaluated at the time points indicated by high content imaging. Data are either expressed as relative to the DMSO control (nuclei count) or as %RESPONDERS (% of cells positive for a given endpoint.)


A
CTG-1458: DSRCT
Day 35
% ΔT/C or REGRESSION
Vehicle 5/5
Cyclo 5/5
Prexa 3/3
C+P 3/3
B
CTG-0926: DSRCT
Day 28
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 6/6
Prexa 5/5
C
SJCRH30: aRMS
Day 41
% ΔT/C or REGRESSION
Vehicle 6/6
Doxo 6/6
Prexa 6/6
D
ST162: aRMS
Day 39
% ΔT/C or REGRESSION
Vehicle 4/4
Doxo 4/4
Prexa 4/4
E
CTG-1213: eRMS
Day 29
% ΔT/C or REGRESSION
Vehicle 5/5
Actino 5/5
Prexa 5/5
F
CTG-1116: eRMS
Day 17
% ΔT/C or REGRESSION
Vehicle 4/4
Doxo 4/4
Prexa 4/4
G
CTG-0994: Ewing's sarcoma
Day 21
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 4/6
Prexa 4/5
H
CTG-0242: Osteosarcoma
Day 39
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 5/5
Prexa 5/5
I
CTG-0816: Ewing's sarcoma
Day 46
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 5/5
Prexa 5/5
J
CTG-0241: Osteosarcoma
Day 32
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 5/5
Prexa 5/5
K
CTG-0243: Osteosarcoma
Day 16
% ΔT/C or REGRESSION
Vehicle 5/5
Doxo 6/6
Prexa 5/5
L
RD-ES: Ewing's sarcoma
Day 25
% ΔT/C or REGRESSION
Vehicle 8/8
Doxo 8/8
Cyclo 8/8
Prexa 8/8



Supplementary Figure S4. Waterfall plots for evaluation of prexasertib as a monotherapy or in combination with chemotherapy. A-L, Animals bearing pediatric sarcoma xenografts were treated with vehicle, prexasertib, or chemotherapy as single agents. **A-F** correspond to tumor growth curves in Figure 2. **G-L** correspond to studies displayed in Figure 3. **M-P,** Animals with pediatric sarcoma tumors were given vehicle, prexasertib, chemotherapy, or a combination of prexasertib and chemotherapy. Panels correspond with tumor growth curves shown in Figure 4. The specific chemotherapy for each study is indicated in each panel. The day of analysis is indicated directly under the model number. The notation under the treatment heading indicates the number of animals evaluable at the time point (x) out of the total *n* included at the start of the study (x/*n*). Unless otherwise indicated, sacrifice prior to analysis was due to excessive tumor burden. Blue bars: progressive disease (>10%); green bars: stable disease (-50% to 10%); red bars: partial response (≤-50% and > 14 mm^3); orange bars: complete response (≤ 14 mm^3). Statistical analyses from these studies are summarized in Supplementary Table S3.

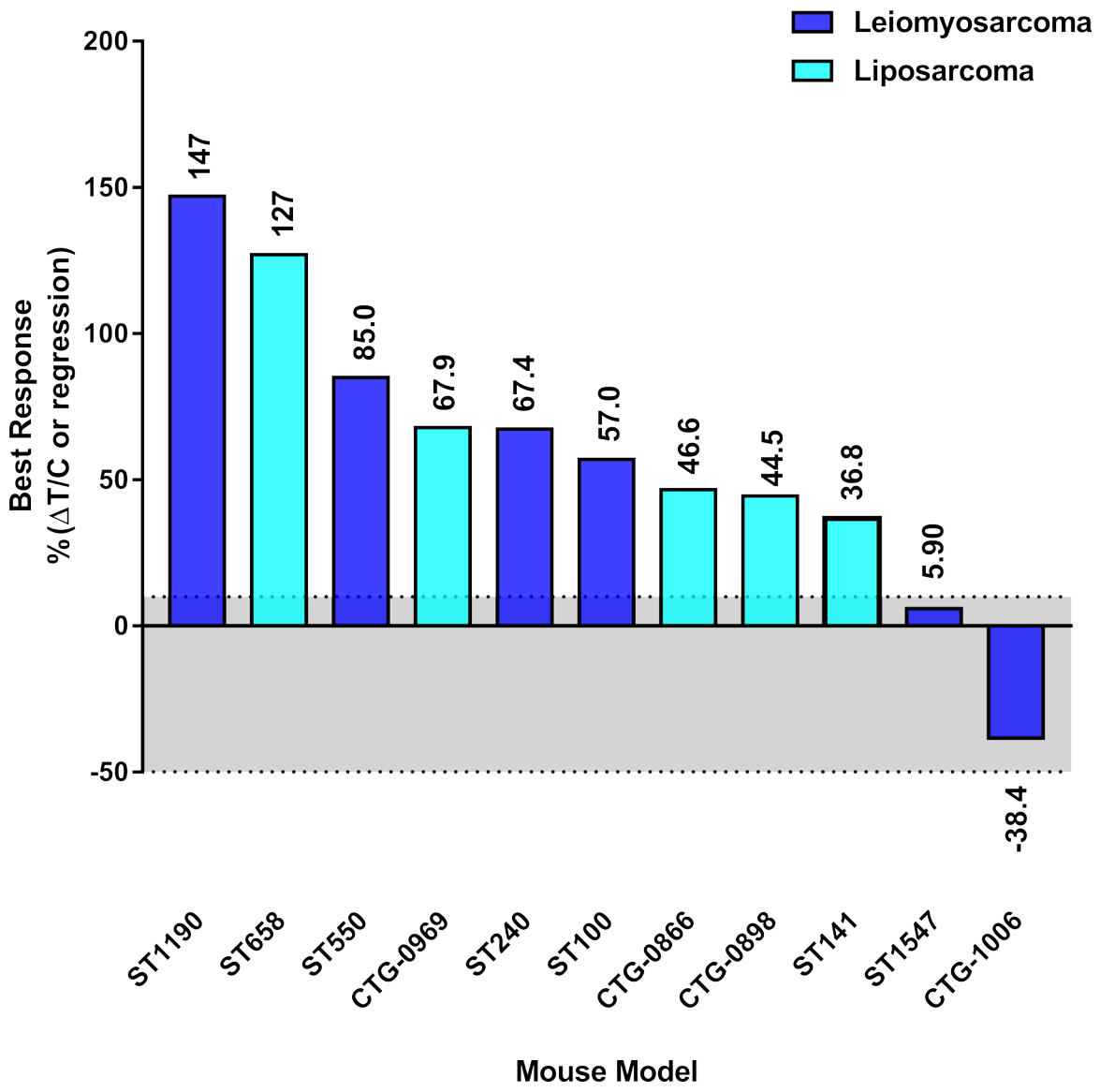




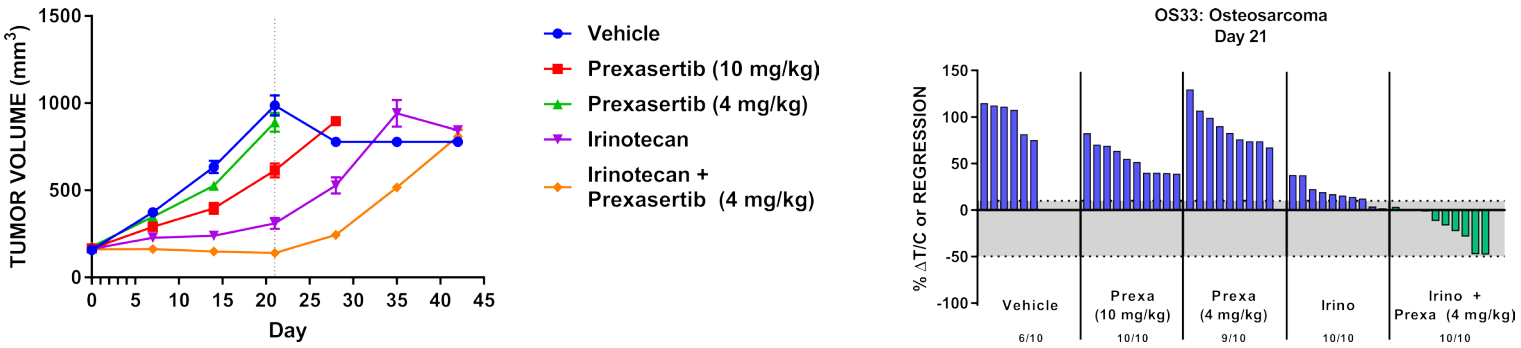
Inhibitor	Dose, Schedule	Prexasertib dose Schedule	Analysis day	Inhibitor	Prexasertib	Combo	Combination effect^
				% ΔT/C or regression on analysis day			
RAFi (LY3074753)	25 mg/kg, BIDx21D	8 mg/kg [BIDx3D, restx4D]x4	28	**146.5**	**-54.80**	**-56.5‡**	No effect
ERKi (LY3214996)	50 mg/kg, BIDx21D			81.3		**-48.5‡**	No effect
PI3K/mTORi (LY3023414)	7.5 mg/kg, BIDx21D			116.4		**-57.5‡**	No effect

$p < 0.05$ compared to vehicle
* $p < 0.05$ compared to prexasertib alone
‡ $p < 0.05$ compared to pathway inhibitor alone
^BLISS independence method

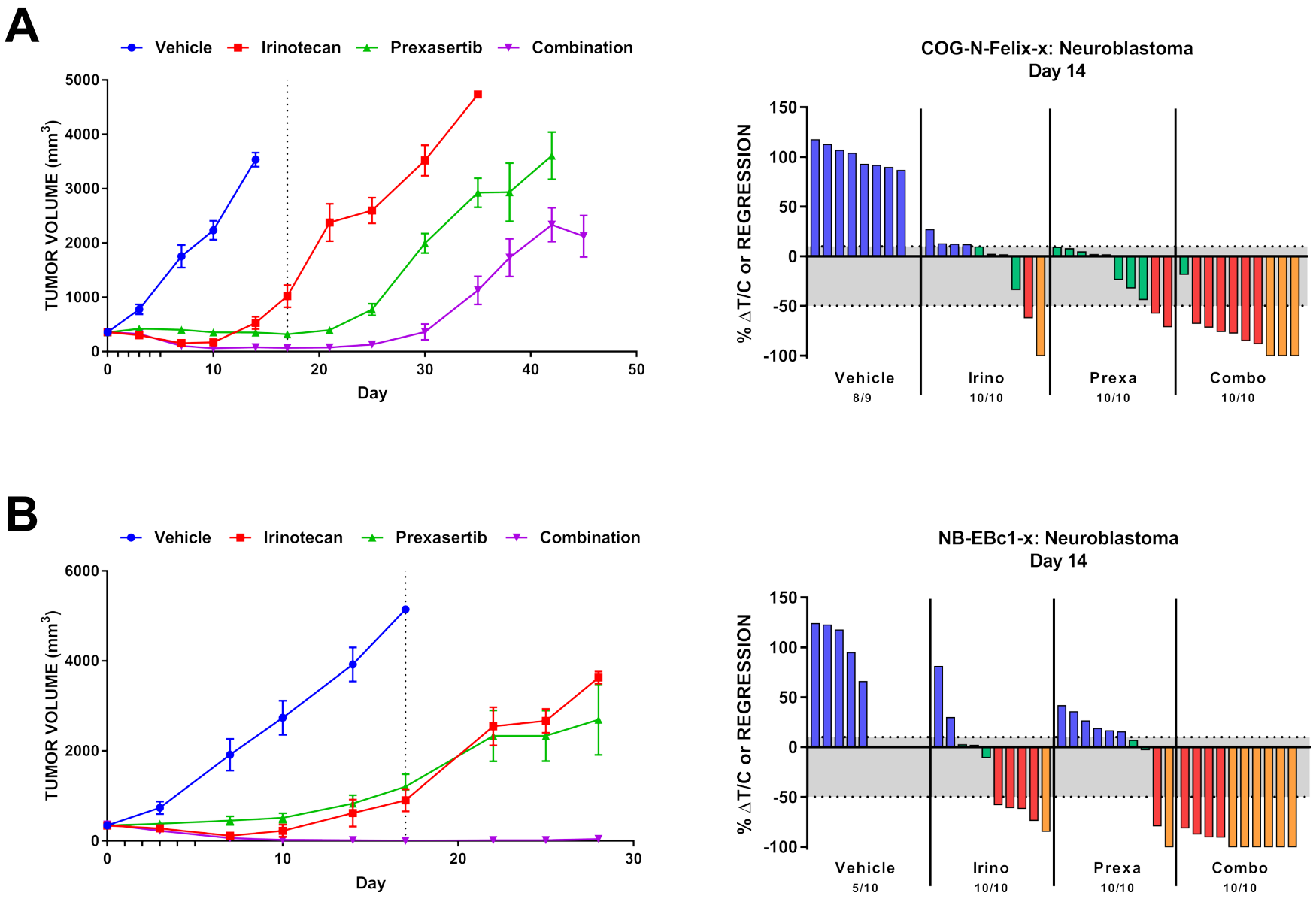
Supplementary Figure S5. PI3K/mTOR and RAS/MAPK signaling is upregulated in prexasertib-resistant SJCRH30 tumors compared to vehicle-treated xenografts but targeted inhibition of these pathways does not prevent acquired resistance to prexasertib. A, Pieces of terminal SJCRH30 alveolar RMS xenografts were lysed and evaluated by immunoblot. Membranes were probed for the indicated total and phosphorylated proteins. **B,** Animals with SJCRH30 tumors were treated with prexasertib (8 mg/kg BIDx3D, restx4D; dose reduced from 10 mg/kg to prevent potential toxicity with combination treatment), a RAF inhibitor (RAFi, LY3074753), a PI3K/mTOR inhibitor (PI3Ki, LY3023414), an ERK inhibitor (ERKi, LY3214669), or a combination of prexasertib plus a pathway inhibitor. (*Top left*) Tumor growth curve. Data are plotted as mean volume (n = 6/group) ± SEM; dotted lines: treatment interval. (*Top right*) Waterfall plot generated at Day 28. Numbers under treatment heading specifies number of animals evaluable at the time point (x) out of the total n included at the start of the study (x/n).
Blue bars: progressive disease (>10%); green bars: stable disease (-50% to 10%); red bars: partial response (≤-50% and > 14 mm^3).
(*Bottom*) Summary of statistical analyses.



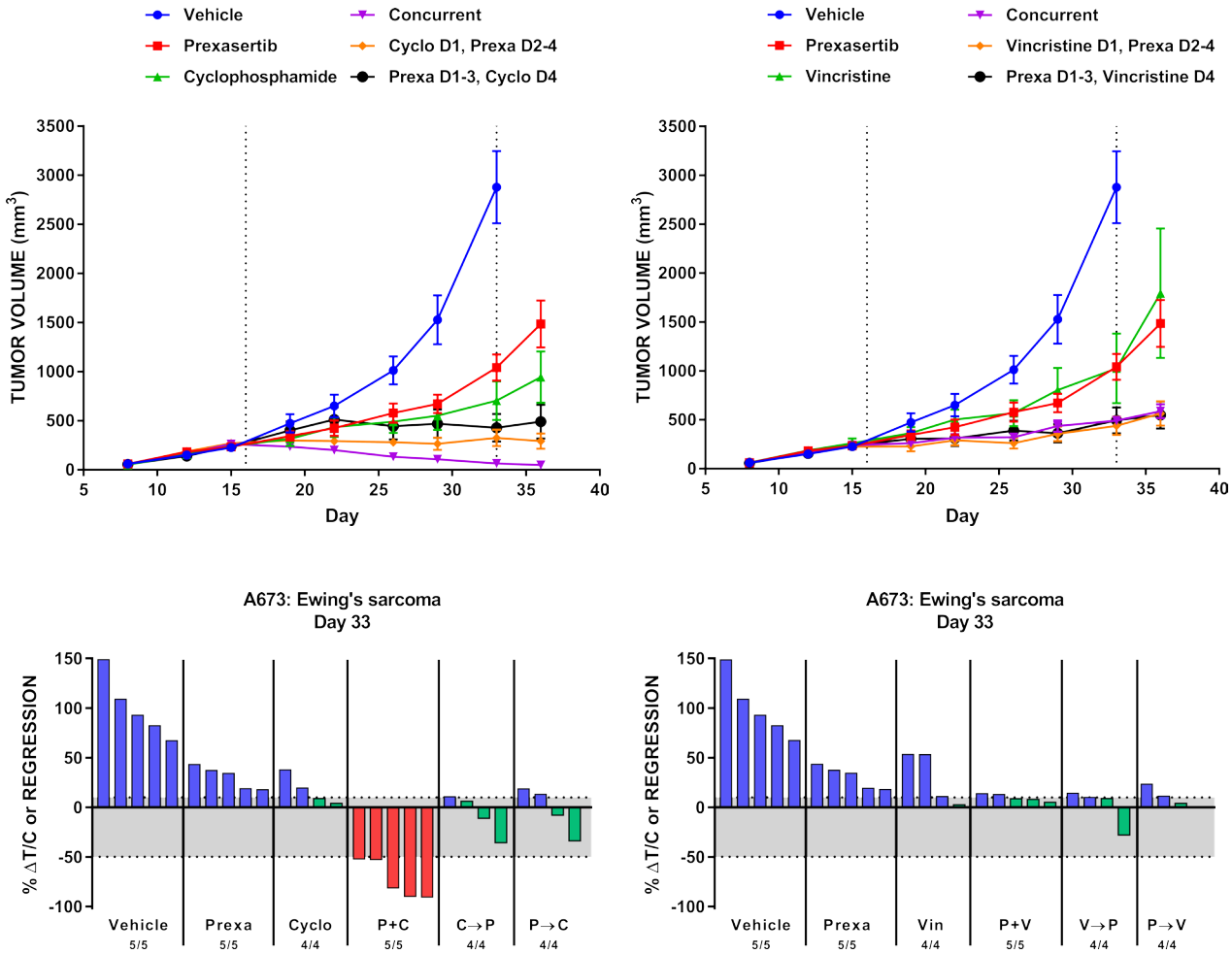
Supplementary Figure S6. Animal PDX models of adult soft tissue sarcoma are resistant to prexasertib. Eleven leiomyosarcoma or liposarcoma PDX models evaluated using an '*n* of 1' study design. Each bar corresponds to the mouse from each model receiving single agent prexasertib. Gray shading indicates stable disease.



Supplementary Figure S7. Concurrent administration of prexasertib and chemotherapy prolongs time to progression in the OS33 mouse model of human osteosarcoma. Animals with osteosarcoma xenografts were treated with vehicle, prexasertib (10 mg/kg BID or 4 mg/kg on Days 1, 3, and 5 for 3 weeks), irinotecan (2.5 mg/kg on Days 1-5), or a combination of 4 mg/kg prexasertib plus irinotecan. Data are plotted as the average tumor volume in each treatment group. Dotted vertical line: end of prexasertib dosing interval; error bars: SEM. Waterfall plot was generated at Day 21; numbers under treatment heading specifies number of animals evaluable at the time point (x) out of the total *n* included at the start of the study (x/*n*). Blue bars: progressive disease (>10%); green bars: stable disease (-50% to 10%). Statistical analyses are presented in Supplementary Table S5.



Supplementary Figure S8. Combination of prexasertib with irinotecan delays neuroblastoma tumor regrowth. Animals with neuroblastoma xenografts were treated with with vehicle, prexasertib (10 mg/kg BID on Days 1, 3, and 5 for 3 weeks), irinotecan (2.5 mg/kg on Days 1-5; indicated by hatch marks on x-axis), or the combination. Data are plotted as the average tumor volume in each treatment group. Dotted vertical line: end of prexasertib dosing interval; error bars: SEM. **A,** COG-N-Felix-x neuroblastoma CDX, *n* = 10/arm. Waterfall plot generated at Day 14. **B,** NB-EBc1-x neuroblastoma CDX, *n* = 10/arm. Waterfall plot generated at Day 14. Waterfall plots: numbers under treatment heading specifies number of animals evaluable at the time point (x) out of the total *n* included at the start of the study (x/*n*). Blue bars: progressive disease (>10%); green bars: stable disease (-50% to 10%); red bars: partial response (≤-50% and > 14 mm^3); orange bars: complete response (≤ 14 mm^3). Statistical analyses are summarized in Supplementary Table S3.



Supplementary Figure S9. Concurrent administration of prexasertib and chemotherapy has comparable or superior activity to sequential combination treatment. (*Top*) One *in vivo* study using the A673 Ewing's sarcoma CDX model is broken into two graphs for easier visualization of the data; the vehicle and prexasertib-treated arms are the same. The dosing interval is bracketed by dotted lines. Animals were given vehicle, prexasertib, or the indicated chemotherapy alone, or a combination of prexasertib and chemotherapy concurrently (purple) or staggered. For sequential treatment, either chemotherapy was given at Day 1 of the cycle, then prexasertib at Days 2-4 BID or prexasertib was administered at Days 1-3 BID with chemotherapy following at Day 4. Error bars: SEM. (*Middle*) Corresponding waterfall plots generated at Day 33. The numbers under the treatment heading for each column specifies the number of animals evaluable at the time point (x) out of the total *n* included at the start of the study (x/*n*). Blue bars: progressive disease (>10%); green bars: stable disease (-50% to 10%); red bars: partial response (≤-50% and > 14 mm^3). (*Bottom*) Results of the statistical analyses conducted for this study.

Chemotherapy		Prexasertib dose	Analysis day	Combo schedule	Chemotherapy	Prexasertib	Combination	Combination effect^
Agent	Dose, Schedule	Schedule			% ΔT/C or regression on analysis day			
Cyclophosphamide	100 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	33	Concurrent	**15.3**	**30.1**	**-78.5***‡	**Greater than additive**
				Chemo D1, Prexasertib D2-4			**2.1***‡	**Additive**
				Prexasertib D1-3, Chemo D4			**4.6***	No effect
Vincristine	1 mg/kg, Q7Dx4	10 mg/kg [BIDx3D, restx4D]x4	33	Concurrent	**22.7**	**30.4**	**10.1***	No effect
				Chemo D1, Prexasertib D2-4			**6.6***‡	**Additive**
				Prexasertib D1-3, Chemo D4			**8.4***	No effect

$p < 0.05$ compared to vehicle
* $p < 0.05$ compared to prexasertib alone
‡ $p < 0.05$ compared to chemotherapy alone
^BLISS independence method