

**Title page****Title:**

Efficacy of combination treatment using YHO-1701, an orally active STAT3 inhibitor, with molecular-targeted agents on cancer cell lines

**Authors' names:**

Keisuke Taniguchi<sup>1</sup>, Hiroaki Konishi<sup>1</sup>, Akiko Yoshinaga<sup>1</sup>, Momomi Tsugane<sup>1</sup>, Hiroyuki Takahashi<sup>2</sup>,  
Fukiko Nishisaka<sup>1,3</sup>, Yoshiyuki Shishido<sup>1</sup>, Akira Asai<sup>3</sup>

**Authors' affiliations:**

1 Yakult Central Institute, Yakult Honsha Co., Ltd., Tokyo; 2 Pharmaceutical Department, Yakult Honsha Co., Ltd., Tokyo; 3 Center for Drug Discovery, Graduate School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan.

**Corresponding author:**

Keisuke Taniguchi, Pharmaceutical Research Department, Yakult Central Institute, Yakult Honsha Co., Ltd., 5-11 Izumi, Kunitachi-shi, Tokyo 186-8650, Japan. Phone: +81425778960. Fax: +81425773020.  
Email: keisuke-taniguchi@yakult.co.jp

## *Supplementary Information*

### **Supplementary Table S1. Cell viability of different cell lines treated with YHO-1701 and/or already-available targeted agents at indicated doses.**

The antiproliferative effects of YHO-1701 and/or molecular-targeted agents were evaluated in a human cancer cell line panel using the WST-8 assay at 48 or 72 h of exposure. Representative data are shown of at least three independent experiments.

<b>K562</b>				
YHO-1701 dose ( $\mu$ M)	Imatinib dose ( $\mu$ M)	% Survival (YHO-1701)	% Survival (Imatinib)	% Survival (Combo)
0.5	0.075	90	88	76
1	0.15	53	63	35
2	0.3	41	34	17
4	0.6	37	19	10
8	1.2	25	15	7

<b>K562</b>				
YHO-1701 dose ( $\mu$ M)	Dasatinib dose (nM)	% Survival (YHO-1701)	% Survival (Dasatinib)	% Survival (Combo)
0.375	0.075	92	84	81
0.75	0.15	67	69	51
1.5	0.3	38	42	18
3	0.6	31	20	8
6	1.2	25	14	9

<b>SUP-B15</b>				
YHO-1701 dose ( $\mu$ M)	Imatinib dose ( $\mu$ M)	% Survival (YHO-1701)	% Survival (Imatinib)	% Survival (Combo)
0.25	0.0875	97	105	96
0.5	0.175	85	75	57
1	0.35	49	57	33
2	0.7	13	50	8
4	1.4	8	47	6

<b>SUP-B15</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Dasatinib dose (nM)	% Survival (YHO-1701)	% Survival (Dasatinib)	% Survival (Combo)
0.25	7.5	94	51	45
0.5	15	84	50	41
1	30	49	45	28
2	60	13	30	10
4	120	9	23	6

<b>H2228</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Crizotinib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Crizotinib)	% Survival (Combo)
2.5	0.25	70	68	48
5	0.5	63	66	33
10	1	48	56	25
20	2	43	43	21
40	4	33	19	6

<b>H2228</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Alectinib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Alectinib)	% Survival (Combo)
2.5	0.025	54	75	47
5	0.05	49	67	33
10	0.1	39	60	28
20	0.2	29	51	21
40	0.4	23	42	20

<b>H2228</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Ceritinib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Ceritinib)	% Survival (Combo)
2.5	0.125	62	75	46
5	0.25	60	73	33
10	0.5	46	65	27
20	1	40	59	21
40	2	30	41	17

<b>H1975</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Osimeertinib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Osimeertinib)	% Survival (Combo)
0.5	0.25	100	53	54
1	0.5	90	52	50
2	1	43	49	34
4	2	28	41	15
8	4	22	28	6

<b>PC-9</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Osimeertinib dose (nM)	% Survival (YHO-1701)	% Survival (Osimeertinib)	% Survival (Combo)
0.25	1.25	94	91	95
0.5	2.5	87	82	70
1	5	42	64	28
2	10	23	42	18
4	20	12	28	12

<b>PC-9</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Gefitinib dose (nM)	% Survival (YHO-1701)	% Survival (Gefitinib)	% Survival (Combo)
0.25	2.5	88	88	101
0.5	5	78	81	78
1	10	42	73	39
2	20	23	58	20
4	40	10	40	12

<b>PC-9</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Erlotinib dose (nM)	% Survival (YHO-1701)	% Survival (Erlotinib)	% Survival (Combo)
0.25	5	95	87	90
0.5	10	81	76	69
1	20	40	57	33
2	40	23	39	17
4	80	10	28	12

<b>PC-9</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Afatinib dose (nM)	% Survival (YHO-1701)	% Survival (Afatinib)	% Survival (Combo)
0.25	0.25	94	90	102
0.5	0.5	85	86	81
1	1	43	63	33
2	2	24	35	20
4	4	12	28	12

<b>SK-BR-3</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Lapatinib dose (nM)	% Survival (YHO-1701)	% Survival (Lapatinib)	% Survival (Combo)
0.25	12.5	75	76	67
0.5	25	63	67	53
1	50	51	57	41
2	100	36	49	28
4	200	25	42	19

<b>BT-474</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Lapatinib dose (nM)	% Survival (YHO-1701)	% Survival (Lapatinib)	% Survival (Combo)
0.25	5	82	99	88
0.5	10	78	69	60
1	20	55	46	32
2	40	35	32	22
4	80	33	22	21

<b>MCF-7</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Everolimus dose (nM)	% Survival (YHO-1701)	% Survival (Everolimus)	% Survival (Combo)
0.5	0.375	94	71	67
1	0.75	80	59	51
2	1.5	47	52	36
4	3	31	47	23
8	6	24	45	20

<b>SAS</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Sorafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Sorafenib)	% Survival (Combo)
0.125	0.75	103	82	75
0.25	1.5	95	70	37
0.5	3	61	47	13
1	6	39	19	14
2	12	15	15	12

<b>K1</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Sorafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Sorafenib)	% Survival (Combo)
1.75	2.5	80	77	80
3.5	5	79	73	44
7	10	46	39	10
14	20	41	8	1
28	40	23	0	3

<b>FTC-133</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Sorafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Sorafenib)	% Survival (Combo)
0.625	2	77	83	64
1.25	4	51	72	43
2.5	8	43	43	27
5	16	43	19	12
10	32	29	1	1

<b>K1</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Vandetanib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Vandetanib)	% Survival (Combo)
1.75	3.75	77	110	79
3.5	7.5	75	111	29
7	15	45	42	3
14	30	39	1	1
28	60	22	0	0

<b>FTC-133</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Vandetanib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Vandetanib)	% Survival (Combo)
0.625	3.75	83	96	77
1.25	7.5	55	91	41
2.5	15	47	44	27
5	30	45	9	5
10	60	32	0	0

<b>COLO205</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Regorafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Regorafenib)	% Survival (Combo)
0.25	0.375	89	92	84
0.5	0.75	69	86	64
1	1.5	47	72	38
2	3	23	53	20
4	6	16	27	16

<b>HCT-15</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Regorafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Regorafenib)	% Survival (Combo)
0.25	1.25	95	89	79
0.5	2.5	81	72	57
1	5	58	48	35
2	10	25	29	16
4	20	18	11	12

<b>SK-MEL-5</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Trametinib dose (nM)	% Survival (YHO-1701)	% Survival (Trametinib)	% Survival (Combo)
0.625	0.5	87	61	54
1.25	1	52	46	34
2.5	2	48	40	24
5	4	35	33	22
10	8	13	33	20

<b>SK-MEL-28</b>				
YHO-1701 dose ( $\mu$ M)	Trametinib dose (nM)	% Survival (YHO-1701)	% Survival (Trametinib)	% Survival (Combo)
2.5	0.5	74	98	72
5	1	65	71	63
10	2	42	47	59
20	4	28	35	37
40	8	21	26	27

<b>A375</b>				
YHO-1701 dose ( $\mu$ M)	Trametinib dose (nM)	% Survival (YHO-1701)	% Survival (Trametinib)	% Survival (Combo)
0.125	0.125	104	100	101
0.25	0.25	101	96	92
0.5	0.5	68	78	57
1	1	43	33	34
2	2	28	22	17

<b>A2058</b>				
YHO-1701 dose ( $\mu$ M)	Trametinib dose (nM)	% Survival (YHO-1701)	% Survival (Trametinib)	% Survival (Combo)
0.175	1.25	99	91	90
0.35	2.5	96	81	75
0.7	5	62	68	52
1.4	10	36	53	30
2.8	20	33	48	25

<b>SK-MEL-5</b>				
YHO-1701 dose ( $\mu$ M)	Vemurafenib dose ( $\mu$ M)	% Survival (YHO-1701)	% Survival (Vemurafenib)	% Survival (Combo)
0.625	0.25	84	56	52
1.25	0.5	53	45	36
2.5	1	50	40	30
5	2	38	36	29
10	4	20	30	22



<b>SK-MEL-28</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Vemurafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Vemurafenib)	% Survival (Combo)
2.5	0.075	75	89	65
5	0.15	66	70	57
10	0.3	42	51	53
20	0.6	28	38	33
40	1.2	22	31	29

<b>A375</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Vemurafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Vemurafenib)	% Survival (Combo)
0.125	0.0125	98	100	100
0.25	0.025	93	82	84
0.5	0.05	63	59	56
1	0.1	42	35	51
2	0.2	30	26	23

<b>A2058</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Vemurafenib dose ( $\mu\text{M}$ )	% Survival (YHO-1701)	% Survival (Vemurafenib)	% Survival (Combo)
0.175	1.25	102	70	71
0.35	2.5	96	59	53
0.7	5	59	47	35
1.4	10	36	37	20
2.8	20	32	19	13

<b>SK-MEL-28</b>				
YHO-1701 dose ( $\mu\text{M}$ )	Dabrafenib dose (nM)	% Survival (YHO-1701)	% Survival (Dabrafenib)	% Survival (Combo)
2.5	2.5	73	81	64
5	5	65	62	58
10	10	39	47	54
20	20	27	36	37
40	40	21	32	29

<b>A375</b>				
YHO-1701 dose ( $\mu$ M)	Dabrafenib dose (nM)	% Survival (YHO-1701)	% Survival (Dabrafenib)	% Survival (Combo)
0.125	1.25	101	76	78
0.25	2.5	90	56	53
0.5	5	56	37	36
1	10	36	25	32
2	20	29	21	17

<b>A2058</b>				
YHO-1701 dose ( $\mu$ M)	Dabrafenib dose ( $\mu$ M)	% Survival (YHO-1701)	% Survival (Dabrafenib)	% Survival (Combo)
0.7	12.5	63	67	55
1.4	25	37	61	40
2.8	50	33	47	18
5.6	100	23	23	5

**Supplementary Table S2. List of cancer cell lines and their origins used in this study.**

<b>Cancer type</b>	<b>Cell line</b>	<b>Supplier</b>	<b>Culture medium</b>
BCR-ABL-positive leukemia	K562	ATCC	IMDM/10% FBS
	SUP-B15	ATCC	IMDM/20% FBS + 0.05mM 2-Mercaptoethanol
EML4-ALK-positive NSCLC	NCI-H2228 (H2228)	ATCC	RPMI1640/10% FBS
EGFR mutation-positive NSCLC	NCI-H1975 (H1975)	ATCC	RPMI1640/10% FBS
	PC-9	ECACC	RPMI1640/10% FBS
Breast	SK-BR-3	ATCC	RPMI1640/10% FBS
	BT-474	ATCC	RPMI1640/10% FBS
	MCF-7	ATCC	DMEM/10% FBS
Oral	SAS	JCRB	DMEM/F12 /10% FBS
Thyroid	K1	ECACC	IMDM/10% FBS
	FTC-133	ECACC	IMDM/10% FBS
Colon	COLO205	ATCC	RPMI 1640/10% FBS
	HCT-15	ATCC	RPMI1640/10% FBS
Melanoma	SK-MEL-5	ATCC	DMEM/10% FBS
	SK-MEL-28	ATCC	DMEM/10% FBS
	A375	ATCC	DMEM/10% FBS
	A2058	ATCC	DMEM/10% FBS

ATCC, American Type Culture Collection (Manassas, VA); ECACC, European Collection of Authenticated Cell Cultures (Salisbury, UK); JCRB, Japanese Collection of Research Bioresources (Osaka, Japan).

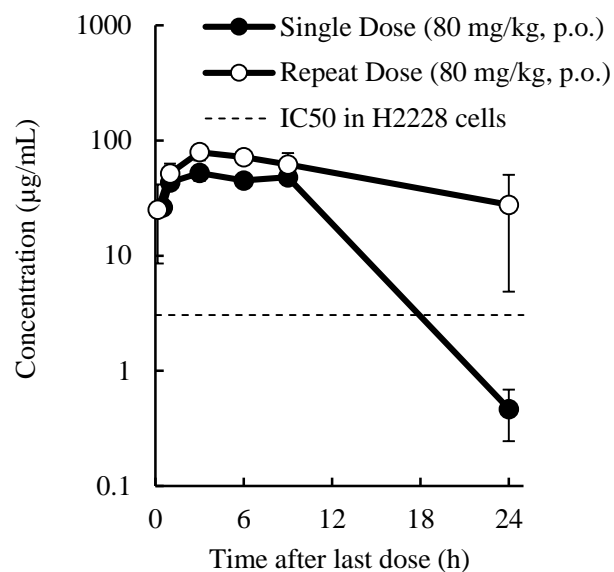
**Supplementary Table S3. List of already-available targeted agents used in this study.**

<b>Molecular-targeted agent</b>	<b>Drug target</b>	<b>Company and catalog number</b>
Imatinib	BCR-ABL	Cell Signaling Technologies (No. 9084)
Dasatinib	BCR-ABL	Bio Vision (No. 1586)
Crizotinib	ALK	LC Laboratories (No. C-7900)
Alectinib	ALK	Selleck Chemicals (No. S2762)
Ceritinib	ALK	Active Biochem (No. A-1189)
Osimertinib	EGFR	Selleck Chemicals (No. S7297)
Gefitinib *	EGFR	Yakult Honsha Co., Ltd.
Everolimus	mTOR	LC Laboratories (No. E-4040)
Erlotinib	EGFR	Santa Cruz Biotechnologies (No. sc-202154)
Afatinib	EGFR	Santa Cruz Biotechnologies (No. sc-364398)
Lapatinib	HER2/EGFR	Santa Cruz Biotechnologies (No. sc-202205)
Sorafenib	Multi Kinases	Cayman Chemical (No. 10009644)
Vandetanib	Multi Kinases	Santa Cruz Biotechnologies (No. sc-220364)
Regorafenib	Multi Kinases	Active Biochem (No. A-1486)
Trametinib	MEK	LC Laboratories (No. T-8123)
Vemurafenib	BRAF	LC Laboratories (No. V-2800)
Dabrafenib	BRAF	Santa Cruz Biotechnologies (No. sc-364477)

\*: Gefitinib was synthesized at Yakult Honsha Co., Ltd.

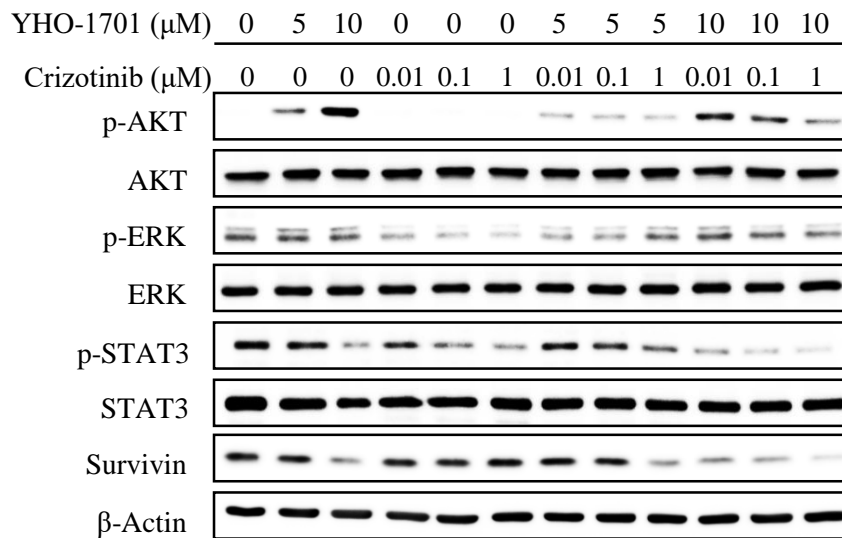
**Supplementary Table S4. List of primary antibodies, including company catalog number.**

<b>Western blotting primary antibody</b>	<b>Dilution</b>	<b>Company and catalog number</b>
Rabbit anti-p-ALK (Y1278/Y1282/Y1283)	1:1000	Cell Signaling Technologies (No. 3983)
Rabbit anti-ALK	1:1000	Cell Signaling Technologies (No. 3633)
Rabbit anti-p-AKT (S473)	1:3000	Cell Signaling Technologies (No. 4058)
Rabbit anti-p-ERK1/2 (T202/Y204)	1:1000	Cell Signaling Technologies (No. 4370)
Rabbit anti-ERK1/2	1:1000	Cell Signaling Technologies (No. 9102)
Rabbit anti-p-STAT3 (Y705)	1:1000	Cell Signaling Technologies (No. 9131)
Rabbit anti-STAT3	1:2000	Cell Signaling Technologies (No. 4904)
Rabbit anti-p-c-Abl (Y245)	1:1000	Cell Signaling Technologies (No. 2861)
Rabbit anti-c-Abl	1:1000	Cell Signaling Technologies (No. 2862)
Rabbit anti-p-EGFR (Y1068)	1:1000	Cell Signaling Technologies (No. 3777)
Rabbit anti-EGFR	1:1000	Cell Signaling Technologies (No. 4267)
Rabbit anti-p-STAT5 (Y694)	1:1000	Cell Signaling Technologies (No. 9351)
Rabbit anti-AKT	1:1000	Santa Cruz Biotechnologies (No. sc-8312)
Rabbit anti-STAT5	1:1000	Santa Cruz Biotechnologies (No. sc-835)
Rabbit anti-survivin	1:1000	R&D Systems (No. AF886)
Mouse anti- $\beta$ -actin	1:5000	Sigma-Aldrich (No. A5316)



**Supplementary Figure S1. YHO-1701 concentration-time profile in plasma after oral administration of single and repeat doses in mice.**

The plasma concentration-time course of YHO-1701 (80 mg/kg) was shown in mice following single (n = 3) and 5-day repeated (n = 6) oral administrations. Blood samples were collected at 6 intervals. Data represent mean  $\pm$  SD. The antiproliferative effect of YHO-1701 was evaluated in H2228 cells using the WST-8 assay after 72 h of exposure. Experiments were performed in triplicates. The horizontal dotted line at 3.05  $\mu\text{g/mL}$  (6.55  $\mu\text{M}$ ) represents the *in vitro*  $\text{IC}_{50}$  for H2228 cells.



**Supplementary Figure S2. Analysis of change in crucial molecules in each pathway when YHO-1701 was treated with crizotinib in H2228 cells.**

Phosphorylation and activation of key molecules in the ALK and STAT3 signaling pathways were examined with western blotting. H2228 cells were left untreated or treated with YHO-1701 and/or crizotinib at the indicated doses for 24 h. The blots were probed with the respective primary antibodies. Cropped images are shown and the full-length blots are presented in Supplementary Fig. S3.

**Supplementary Figure S3. Full-length images of blots presented in the main figures.**

The full blot of p-BCR-ABL in Figure 3B



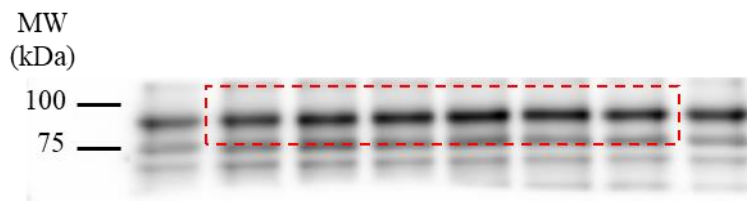
The full blot of BCR-ABL in Figure 3B



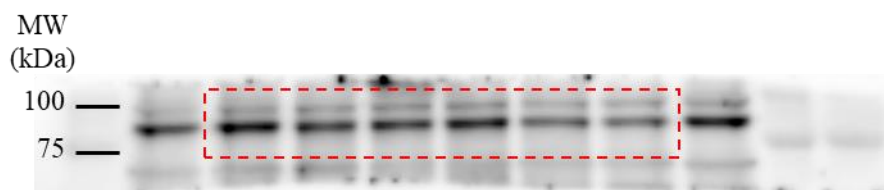
The full blot of p-STAT5 in Figure 3B



The full blot of STAT5 in Figure 3B

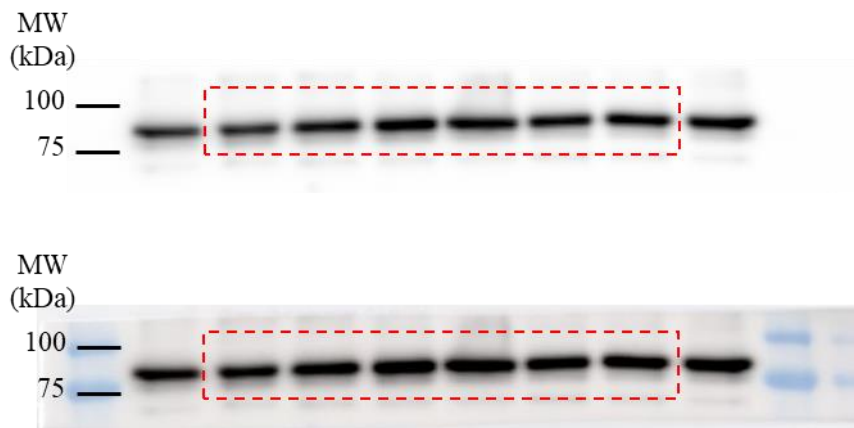


The full blot of p-STAT3 in Figure 3B





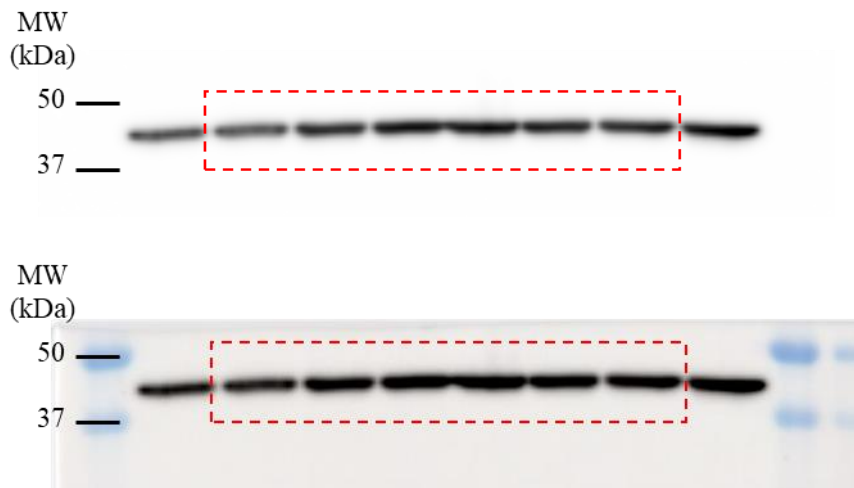
The full blot of STAT3 in Figure 3B [high contrast (upper); low contrast (lower)]



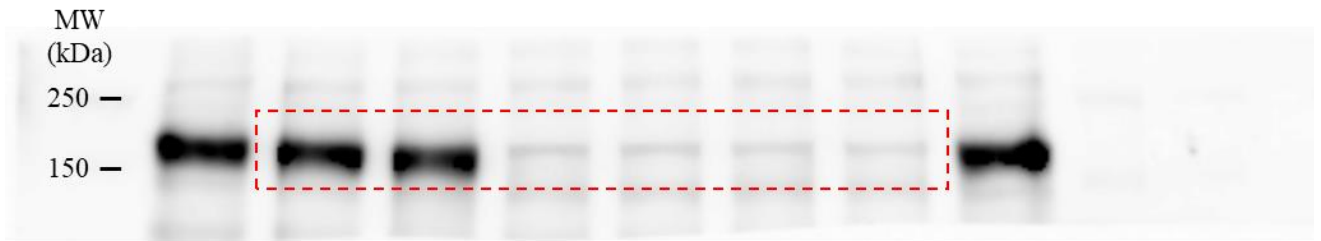
The full blot of survivin in Figure 3B



The full blot of  $\beta$ -Actin in Figure 3B [high contrast (upper); low contrast (lower)]



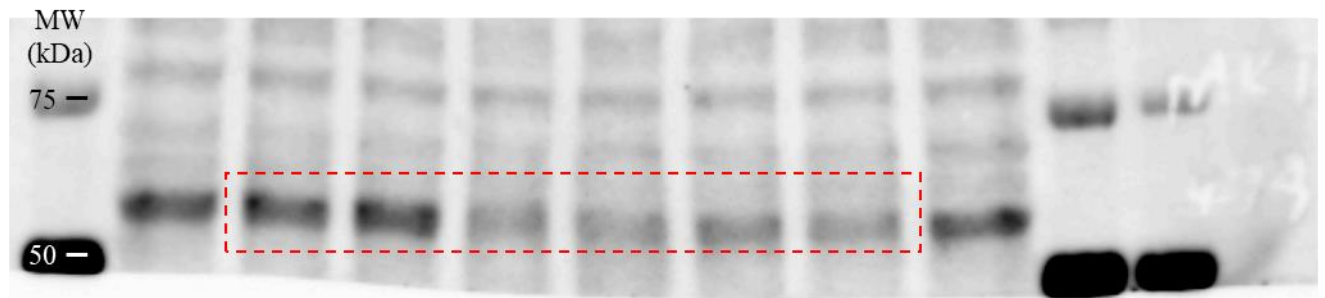
The full blot of p-EGFR in Figure 4B



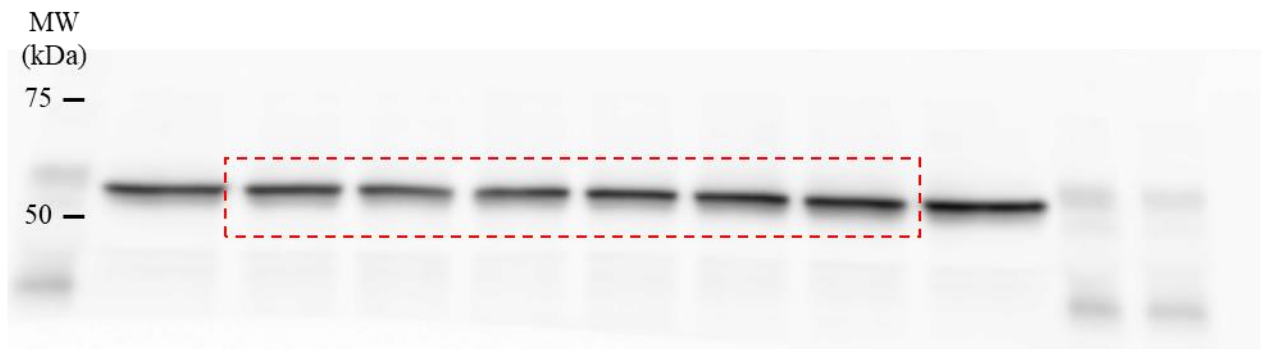
The full blot of EGFR in Figure 4B



The full blot of p-AKT in Figure 4B



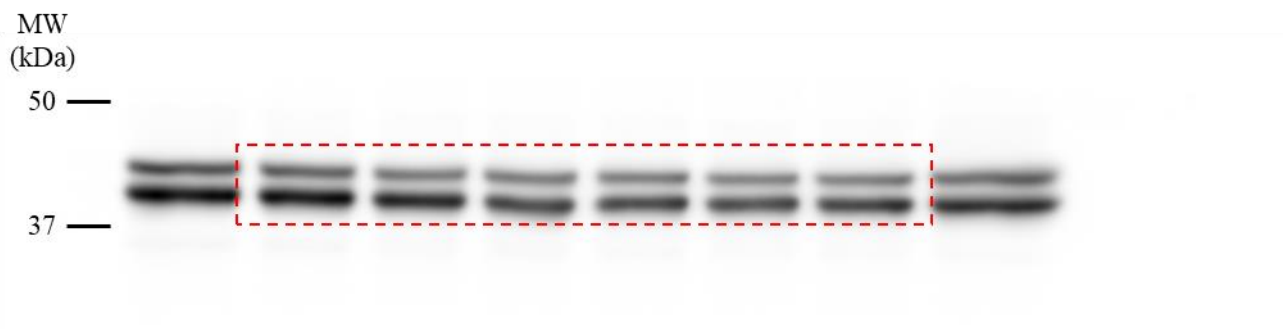
The full blot of AKT in Figure 4B



The full blot of p-ERK in Figure 4B



The full blot of ERK in Figure 4B



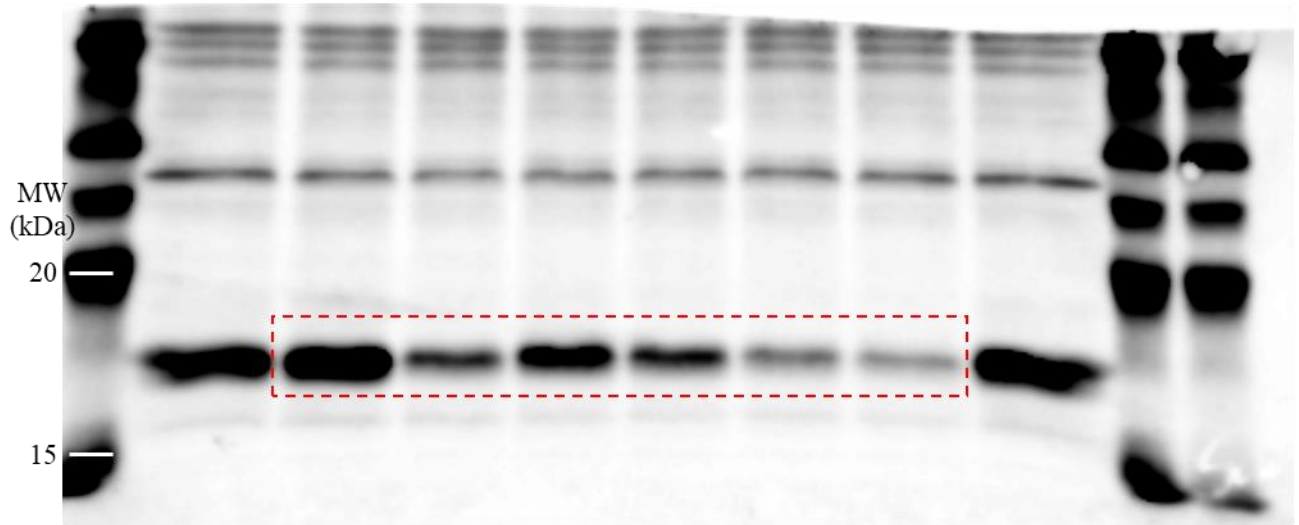
The full blot of p-STAT3 in Figure 4B



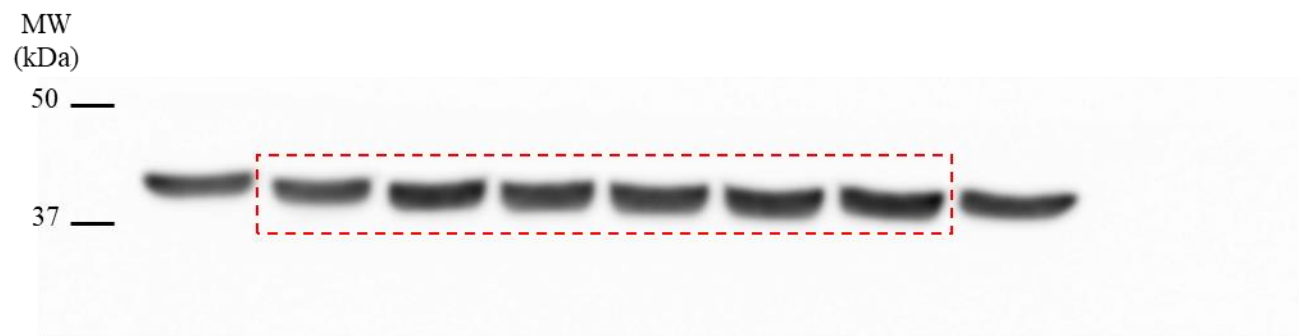
The full blot of STAT3 in Figure 4B



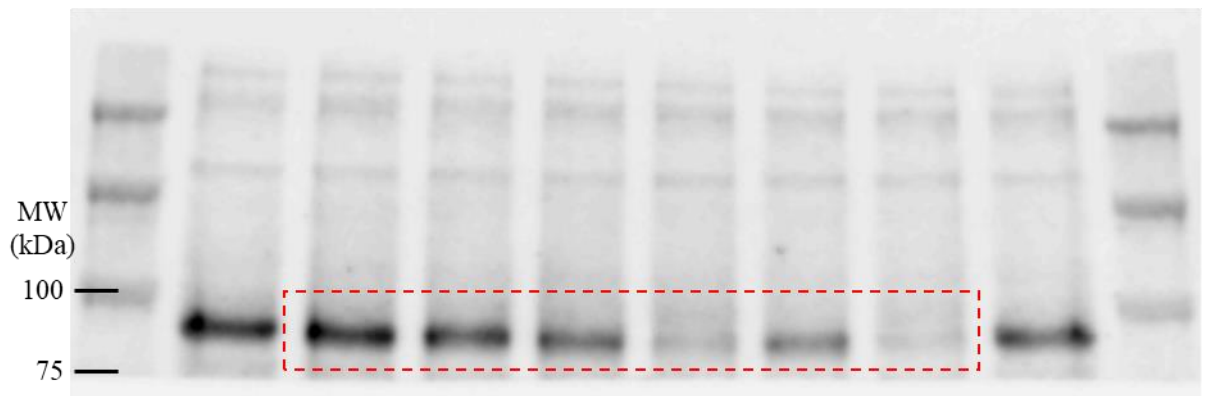
The full blot of survivin in Figure 4B



The full blot of  $\beta$ -Actin in Figure 4B



The full blot of p-ALK in Figure 5B



The full blot of ALK in Figure 5B



The full blot of p-AKT in Figure 5B



The full blot of AKT in Figure 5B



The full blot of p-ERK in Figure 5B



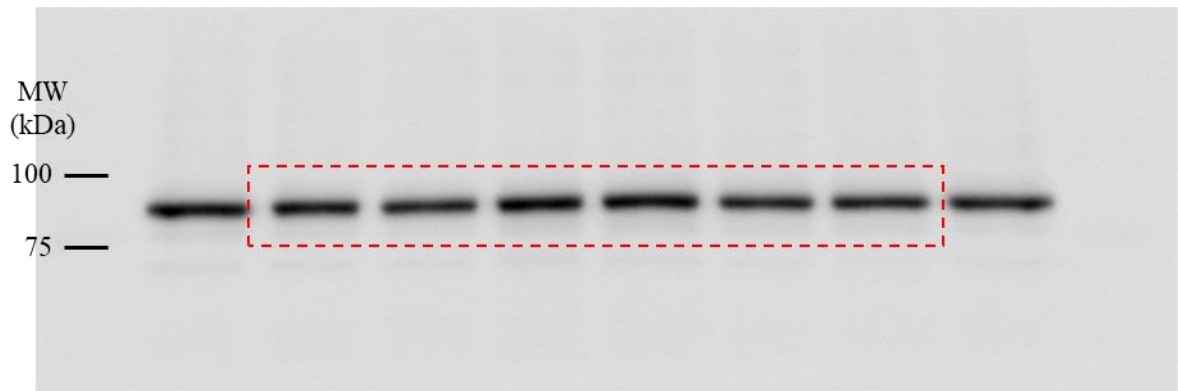
The full blot of ERK in Figure 5B



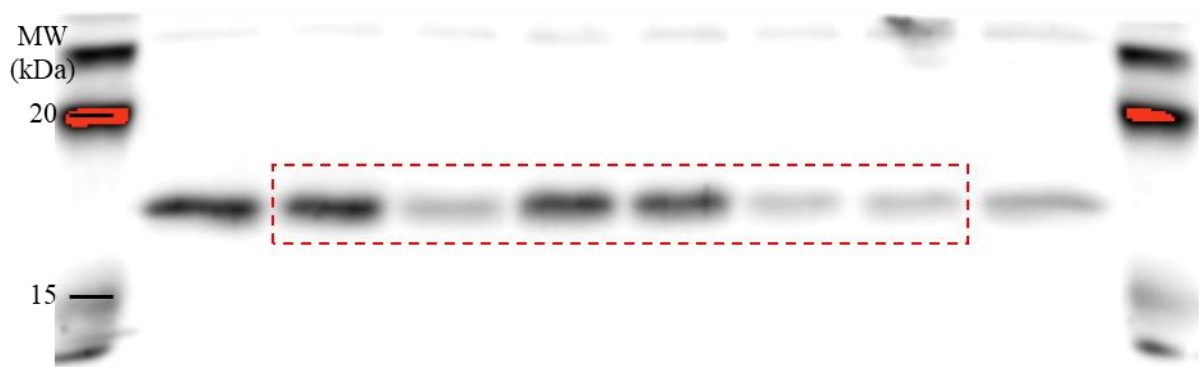
The full blot of p-STAT3 in Figure 5B



The full blot of STAT3 in Figure 5B



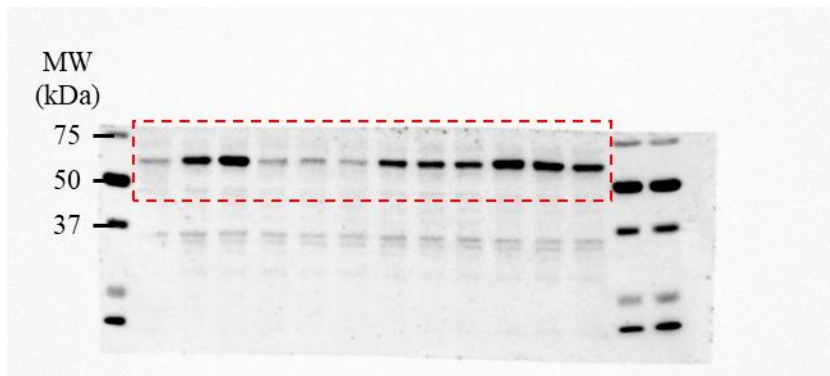
The full blot of survivin in Figure 5B



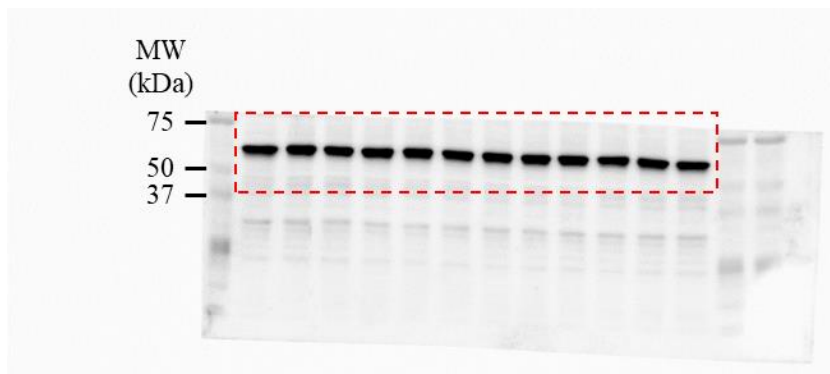
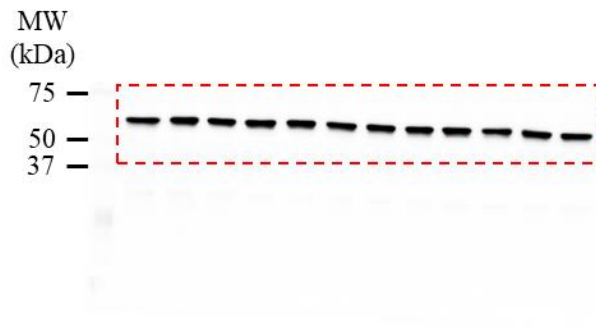
The full blot of  $\beta$ -Actin in Figure 5B



The full blot of p-AKT in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]

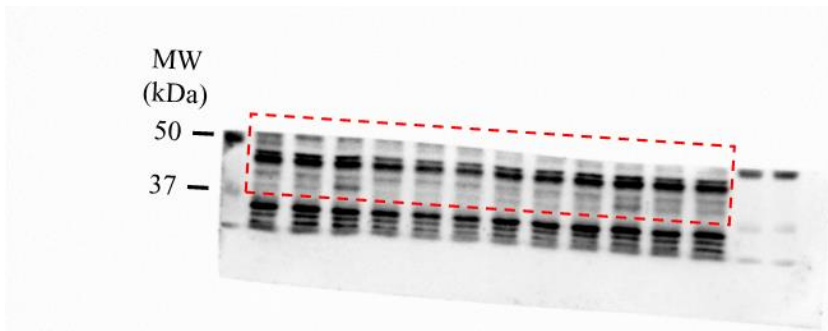
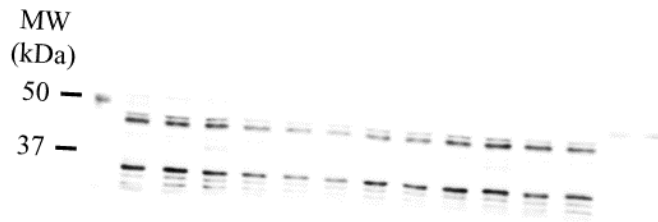


The full blot of AKT in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]

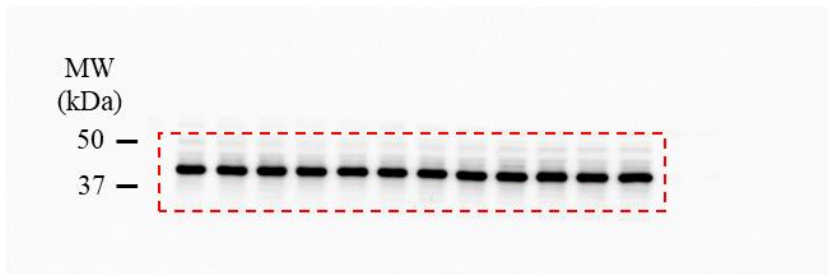




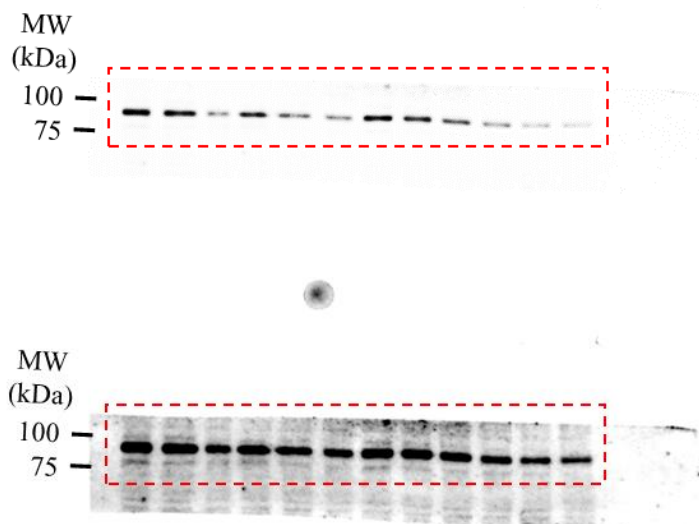
The full blot of p-ERK in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]



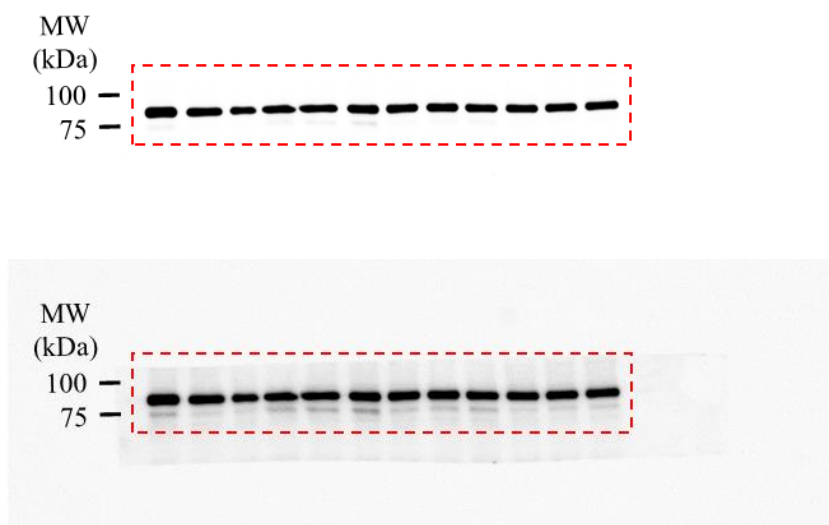
The full blot of ERK in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]



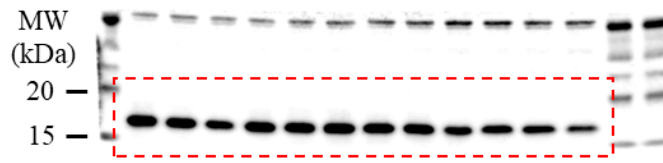
The full blot of p-STAT3 in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]



The full blot of STAT3 in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]



The full blot of survivin in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]



The full blot of  $\beta$ -Actin in Supplementary Figure S2 [high contrast (upper); low contrast (lower)]

