Figure S1. Induction of apoptosis of FLT3-ITD-expressing cells by AFG206 and AFG210. Induction of apoptosis following an approximately 3-day treatment of FLT3-ITD-Ba/F3 cells (+/- IL-3) with AFG206 (A), AFG210 (B).

Figure S2. Induction of apoptosis of FLT3-ITD-expressing cells by AHL196. Induction of apoptosis following an approximately 3-day treatment of FLT3-ITD-Ba/F3 cells (+/- IL-3) with AHL196 (C).

Figure S3 (A-C). Inhibition of D835Y-FLT3 by first generation FLT3 inhibitors, AFG206 and AFG210: Effects of drug treatment on cell proliferation and FLT3 kinase activity. Approximately 3-day treatment of D835Y-Ba/F3 cells (+/- IL-3) with AFG206 (A) and AFG210 (B). (C) FLT3 I.P./western.

Figure S3 (D-E). Inhibition of STAT and MAPK signaling in FLT3-ITD-Ba/F3 cells by AFG206, AFG210, AUZ454, and PKC412. Whole cell lysate western, showing approximately 1.5 hr treatments of FLT3-ITD-Ba/F3 cells with AFG206 and AFG210 at 1 μM, each, and AUZ454 and PKC412 at 0.1 μM each. (D) pSTAT5 and total STAT5 immunoblot. (E) pMAPK and total MAPK immunoblot. FLT3 expression is shown in (D) and (E). β-actin was used as a loading control.

Figure S4. Inhibition of FLT3-ITD and D835Y-FLT3 by first generation FLT3 inhibitor, AHL196: Effects of drug treatment on cell proliferation and FLT3 kinase activity. (A) Approximately 3-day treatment of Ba/F3 cells and FLT3-ITD-Ba/F3 cells (+/- IL-3) with AHl196. (B) FLT3 I.P./western. (C) Approximately 3-day treatment of D835Y-Ba/F3 cells (+/- IL-3) with AHL196. (D) FTL3 I.P./western.

Figure S5. Induction of apoptosis of D835Y-expressing cells by AFG206 and AFG210. Induction of apoptosis following an approximately 3-day treatment of D835Y-Ba/F3 cells (+/- IL-3) with AFG206 (A), AFG210 (B).

Figure S6. Effects of combining AFG206 and PKC412 against FLT3-ITD-expressing cells. (A,B) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG206 +/- PKC412. Combination indices derived through Calcusyn analysis: For (A): ED25: 1.17352; ED50: 0.98134; ED75: 0.84594; ED90: 0.75205. For (B) ED25: 1.26327; ED50: 0.99946; ED75: 0.86489; ED90: 0.82517. Values 0.3-0.7 indicate synergy. Values 0.7-0.85 indicate moderate synergy. Values 0.85-0.90 indicate slight synergy. Values 0.9-1.1 indicate nearly additive effects. Values 1.10-1.20 indicate slight antagonism. Values 1.20-1.45 indicate moderate antagonism. Values 1.45-3.3 indicate antagonism. (C,D) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG206 +/- PKC412.

Figure S7. Effects of combining AFG210 and PKC412 against FLT3-ITD-expressing cells. (A,B) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG210 +/- PKC412. Combination indices derived through Calcusyn analysis: For (A): ED25: 0.71176; ED50: 0.75521; ED75: 0.80562; ED90: 0.86468. For (B): ED25: 1.38015; ED50: 1.19545; ED75: 1.03756; ED90: 0.90241. Values 0.3-0.7 indicate synergy. Values 0.7-0.85 indicate moderate synergy. Values 0.85-0.90 indicate slight synergy. Values 0.9-1.1 indicate nearly additive effects. Values 1.10-1.20 indicate slight antagonism. Values 1.20-1.45 indicate

moderate antagonism. Values 1.45-3.3 indicate antagonism. (C,D) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG210 +/- PKC412.

Figure S8. Inhibition of N841I-FLT3 by second generation FLT3 inhibitors, NVP-AUZ454 and NVP-ATH686: Effects on cell proliferation and FLT3 kinase activity. (A,B) Three-day treatment of FLT3-N841I-Ba/F3 cells, in the presence and absence of IL-3, with NVP-AUZ454 (A) and ATH686 (B). (C) Treatment of FLT3-N841I-Ba/F3 cells for 15 minutes with 10 or 100 nM NVP-ATH686 or NVP-AUZ454, respectively.

Figure S9. Inhibition of D835Y-FLT3 by second generation FLT3 inhibitors, NVP-AUZ454 and NVP-ATH686: Effects on cell proliferation and viability. Three-day treatment of D835Y-Ba/F3 cells, in the presence and absence of IL-3, with NVP-AUZ454 (A) and NVP-ATH686 (D). Parental Ba/F3 cells are treated as a control by both agents (A,D). Effects of NVP-AUZ454 (B) and NVP-ATH686 (B) on viability of D835Y-Ba/F3 cells, in the absence of IL-3. Effects of NVP-AUZ454 (C) and NVP-ATH686 (F) on viability of parental Ba/F3 cells following 3 days of treatment.

Figure S10. Human serum protein binding capacity of second generation FLT3 inhibitors. (A) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG206 in the presence of 10% human serum or 10% fetal calf serum. (B) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AFG210 in the presence of 10% human serum or 10% fetal calf serum. (C) Approximately 3-day treatment of FLT3-ITD-Ba/F3 cells with AHL196 in the presence of 10% human serum or 10% fetal calf serum. (D) Efficacy of NVP-AUZ454 against FLT3-ITD-Ba/F3 cells cultured for 66 hours in presence of fetal calf serum versus human serum. (E) Efficacy of NVP-ATH686 against FLT3-ITD-Ba/F3 cells cultured for 66 hours in presence of fetal calf serum versus human serum.

Figure S11. Inhibition of proliferation of mutant FLT3-positive AML patient cells by NVP-AUZ454 and NVP-ATH686. (A, B) Three day treatment of AML FLT3-ITD-expressing patient cells versus FLT3-ITD-Ba/F3 cells with NVP-AUZ454 (A) or NVP-ATH686 (B).

AML patient FLT3 mutation status (published previously, Weisberg et al., 2008)

AML4: ITD (42 bp), 89% variant allele

AML5: TKD, D835Y, 4% variant allele

AML patient disease status

Sample	Blood/Marrow	PT-ID	FAB	WBC	PB BLAST	HCT	BM BLAST	CHROM
AML4	marrow	63F	M4	179.4	72%	19.4	95%	NL
AML5	marrow	77M	M1	26.3	27%	23.2	92%	NL

Figure S12. Inhibition of FLT3-ITD and D835Y-FLT3 by "type I" FLT3 inhibitor, AAE871: Effects on cell proliferation and FLT3 kinase activity.

Figure S13 (A-B). Over-expression of mutant FLT3 in PKC412- and AAE871-resistant cells.

Figure S13 (C-D). STAT and MAPK signaling in AAE871-resistant cells.

Figure S14. Cross-resistance of "type I" FLT3 inhibitor-resistant mutant FLT3-expressing cells to "type II" first generation FLT3 inhibitor, AHL196. (A) Approximately 3-day treatment of FLT3-ITD-Ba/F3 and PKC412-resistant cells with AHL196.

Fig S1

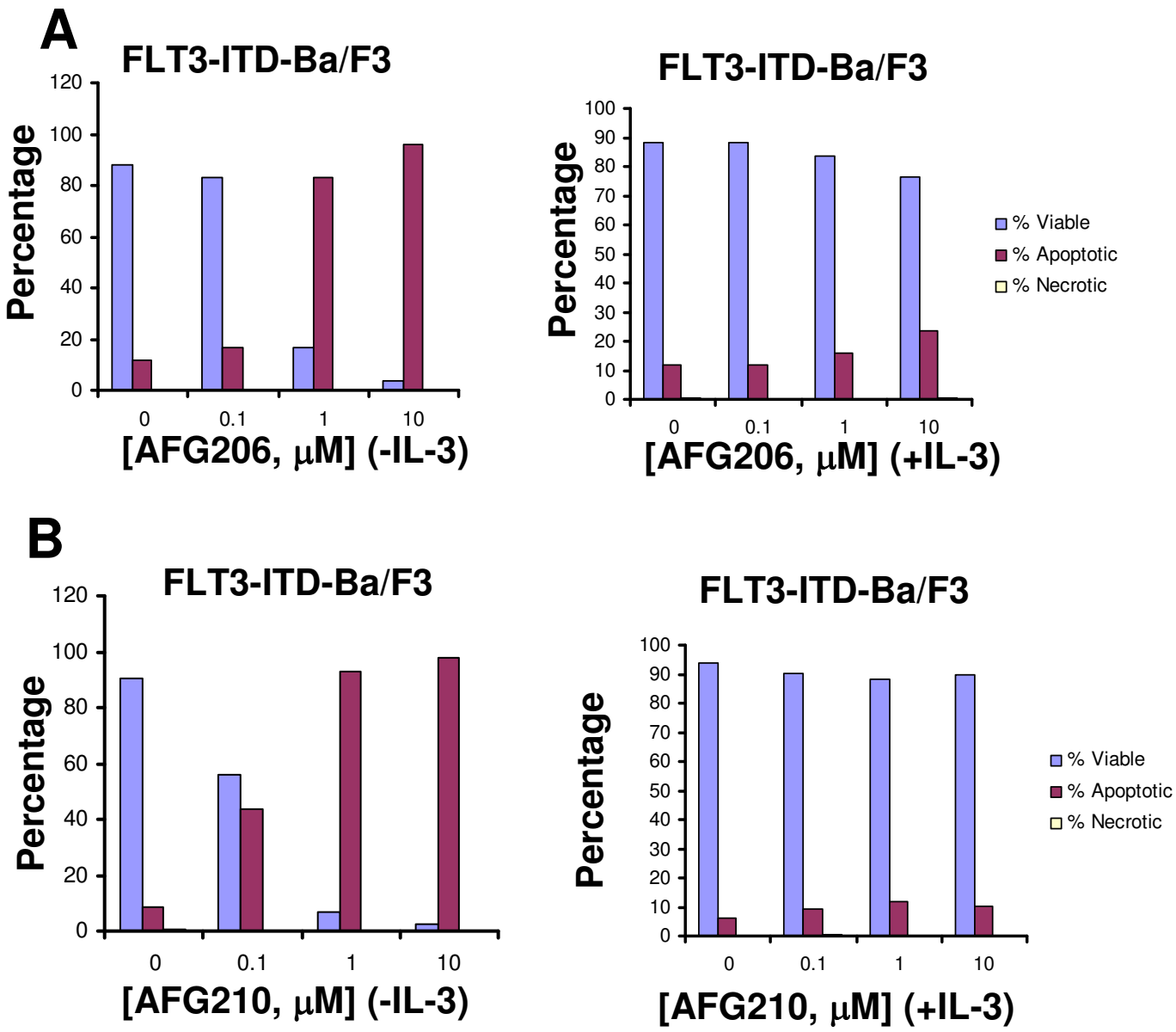

A
FLT3-ITD-Ba/F3
Percentage
120
100
80
60
40
20
0
0
0.1
1
10
[AFG206, μM] (-IL-3)
FLT3-ITD-Ba/F3
Percentage
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
[AFG206, μM] (+IL-3)
% Viable
% Apoptotic
% Necrotic
B
FLT3-ITD-Ba/F3
Percentage
120
100
80
60
40
20
0
0
0.1
1
10
[AFG210, μM] (-IL-3)
FLT3-ITD-Ba/F3
Percentage
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
[AFG210, μM] (+IL-3)
% Viable
% Apoptotic
% Necrotic

Fig S2

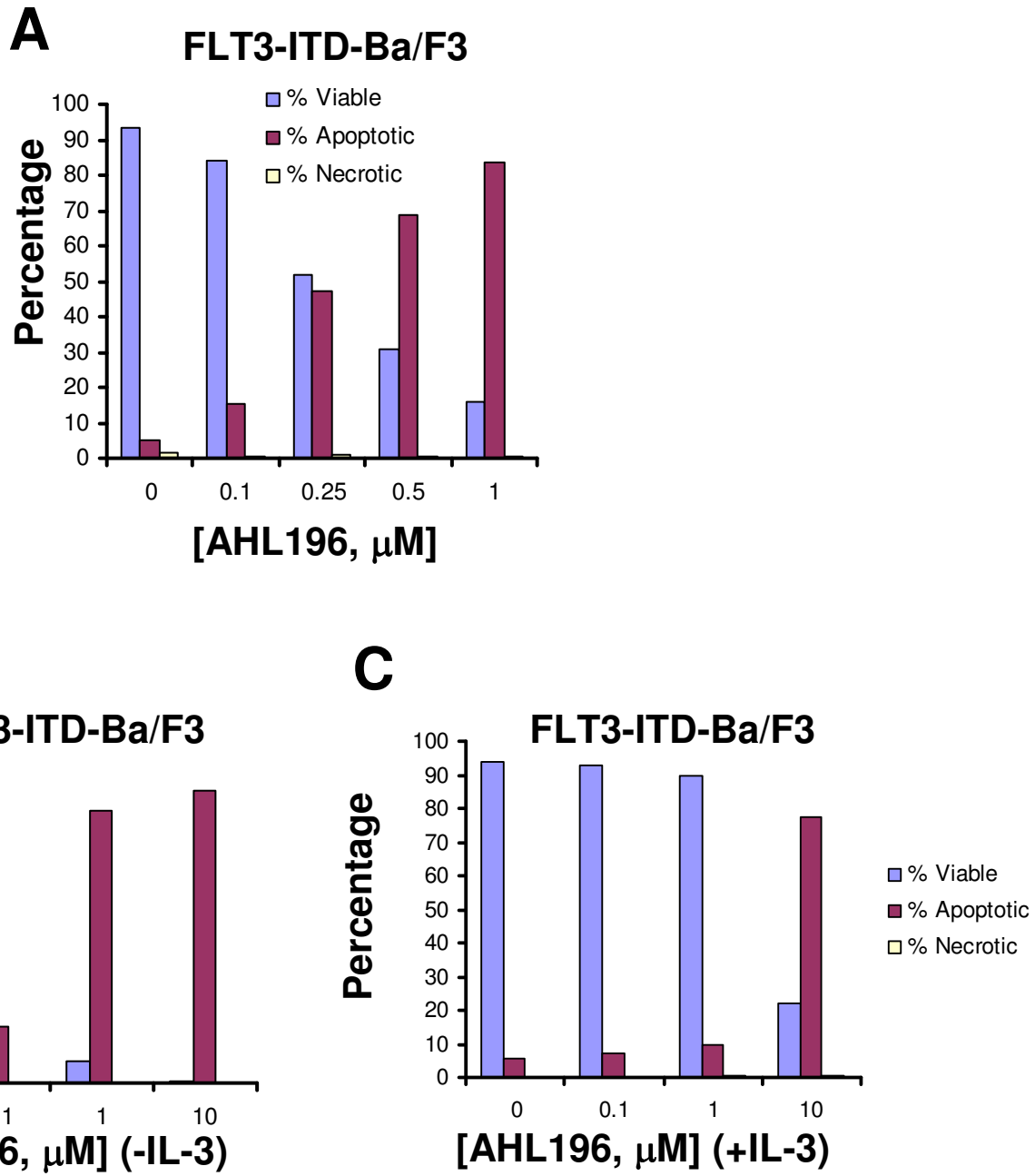

A
FLT3-ITD-Ba/F3
% Viable
% Apoptotic
% Necrotic
Percentage
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.25
0.5
1
[AHL196, μM]
B
FLT3-ITD-Ba/F3
Percentage
120
100
80
60
40
20
0
0
0.1
1
10
[AHL196, μM] (-IL-3)
C
FLT3-ITD-Ba/F3
Percentage
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
[AHL196, μM] (+IL-3)
% Viable
% Apoptotic
% Necrotic

Fig S3 (A-C)

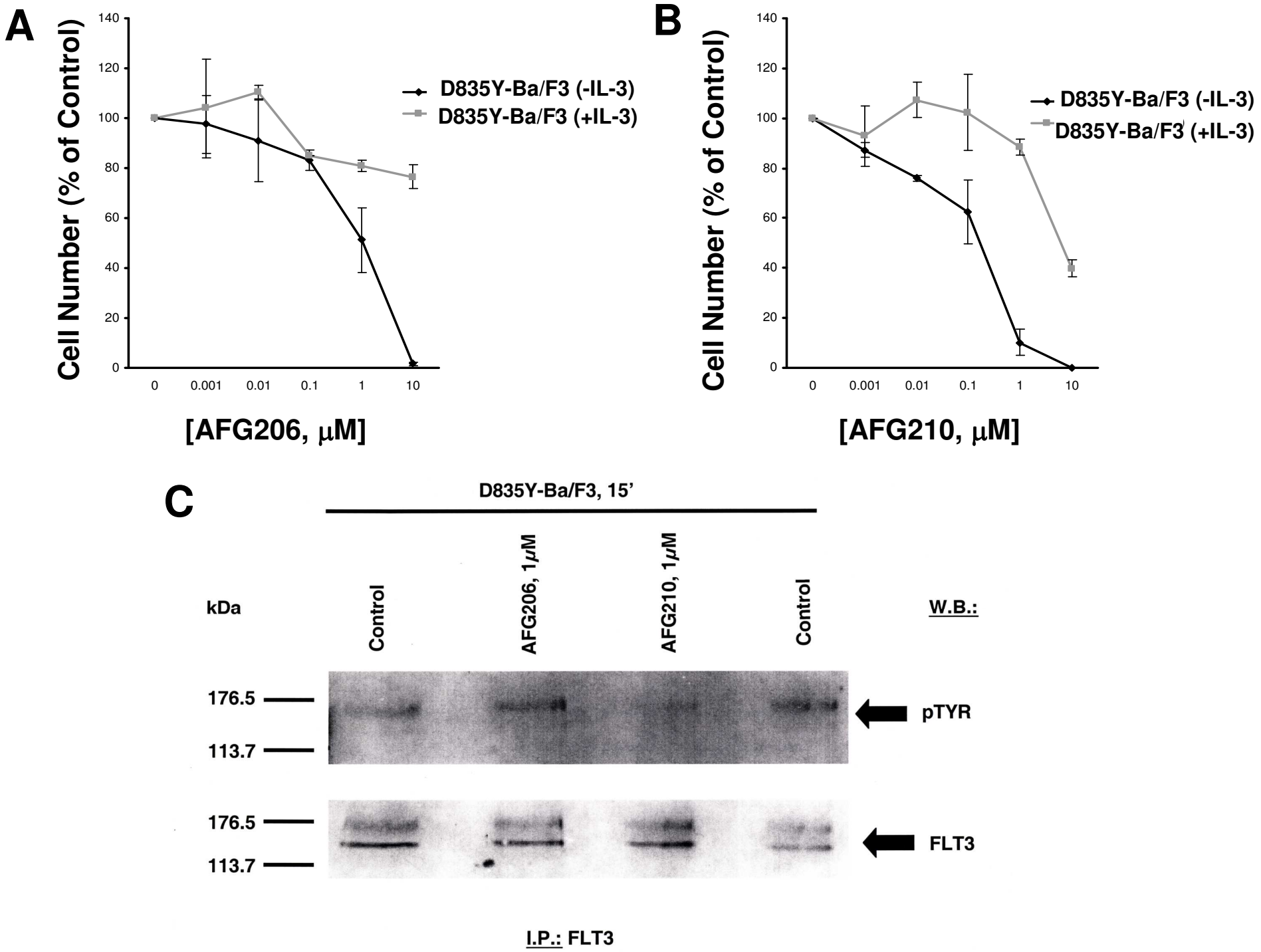

A
Cell Number (% of Control)
140
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
[AFG206, μM]
D835Y-Ba/F3 (-IL-3)
D835Y-Ba/F3 (+IL-3)
B
Cell Number (% of Control)
[AFG210, μM]
D835Y-Ba/F3 (-IL-3)
D835Y-Ba/F3 (+IL-3)
C
D835Y-Ba/F3, 15'
kDa
Control
AFG206, 1μM
AFG210, 1μM
Control
W.B.:
176.5
113.7
pTYR
176.5
113.7
FLT3
I.P.: FLT3

Fig S3 (D-E)

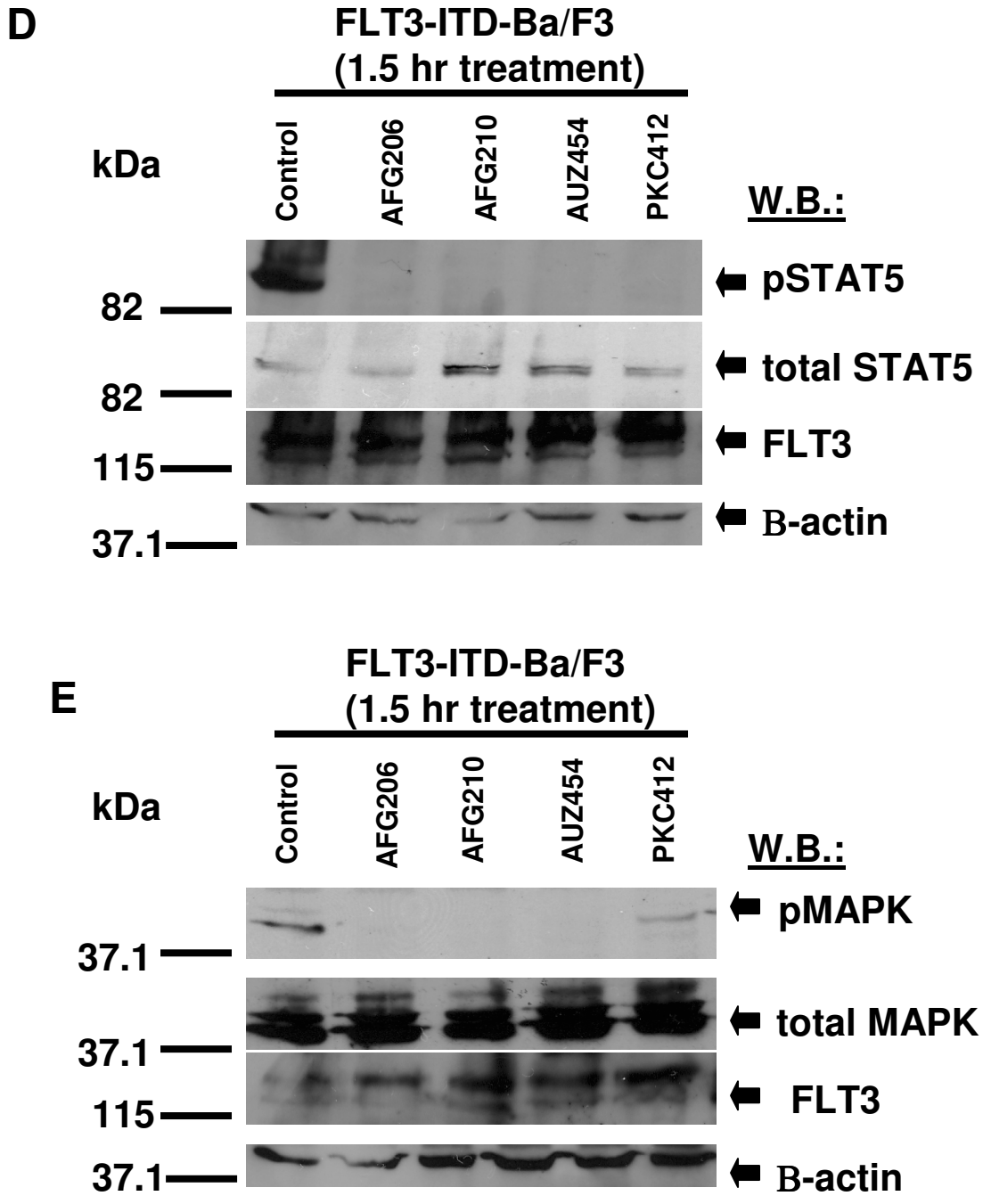

D
FLT3-ITD-Ba/F3
(1.5 hr treatment)
kDa
Control
AFG206
AFG210
AUZ454
PKC412
W.B.:
82
pSTAT5
82
total STAT5
115
FLT3
37.1
B-actin
E
FLT3-ITD-Ba/F3
(1.5 hr treatment)
kDa
Control
AFG206
AFG210
AUZ454
PKC412
W.B.:
37.1
pMAPK
37.1
total MAPK
115
FLT3
37.1
B-actin

Fig S4

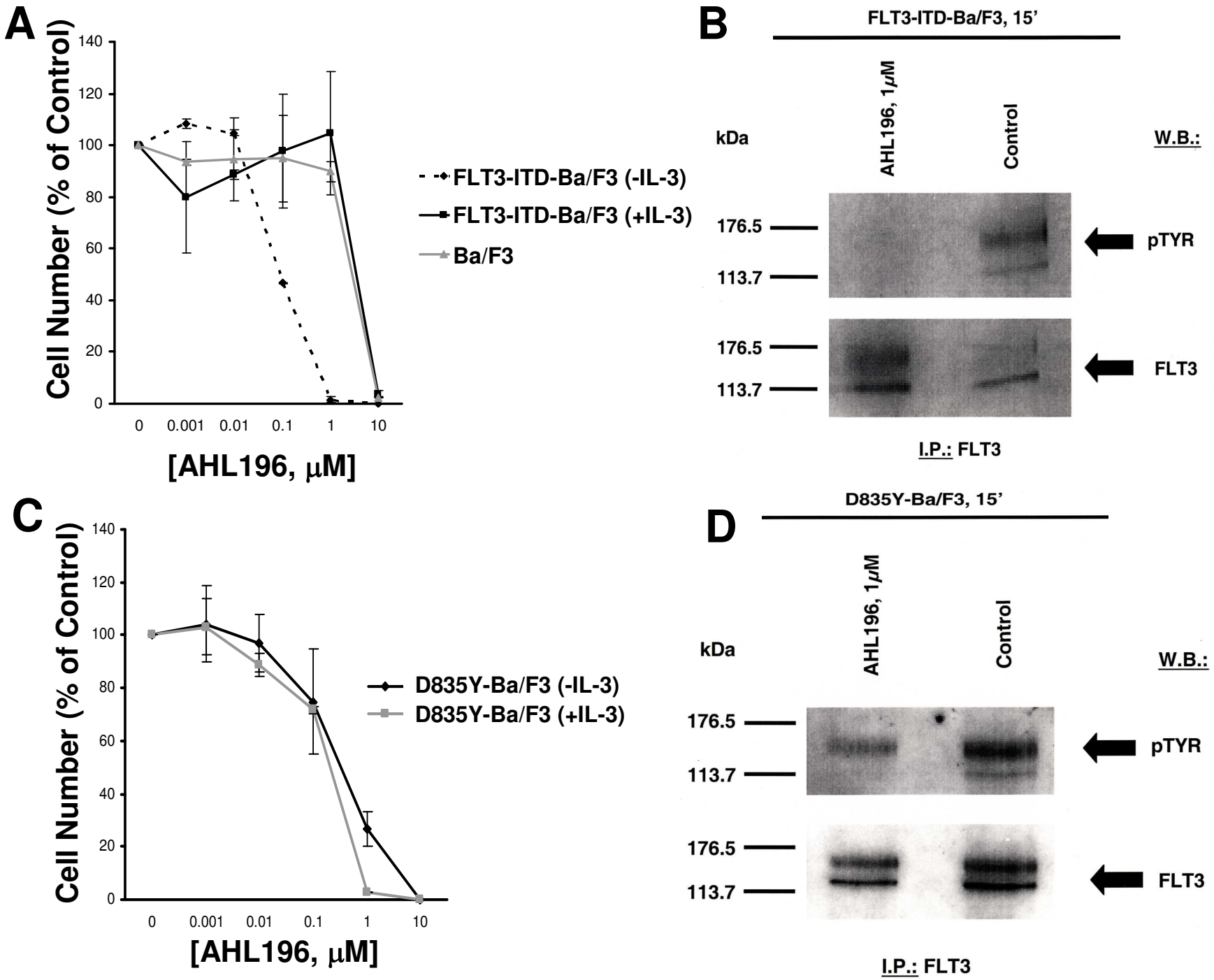

A
Cell Number (% of Control)
140
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
[AHL196, μM]
FLT3-ITD-Ba/F3 (-IL-3)
FLT3-ITD-Ba/F3 (+IL-3)
Ba/F3
B
FLT3-ITD-Ba/F3, 15'
AHL196, 1μM
Control
kDa
W.B.:
176.5
113.7
pTYR
176.5
113.7
FLT3
I.P.: FLT3
C
Cell Number (% of Control)
140
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
[AHL196, μM]
D835Y-Ba/F3 (-IL-3)
D835Y-Ba/F3 (+IL-3)
D
D835Y-Ba/F3, 15'
AHL196, 1μM
Control
kDa
W.B.:
176.5
113.7
pTYR
176.5
113.7
FLT3
I.P.: FLT3

Fig S5

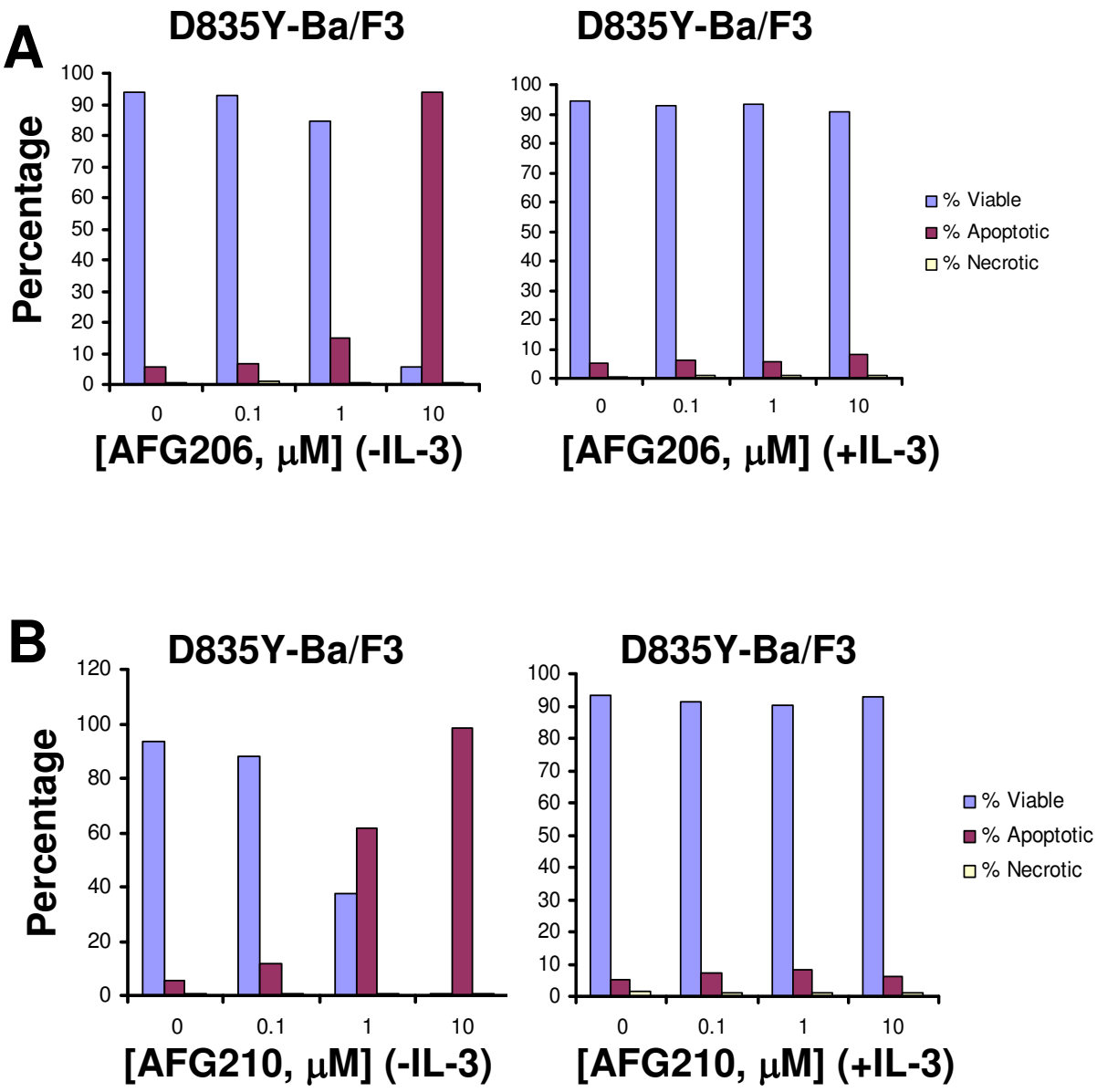

A
D835Y-Ba/F3
D835Y-Ba/F3
Percentage
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
[AFG206, μM] (-IL-3)
[AFG206, μM] (+IL-3)
% Viable
% Apoptotic
% Necrotic
B
D835Y-Ba/F3
D835Y-Ba/F3
Percentage
120
100
80
60
40
20
0
[AFG210, μM] (-IL-3)
[AFG210, μM] (+IL-3)

Fig S6

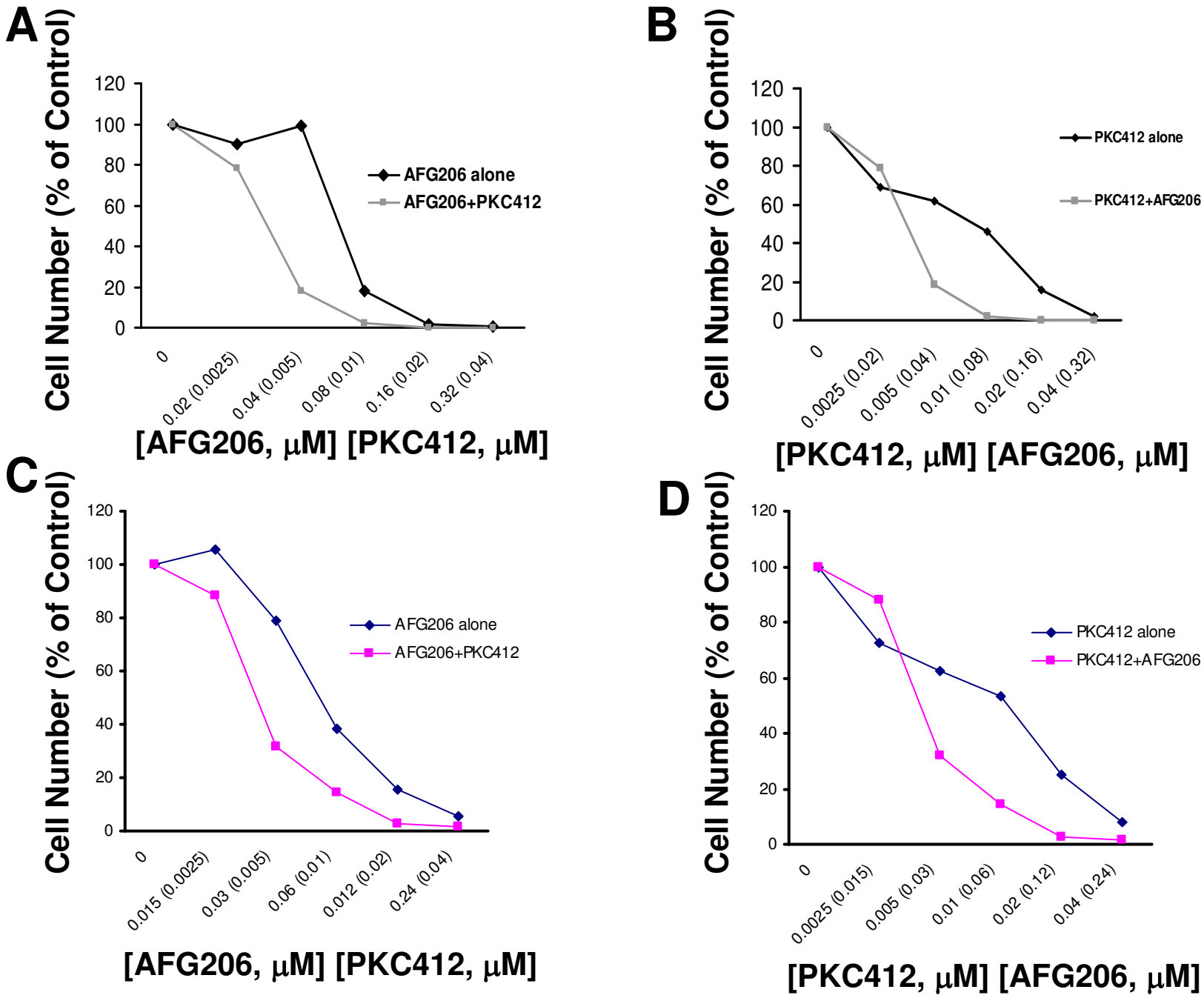

A
Cell Number (% of Control)
120
100
80
60
40
20
0
AFG206 alone
AFG206+PKC412
0
0.02 (0.0025)
0.04 (0.005)
0.08 (0.01)
0.16 (0.02)
0.32 (0.04)
[AFG206, μM] [PKC412, μM]
B
Cell Number (% of Control)
120
100
80
60
40
20
0
PKC412 alone
PKC412+AFG206
0
0.0025 (0.02)
0.005 (0.04)
0.01 (0.08)
0.02 (0.16)
0.04 (0.32)
[PKC412, μM] [AFG206, μM]
C
Cell Number (% of Control)
120
100
80
60
40
20
0
AFG206 alone
AFG206+PKC412
0
0.015 (0.0025)
0.03 (0.005)
0.06 (0.01)
0.012 (0.02)
0.24 (0.04)
[AFG206, μM] [PKC412, μM]
D
Cell Number (% of Control)
120
100
80
60
40
20
0
PKC412 alone
PKC412+AFG206
0
0.0025 (0.015)
0.005 (0.03)
0.01 (0.06)
0.02 (0.12)
0.04 (0.24)
[PKC412, μM] [AFG206, μM]

Fig S7

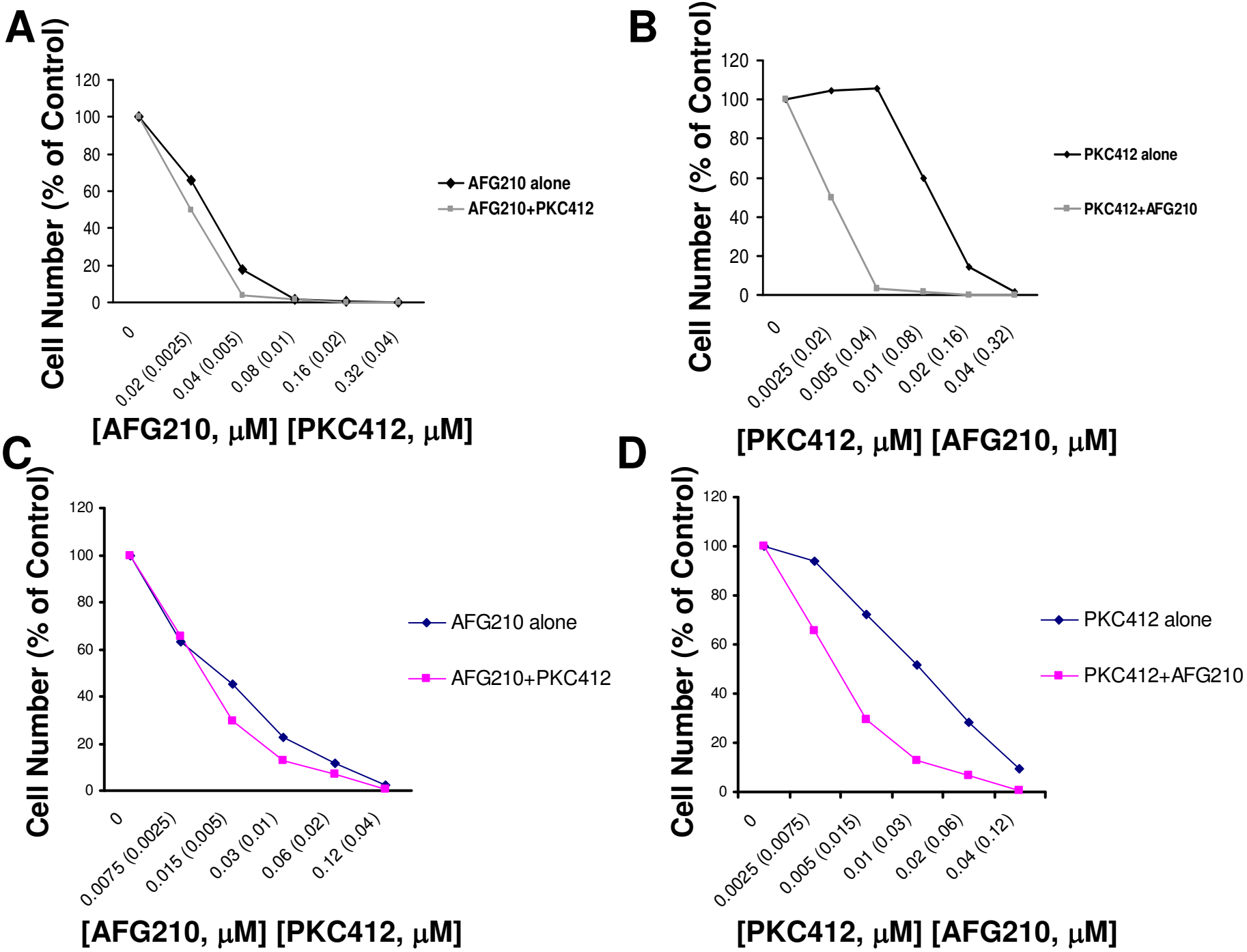

A
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.02 (0.0025)
0.04 (0.005)
0.08 (0.01)
0.16 (0.02)
0.32 (0.04)
[AFG210, μM] [PKC412, μM]
AFG210 alone
AFG210+PKC412
B
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.0025 (0.02)
0.005 (0.04)
0.01 (0.08)
0.02 (0.16)
0.04 (0.32)
[PKC412, μM] [AFG210, μM]
PKC412 alone
PKC412+AFG210
C
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.0075 (0.0025)
0.015 (0.005)
0.03 (0.01)
0.06 (0.02)
0.12 (0.04)
[AFG210, μM] [PKC412, μM]
AFG210 alone
AFG210+PKC412
D
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.0025 (0.0075)
0.005 (0.015)
0.01 (0.03)
0.02 (0.06)
0.04 (0.12)
[PKC412, μM] [AFG210, μM]
PKC412 alone
PKC412+AFG210

Fig S8

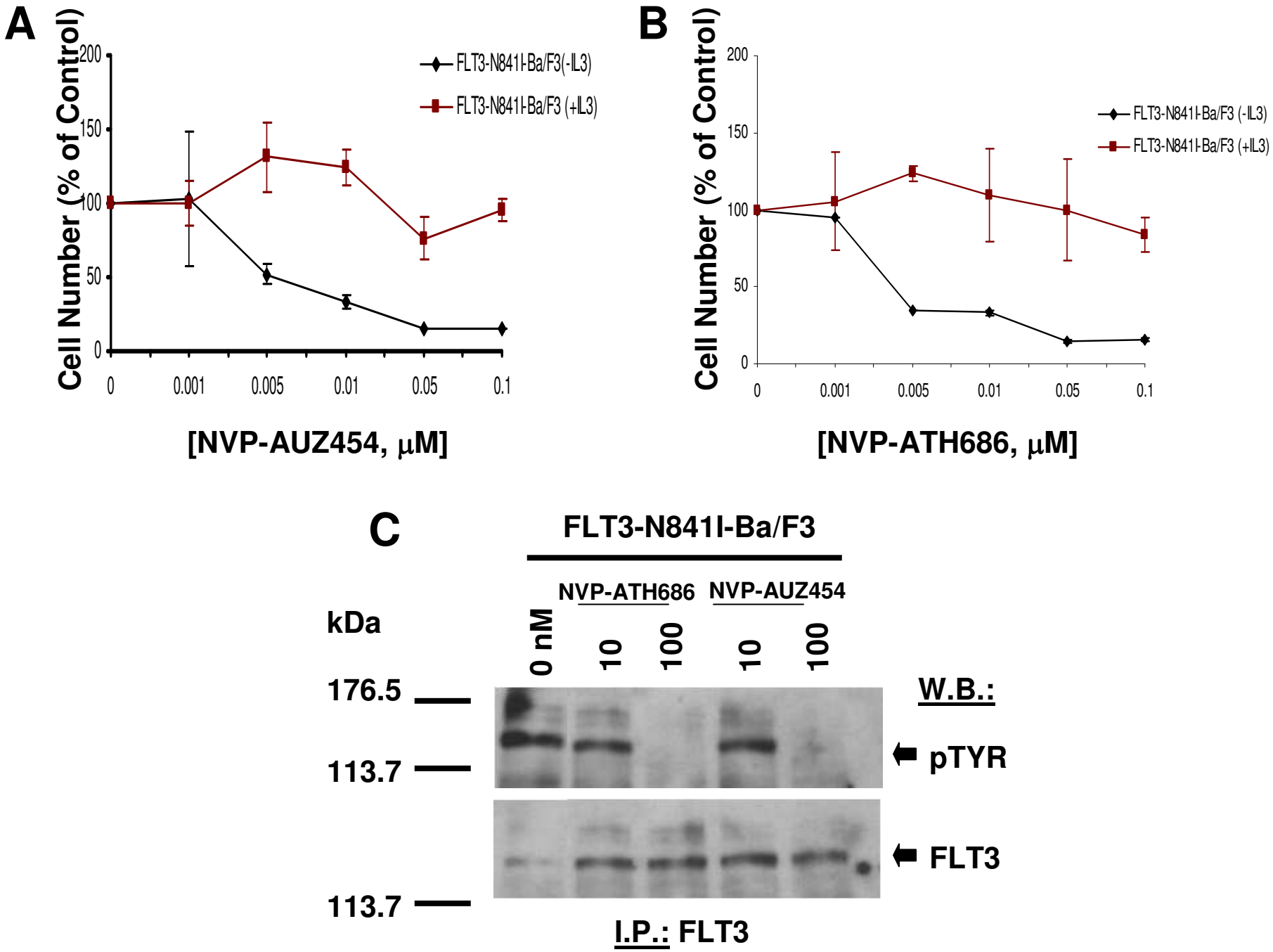

A
Cell Number (% of Control)
200
150
100
50
0
0
0.001
0.005
0.01
0.05
0.1
FLT3-N841I-Ba/F3(-IL3)
FLT3-N841I-Ba/F3 (+IL3)
[NVP-AUZ454, μM]
B
Cell Number (% of Control)
200
150
100
50
0
0
0.001
0.005
0.01
0.05
0.1
FLT3-N841I-Ba/F3 (-IL3)
FLT3-N841I-Ba/F3 (+IL3)
[NVP-ATH686, μM]
C
FLT3-N841I-Ba/F3
NVP-ATH686
NVP-AUZ454
kDa
0 nM
10
100
10
100
176.5
113.7
113.7
W.B.:
pTYR
FLT3
I.P.: FLT3

Fig S9

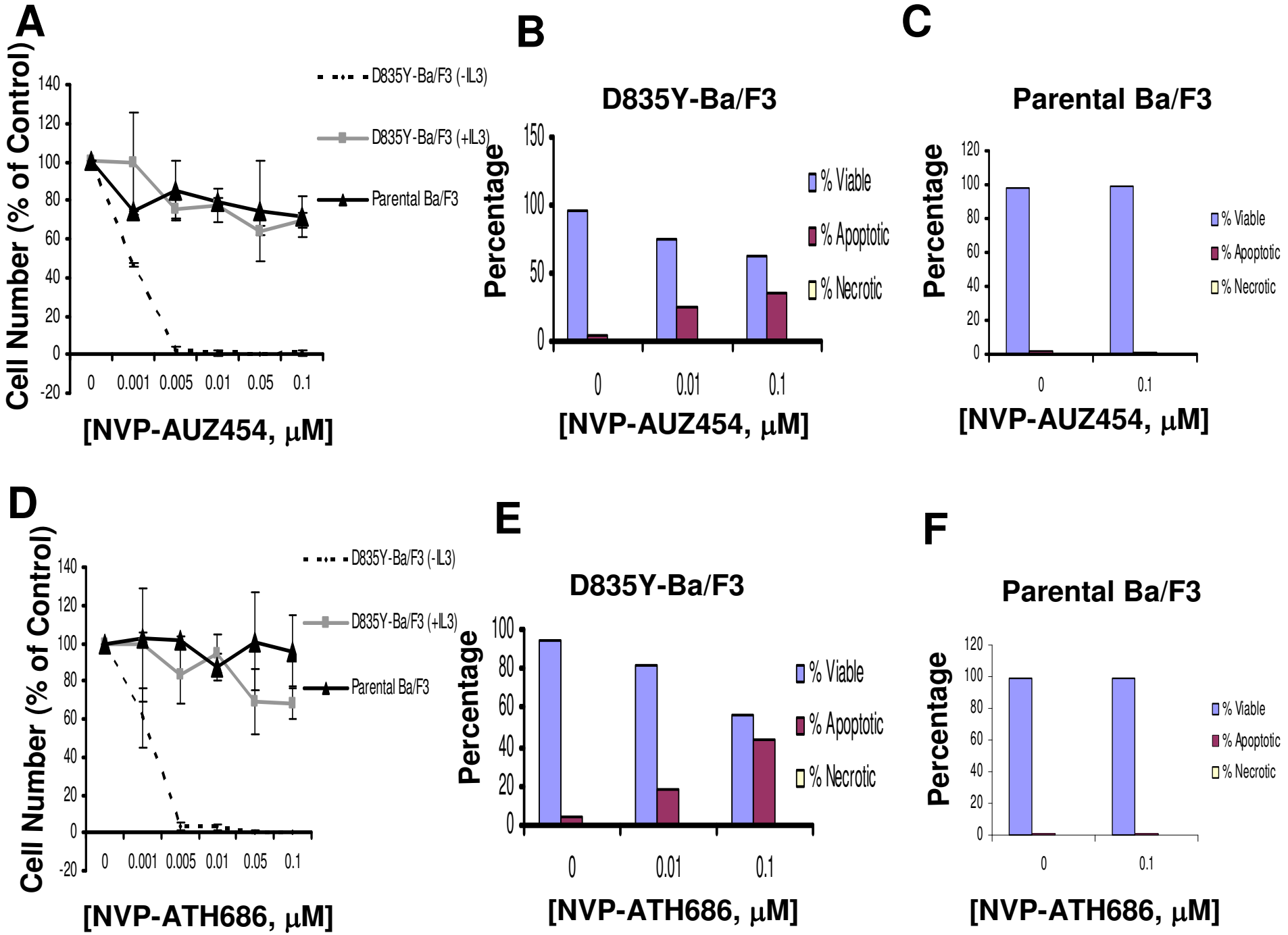

A
Cell Number (% of Control)
D835Y-Ba/F3 (-IL3)
D835Y-Ba/F3 (+IL3)
Parental Ba/F3
[NVP-AUZ454, μM]
B
D835Y-Ba/F3
Percentage
% Viable
% Apoptotic
% Necrotic
[NVP-AUZ454, μM]
C
Parental Ba/F3
Percentage
% Viable
% Apoptotic
% Necrotic
[NVP-AUZ454, μM]
D
Cell Number (% of Control)
D835Y-Ba/F3 (-IL3)
D835Y-Ba/F3 (+IL3)
Parental Ba/F3
[NVP-ATH686, μM]
E
D835Y-Ba/F3
Percentage
% Viable
% Apoptotic
% Necrotic
[NVP-ATH686, μM]
F
Parental Ba/F3
Percentage
% Viable
% Apoptotic
% Necrotic
[NVP-ATH686, μM]

Fig S10

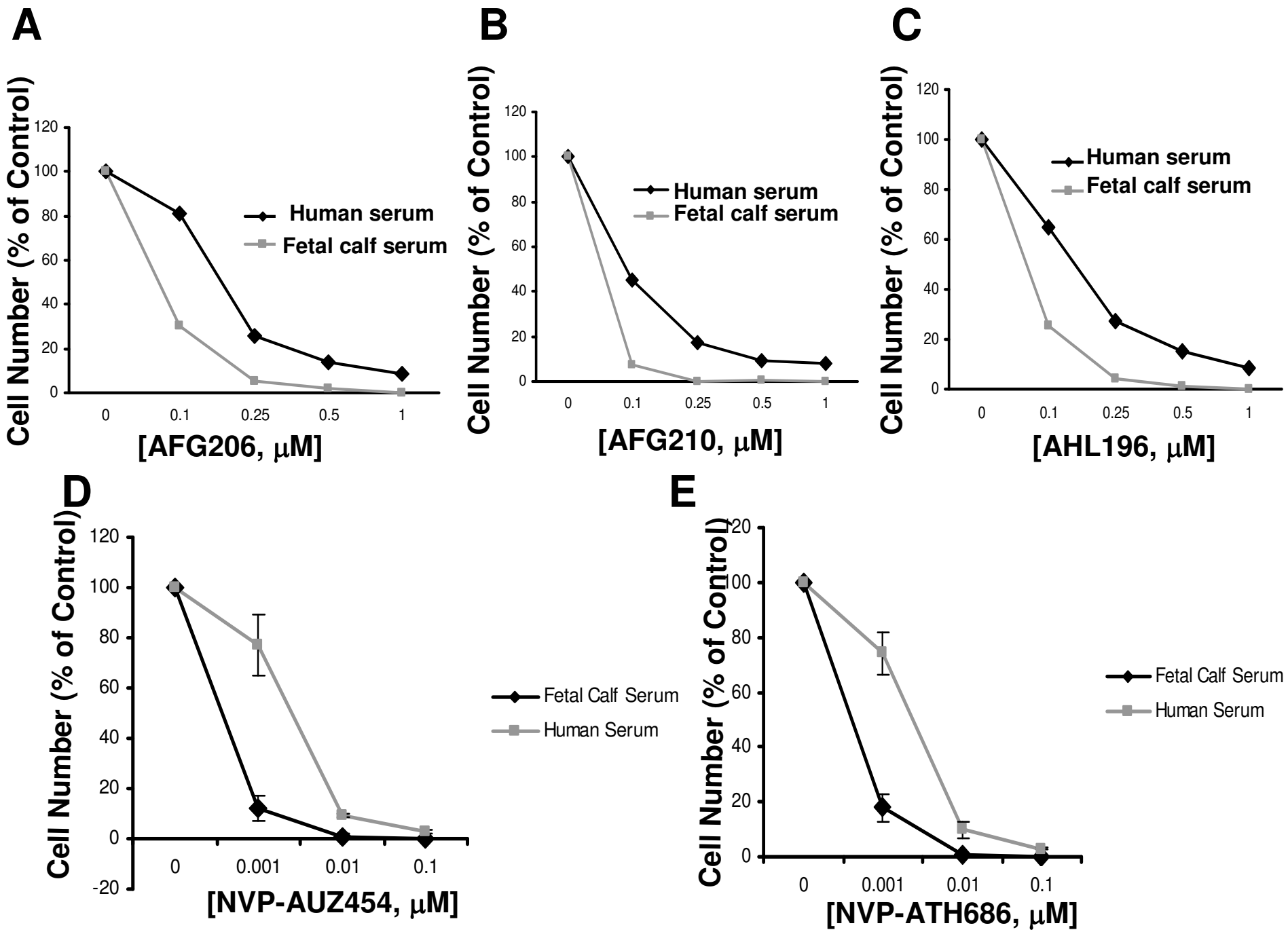

A
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.1
0.25
0.5
1
[AFG206, μM]
Human serum
Fetal calf serum
B
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.1
0.25
0.5
1
[AFG210, μM]
Human serum
Fetal calf serum
C
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.1
0.25
0.5
1
[AHL196, μM]
Human serum
Fetal calf serum
D
Cell Number (% of Control)
120
100
80
60
40
20
0
-20
0
0.001
0.01
0.1
[NVP-AUZ454, μM]
Fetal Calf Serum
Human Serum
E
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.001
0.01
0.1
[NVP-ATH686, μM]
Fetal Calf Serum
Human Serum

Fig S11

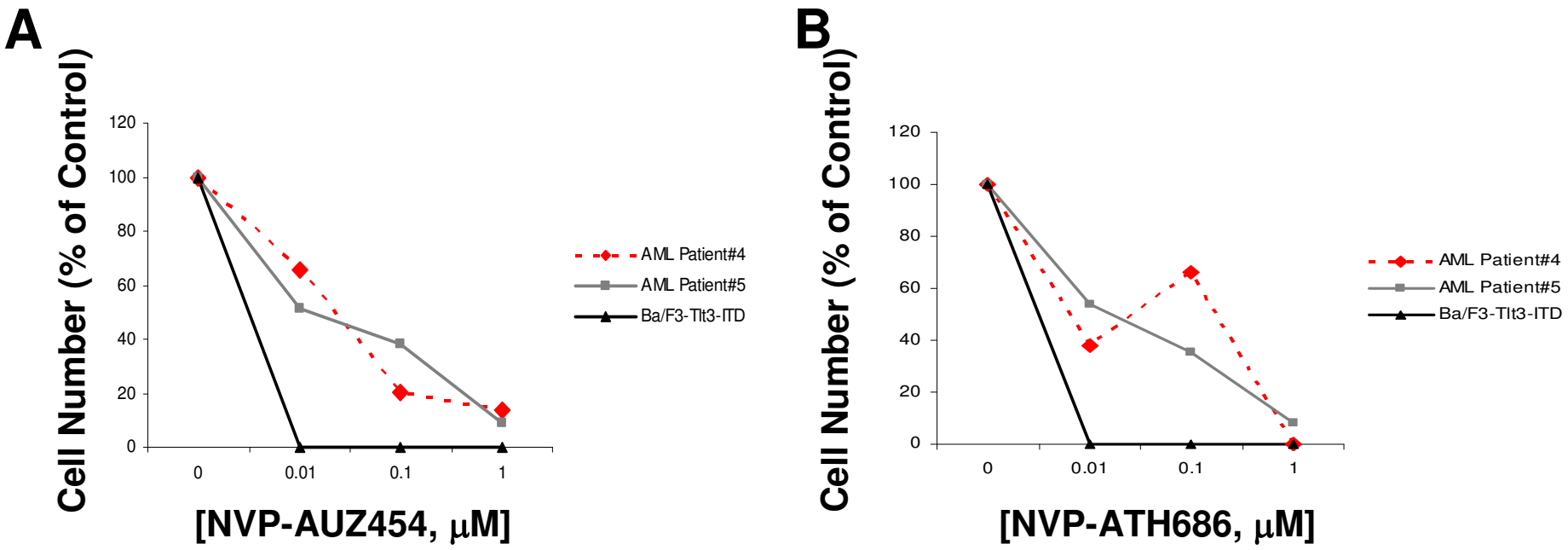
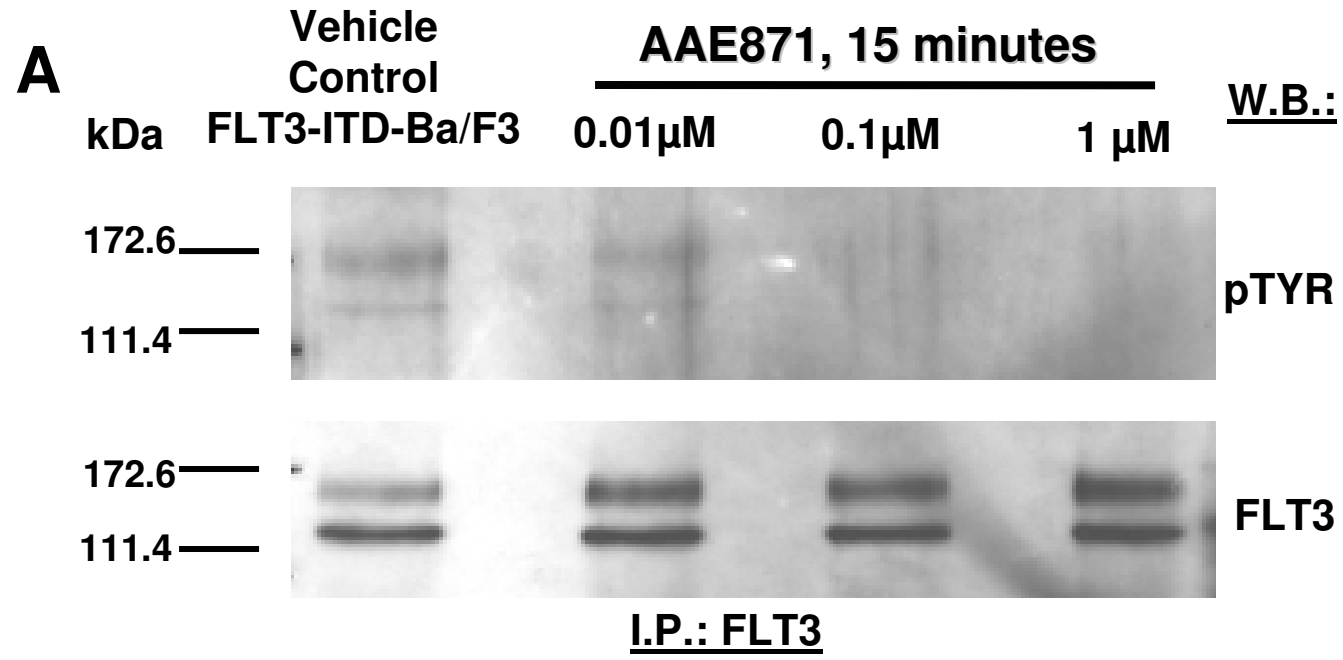
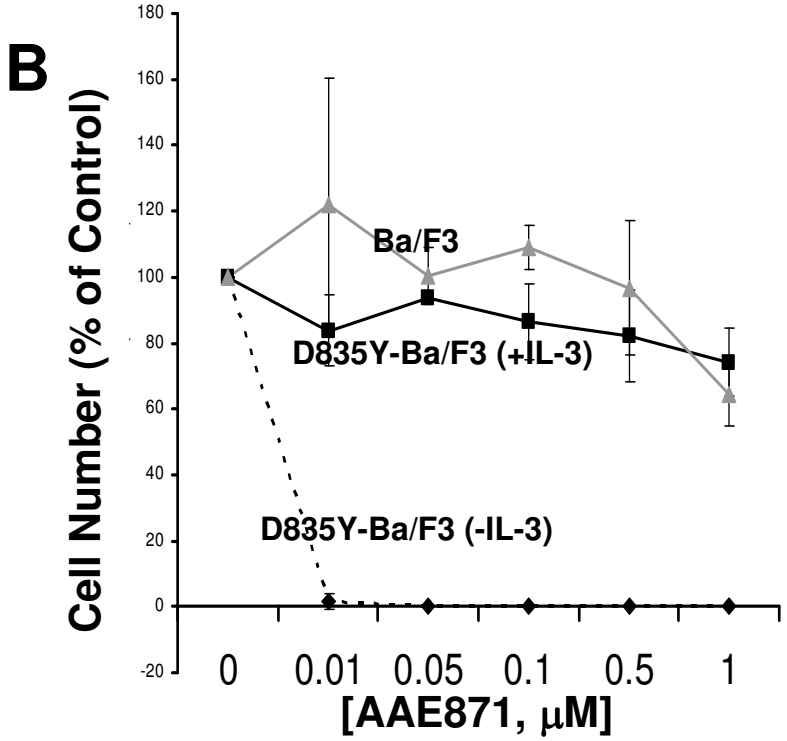

A
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.01
0.1
1
[NVP-AUZ454, μM]
AML Patient#4
AML Patient#5
Ba/F3-Tlt3-ITD
B
Cell Number (% of Control)
120
100
80
60
40
20
0
0
0.01
0.1
1
[NVP-ATH686, μM]
AML Patient#4
AML Patient#5
Ba/F3-Tlt3-ITD

Fig S12


A
Vehicle Control FLT3-ITD-Ba/F3
AAE871, 15 minutes
kDa
0.01μM
0.1μM
1 μM
W.B.:
172.6
111.4
pTYR
172.6
111.4
FLT3
I.P.: FLT3


B
Cell Number (% of Control)
180
160
140
120
100
80
60
40
20
0
-20
Ba/F3
D835Y-Ba/F3 (+IL-3)
D835Y-Ba/F3 (-IL-3)
0
0.01
0.05
0.1
0.5
1
[AAE871, μM]

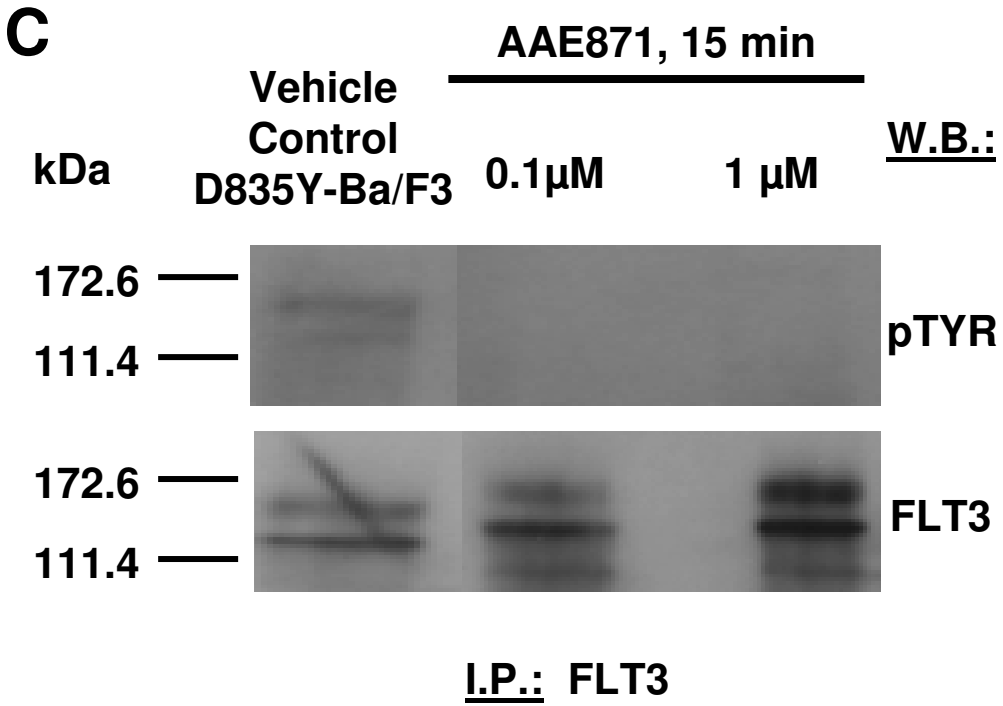
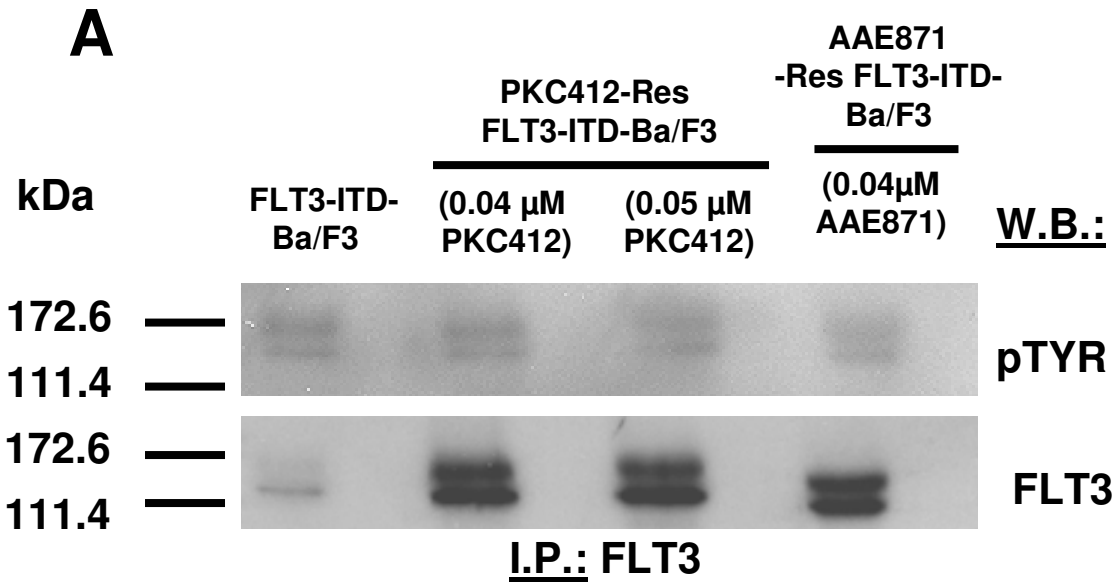

C
AAE871, 15 min
Vehicle Control D835Y-Ba/F3
kDa
0.1μM
1 μM
W.B.:
172.6
111.4
pTYR
172.6
111.4
FLT3
I.P.: FLT3

Fig S13 (A-B)


A
PKC412-Res FLT3-ITD-Ba/F3
AAE871 -Res FLT3-ITD-Ba/F3
kDa
FLT3-ITD-Ba/F3
(0.04 µM PKC412)
(0.05 µM PKC412)
(0.04µM AAE871)
W.B.:
172.6
111.4
pTYR
172.6
111.4
FLT3
I.P.: FLT3

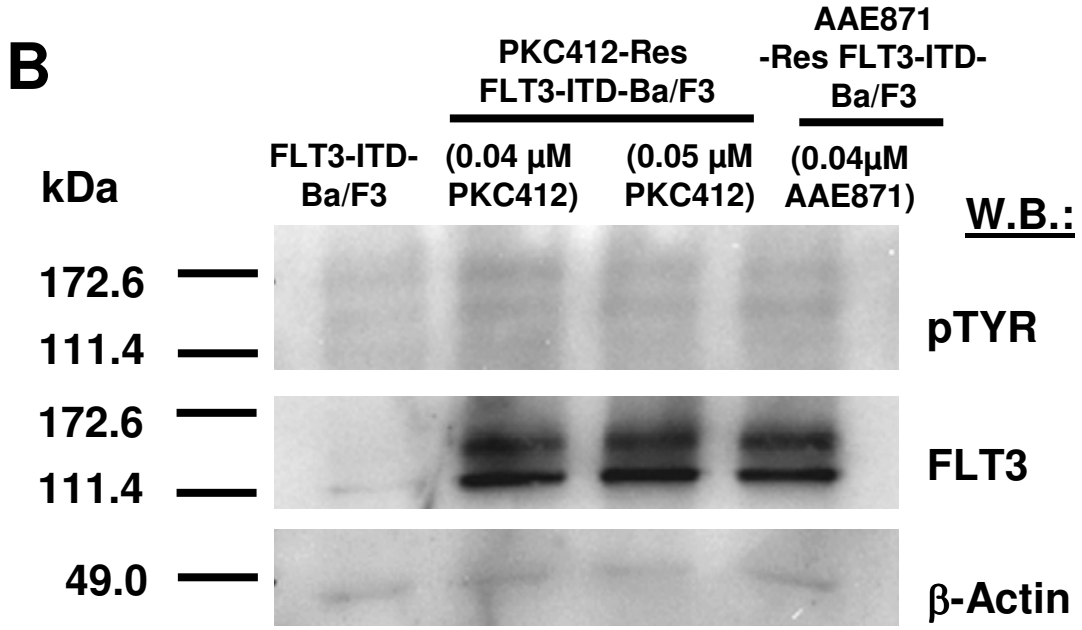

B
PKC412-Res FLT3-ITD-Ba/F3
AAE871 -Res FLT3-ITD-Ba/F3
kDa
FLT3-ITD-Ba/F3
(0.04 µM PKC412)
(0.05 µM PKC412)
(0.04µM AAE871)
W.B.:
172.6
111.4
pTYR
172.6
111.4
FLT3
49.0
β-Actin

Fig S13 (C-D)

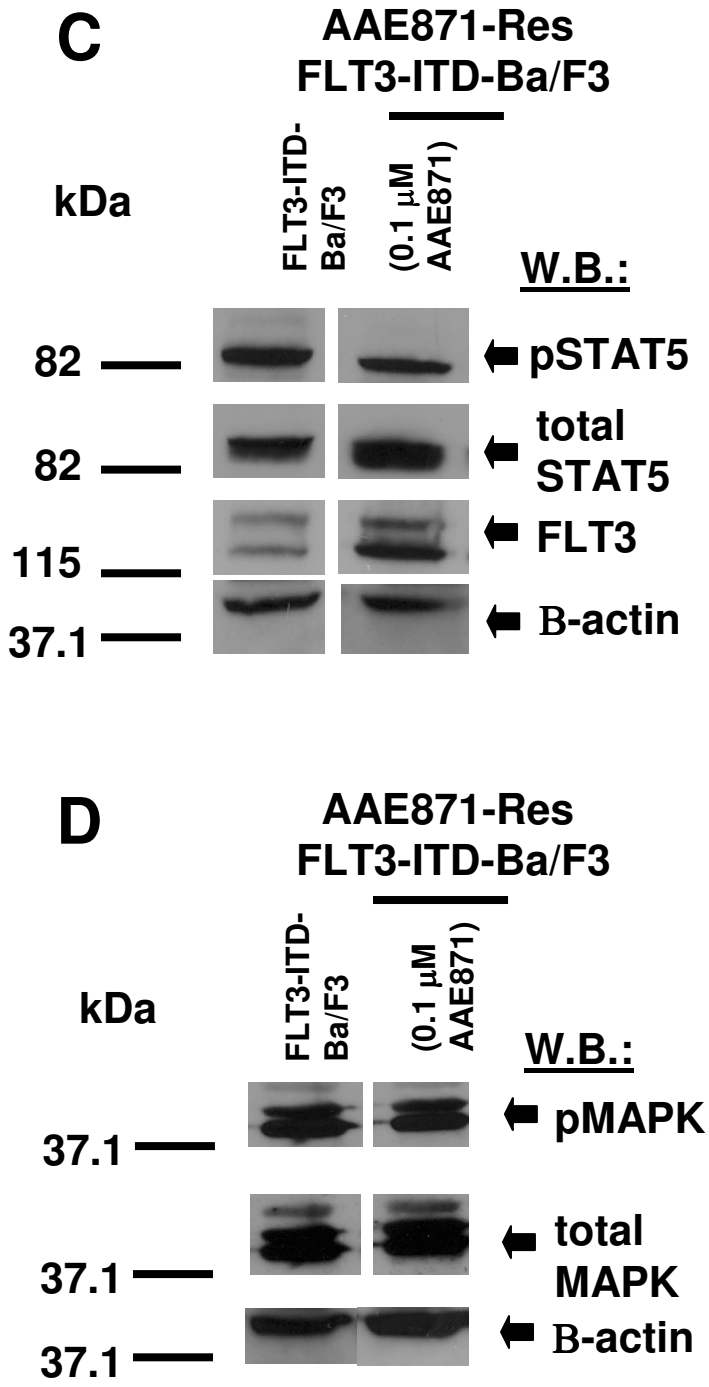
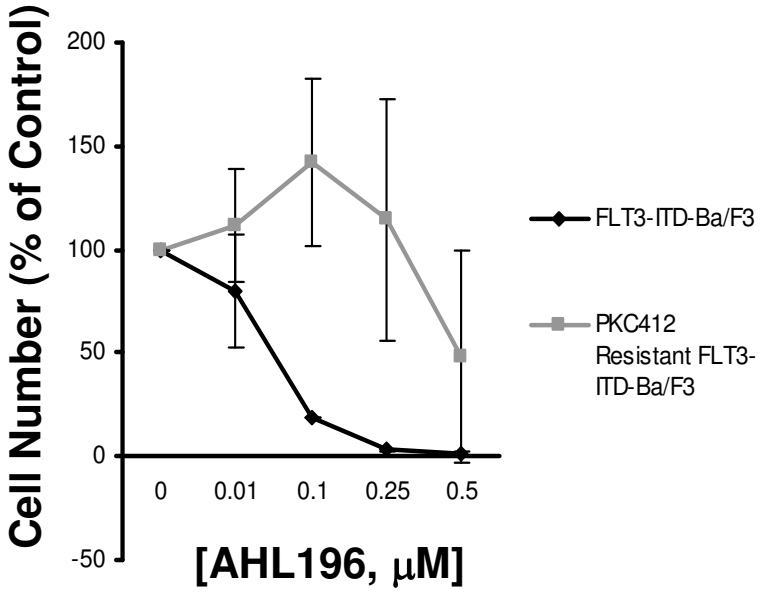

C
AAE871-Res
FLT3-ITD-Ba/F3
kDa
FLT3-ITD-Ba/F3
(0.1 μM AAE871)
W.B.:
82
pSTAT5
82
total STAT5
115
FLT3
37.1
B-actin
D
AAE871-Res
FLT3-ITD-Ba/F3
kDa
FLT3-ITD-Ba/F3
(0.1 μM AAE871)
W.B.:
37.1
pMAPK
37.1
total MAPK
37.1
B-actin

Fig S14


Cell Number (% of Control)
200
150
100
50
0
-50
0
0.01
0.1
0.25
0.5
[AHL196, μM]
FLT3-ITD-Ba/F3
PKC412 Resistant FLT3-ITD-Ba/F3