SUPPLEMENTARY APPENDIX

Targeted activation of the SHP-1/PP2A signaling axis elicits apoptosis of chronic lymphocytic leukemia cells

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SUPPLEMENTAL DATA

MATERIALS

PARP polyclonal antibody was from Roche Applied Science (Mannheim, Germany). Anti-PP2A-Cα/ß, anti-p-PP2A-Cα/ß (Tyr307), anti-I2PP2A/SET and anti-caspase 3 antibodies, protein G PLUS-Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pS591-SHP1 and anti-pY536 SHP-1 were from ECM Biosciences (Versailles, KY, USA). Anti-procasp8, anti-phosphotyrosine (pY) and anti-SHP-1 antibodies, SHP-1 Inhibitor-I (PTP-I-I), and Ser/Thr Phosphatase Assay Kit 1 (K-R-pT-I-R-R) were from Millipore-Merck (Billerica, MA, USA). Anti-phospho-Tyr380 procasp8 (anti-p-casp8) antibody was from Abcam (Cambridge, UK). Anti-ß-actin antibody, protein A-Sepharose and okadaic acid were from Sigma-Aldrich (St Louis, MO, USA). Pierce ECL Western Blotting Substrate, Protease inhibitor cocktail were from Thermo Fisher Scientific (Strasbourg, France). The SHP-1 siRNA and negative control siRNA were provided by Santa Cruz Biotechnology (Santa Cruz, CA, catalog number sc-29478). [γ^{32}P]ATP was from Perkin Elmer (Milan, Italy). Nintedanib, Necrostatin-1 and Everolimus were from Selleckchem (Houston, TX, USA). Caspase-8 Colorimetric Assay Kit was from Alexis Corporation, (Lausen, CH).

METHODS

Cell lysis and subcellular fractionation

For total lysates, normal and CLL cells (5×10^5 for each assay) were rapidly lysed as previously described[1,2]. For subcellular fractionation, CLL cells (15×10^6 for each assay) were disrupted and homogenized as previously described[1]. Homogenates were centrifuged 10 min at 10,000g to separate unbroken cells and debris from the supernatant, which underwent ultracentrifugation at 105,000g for 1 h to separate cytosol from microsomes (particulate fraction)[3].

Immunoprecipitation of SHP-1

Cells were disrupted on ice by sonication (three cycles of 5 s at 22 Hz intervalled by 15 s) in isotonic buffer containing 1% NP-40, 20 mM Tris–HCl, pH 7.4, 250 mM Sucrose, 2 mM EGTA, 150 mM NaCl, phosphatase and protease inhibitor cocktails. The lysates were centrifuged at 15 000 *g* for 10 min at 4 °C. The supernatants were immunoprecipitated for 2 h at 4 °C with the anti-SHP-1 antibody and immune complexes were recovered by incubation for 1 h with protein A-Sepharose previously saturated with bovine serum albumine. The immunocomplexes were washed three times in 50 mMTris/HCl (pH 7.5), 0.05% NP-40, protease inhibitor cocktail and submitted to SHP-1 activity assays.

[^{32}P]-phospho band 3 preparation

Band 3 was phosphorylated by incubating erythrocyte ghosts (15 μg) at 30°C with the tyrosine kinases Syk and Lyn in the presence of [γ^{32}P]ATP as elsewhere described[4]. After 10-min incubation, the sample was centrifuged at 14,000 *g* and the pellet washed 3 times with 25 mM Tris, pH 8.0, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 10 mM β-mercaptoethanol, 10 mg/ml leupeptin, and 50 mM phenylmethylsulphonyl fluoride and then resuspended in the same buffer for the phosphatase activity assay.

SHP-1 activity assay

SHP-1 activity was tested in SHP-1 immunoprecipitates using 0.3μg of [^{32}P]-Band3 as a substrate [4]. After 10-min incubation at 30°C, the assays were stopped and subjected to SDS-PAGE. The extent of [^{32}P]-Band 3 dephosphorylation was evaluated either by analysis on a Packard Cyclone or by autoradiography and, after excision of the Band 3, by liquid scintillation counting.

PP2A activity assays

PP2A activity assays from samples undergoing the various treatments utilized throughout the study was measured by using the Malachite Green-based Phosphatase Assay Kit 1(K-R-pT-I-R-R) following the manufacturer's instructions.

Casp8 activity assay

Casp8 activity from samples undergoing the various treatments utilized throughout the study was measured using a Caspase-8 Colorimetric Assay Kit according to the manufacturer's instructions.

Apoptosis assays

CLL cells subjected to the various treatments applied throughout the study were collected forflow cytometric analysis. Ten thousand cells per sample were acquired with the use of BD FACS Diva software (version 7.0), and data were analyzed by plotting on an annexin V–PI logarithmic scattergram.

Western blotting

Whole cell lysates and different cell fractions were run in 10% SDS-PAGE and transferred to nitrocellulose membranes. After 1 hour of treatment with 3% bovine serum albumine at room temperature, membranes were incubated with the appropriate antibodies overnight.

Immunodetection was carried out with the ECL Western Blotting Substrate on the Kodak Image Station 4000mm Pro Digital System (Eastman Kodak, Rochester, NY, USA). Membranes, when required, were reprobed with other primary antibodies after stripping with 0.1 M glycine (pH 2.5), 0.5 M NaCl, 0.1% Tween 20, 1% β-mercaptoethanol and 0.1% NaN3 for 2 x 10 minutes.

Cell transfection

siRNA transfection into CLL cells was performed using the AMAXA nucleofection system (AMAXA, Cologne, Germany). Briefly, cells ($5x10^6$) were resuspended in 100 µl of Nuclefector Solution, mixed with 300 nM of siRNA duplex (control or against SHP-1), and electroporated using program U-015 on AMAXA nucleofector device. Cells were then transferred to 37°C preheated medium and incubated for 48 h at 37°C, 5% CO_2.

Coculture conditions

$2x10^5$/well bone mesenchymal stromal cells from CLL patients were seeded into 12-well plates and incubated for several days before the experiment at 37°C in 5% CO_2 up to confluence. Then CLL B cells were added to the MSC layer at a ratio of 20:1, as described elsewhere [5] and subjected to the treatments described throughout the text.

In vitro tyrosine kinase assays

To analyze cellular tyrosine kinase activity, CLL cells (4×10^5 for each assay) were lysed by adding 0.5% Triton X-100 and phosphatase and protease inhibitor cocktails in a 25-µl volume for 15 minutes. The tyrosine kinase activity was then tested in 50 µl of phosphorylation medium containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 30 µM [γ^{32}P]ATP (Amersham Pharmacia Biotech) (specific activity 1,000 cpm/pmol), 200 µM sodium orthovanadate, and either 1 mg/ml random polymer polyGlu$_4$Tyr (Sigma-Aldrich) or 200 µM cdc2(6–20) peptide used as exogenous substrates. After 10 minutes of incubation at 30°C, the reactions were stopped and the samples were loaded on SDS/PAGE. Substrate ^{32}P-phosphorylation was quantified on a Packard InstantImager[1].

Statistical analysis

The data presented as mean ± SD were compared using one-way analysis of variance followed by Bonferroni post hoc test or Student's t-test. A P-value ≤ 0.01 was considered as statistically significant. All statistics were performed using GraphPad Prism (version 5) statistical software (GraphPad Software; San Diego, CA, USA).

References

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2. Trentin L, Frasson M, Donella-Deana A et al. Geldanamycin-induced Lyn dissociation from aberrant Hsp90-stabilized cytosolic complex is an early event in apoptotic mechanisms in B-chronic lymphocytic leukemia. Blood 2008; 112: 4665-4674.
3. Tibaldi E, Brunati AM, Zonta F et al.Lyn-mediated SHP-1 recruitment to CD5 contributes to resistance to apoptosis of B-cell chronic lymphocytic leukemia cells. Leukemia 2011; 25: 1768-1781.
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6. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975; 46: 219–234.

Figure S1. In-vitro effect of nintedanib on the differently phosphorylated forms of SHP-1 pulled down from CLL cells. (A) Phosphorylation state of SHP-1 in total cell lysates, particulate and cytosol of CLL cells of patients #22, #24, and #34. Anti-LDH (cytosolic marker), anti-PMCA (plasma membrane marker). **(B)** Tyrosine phosphatase activity of SHP-1 immunoprecipitated from particulate and cytosol of CLL of patients #22, #24, and #34 measured as [^{32}P] released from in-vitro [^{32}P]-Band 3.

Figure S2. Aberrant phosphorylation state and localization of Lyn in CLL. Total cell lysates (lanes 1 and 4), or alternatively particulate (lanes 2 and 5) and cytosol (lanes 3 and 6) from CLL cells of normal donor (lanes 1-3) and the CLL patient (lanes 4-6) underwent Wb analysis with anti-pY396-Lyn, anti-pY507-Lyn antibodies and, after stripping, were reprobed with anti-Lyn antibody. Anti-LDH (cytosolic marker), anti-PMCA (plasma membrane marker).The figure is representative of experiments performed in triplicate on samples from 4 normal donor and 8 CLL patients.

Figure S3. Effect of nintedanib on the tyrosine phosphorylation of CLL cells. (A) Expression of SHP-1 in CLL cells transfected with scrambled and SHP-1 siRNAs. **(B)** Phosphorylation pattern of CLL cells of patient #35 transfected with scrambled or SHP-1 siRNAs and cultured in the absence or presence of increasing concentrations of nintedanib for 1 hour. **(C)** Global tyrosine kinase activity and specific Src activity in CLL cells cultured as in (B) determined by [^{32}P] incorporation into the nonspecific random polymer polyGlu_4Tyr (top panel) or the specific peptide substrate cdc2(6-20) (bottom panel). **(D)** Activation state of Lyn in CLL cells cultured as in (B) determined by Wb with anti-pTyr396-Lyn antibody. **(E)** Phosphorylation state of CD5 as assessed by Wb with anti-pTyr antibody of CD5 immunoprecipitates from total cell lysates of CLL cells treated as in (B). Data are mean ± SD of 3 experiments performed in triplicate (*$P \leq 0.05$).

Figure S4. Effect of nintedanib on the tyrosine phosphorylation of CLL cells promoted by BCR cross-linking. Wb analysis with anti-pTyr antibody of total lysates of CLL cells pre-incubated without and with 15 µM nintedanib for 1 hour and then stimulated over time by BCR crosslinking with anti-IgM antibody. The figure is representative of 8 experiments performed in triplicate.

Figure S5. Pro-apoptotic effect of nintedanib on cultured CLL cells. Apoptosis of CLL cells from 10 patients induced by 24 hours' incubation without (spontaneous apoptosis) or with 10 µM nintedanib measured by annexin V-PI flow cytometry.

Figure S6. Effect of nintedanib on the viability of normal B cells. Determination of apoptosis of normal B cell after treatment with increasing concentration of nintedanib for 24 hours as measured by annexin V-PI flow cytometry.

Figure S7. Lack of contribution of death cascades in CLL cells alternative to apoptosis on response to nintedanib. (A) Quadrant analysis of annexin V-PI flow cytometry of CLL cells incubated in the absence (control) or presence of 15 µM nintedanib supplemented without or with the pan-caspase inhibitor z-VADfmk (left-hand panel, patient #24) for 24 hours and pooled data collected as mean percentage of early and late apoptosis ± SD of further three separate experiments performed in triplicate on CLL cells from 8 patients (right-hand panel) to assess residual cell death independent of apoptosis such as necroptosis. **(B)** Apoptosis of CLL cells from 10 patients after 0 and 24 hours' incubation induced by 10 µM nintedanib in the absence or presence of necrostatin-1 as determined by annexin V-PI flow cytometry. **(C)** Expression of p62/SQSTM1, a marker of autophagy, as assessed by Wb analysis with relevant antibody, on response to nintedanib treatment over time. **(D)** Expression of p62/SQSTM1 assessed by Wb analysis as in (C) after treatment of CLL cells with everolimus, mTOr inhibitor, over time.

Figure S8. Effect of nintedanib on the viability of CLL cells co-cultured with bone marrow mesenchymal stromal cells (MSCs). (A) Apoptosis of CLL cells co-cultured with MSCs in the presence of increasing concentrations of nintedanib for 24 as measured by annexin V–PI flow cytometry. Data are mean percentage of early and late apoptosis ± SD from three separate experiments performed in triplicate (*$P \leq 0.01$). **(B)** Wb analysis of total lysates of MSCs (upper panel) or CLL cells previously co-cultured with MSCs (lower panel) with anti-PARP antibody to monitor caspase-dependent apoptosis after incubation with 15 µM nintedanib over time, and anti-β-actin antibody as a loading control.

Figure S9. Lack of contribution of death cascades in CLL cells alternative to apoptosis on response to MP07-66. (A) Quadrant analysis of annexin V-PI flow cytometry of CLL cells incubated in the absence (control) or presence of 8 μM MP07-66 supplemented without or with the pan-caspase inhibitor z-VADfmk (left-hand panel, patient #24) for 24 hours and pooled data collected as mean percentage of early and late apoptosis ± SD of further three separate experiments performed in triplicate on CLL cells from 8 patients (right-hand panel) to assess residual cell death independent of apoptosis such as necroptosis. **(B)** Apoptosis of CLL cells of 10 patients after 0 and 24 hours' incubation induced by 8 μM MP07-66 in the absence or presence of necrostatin-1 as determined by annexin V-PI flow cytometry. **(C)** Expression of p62/SQSTM1, a marker of autophagy, as assessed by Wb with relevant antibody, on response to MP07-66 treatment over time.

Figure S10 Effect of nintedanib on the viability of normal B cells. Determination of apoptosis of normal B cell after treatment with increasing concentration of MP07-66 for 24 hours as measured by annexin V-PI flow cytometry.

Figure S11. Effect of MP 07-66 on SHP-1 activity. (A) Tyrosine phosphatase activity of SHP-1 immunoprecipitated from the cytosol of CLL cells of 10 patients cultured in the presence of increasing concentrations of MP07-66 for 1 hour and measured as [^{32}P] released from in-vitro [^{32}P]-Band 3. Data are expressed as mean ± SD from 1 experiment performed in triplicate (*P ≤ 0.01). **(B)** Pooled densitometric analysis (arbitrary units) of Western blot bands corresponding to the expression of SHP-1 (lower histogram) and the phosphorylation state of S591-SHP-1 (upper histogram) in CLL cells of 16 patients incubated with increasing concentrations of MP07-66 from 4 independent experiments performed in triplicate. Data are expressed as mean ± SD from 4 experiments (*P ≤ 0.01). **(C)** Pooled densitometric analysis (arbitrary units) of Western blot bands corresponding to the expression and phosphorylation state of procasp8 and PP2Ac, respectively, in CLL cells of 16 patients incubated in the absence (lanes 1 and 5) and presence of 8 μM MP07-66 supplemented with 5 nM OA (lanes 3 and 7) or 25 μM PTP-I-I (lanes 4 and 8) for 0 and 6 hr. Data are mean ± SD from 4 experiments performed in triplicate (*P ≤ 0.01).

Figure S12. Pro-apoptotic effect of nintedanib and MP07-66 in combination on cultured CLL cells. Apoptosis of CLL cells from 10 patients induced by 24 hours' incubation without (spontaneous apoptosis) or with 15 µM nintedanib and 8 µM MP07-66 measured by annexin V-PI flow cytometry.

Figure S13. Effect of nintedanib and MP07-66 in combination on the viability of CLL cells co-cultured with bone marrow mesenchymal stromal cells (MSCs). (A) Apoptosis of CLL cells co-cultured with MSCs in the absence and presence of 15 µM nintedanib, 8 µM MP07-66 or both over time analyzed by annexin V–PI flow cytometry. Data are mean percentage of early and late apoptosis ± SD from three separate experiments performed in triplicate (*$P \leq 0.01$). **(B)** Wb analysis of total lysates of MSCs (upper panel) or CLL cells previously co-cultured with MSCs (lower panel) with anti-PARP antibody to monitor caspase-dependent apoptosis after incubation with 15 µM nintedanib and 8 µM MP07-66 over time, and anti-β-actin antibody as a loading control.

Table S1. Biological and clinical characteristics of the patients[6]

Patient no.	Age, y	Sex	Rai stage*	WBC count/mm^3	Lymphocytes, %	V_H mutational status†	Zap-70 expression‡	FISH
1	84	F	0	48630	95,2	Unmut	POS	13q-
2	78	M	0	34910	62,7	Unmut	POS	11q-;13q-;12+
3	65	M	0	79430	98,5	Mut	POS	13q-
4	57	F	0	109500	97	Unmut	NEG	13q-
5	56	M	0	85150	89	Unmut	NEG	11q-;12+
6	81	M	0	112000	80,8	Mut	POS	13q-
7	89	F	0	50300	63	Unmut	POS	nd
8	65	M	0	51180	96,8	Mut	POS	13q-
9	79	F	I	53400	76	Mut	NEG	13q-
10	69	F	I	45150	94,4	Mut	NEG	13q-
11	54	M	I	57110	82,6	Unmut	NEG	12+
12	67	F	I	61660	92,9	Unmut	NEG	12+
13	76	M	I	158000	97	Mut	NEG	N
14	82	M	I	31010	51,2	Mut	POS	12+
15	82	F	I	145700	95	Unmut	POS	12+
16	67	F	I	61660	92,9	Unmut	NEG	12+
17	77	M	I	35140	83,5	Unmut	NEG	17p-;11q-
18	64	F	I	57600	93	Mut	NEG	13q-
19	73	F	I	58100	83,9	Mut	NEG	13q-
20	73	F	II	35990	82	Mut	POS	nd
21	66	F	II	47180	92,6	Unmut	POS	13q-
22	76	M	II	55600	87	Unmut	POS	17p-
23	76	M	II	25290	76	Mut	NEG	N
24	64	M	II	74610	89	Mut	POS	13q-
25	66	M	II	99600	91	Unmut	NEG	11q-
26	77	F	II	62110	84	Unmut	NEG	13q-
27	80	F	II	155800	94,5	Mut	NEG	N
28	63	M	III	93290	91,3	Mut	NEG	12+
29	77	F	III	43290	93,9	Mut	POS	13q-
30	63	M	III	90170	85,5	Mut	NEG	12+
31	76	M	IV	122000	94	Unmut	NEG	13q-
32	79	M	IV	69370	83	Unmut	NEG	N
33	72	M	IV	91950	95	Mut	POS	13q-
34	69	M	IV	65600	89	Mut	NEG	17p-;13q-
35	64	M	IV	97970	63	Mut	POS	13q-;12+
36	83	F	IV	26590	91	Unmut	POS	13q-
37	57	F	IV	82520	55	Unmut	NEG	N

NEG, negative; POS, positive; WBC, white blood cell; N, CLL with a normal karyotype; nd, not determined.

* Staging system developed by Rai et al.

† Mutated was defined as having a frequency of mutations >2% from germline V_H sequence.

‡ As determined by western blot analysis on purified B cells (purity at least 98%).

Fig. S1

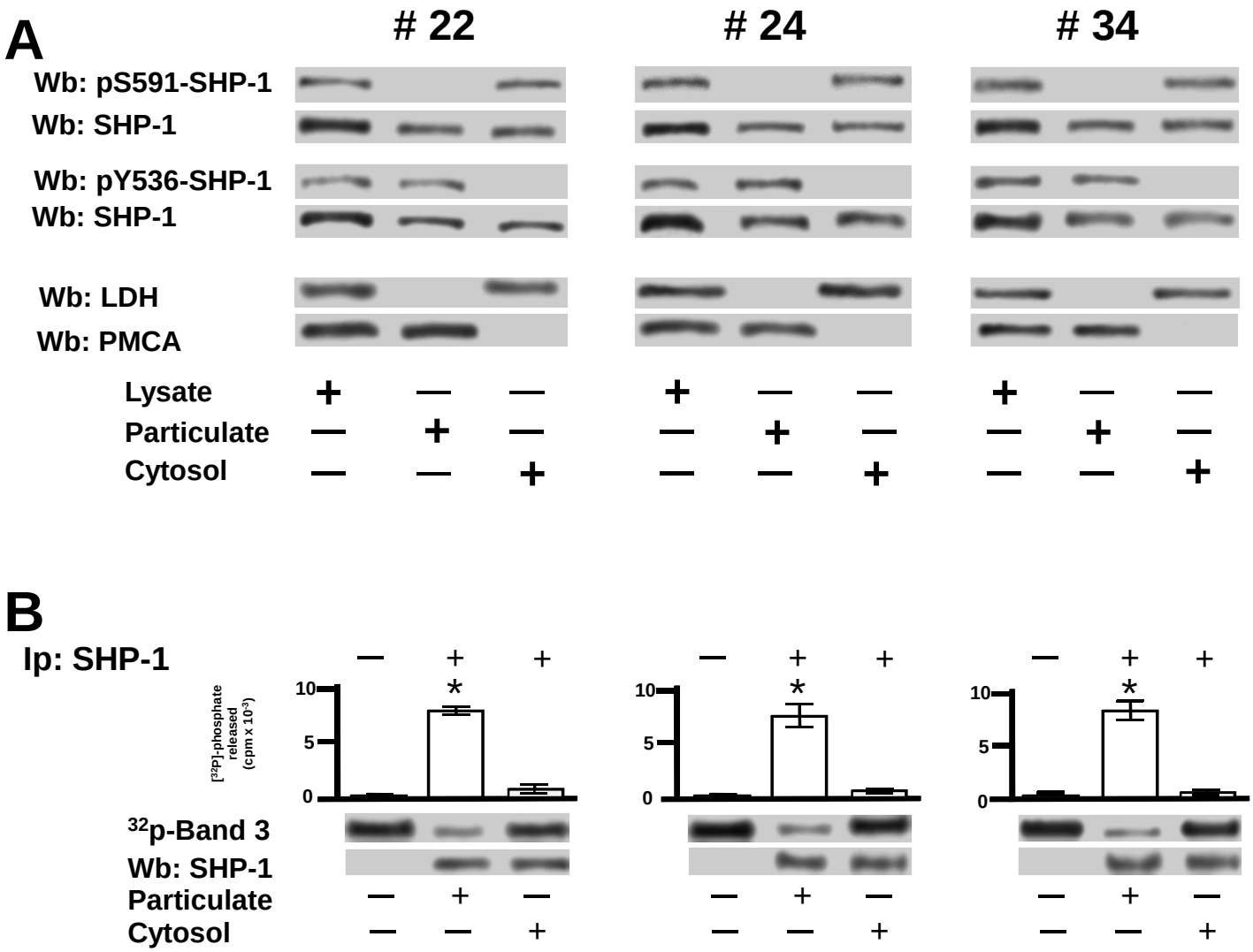

A
22
24
34
Wb: pS591-SHP-1
Wb: SHP-1
Wb: pY536-SHP-1
Wb: SHP-1
Wb: LDH
Wb: PMCA
Lysate
Particulate
Cytosol
B
Ip: SHP-1
[32P]-phosphate released (cpm x 10-3)
32p-Band 3
Wb: SHP-1
Particulate
Cytosol

Fig. S2

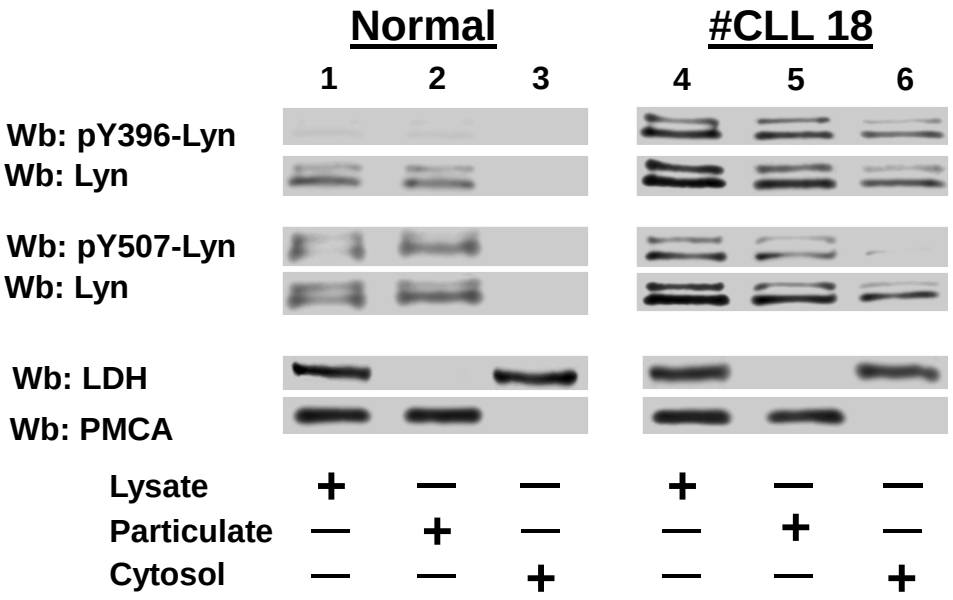

Normal
#CLL 18
1
2
3
4
5
6
Wb: pY396-Lyn
Wb: Lyn
Wb: pY507-Lyn
Wb: Lyn
Wb: LDH
Wb: PMCA
Lysate + — — + — —
Particulate — + — — + —
Cytosol — — + — — +

Fig. S3

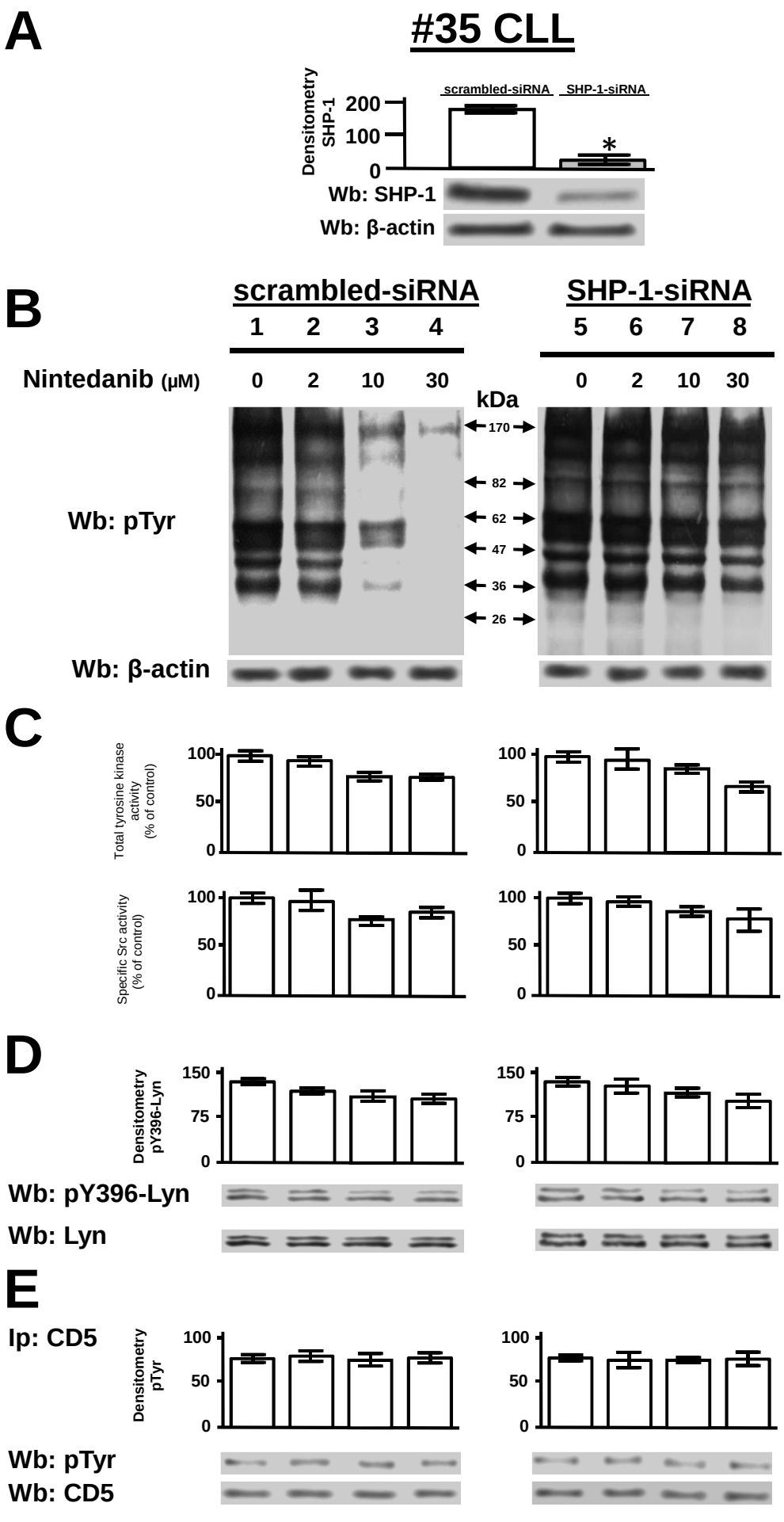

A
#35 CLL
Densitometry SHP-1
200
100
0
scrambled-siRNA
SHP-1-siRNA
*
Wb: SHP-1
Wb: β-actin
B
scrambled-siRNA
SHP-1-siRNA
1 2 3 4
5 6 7 8
Nintedanib (μM)
0 2 10 30
0 2 10 30
kDa
170
82
62
47
36
26
Wb: pTyr
Wb: β-actin
C
Total tyrosine kinase activity (% of control)
Specific Src activity (% of control)
100
50
0
D
Densitometry pY396-Lyn
150
75
0
Wb: pY396-Lyn
Wb: Lyn
E
Ip: CD5
Densitometry pTyr
Wb: pTyr
Wb: CD5

Fig. S4

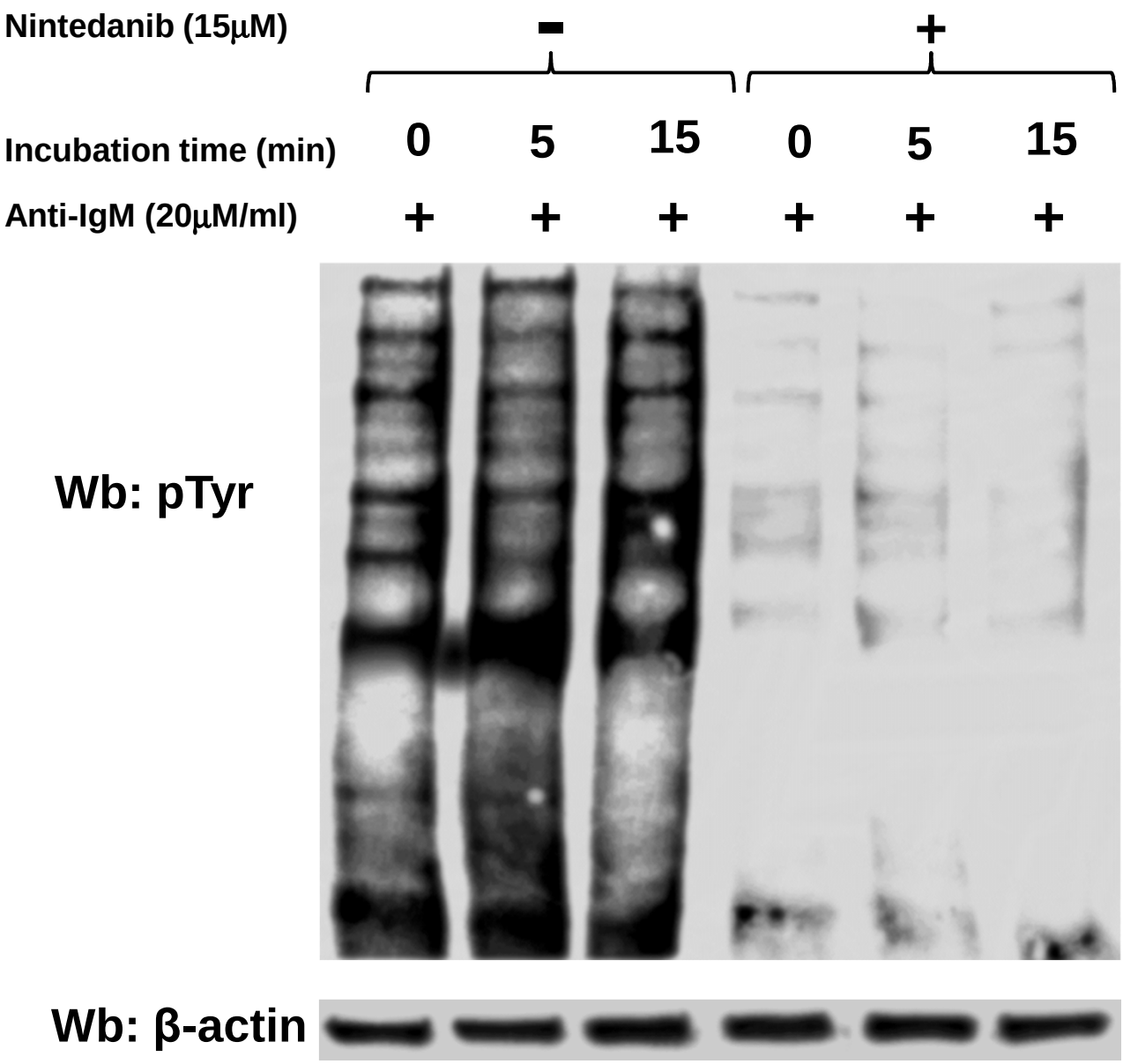

Nintedanib (15μM)
-
+
Incubation time (min)
0
5
15
0
5
15
Anti-IgM (20μM/ml)
+
+
+
+
+
+
Wb: pTyr
Wb: β-actin

Fig. S5

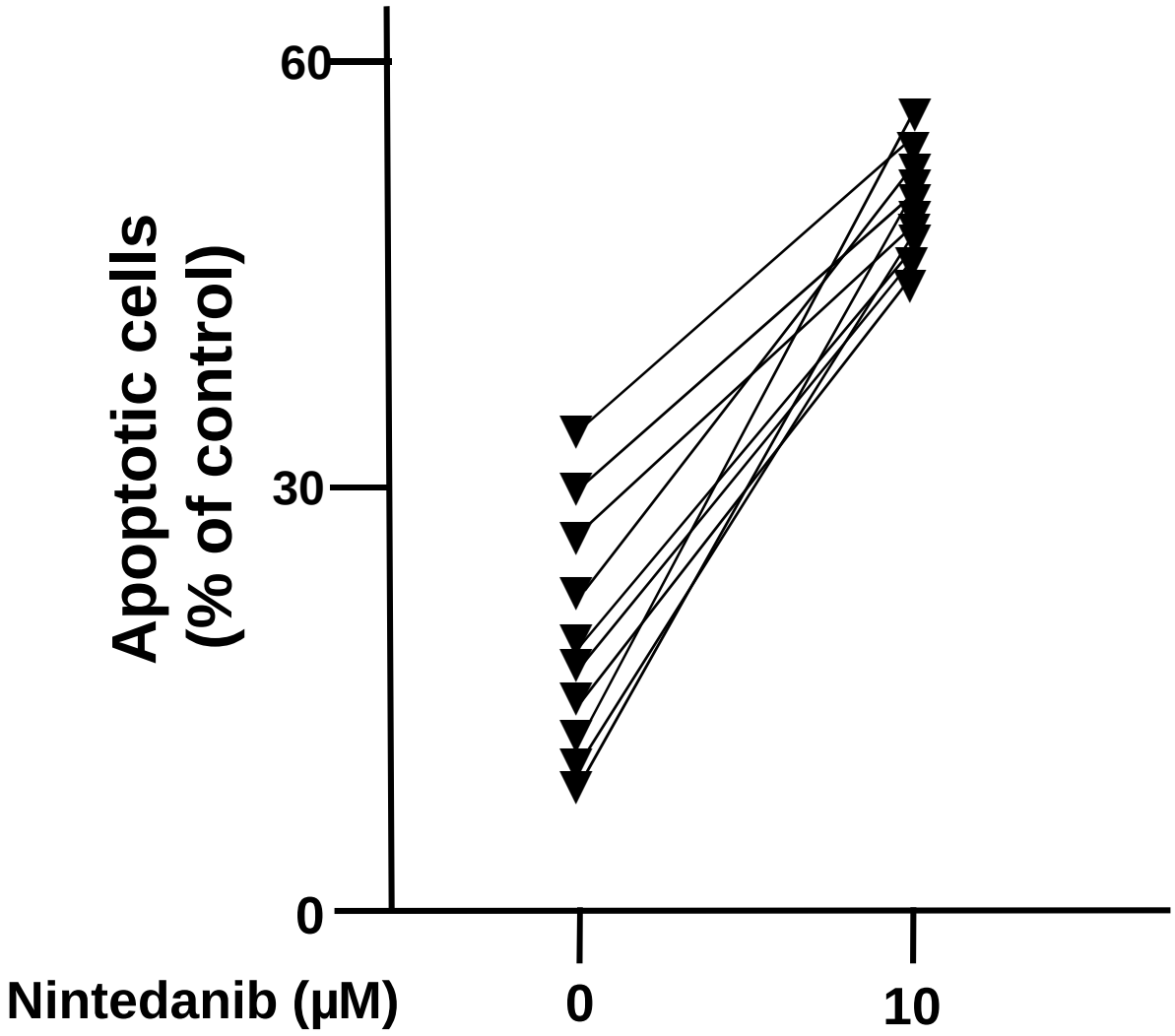


Fig. S6

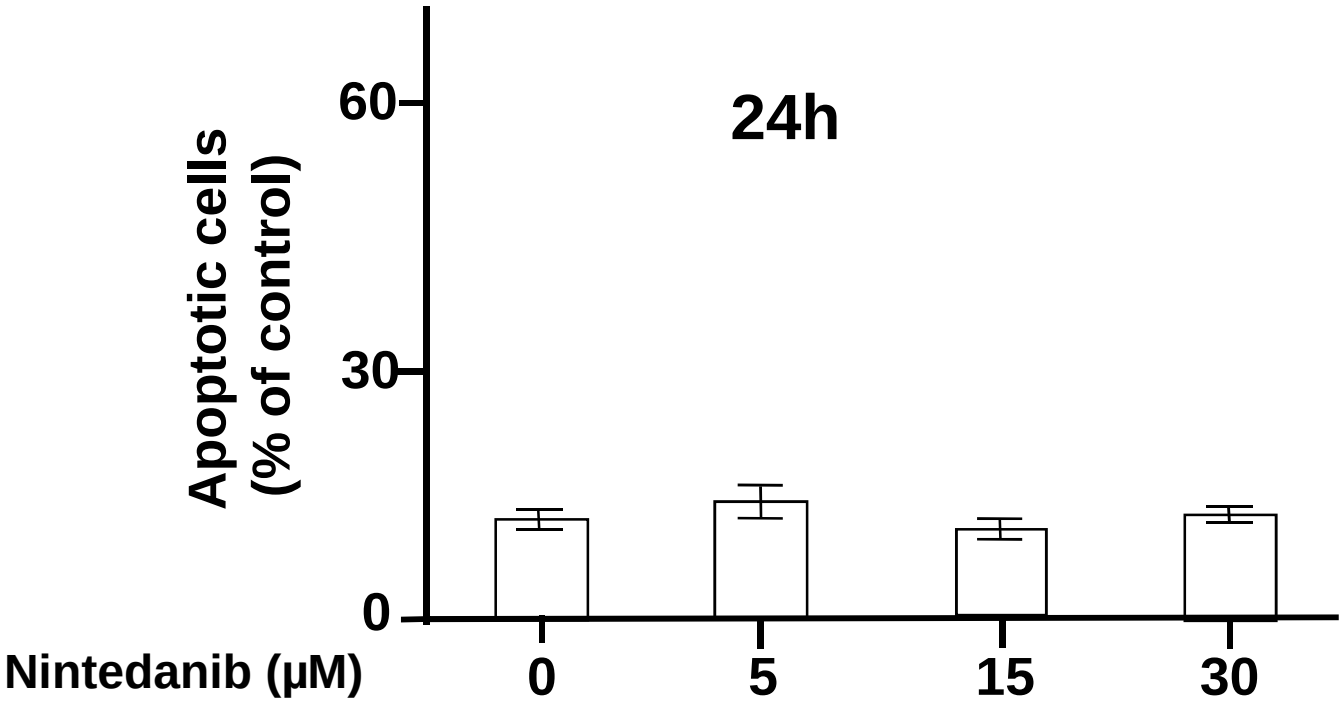
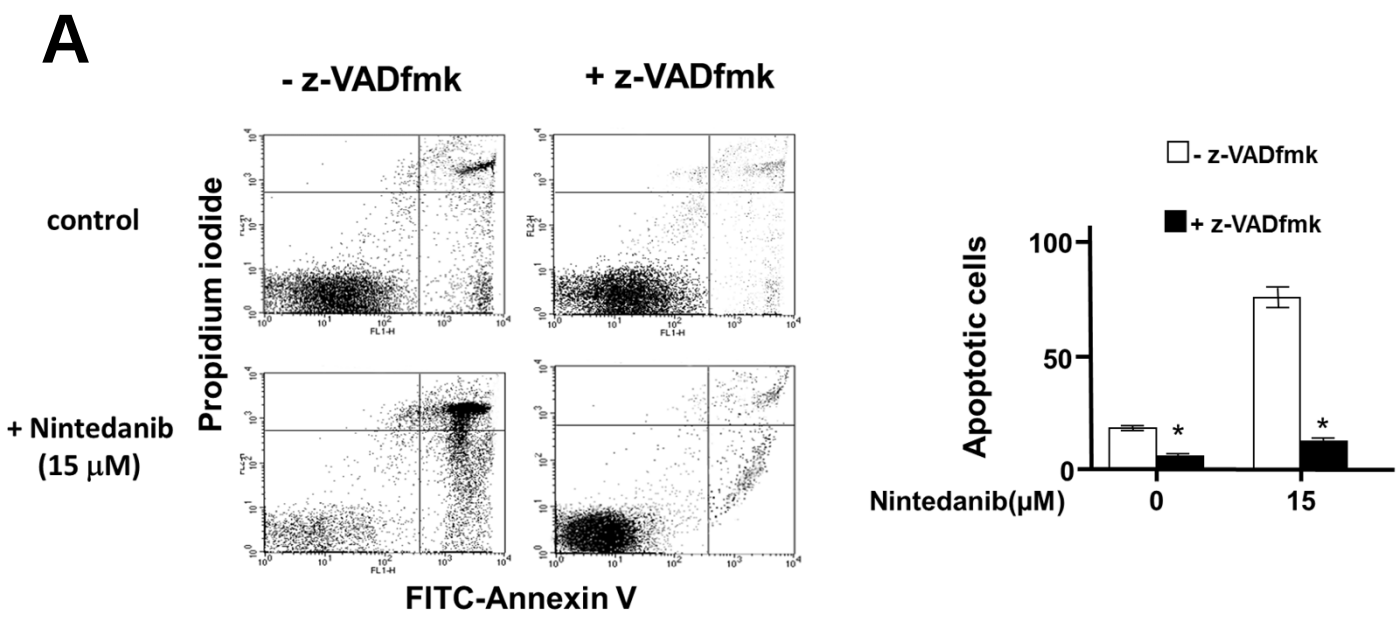
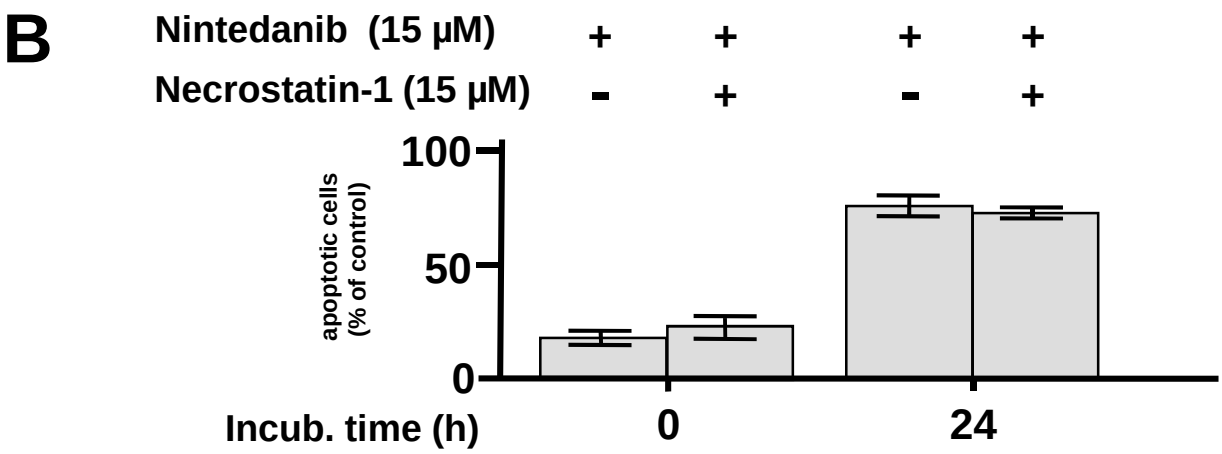
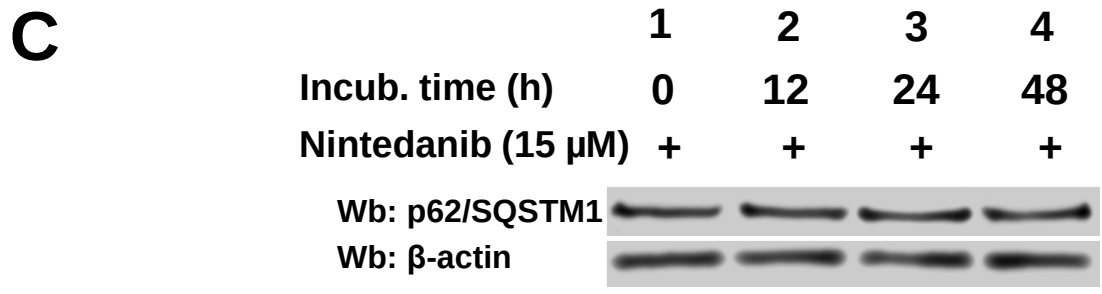


Fig. S7


A
- z-VADfmk
+ z-VADfmk
control
+ Nintedanib
(15 μM)
Propidium iodide
FITC-Annexin V
- z-VADfmk
+ z-VADfmk
Apoptotic cells
100
50
0
*
*
Nintedanib(μM)
0
15


B
Nintedanib (15 μM) + + + +
Necrostatin-1 (15 μM) - + - +
apoptotic cells
(% of control)
100
50
0
Incub. time (h)
0
24


C
1 2 3 4
Incub. time (h) 0 12 24 48
Nintedanib (15 μM) + + + +
Wb: p62/SQSTM1
Wb: β-actin

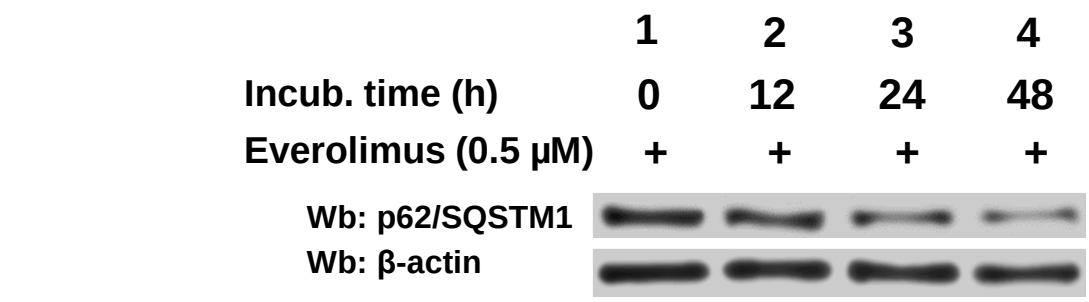

D
1 2 3 4
Incub. time (h) 0 12 24 48
Everolimus (0.5 μM) + + + +
Wb: p62/SQSTM1
Wb: β-actin

Fig. S8

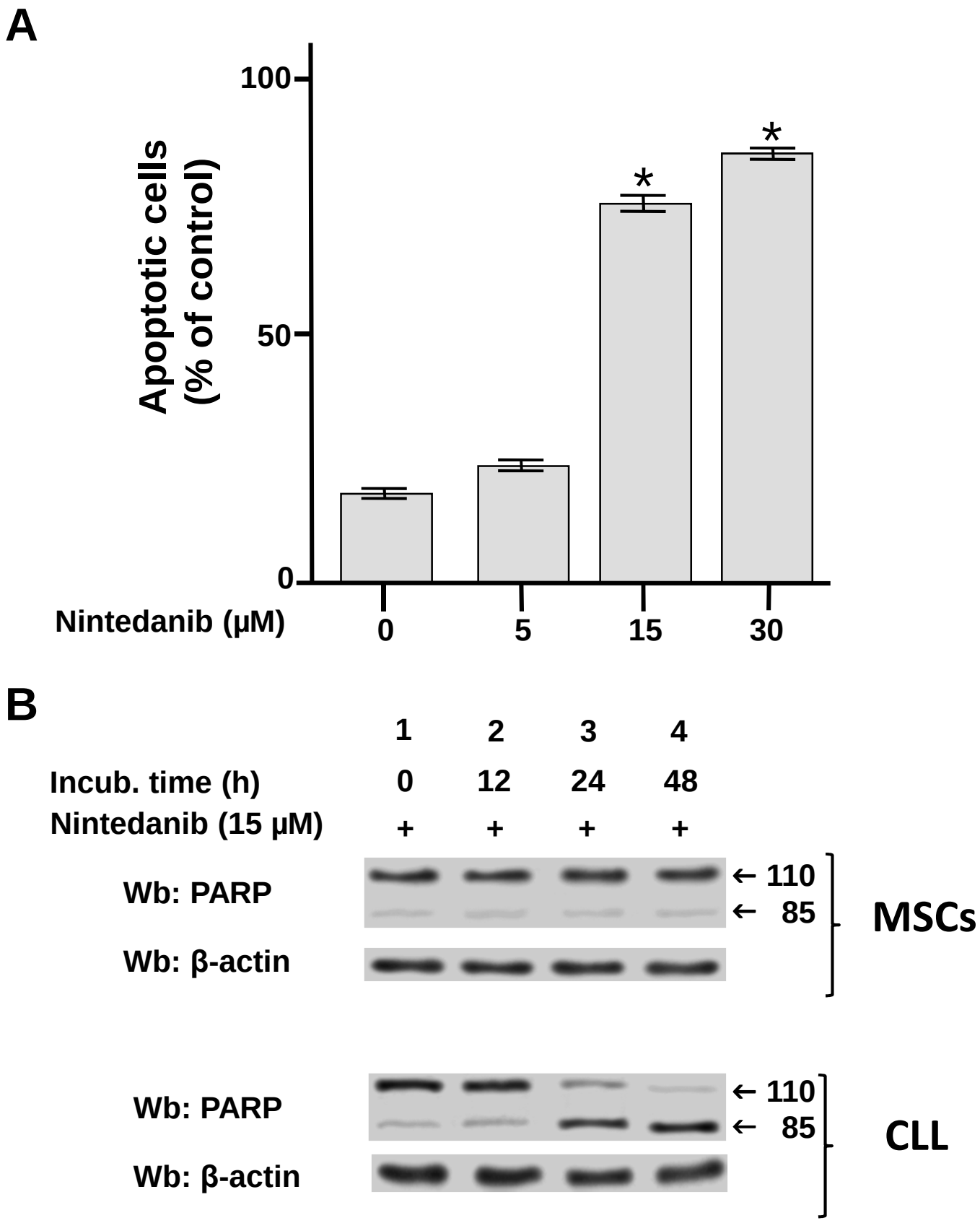
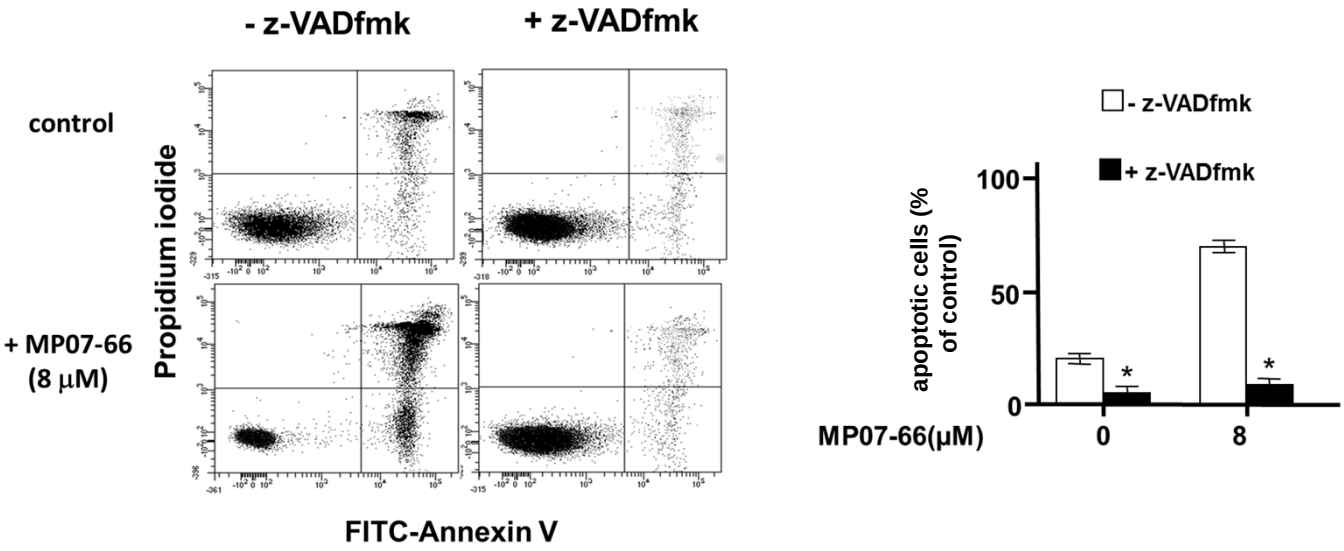
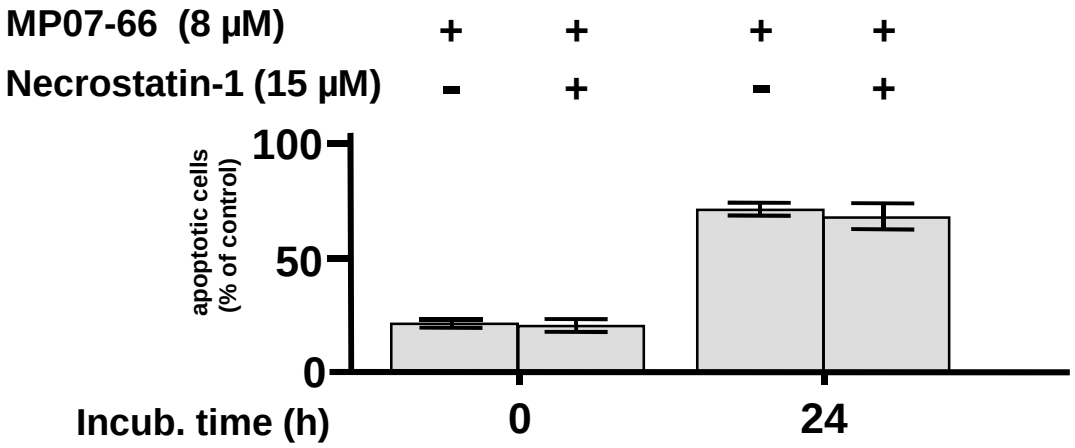

A
Apoptotic cells
(% of control)
100
50
0
*
*
Nintedanib (μM)
0
5
15
30
B
1
2
3
4
Incub. time (h)
0
12
24
48
Nintedanib (15 μM)
+
+
+
+
Wb: PARP
← 110
← 85
Wb: β-actin
MSCs
Wb: PARP
← 110
← 85
Wb: β-actin
CLL

Fig. S9

A


- z-VADfmk
+ z-VADfmk
control
+ MP07-66
(8 μM)
Propidium iodide
FITC-Annexin V
- z-VADfmk
+ z-VADfmk
apoptotic cells (%
of control)
100
50
0
*
*
MP07-66(μM)
0
8

B


MP07-66 (8 μM)
+
+
+
+
Necrostatin-1 (15 μM)
-
+
-
+
apoptotic cells
(% of control)
100
50
0
Incub. time (h)
0
24

C

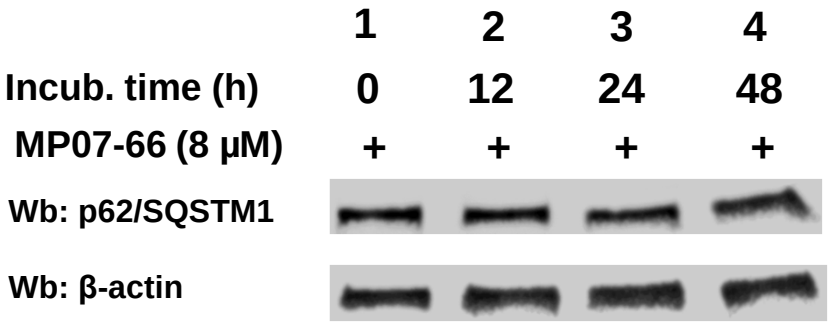

1
2
3
4
Incub. time (h)
0
12
24
48
MP07-66 (8 μM)
+
+
+
+
Wb: p62/SQSTM1
Wb: β-actin

Fig. S10

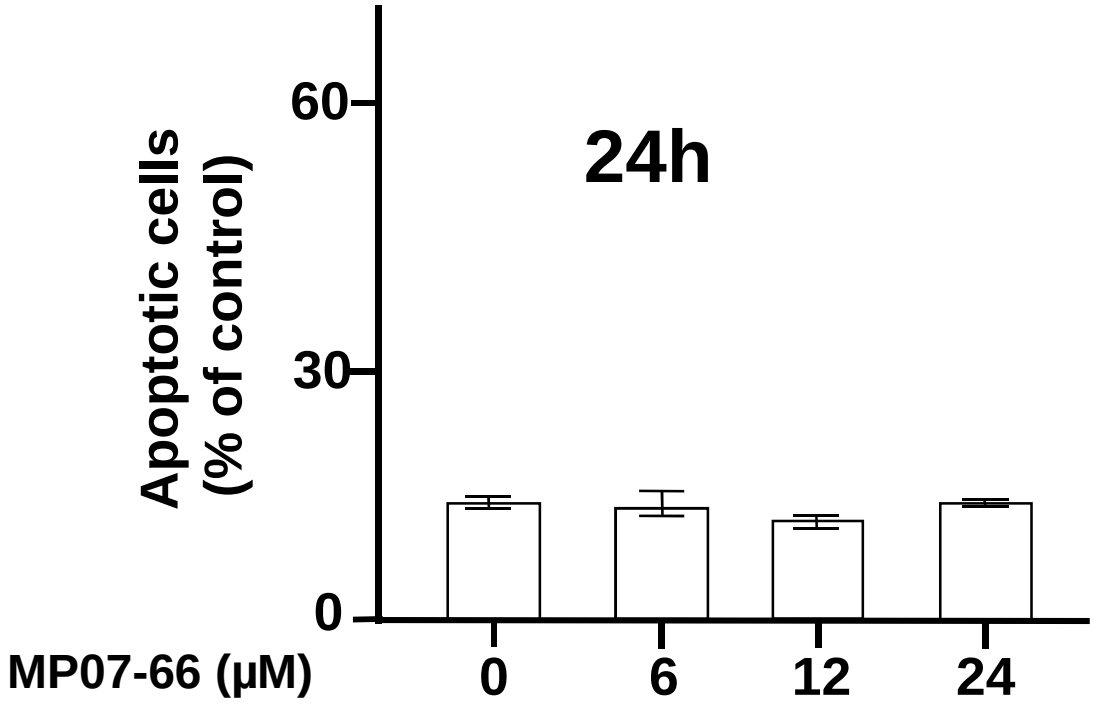


Fig. S11

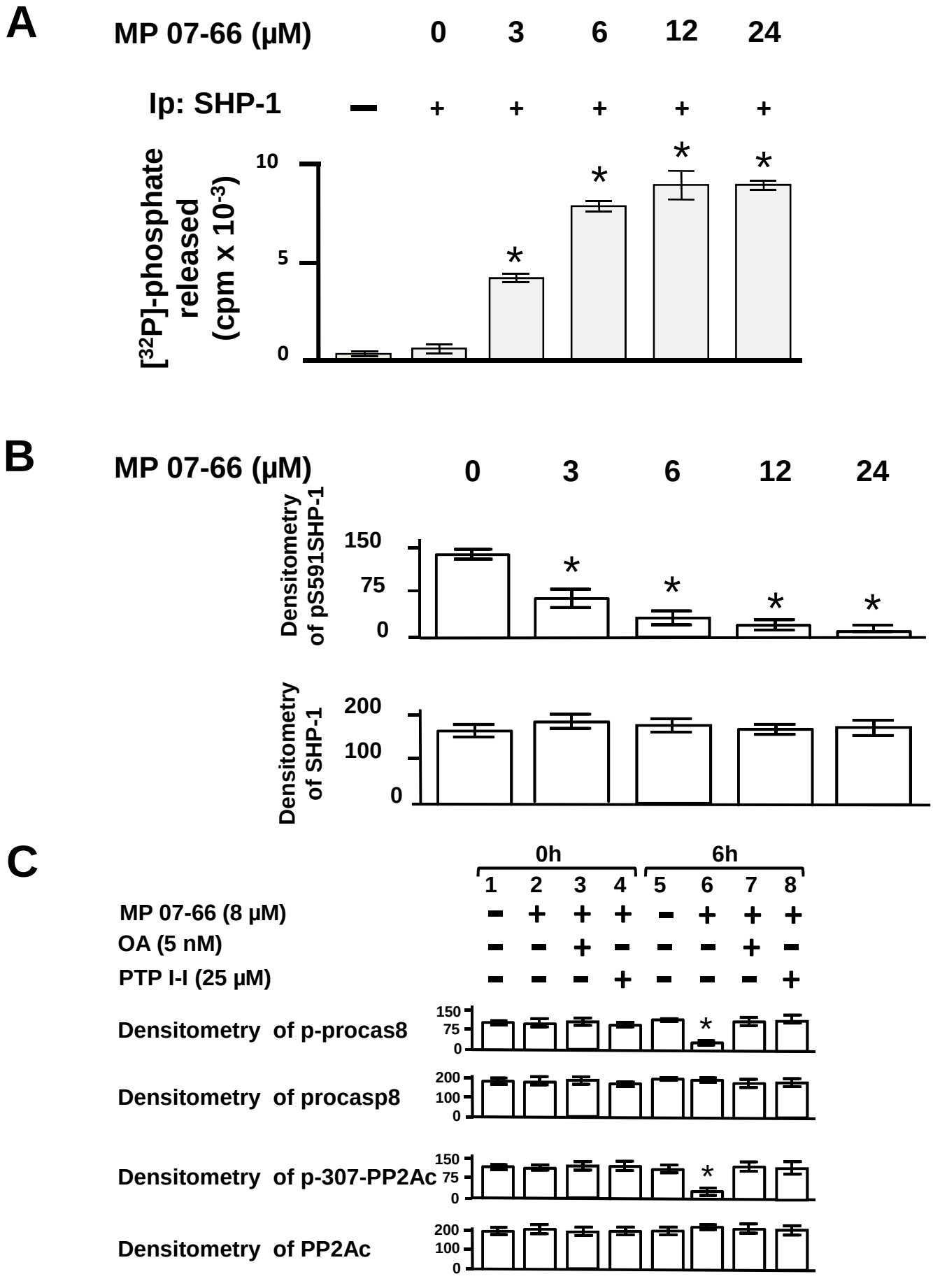

A
MP 07-66 (μM)
0
3
6
12
24
Ip: SHP-1
[32P]-phosphate released (cpm x 10-3)
10
5
0
B
MP 07-66 (μM)
0
3
6
12
24
Densitometry of pS591SHP-1
150
75
0
Densitometry of SHP-1
200
100
0
C
0h
6h
1 2 3 4 5 6 7 8
MP 07-66 (8 μM)
OA (5 nM)
PTP I-I (25 μM)
Densitometry of p-procas8
Densitometry of procasp8
Densitometry of p-307-PP2Ac
Densitometry of PP2Ac

Fig.S12

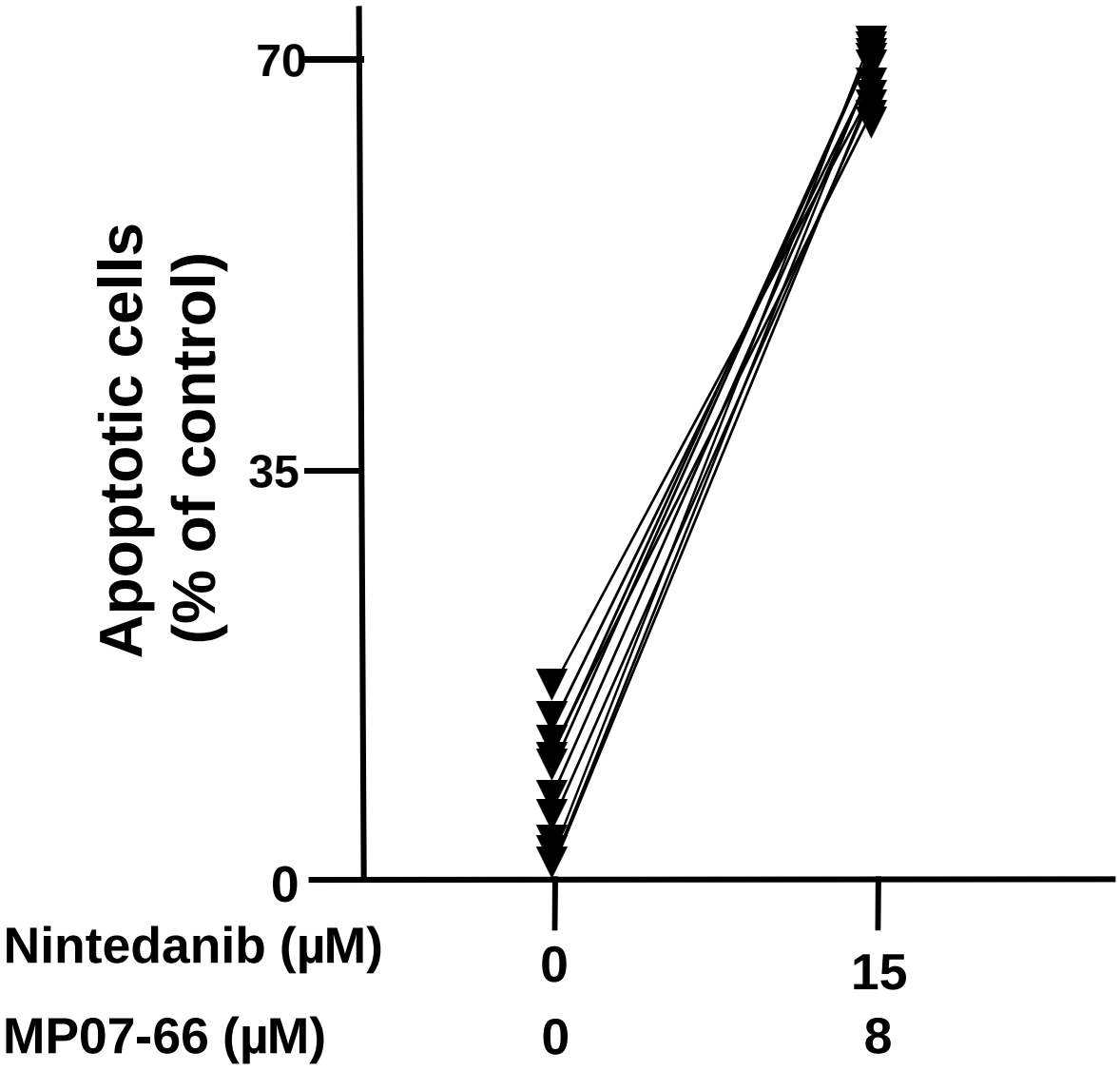
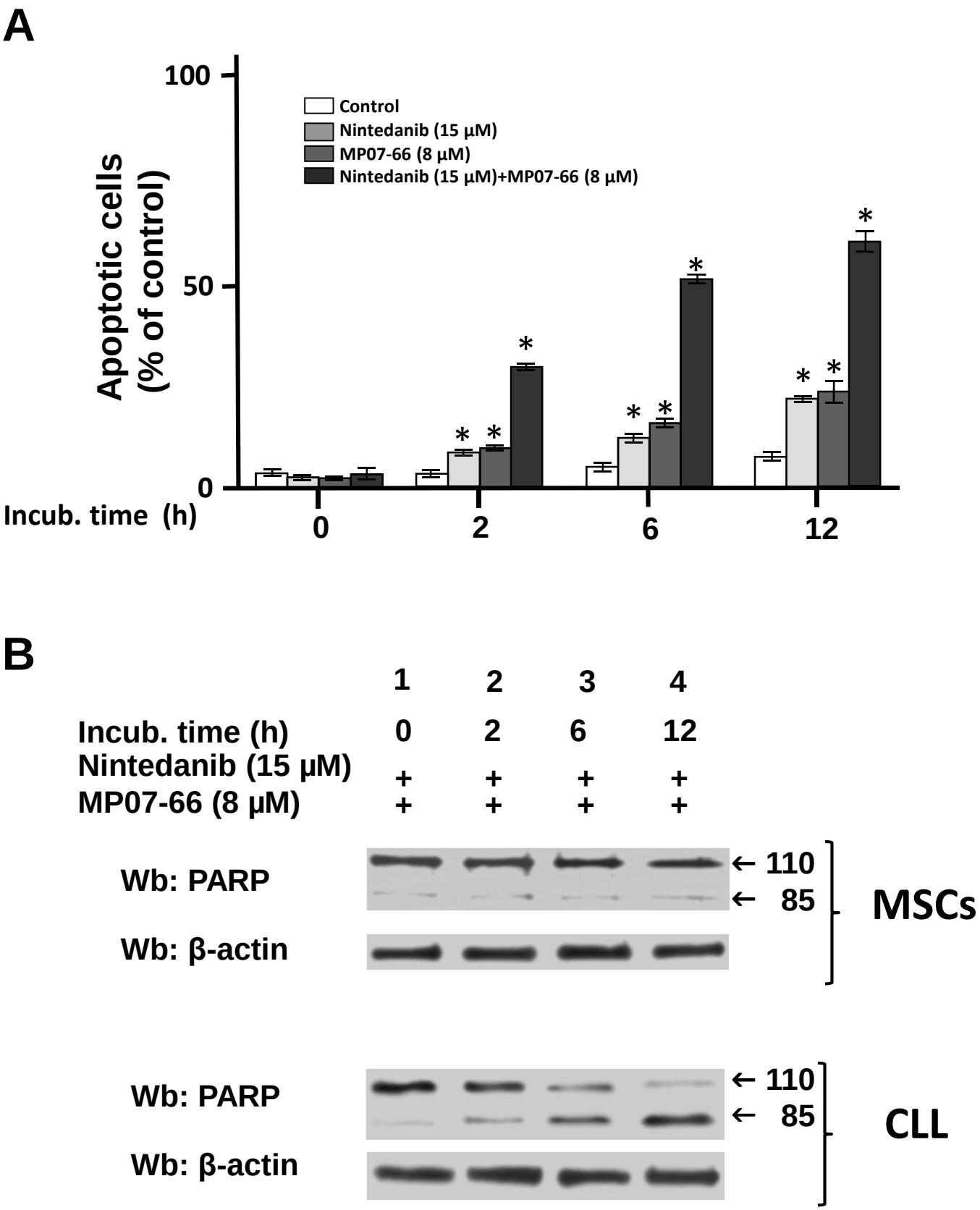

70
35
0
Apoptotic cells
(% of control)
Nintedanib (μM)
0
15
MP07-66 (μM)
0
8

Fig. S13


A
100
50
0
Apoptotic cells
(% of control)
Control
Nintedanib (15 μM)
MP07-66 (8 μM)
Nintedanib (15 μM)+MP07-66 (8 μM)
Incub. time (h)
0
2
6
12
B
1
2
3
4
Incub. time (h)
0
2
6
12
Nintedanib (15 μM)
+
+
+
+
MP07-66 (8 μM)
+
+
+
+
Wb: PARP
← 110
← 85
Wb: β-actin
MSCs
Wb: PARP
← 110
← 85
Wb: β-actin
CLL