



ALPHAMAB

R&D Report

Title: Anti-tumor efficacy of KN035 on
NOD-SCID xenograft tumor model of
A375-PDL1/human PBMC at different
frequencies

Report#: RDR-KN035-PD-2015-023

Rev#: 01

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Revision history

Rev #	Date	Section(s)	Description of Revision	Reason for Revision
01	See Title Page	N/A	N/A	N/A

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Abstract

KN035 is a humanized single domain antibody targeting human PD-L1, which does not bind to mouse PD-L1. The anti-tumor efficacy of the drug KN035 depends on immune cells activation. So here we established a xenograft tumor model, by subcutaneous inoculation of mixed human tumor cells and PBMCs into immune-deficient NOD-SCID mice, to directly evaluate the anti-tumor efficacy of humanized antibody drug.

In this study, human melanoma cell line A375 which was stably transfected with human PD-L1 (A375-hPD-L1) were mixed with human PBMCs as 4:1, and were subcutaneously inoculated to NOD-SCID mice. Four hours later, the mice received KN035 (0.3mg/kg) by i.p. injection. PBS was administrated on the 1st, 4th, 7th and 10th day by i.p. injection. Mice in KN035 0.3mg/kg group were divided into four sub-groups which administrated with 0.3mg/kg of KN035 on the 1st day (1); 1st and 4th days (1, 4); 1st, 4th, and 7th days (1, 4, 7); 1st, 4th, 7th, and 10th days (1, 4, 7, 10) respectively. The results showed that the mice in the group of KN035 0.3mg/kg (1) have significantly decreased tumor volume on the 8th, 11th, 25th days; KN035 0.3mg/kg (1, 4) group on the 8th, 11th, 15th, 22nd, 25th, 29th, 32nd days; KN035 0.3mg/kg (1, 4, 7) group on the 8th, 11th, 15th, 25th, 32nd days; KN035 0.3mg/kg (1, 4, 7, 10) group on the 8th, 11th, 15th, 22nd, 25th days after drug administration have significantly decreased tumor volume. The four groups had tumor growth inhibition rate (TGI, %) of 36.57%, 60.26%, 59.93%, and 50.65% respectively on the 32nd day.




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1. PURPOSE

Study the antitumor effect of different times of treatments of KN035 using an A375-hPDL1/human PBMC-NOD/SCID mice xenograft model, in which A375-hPDL1 and human PBMCs mixture was inoculated subcutaneously to NOD-SCID mice.

2 Test substance information

Substance	Lot. No.	Manufacturer	Storage
KN035	20150502FB	Alphamab Co. Ltd	2-8°C

3 Test substance preparation


Substance: KN035 196.78 mg/mL

Solvent: PBS

Gradient dilution method:

3mg/mL of KN035: Add KN035 (196.78 mg/mL) 0.027 mL to PBS to make a total volume of 1.8mL. The final concentration is 3mg/mL;

0.03mg/mL of KN035: Add KN035 (3mg/mL) 0.018 mL to PBS to make a total volume up to 1.8mL. The final concentration is 0.03mg/mL.

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4 Animal housing

4.1 Animal

Strain: NOD-SCID mouse

Grade: SPF

Vendor: Vital River Laboratory Animal Technology Co. Ltd.

Animal Certificate Number: 11400700104909

Age (at the beginning): 6-8 weeks

Body weight (at the beginning): 18-22 g

Amount and Gender: 30 females, 6 mice/group


Animal housing: Standard operating procedure

4.2 Diet and Water

Animal food was supplied by Kangmaibo (SuZhou) Technology Inc. Animals had free access to food and autoclaved water.

4.3 Laboratory environment and acclimation

Experiments were performed in the SPF grade Animal Facility (KangMaiBo, Co. Ltd.) with environment of temperature $23\pm 2^{\circ}\text{C}$, humidity 40-70%, 12:12 hours light: dark cycle. Animals were housed before the experiment to acclimate to the environment.

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4.4 Animal selection and fasting

Animals that were health and acclimated to the environment were selected for the experiments. The mice should be free to access regular food and autoclaved water.

5 Experiment

5.1 Cell culture


5.1.1 A375-hPDL1 culture

A375-hPDL1 melanoma cell line was provided by DingFu Target Biotechnology Co.Ltd. Cells were cultured in DMEM (Hyclone, Lot. No. NAA1324) supplemented with 10% FBS in sterile conditions. Cell cultures were maintained in incubator at 37°C, 5% CO₂. Tumor cells were sub-cultured twice a week using 0.25% trypsin-EDTA. Cells in log-phase growth were harvested and counted for inoculation.

5.1.2 PBMC isolation

60mL fresh blood was provided by one donor, and PBMCs was isolated as following:

1. Add the same volume of PBS to the blood.
2. Add 10 ml human lymphocyte separation medium to six 50-ml tubes respectively. Carefully layer the 20ml diluted blood over the lymphocyte separation medium using pipette. Do not disturb the surface of the medium.
3. Carefully place the tubes in a desktop centrifuge. Centrifuge at 1500 rpm for 15 min at room temperature. Acceleration 0, no brake.

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4. Carefully remove the tubes from the centrifuge and transfer the mononuclear cell layer at the interphase (about 5-12 ml) to a new tube. Fill the tube with sterile PBS making up the volume to 50 ml. Aliquot the fluid and each tube less than 20 ml.
5. Centrifuge at room temperature on a desktop horizontal centrifuge at 1200 rpm for 10 min.
6. Discard the supernatant; wash the cells by adding 20 ml PBS. Centrifuge at 1200 rpm for 10 min.
7. Discard the supernatant; re-suspend the cells using RPMI1640. Count the cells and diluted the cells to 4×10^7 /ml using PRMI1640.
8. Place the cells on ice for later use.

5.2 Grouping and dosing

NOD-SCID mice were inoculated with 4×10^6 A375-hPDL1(50 μ L) mixed with 1×10^6 human PBMCs (50 μ L) subcutaneously under the armpit. The mice were randomized into five groups, 6 mice each group, including one PBS treated group and four KN035 0.3mg/kg treatment groups with different dosing frequency. Four hours after inoculation, KN035 0.3mg/kg was administrated to the mice by i.p. injection. The day was marked with the 1st day. PBS was administrated on the 1st, 4th, 7th and the 10th day by i.p. injection. Mice in KN035 0.3mg/kg group were injected with 0.3mg/kg of KN035 on the 1st day; 1st and 4th day; 1st, 4th and 7th day; 1st, 4th, 7th and 10th days respectively. We measured the tumor twice a week and weighed the mice when the tumor volume reached approximately 70 mm³. Euthanasia the mice using CO₂ after the tumor volume exceeded 2000mm³. Mice were grouped as [Table 1](#).

Table 1 Animal group and drug administration

Group	Route	Number of doses	Dose (mg/kg)	Volume (mL/kg)	Concentration (mg/mL)
A PBS	IP	D1、4、 7、 10, 4 times	-	-	-

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B KN035	IP	D1, 1 time	0.3	10	0.03
C KN035		D1, 4, twice			
D KN035		D1, 4, 7, three times			
E KN035		D1, 4, 7, 10, four times			

5.3 Evaluation

We weigh the mice twice a week, and measure the tumors in two dimensions, the length (Y) and the width (X). Calculate the tumor volume and TGI % as the following formulas:

$$V = (X^2Y)/2$$

$$TGI\% = (V_{\text{tumor PBS}} - V_{\text{tumor KN035}}) / (V_{\text{tumor PBS}}) * 100\%$$

The tumor volume in PBS group mice was exceeding 2000mm³ on the 30th day. These mice were sacrificed using CO₂ and the tumors were weighed. The mice in other groups were sacrificed on the 34th day. Tumors were dissected and weighed.

5.4 Inspection

Animal clinical reactions were recorded during the experiment.


6 Results

6.1 Clinical symptoms

All the mice clinical performances were normal.

6.2 Effect on the body weight of KN035 on A375-hPDL1/human PBMC xenografted NOD-SCID mice model

The body weight of the mice in KN035 0.3mg/kg (1, 4) group significantly decreased on the 8th

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and 25th day after treatment ($P < 0.05$), and KN035 0.3mg/kg (1, 4, 7) group decreased on the 32nd day after treatment compared to the PBS group ($P < 0.01$), other groups had normal body weights. (Table 2, Figure 1)

6.3 Effect on the tumor growth of KN035 on A375-hPDL1/human PBMC xenografted NOD-SCID mice model

The results showed that the mice in the group of KN035 0.3mg/kg (1) on the 8th, 11th, 25th days; KN035 0.3mg/kg (1, 4) group on the 8th, 11th, 15th, 22nd, 25th, 29th, 32nd days; KN035 0.3mg/kg (1, 4, 7) group on the 8th, 11th, 15th, 25th, 32nd days; KN035 0.3mg/kg (1, 4, 7, 10) group on the 8th, 11th, 15th, 22nd, 25th days after drug administration have significantly decreased tumor volume compared to PBS group. ($P < 0.05$, $P < 0.01$) (Table 3, Figure 2)

The mice in groups of KN035 0.3mg/kg (1), KN035 0.3mg/kg (1, 4), KN035 0.3mg/kg (1, 4, 7), KN035 0.3mg/kg (1, 4, 7, 10) had tumor growth inhibition rate (TGI%) of 36.57%, 60.26%, 59.93%, 50.65% respectively on the 32nd day compared to those in the PBS group. (Table 4, Figure 3)

7 Conclusion

In this study, we established a mouse model by subcutaneous inoculation of mixed 4×10^6 A375-hPDL1 (50 μ L) and 1×10^6 of human PBMCs (50 μ L) on NOD-SCID mice. Four hours later, the mice received KN035 (0.3mg/kg) by i.p. injection. The mice in KN035 0.3mg/kg group were received KN035 with different frequencies of treatments; those are on the 1st day; 1st and 4th day; 1st, 4th and 7th day; 1st, 4th, 7th and 10th days respectively. The results showed that the mice in the group of KN035 0.3mg/kg (1, 4) group and KN035 0.3mg/kg (1, 4, 7) group exhibited a better and equivalent tumor inhibition rate with the tumor growth inhibition rate (TGI, %) of 60.26%, 59.93%, respectively, on the 32nd day; KN035 0.3mg/kg (1, 4, 7, 10) group had a moderate lower tumor growth inhibition rate (TGI, %) of 50.65% on the 32nd day; and KN035 0.3mg/kg (1) group had the lowest TGI% of 36.57%.



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8 Tables

Table 2 Effect on the body weight of KN035 on A375-hPDL1/human PBMC xenografted model (g,Mean±SEM)

Day	Group				
	A PBS 1, 4, 7, 10	B KN035 0.3mg/kg 1	C KN035 0.3mg/kg 1, 4	D KN035 0.3mg/kg 1, 4, 7	E KN035 0.3mg/kg 1, 4, 7, 10
3	24.5±0.54	24.15±0.48	23.23±0.68	23.95±0.42	24.78±0.64
8	24.22±0.57	23.57±0.48	22.57±0.44*	23.02±0.38	23.63±0.8
11	24.23±0.61	23.83±0.34	22.87±0.58	23.52±0.3	24.23±0.75
15	25.02±0.76	24.42±0.34	23.70±0.6	24.20±0.55	24.57±0.99
18	25.52±0.68	24.78±0.41	24.03±0.71	24.38±0.58	24.32±0.96
22	25.73±0.68	25.15±0.34	24.07±0.74	24.12±0.67	24.03±0.93
25	26.20±0.72	25.58±0.45	23.58±0.9*	24.22±0.61	24.22±0.99
29	26.40±0.83	25.52±0.45	24.25±0.94	24.45±0.53	24.38±1.25
32	26.82±0.68	26.28±0.49	24.52±0.93	22.77±0.76**	24.60±1.28

T test vs PBS, *P<0.05, **P<0.01


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Table 3 Effect on the tumor volume of KN035 on A375-hPDL1/human PBMC xenograft model (mm³, Mean±SEM)

Day	Group				
	A PBS 1, 4, 7, 10	B KN035 0.3mg/kg 1	C KN035 0.3mg/kg 1, 4	D KN035 0.3mg/kg 1, 4, 7	E KN035 0.3mg/kg 1, 4, 7, 10
3	77.33±4.34	70.79±8.19	69.64±4.65	67.96±2.28	64.46±5.58
8	143.92±17.25	63.48±8.76**	55.67±7.03**	48.77±11.24**	41.54±2.64**
11	152.66±9.00	89.17±10.68**	65.71±6.49**	67.01±14.50**	56.07±4.68**
15	163.19±35.91	101.85±13.16	73.24±6.92*	78.08±14.25	61.21±2.30*
18	149.03±48.83	131.08±27.01	75.48±10.62	100.56±16.22	81.19±1.70
22	293.53±84.45	162.54±42.82	86.54±13.96*	124.27±20.39	101.62±8.53*
25	515.07±110.85	204.97±51.72*	167.37±30.85*	188.96±34.77*	186.19±31.31*
29	976.26±237.96	462.10±95.18	357.04±105.75*	479.6±158.83	435.72±80.83
32	1595.82±355.35	1012.29±199.44	634.14±164.94*	639.44±196.18*	787.56±119.52

T test vs PBS, *P<0.05, **P<0.01



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Table 4 Effect on the tumor inhibition rate of KN035 on A375-hPDL1/human PBMC xenograft model TGI(%)

Day	Group			
	B KN035 0.3mg/kg1	C KN035 0.3mg/kg 1, 4	D KN035 0.3mg/kg 1, 4, 7	E KN035 0.3mg/kg 1, 4 , 7, 10
8	55.90	61.32	66.11	71.14
11	41.59	56.96	56.11	63.28
15	37.59	55.12	52.15	62.49
18	12.05	49.35	32.52	45.52
22	44.63	70.52	57.66	65.38
25	60.21	67.51	63.31	63.85
29	52.67	63.43	50.87	55.37
32	36.57	60.26	59.93	50.65

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9 Figures

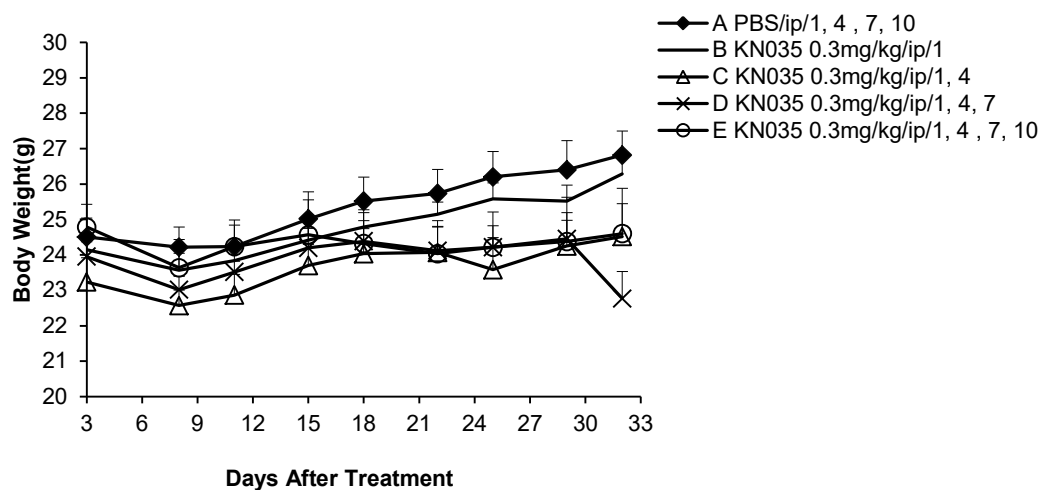


Figure 1 Effect on the body weight of KN035 on A375-hPDL1/human PBMC xenograft model

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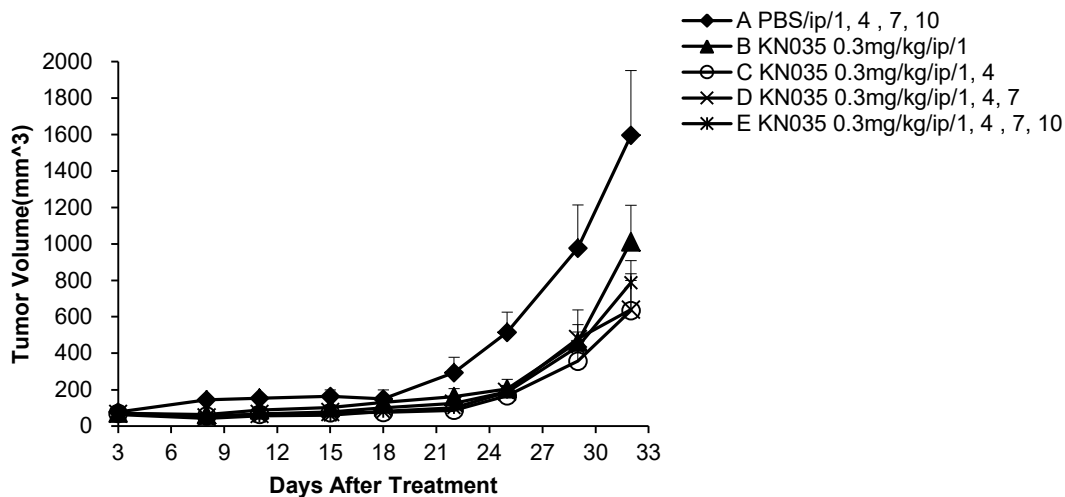


Figure 2 Effect on the tumor volume of KN035 on A375-hPDL1/human PBMC xenograft model

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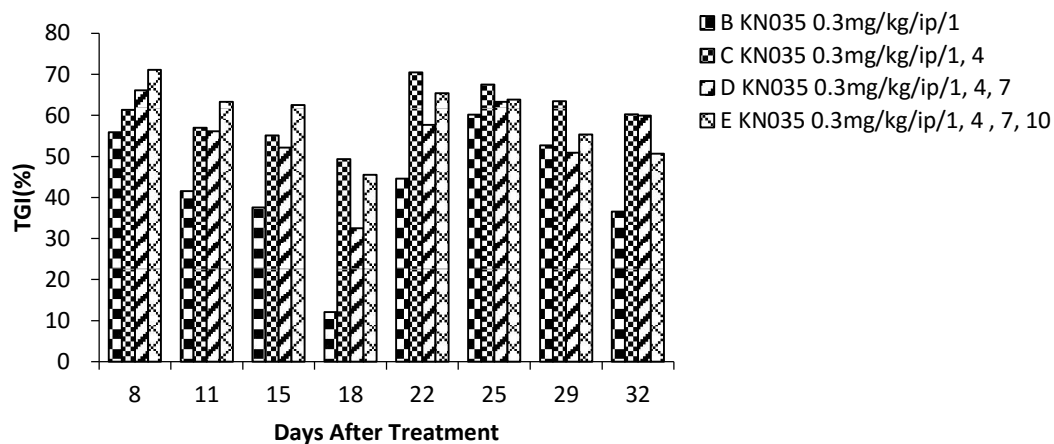



Figure 3 Effect on the tumor inhibitory rate (TGI%) of KN035 on A375-hPDL1/human PBMC xenograft model

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10 Appendix

Appendix 1 Body weight of the mice in this study (g)

Group	Animal #	Days Post Treatment								
		3	8	11	15	18	22	25	29	32
A PBS 1, 4 , 7, 10	1	24.8	25.2	24.8	26.4	26.1	26.5	26.9	26.7	26.8
	2	24.4	24.5	25.0	25.3	25.9	25.7	26.2	27.3	27.6
	3	24.1	23.2	23.3	22.8	23.7	24.5	24.7	24.3	24.8
	4	26.9	26.4	26.6	27.7	28.3	28.1	28.9	29.1	29.1
	5	23.0	23.1	22.9	24.6	25.0	26.3	26.6	27.3	27.6
	6	23.8	22.9	22.8	23.3	24.1	23.3	23.9	23.7	25.0
	Mean	24.50	24.22	24.23	25.02	25.52	25.73	26.20	26.40	26.82
	SD	1.32	1.40	1.50	1.86	1.66	1.67	1.76	2.03	1.66
	SEM	0.54	0.57	0.61	0.76	0.68	0.68	0.72	0.83	0.68
B KN035 0.3mg/kg 1	1	24.2	23.6	24.4	24.7	24.9	25.8	25.6	25.6	26.1
	2	24.8	23.5	23.2	23.8	23.6	25.4	24.9	24.6	25.2
	3	25.8	25.8	25.2	25.4	26.4	25.7	26.4	25.7	26.7
	4	24.1	22.6	23.5	23.5	24.2	24.2	25.3	25.6	26.6
	5	23.7	23.3	23.7	25.3	25.4	25.8	27.2	27.4	28.2
	6	22.3	22.6	23.0	23.8	24.2	24.0	24.1	24.2	24.9
	Mean	24.15	23.57	23.83	24.42	24.78	25.15	25.58	25.52	26.28
	SD	1.16	1.18	0.83	0.83	1.01	0.83	1.10	1.11	1.19
	SEM	0.48	0.48	0.34	0.34	0.41	0.34	0.45	0.45	0.49



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C KN035 0.3mg/kg 1, 4	1	25.2	23.8	24.7	25.4	26.5	26.2	25.8	26.9	27.1
	2	20.7	20.9	20.6	21.1	21.2	21.3	20.9	20.7	21.2
	3	24.3	22.9	22.5	23.7	23.9	23.1	20.8	22.9	22.8
	4	22.0	21.6	22.4	23.2	23.5	23.6	24.0	24.0	24.1
	5	24.1	23.0	23.9	24.4	24.3	24.5	24.6	24.6	25.2
	6	23.1	23.2	23.1	24.4	24.8	25.7	25.4	26.4	26.7
	Mean	23.23	22.57	22.87	23.70	24.03	24.07	23.58	24.25	24.52
	SD	1.66	1.09	1.41	1.48	1.74	1.80	2.21	2.29	2.28
	SEM	0.68	0.44	0.58	0.60	0.71	0.74	0.90	0.94	0.93
D KN035 0.3mg/kg 1, 4, 7	1	24.6	24.4	24.6	25.7	25.9	25.1	25.6	25.5	24.0
	2	25.4	23.7	23.3	26.0	26.4	26.9	26.1	26.5	25.6
	3	22.8	22.1	23.2	23.5	23.9	23.9	24.4	24.2	23.0
	4	22.8	22.1	22.7	22.9	23.1	23.0	23.5	23.5	20.4
	5	24.3	23.3	24.2	24.1	23.8	23.4	23.7	24.0	21.5
	6	23.8	22.5	23.1	23.0	23.2	22.4	22.0	23.0	22.1
	Mean	23.95	23.02	23.52	24.20	24.38	24.12	24.22	24.45	22.77
	SD	1.03	0.94	0.73	1.35	1.41	1.64	1.50	1.31	1.86
	SEM	0.42	0.38	0.30	0.55	0.58	0.67	0.61	0.53	0.76
E KN035 0.3mg/kg 1, 4, 7, 10	1	27.2	27.2	27.5	29.0	28.9	28.4	28.7	29.8	30.2
	2	26.1	24.5	25.2	25.5	23.6	23.5	22.0	21.9	20.9
	3	24.1	22.4	22.5	22.1	22.5	22.6	23.5	23.3	23.5
	4	23.4	23.3	23.3	23.2	23.3	22.8	23.0	23.5	24.8
	5	24.7	22.4	23.8	23.7	23.0	22.4	23.0	21.8	23.0
	6	23.2	22.0	23.1	23.9	24.6	24.5	25.1	26.0	25.2
	Mean	24.78	23.63	24.23	24.57	24.32	24.03	24.22	24.38	24.60
	SD	1.58	1.96	1.84	2.44	2.35	2.27	2.42	3.06	3.14
	SEM	0.64	0.80	0.75	0.99	0.96	0.93	0.99	1.25	1.28



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Appendix 2 Tumor volume data in this study (mm³)

Group	Animal #	Days Post Treatment								
		3	8	11	15	18	22	25	29	32
A PBS 1, 4, 7, 10	1	78.11	96.61	144.88	0.00	0.00	74.30	289.27	549.10	1078.20
	2	75.96	164.72	179.92	198.80	177.45	321.48	667.12	1203.75	2271.27
	3	88.00	214.91	139.39	132.17	0.00	0.00	71.10	0.00	97.29
	4	66.74	148.31	120.69	227.38	226.87	474.90	739.50	1433.69	2028.63
	5	64.59	119.71	172.50	185.42	214.49	446.22	706.63	1484.04	2380.64
	6	90.58	119.28	158.61	235.34	275.37	444.26	616.77	1186.99	1718.91
	Mean	77.33	143.92	152.66	163.19	149.03	293.53	515.07	976.26	1595.82
	SD	10.64	42.24	22.05	87.96	119.61	206.85	271.52	582.89	870.42
	SEM	4.34	17.25	9.00	35.91	48.83	84.45	110.85	237.96	355.35
B KN035 0.3mg/kg 1	1	76.39	93.32	108.27	108.60	124.20	143.95	192.78	638.06	1112.81
	2	73.17	57.00	86.84	90.13	127.14	87.01	125.42	305.81	585.03
	3	105.56	87.73	131.95	164.22	260.14	372.18	440.14	643.17	1759.70
	4	64.04	49.41	72.68	80.94	80.05	107.54	94.38	191.33	475.41
	5	58.82	49.90	71.30	86.76	109.71	140.56	135.20	264.91	799.22
	6	46.79	43.49	64.01	80.46	85.21	123.97	241.89	729.32	1341.55



ALPHAMAB

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	Mean	70.79	63.48	89.17	101.85	131.08	162.54	204.97	462.10	1012.29
	SD	20.05	21.46	26.17	32.24	66.16	104.88	126.68	233.14	488.54
	SEM	8.19	8.76	10.68	13.16	27.01	42.82	51.72	95.18	199.44
C KN035 0.3mg/kg 1, 4	1	58.74	40.46	35.88	54.50	34.73	51.00	251.57	427.28	988.69
	2	76.25	88.30	76.55	101.03	85.11	114.48	167.86	307.99	600.94
	3	60.24	57.71	76.10	75.15	104.46	73.54	92.27	91.68	206.42
	4	68.82	54.83	73.97	79.97	93.65	120.19	230.81	731.71	1052.57
	5	89.00	44.42	72.25	71.71	54.56	45.24	63.28	58.49	102.17
	6	64.78	48.28	59.51	57.08	80.35	114.77	198.41	525.10	854.04
	Mean	69.64	55.67	65.71	73.24	75.48	86.54	167.37	357.04	634.14
	SD	11.40	17.22	15.90	16.95	26.02	34.20	75.57	259.04	404.03
	SEM	4.65	7.03	6.49	6.92	10.62	13.96	30.85	105.75	164.94
D KN035 0.3mg/kg 1, 4, 7	1	66.05	103.28	136.93	148.96	165.75	213.59	339.71	1242.27	1522.76
	2	72.87	27.68	36.92	58.34	64.22	124.74	144.77	274.19	673.29
	3	58.07	43.13	65.74	64.05	110.93	119.14	205.00	476.08	602.83
	4	69.09	32.75	51.23	62.09	115.74	95.00	163.13	394.42	620.72
	5	73.37	39.97	55.35	66.74	57.51	64.34	85.85	156.05	218.14
	6	68.33	45.82	55.87	68.30	89.23	128.83	195.27	334.58	198.93
	Mean	67.96	48.77	67.01	78.08	100.56	124.27	188.96	479.60	639.44



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	SD	5.59	27.54	35.51	34.90	39.72	49.95	85.18	389.06	480.55
	SEM	2.28	11.24	14.50	14.25	16.22	20.39	34.77	158.83	196.18
E KN035 0.3mg/kg 1, 4, 7, 10	1	46.87	48.22	57.63	66.45	88.31	135.24	298.17	678.48	1127.89
	2	60.53	44.66	58.07	59.91	83.91	98.71	137.12	210.02	379.49
	3	88.96	39.57	47.05	53.91	78.30	71.93	96.55	181.73	482.08
	4	65.35	34.97	39.03	55.41	78.30	92.31	135.02	474.42	974.78
	5	62.89	48.24	70.92	66.28	77.86	110.24	205.36	549.96	863.80
	6	62.18	33.58	63.70	65.28	80.47	101.31	244.92	519.71	897.35
	Mean	64.46	41.54	56.07	61.21	81.19	101.62	186.19	435.72	787.56
	SD	13.66	6.47	11.46	5.63	4.16	20.89	76.70	197.98	292.77
	SEM	5.58	2.64	4.68	2.30	1.70	8.53	31.31	80.83	119.52