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Supporting Information

Synergy and Antagonism between Allosteric and Active-Site Inhibitors of Abl Tyrosine Kinase

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I. Materials and Methods.

General Biochemical Methods. Black, opaque-bottom 96 well plates were purchased from Nunc. All proteins were expressed in *E.coli* using previously published procedures.[24] Data were obtained using Biotek Synergy Mx and Biotek Synergy 4 plate readers. Curve fitting was done using Graphpad Prism 6 software.

General Procedure for Proteolysis Half-Life Determination. Assays employed a final concentration of 2 μM abl, 10 μM compound, and 60 nM thermolysin (Promega, V4001) in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM $CaCl_2$. Compounds and enzyme were allowed to equilibrate for 5 minutes at 20 °C prior to the addition of thermolysin. Reactions were sampled at various time points (2, 5, 10, 30, 60, 90, 120, 180, and 240 minutes) and quenched with 12.5 mM EDTA. Samples were analyzed using a PerkinElmer LabChip GX II with LabChip HT Protein Express Chips as per the manufacturer's instructions. Percent protein remaining was plotted versus time and fit to an exponential one phase decay equation using GraphPad Prism software (version 8.2) to obtain half-lives of each protein.

***General procedure for cellular characterization*.**
1. Cell culture and seeding: All Ba/F3 and K562 cell lines were cultured in RPMI 1640 media with 10% FBS. Parental Ba/F3 cell culture additionally contained 15% WEHI-3 conditioned media. An aliquot of the cells was mixed with Trypan Blue solution and the cell number was quantified using a hemocytometer. The cells were plated 100 μL in each well at 30,000 cells/mL so that each well contained 3,000 cells. The cells were plated into sterile, clear bottom 96 well plates and then immediately dosed with compound. Additionally, 3 wells were created containing 100 μL of media with no cells.
2. Dosing: The compounds were made in 100% DMSO at 1,000X the final concentrations that were desired for the assay generally covering a concentration range of 6 log units. These DMSO stocks were diluted 10X in RPMI 1640 media. 1 μL of the compound diluted in media was added to each well for a final concentration of 0.1% DMSO. The wells containing only media were not dosed. In general, each compound concentration was dosed in triplicate wells. The plates were returned to normal culture conditions (per ATCC) for 72 hours.
3. Assay: After 72 hours, the plates were removed from the incubator, and 10 μL of WST-1 reagent was added to each well. The plates were returned to the incubator and the color change was visually monitored for 0.5 – 2 hours. When sufficient color change had occurred, the plates were shaken on a plate shaker for 30 seconds, and absorbance at 450 and 630 nm was read in a Biotek Synergy 4 plate reader. The absorbance at 630 nm was subtracted from the absorbance at 450 nm.
4. Data Analyses: The average absorbance value from wells containing media without cells was subtracted from the absorbance value for all the wells containing cells. The absorbance values were then taken as a percentage of the absorbance for the vehicle wells (0.1% DMSO - no compound). The percent compared to vehicle was then plotted vs. log(Concentration). Data analyses and curve fitting were performed using Graphpad Prism 6. For each compound, there were n = 3 data points for each concentration. For curves that did not reach full inhibition, the bottom was set to -10.

General procedure for cellular synergy.
1. Cell culture and seeding: All Ba/F3 and K562 cell lines were cultured in RPMI 1640 media with 10% FBS. Parental Ba/F3 cell culture additionally contained 15% WEHI-3 conditioned

media. An aliquot of the cells was mixed with trypan blue solution and the cell number was quantified using a hemacytometer. The cells were plated 100 µL in each well at 30,000 cells/mL so that each well contained 3,000 cells. The cells were plated into sterile, clear bottom 96 well plates and then immediately dosed with compound.

2. Dosing: The compounds dilutions (2X) and combinations were made in 100% DMSO at 1,000X the final concentrations that were desired for the assay. These DMSO stocks were diluted 10X in RPMI 1640 media. 1 µL of the compound diluted in media was added to each well for a final concentration of 0.1% DMSO. The wells containing only media were not dosed. In general, each compound concentration was dosed in triplicate wells. The plates were returned to normal culture conditions (per ATCC) for 72 hours.

3. Assay: After 72 hours, the plates were removed from the incubator and 10 µL of WST-1 reagent was added to each well. The plates were returned to the incubator and the color change was visually monitored for 0.5 – 2 hours. When sufficient color change had occurred, the plates were shaken on a plate shaker for 60 seconds and read in a Biotek Synergy 4 plate reader.

4. Data Analyses: The average absorbance value from wells containing media without cells was subtracted from the absorbance value for all the wells containing cells. The data were then calculated as a fraction of the vehicle well (1% DMSO) and subtracted from 1 in order to represent the data as the fraction of population affected by the treatment at each given dose. The data were then analyzed using Compusyn to determine the combination indices.

Equation for Determination of Combination Index (CI)

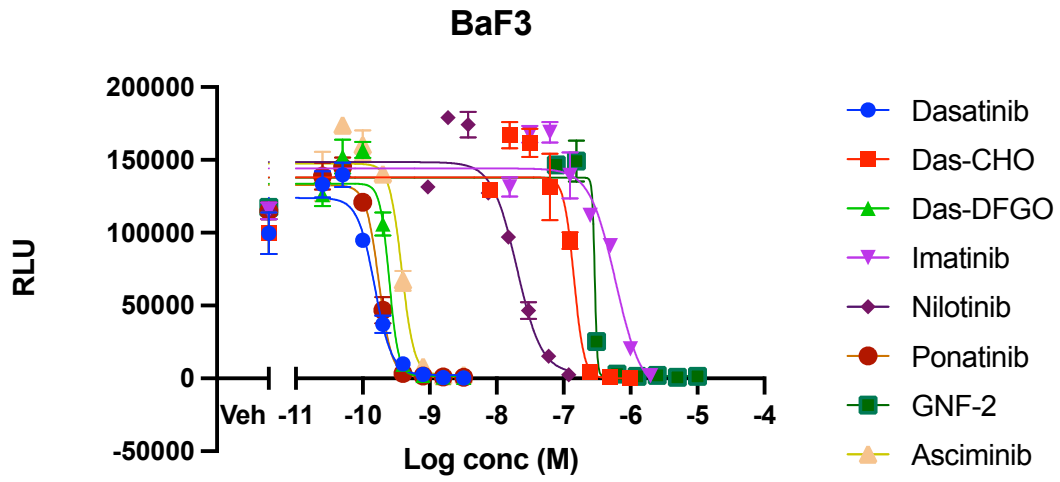
$$\mathrm{CI} = \frac{(\mathrm{D})_1}{(\mathrm{D}_x)_1} + \frac{(\mathrm{D})_2}{(\mathrm{D}_x)_2} = \frac{(\mathrm{D})_1}{(\mathrm{D}_m)_1\left[{}^{fa}/_{(1-f_a)}\right]^{1/m_1}} + \frac{(\mathrm{D})_2}{(\mathrm{D}_m)_2\left[{}^{fa}/_{(1-f_a)}\right]^{1/m_2}} \qquad (1)^{1}$$

where $(D)_1$ and $(D)_2$ are the doses of drugs 1 and 2, D_m is the dose required to produce the median effect (analogous to IC_{50}, ED_{50}, or LD_{50} values), m is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve, and f_a is fraction affected[1]

II. Single drug dose-response curves

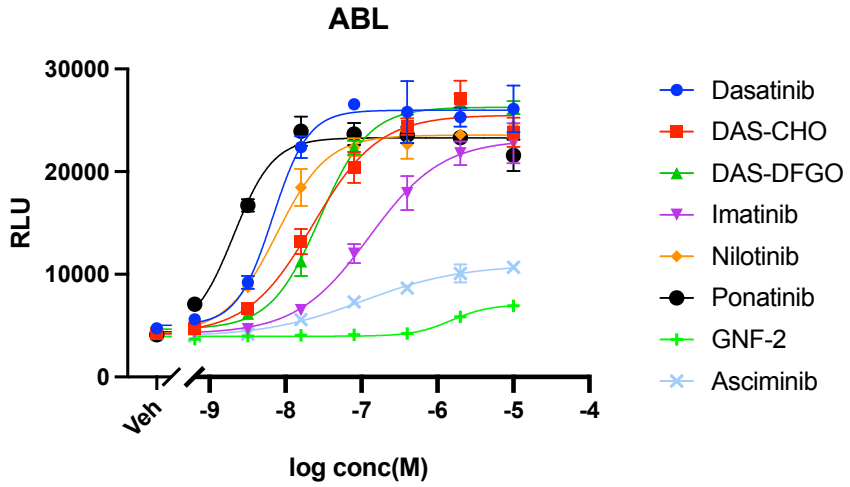
These dose-response curves were used to aid in the selection of optimal doses for the Chou-Talalay synergy experiments.

BCR-Abl/BaF3:



	Dasatinib	Das-CHO	Das-DFGO	Imatinib	Nilotinib	Ponatinib	GNF-2	Asciminib
IC50	1.508e-010	1.427e-007	2.485e-010	5.972e-007	2.004e-008	1.736e-010	~ 2.926e-007	3.821e-010

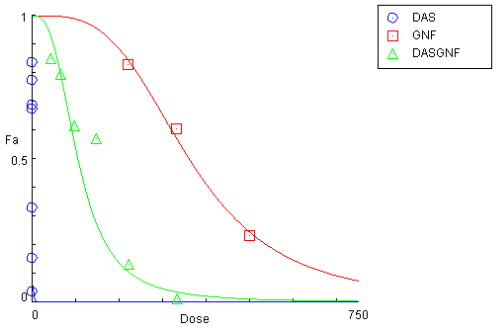
InCELL Pulse CETSA:

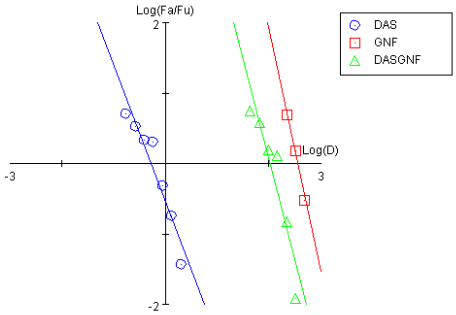


	Dasatinib	DAS-CHO	DAS-DFGO	Imatinib	Nilotinib	Ponatinib	GNF-2	Asciminib
IC50	6.758e-009	2.303e-008	2.833e-008	1.267e-007	7.638e-009	2.084e-009	1.520e-006	1.044e-007

III. Analytical Data for BCR-Abl/BaF3 Cellular Synergy

Dasatinib-GNF2



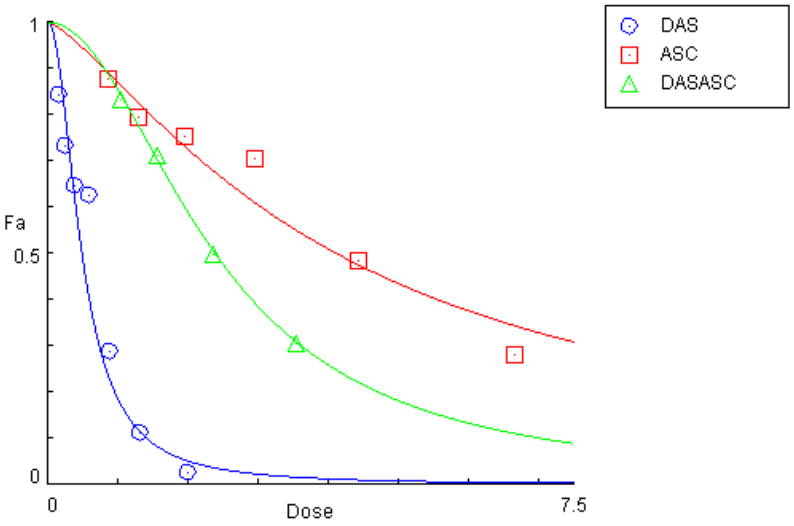


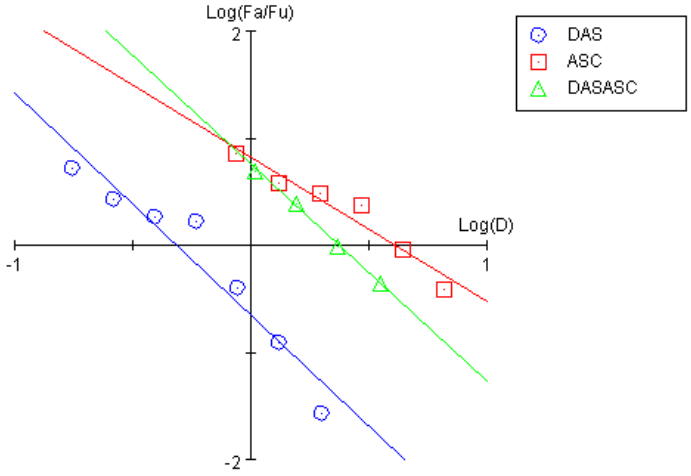
Drug/Combo	Dm	m	r
DAS	0.53773	-1.9520	-0.9527
GNF	360.769	-3.4430	-0.9956
DASGNF	105.274	-2.8634	-0.9523

CI values at:

Combo	ED50	ED75	ED90	ED95
DASGNF	1.07062	1.20546	1.37146	1.50509

Dasatinib-Asciminib



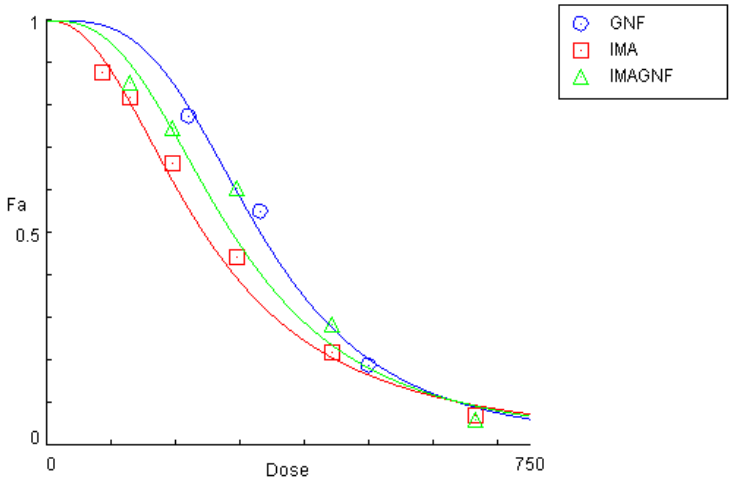


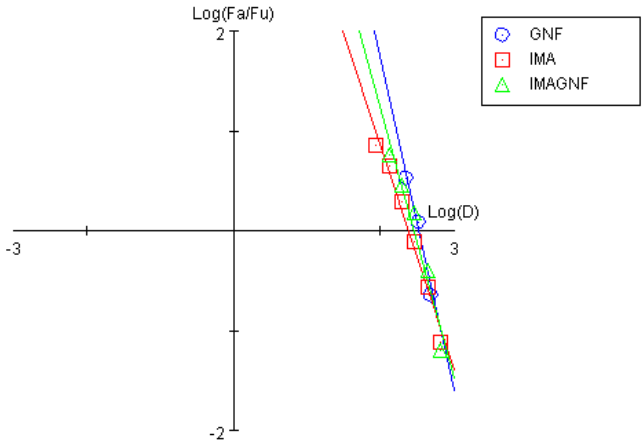
Drug/Combo	Dm	m	r
DAS	0.49012	-2.0722	-0.9595
ASC	4.12004	-1.3464	-0.9695
DASASC	2.38456	-2.0298	-0.9990

CI values at:

Combo	ED50	ED75	ED90	ED95
DASASC	1.29319	1.43674	1.62859	1.79427

Imatinib-GNF2



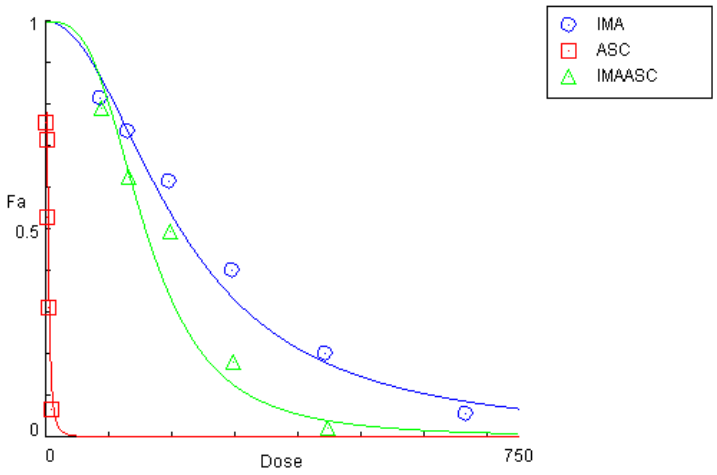


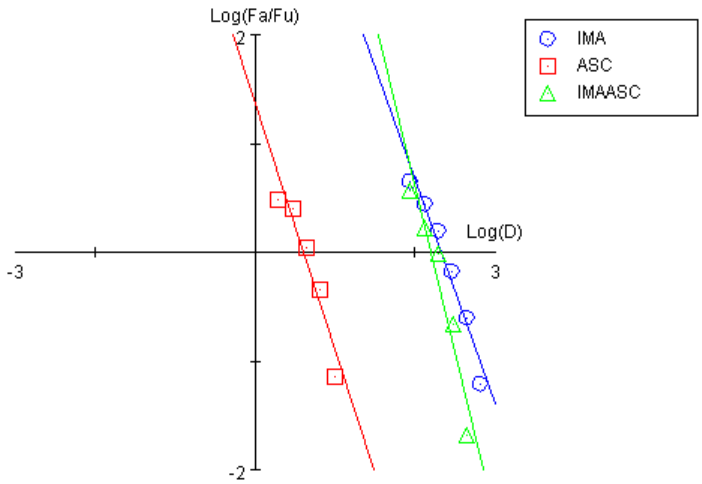
Drug/Combo	Dm	m	r
GNF	331.684	-3.3294	-0.9905
IMA	243.617	-2.2497	-0.9898
IMAGNF	287.416	-2.7159	-0.9727

CI values at:

Combo	ED50	ED75	ED90	ED95
IMAGNF	1.07537	1.12340	1.17893	1.22120

Imatinib-Asciminib



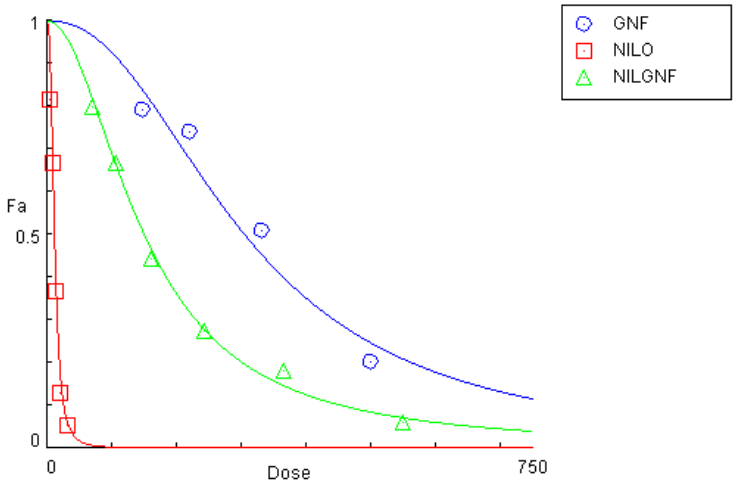


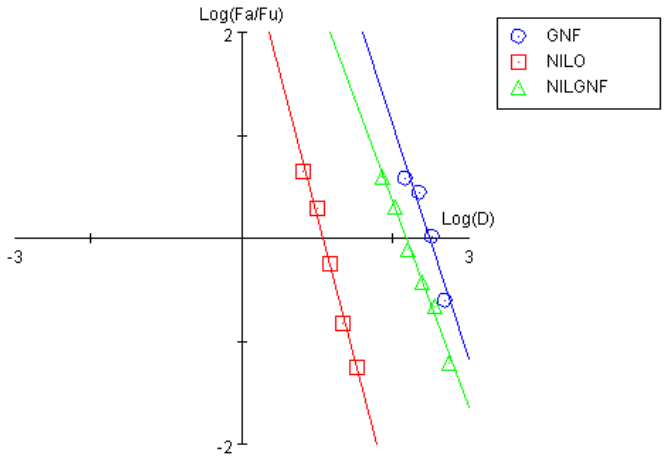
Drug/Combo	Dm	m	r
IMA	213.299	-2.0714	-0.9806
ASC	3.99456	-2.2695	-0.9544
IMAASC	158.173	-3.0529	-0.9606

CI values at:

Combo	ED50	ED75	ED90	ED95
IMAASC	1.12626	1.31462	1.53521	1.70650

Nilotinib-GNF2



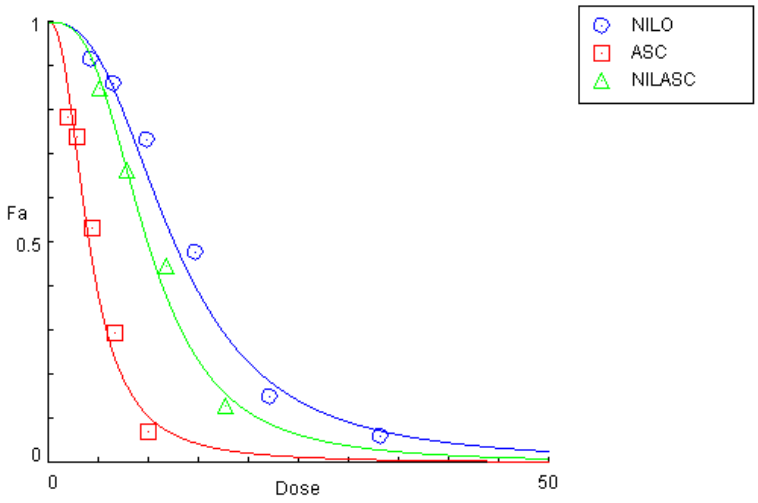


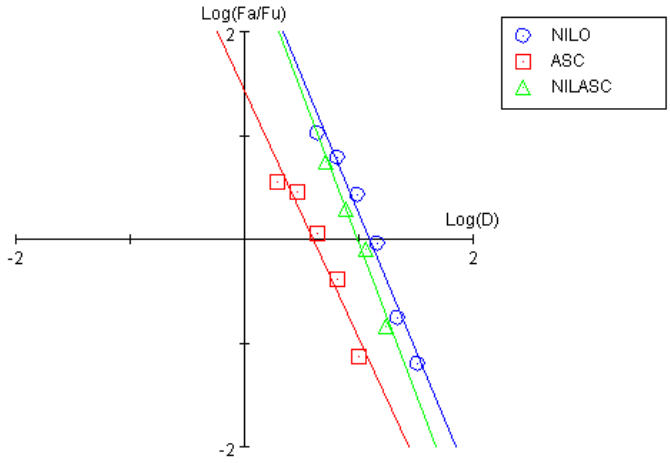
Drug/Combo	Dm	m	r
GNF	305.060	-2.2627	-0.9637
NILO	11.8307	-2.7963	-0.9969
NILGNF	149.888	-1.9798	-0.9948

CI values at:

Combo	ED50	ED75	ED90	ED95
NILGNF	1.59844	1.39624	1.22181	1.11698

Nilotinib-Asciminib



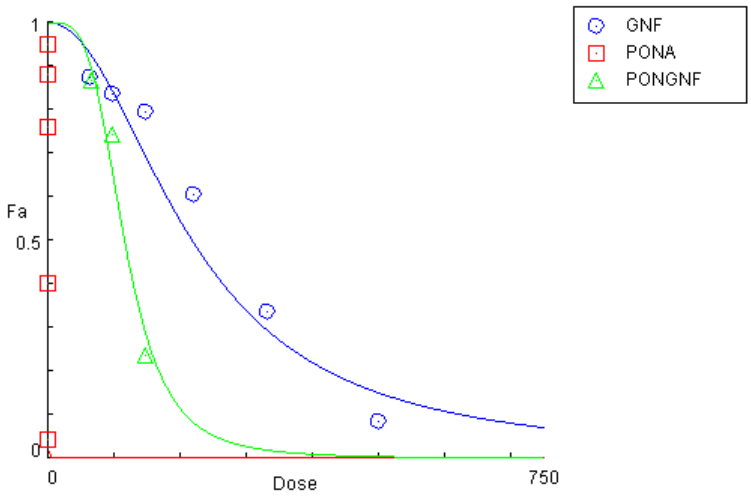


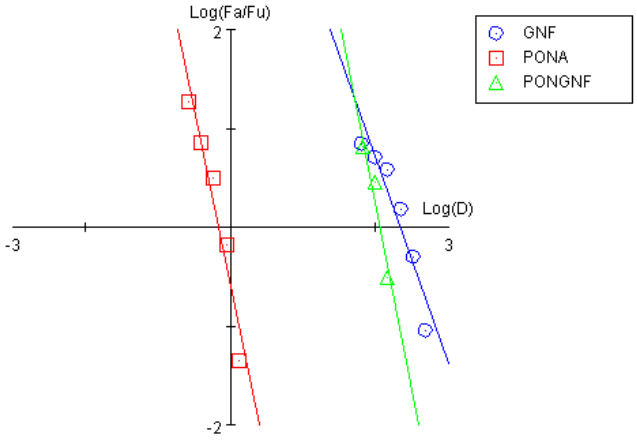
Drug/Combo	Dm	m	r
NILO	12.5815	-2.6289	-0.9873
ASC	4.08488	-2.3880	-0.9650
NILASC	9.92739	-2.9080	-0.9892

CI values at:

Combo	ED50	ED75	ED90	ED95
NILASC	1.06259	1.12423	1.18995	1.23713

Ponatinib-GNF2



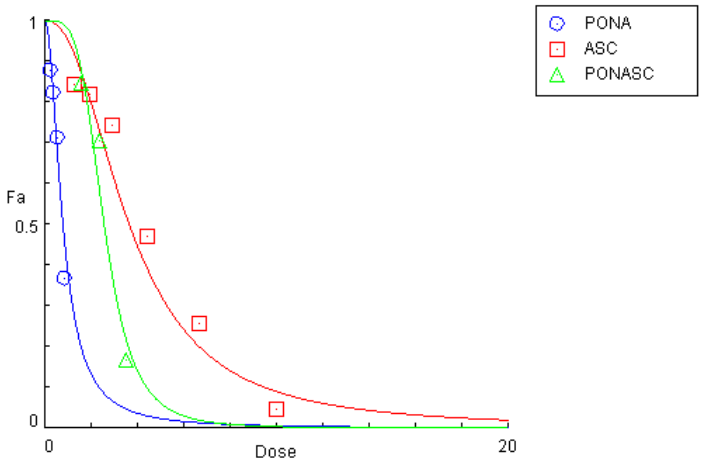


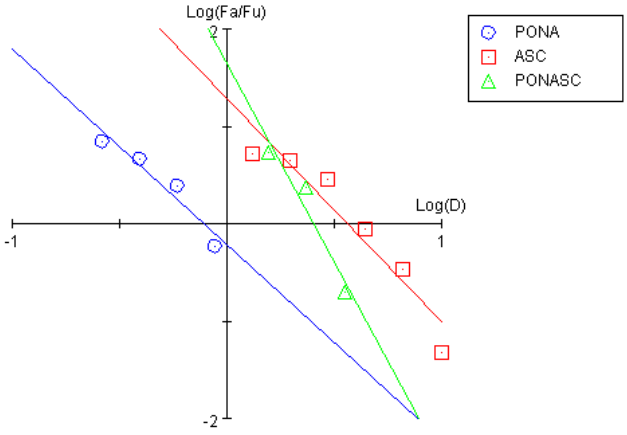
Drug/Combo	Dm	m	r
GNF	217.943	-2.0843	-0.9505
PONA	0.68557	-3.5580	-0.9672
PONGNF	116.107	-3.7661	-0.9661

CI values at:

Combo	ED50	ED75	ED90	ED95
PONGNF	1.20535	1.35779	1.54781	1.70349

Ponatinib-Asciminib



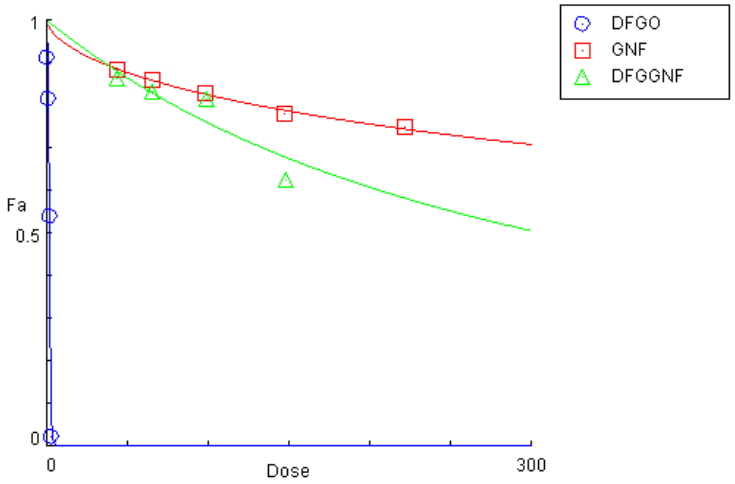


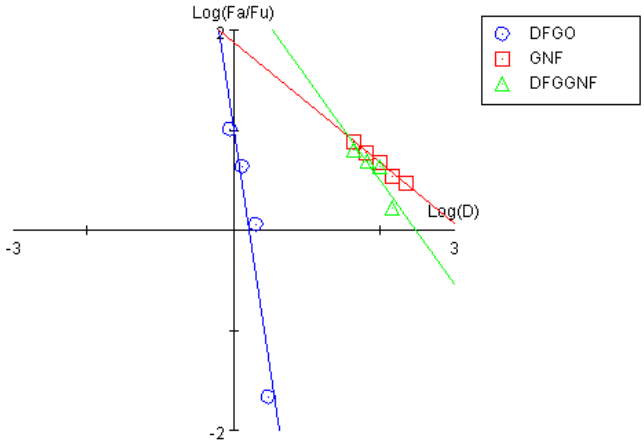
Drug/Combo	Dm	m	r
PONA	0.78362	-2.0072	-0.9606
ASC	3.64145	-2.2926	-0.9530
PONASC	2.56180	-4.0787	-0.9610

CI values at:

Combo	ED50	ED75	ED90	ED95
PONASC	1.13112	1.44261	1.84201	2.17656

DAS-DFGO-II – GNF2



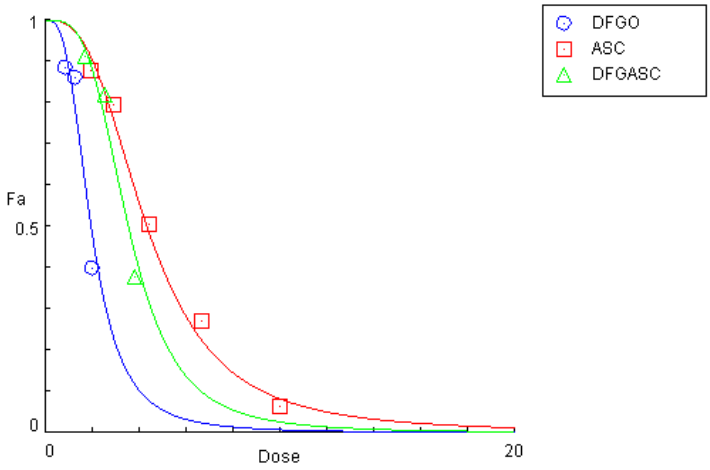


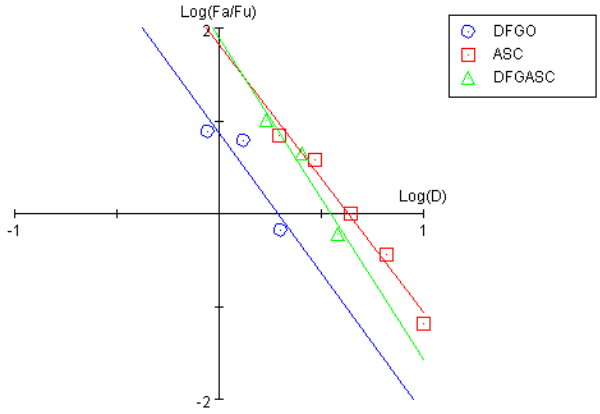
Drug/Combo	Dm	m	r
DFGO	1.64395	-4.8848	-0.9373
GNF	1313.29	-0.6030	-0.9969
DFGGNF	307.442	-1.0236	-0.9510

CI values at:

Combo	ED50	ED75	ED90	ED95
DFGGNF	1.34809	0.96943	1.24430	1.84496

DAS-DFGO-II – Asciminib



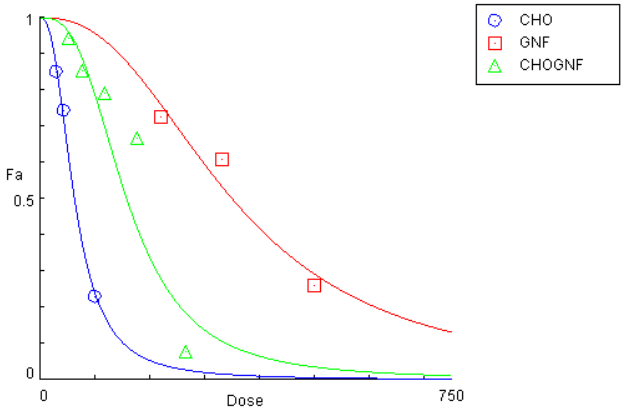


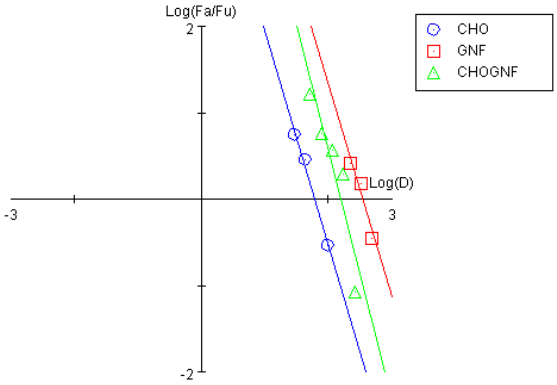
Drug/Combo	Dm	m	r
DFGO	1.95313	-3.0281	-0.9544
ASC	4.33040	-2.8843	-0.9888
DFGASC	3.53858	-3.4850	-0.9709

CI values at:

Combo	ED50	ED75	ED90	ED95
DFGASC	1.04667	1.10970	1.17662	1.22448

DAS-CHO-II – GNF2



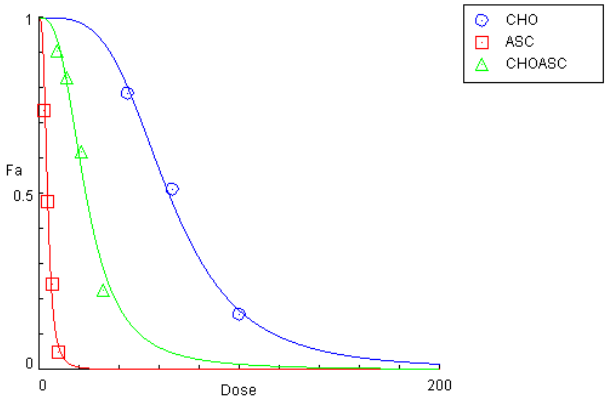


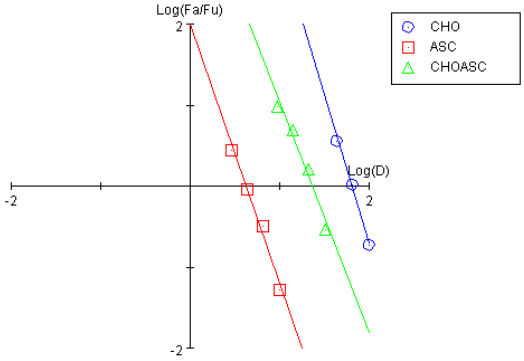
Drug/Combo	Dm	m	r
CHO	62.8500	-2.4836	-0.9935
GNF	350.039	-2.4789	-0.9646
CHOGNF	158.014	-2.8688	-0.9513

CI values at:

Combo	ED50	ED75	ED90	ED95
CHOGNF	0.79521	0.84420	0.89621	0.93340

DAS-CHO-II – Asciminib





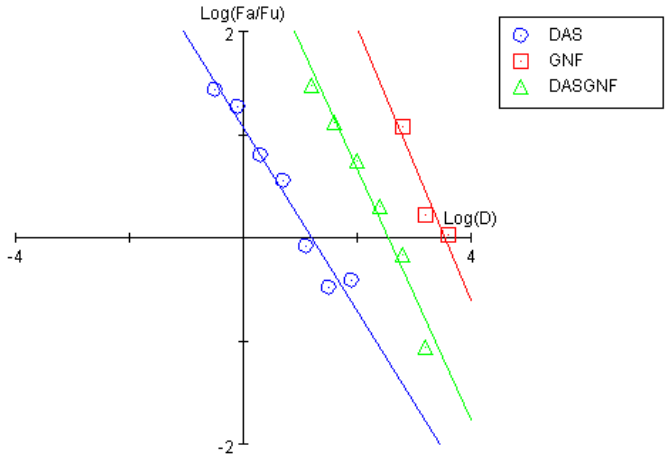
Drug/Combo	Dm	m	r
CHO	64.7249	-3.6691	-0.9960
ASC	4.25424	-3.1862	-0.9913
CHOASC	23.3313	-2.8485	-0.9801

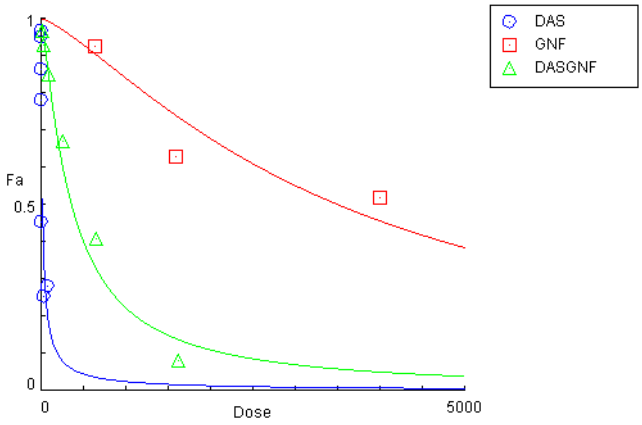
CI values at:

Combo	ED50	ED75	ED90	ED95
CHOASC	0.82627	0.77921	0.73520	0.70689

V. Analytical Data for InCELL Pulse CETSA Synergy

Dasatinib-GNF2



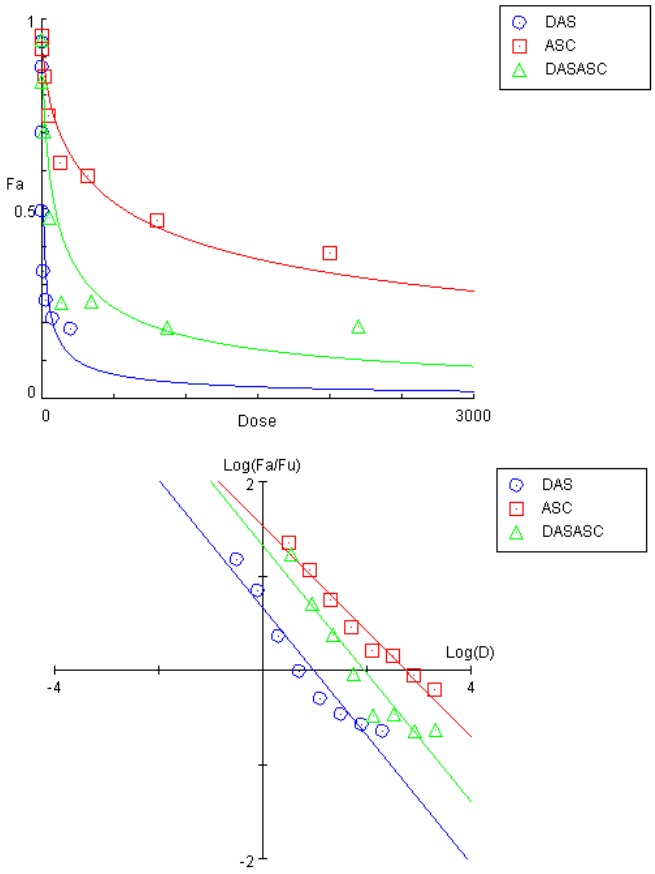


Drug/Combo	Dm	m	r
DAS	16.2394	-0.8918	-0.9798
GNF	3507.01	-1.3245	-0.9506
DASGNF	358.707	-1.2181	-0.9838

CI values at:

Combo	ED95
DASGNF	1.13144

Dasatinib-Asciminib

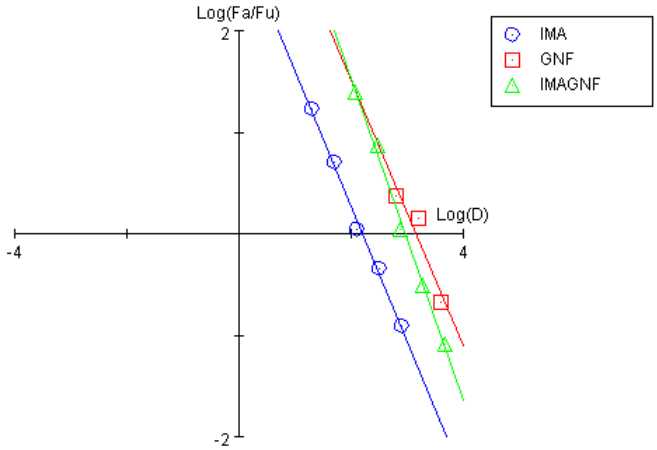


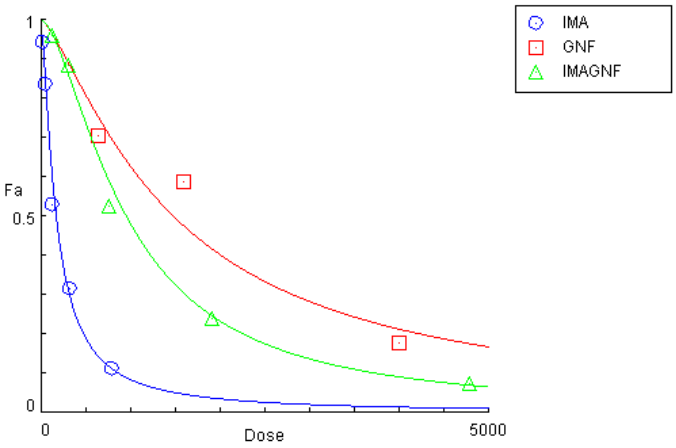
Drug/Combo	Dm	m	r
DAS	9.69257	-0.6764	-0.9662
ASC	570.043	-0.5555	-0.9852
DASASC	92.5031	-0.6796	-0.9496

CI values at:

Combo	ED95
DASASC	1.27397

Imatinib-GNF2



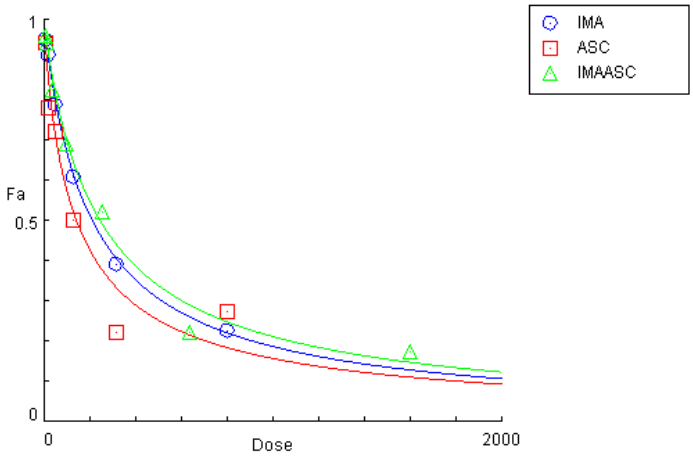


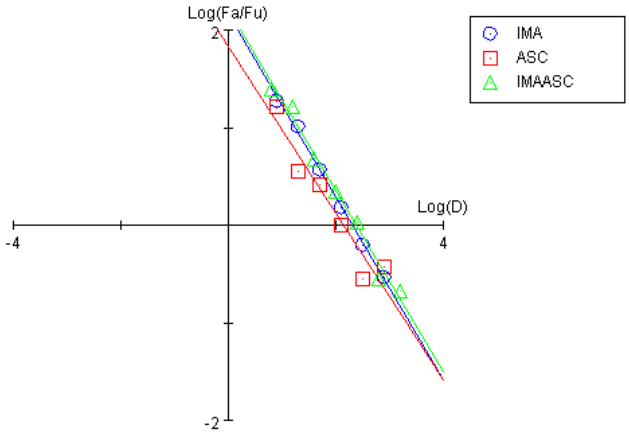
Drug/Combo	Dm	m	r
IMA	166.378	-1.3318	-0.9978
GNF	1479.10	-1.3152	-0.9496
IMAGNF	948.220	-1.5973	-0.9974

CI values at:

Combo	ED95
IMAGNF	2.16487

Imatinib-Asciminib



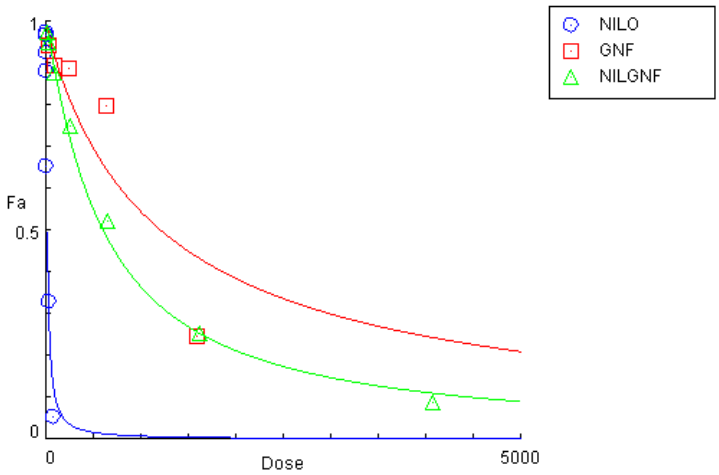


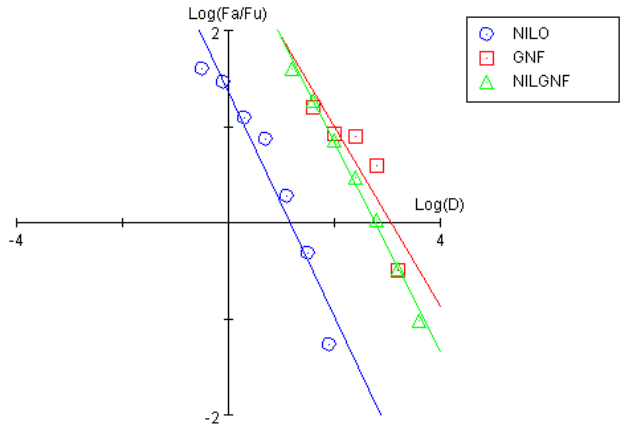
Drug/Combo	Dm	m	r
IMA	209.310	-0.9389	-0.9985
ASC	140.775	-0.8559	-0.9622
IMAASC	243.698	-0.9304	-0.9928

CI values at:

Combo	ED95
IMAASC	1.70554

Nilotinib-GNF2



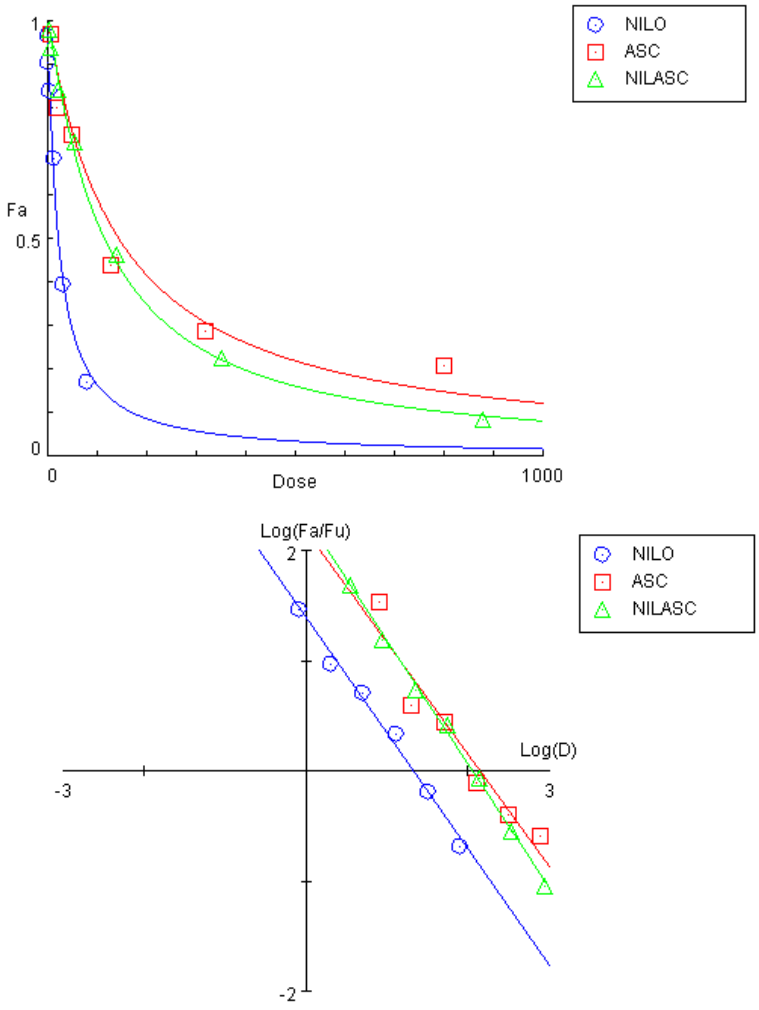


Drug/Combo	Dm	m	r
NILO	14.8299	-1.1627	-0.9654
GNF	1211.85	-0.9354	-0.9514
NILGNF	601.377	-1.0918	-0.9967

CI values at:

Combo	ED95
NILGNF	1.4382

Nilotinib-Asciminib

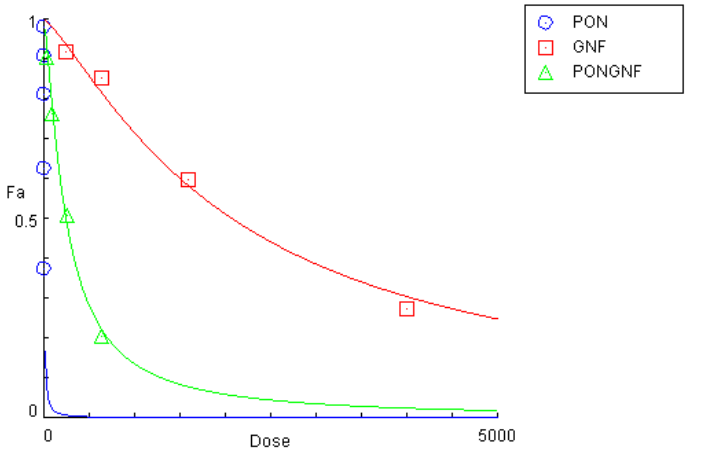


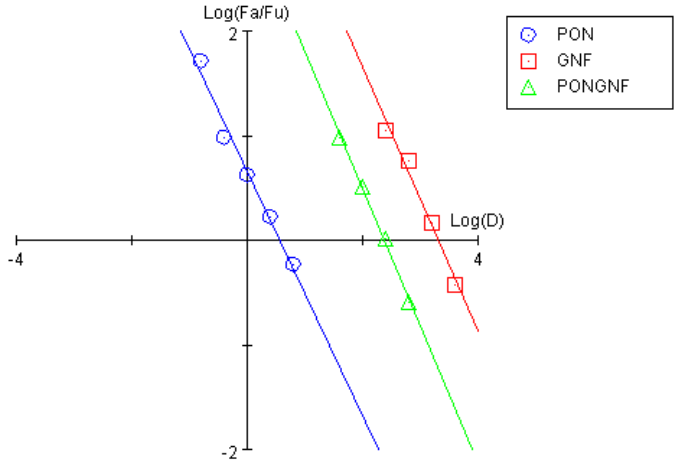
Drug/Combo	Dm	m	r
NILO	21.2276	-1.0496	-0.9951
ASC	143.412	-1.0143	-0.9679
NILASC	114.248	-1.1152	-0.9988

CI values at:

Combo	ED95
NILASC	1.51862

Ponatinib-GNF2



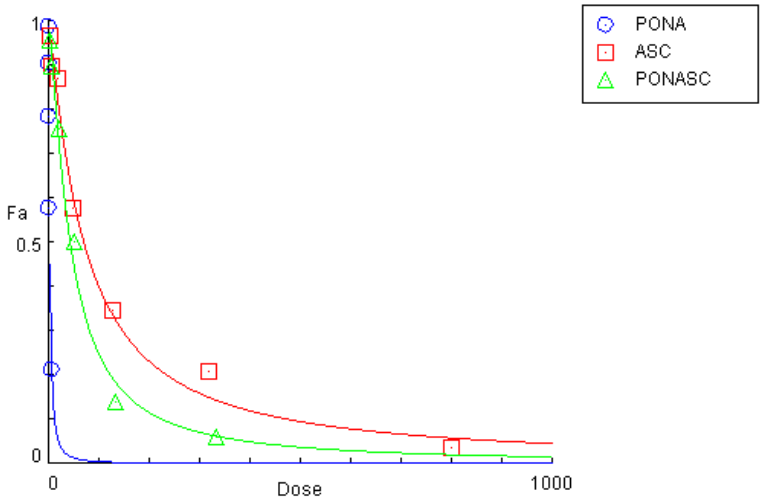


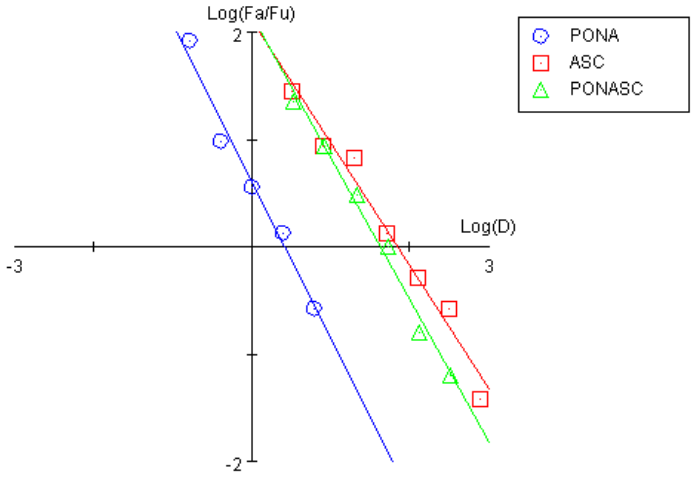
Drug/Combo	Dm	m	r
PON	3.81974	-1.1703	-0.9913
GNF	2079.03	-1.2593	-0.9894
PONGNF	244.710	-1.3122	-0.9982

CI values at:

Combo	ED95
PONGNF	0.96071

Ponatinib-Asciminib



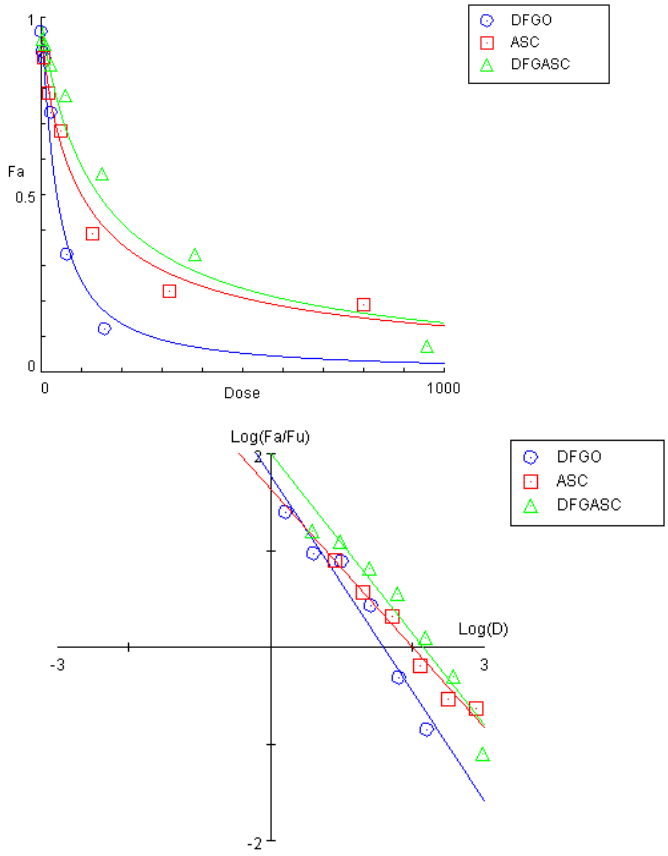


Drug/Combo	Dm	m	r
PONA	2.64755	-1.4674	-0.9883
ASC	70.0436	-1.1453	-0.9878
PONASC	43.0758	-1.3255	-0.9943

CI values at:

Combo	ED95
PONASC	1.45577

DAS-DFGO-II – Asciminib

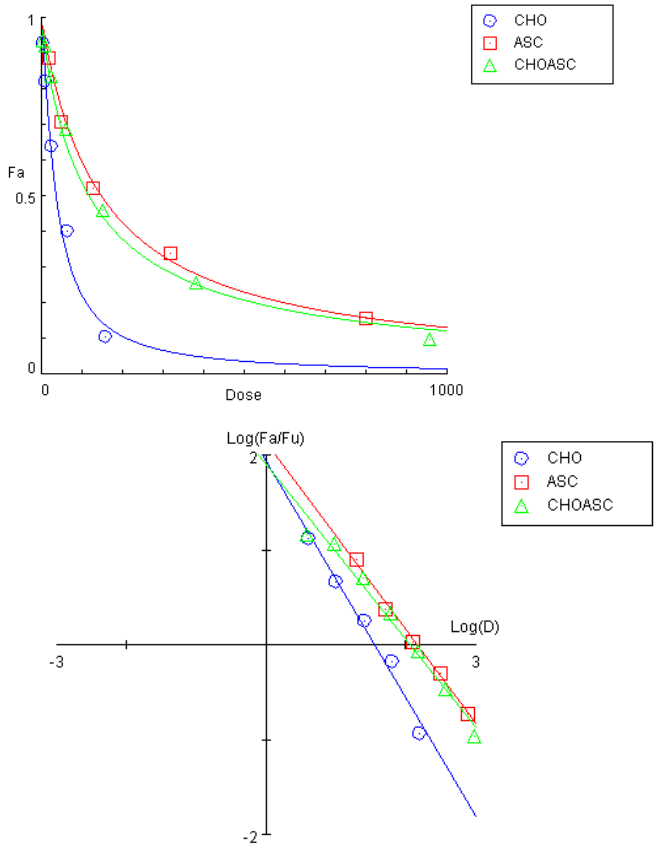


Drug/Combo	Dm	m	r
DFGO	38.7354	-1.1178	-0.9754
ASC	99.9675	-0.8204	-0.9879
DFGASC	142.498	-0.9308	-0.9689

CI values at:

Combo	ED95
DFGASC	2.17956

DAS-CHO-II – Asciminib



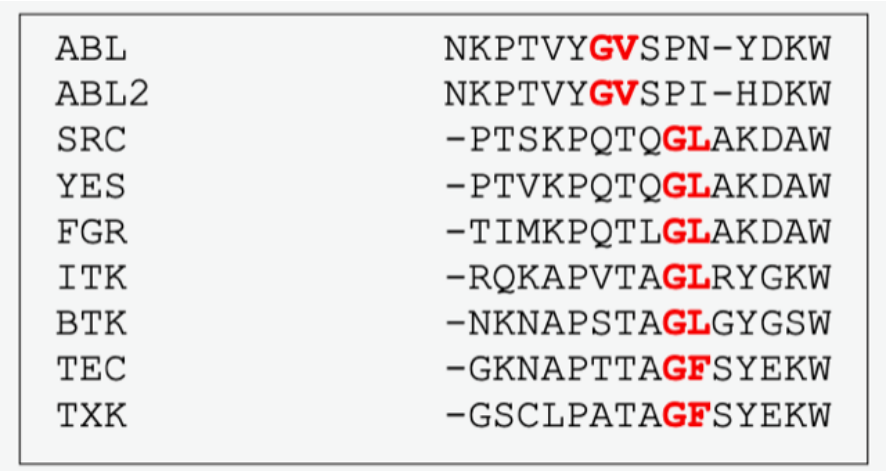
Drug/Combo	Dm	m	r
CHO	36.2497	-1.2448	-0.9919
ASC	147.585	-0.9862	-0.9969
CHOASC	117.076	-0.9208	-0.9905

CI values at:

Combo	ED95
CHOASC	0.76886

V. Data for cleavage of Abl by thermolysin

Abl is selectively cleaved after the GV residues in the kinase-SH2 linker:



FULL LENGTH

Theoretical pI/Mw (average) for the user-entered sequence:

```
         10         20         30         40         50         60
GHMARWNSKE NLLAGPSEND PNLFVALYDF VASGDNTLSI TKGEKLRVLG YNHNGEWCEA

         70         80         90        100        110        120
QTKNGQGWVP SNYITPVNSL EKHSWYHGPV SRNAAEYLLS SGINGSFLVR ESESSPGQRS

        130        140        150        160        170        180
ISLRYEGRVY HYRINTASDG KLYVSSESRF NTLAELVHHH STVADGLITT LHYPAPKRNK

        190        200        210        220        230        240
PTVYGVSPNY DKWEMERTDI TMKHKLGGGQ YGEVYEGVWK KYSLTVAVKT LKEDTMEVEE

        250        260        270        280        290        300
FLKEAAVMKE IKHPNLVQLL GVCTREPPFY IITEFMTYGN LLDYLRECNR QEVNAVVLLY

        310        320        330        340        350        360
MATQISSAME YLEKKNFIHR DLAARNCLVG ENHLVKVADF GLSRLMTGDT YTAHAGAKFP

        370        380        390        400        410        420
IKWTAPESLA YNKFSIKSDV WAFGVLLWEI ATYGMSPYPG IDLSQVYELL EKDYRMERPE

        430        440        450        460        470
GCPEKVYELM RACWQWNPSD RPSFAEIHQA FETMFQESSI SDEVEKELGK QGV
```

Mw: 53847.91

CUT N_TERM

Theoretical pI/Mw (average) for the user-entered sequence:

```
        10         20         30         40         50         60
GHMARWNSKE NLLAGPSEND PNLFVALYDF VASGDNTLSI TKGEKLRVLG YNHNGEWCEA

        70         80         90        100        110        120
QTKNGQGWVP SNYITPVNSL EKHSWYHGPV SRNAAEYLLS SGINGSFLVR ESESSPGQRS

       130        140        150        160        170        180
ISLRYEGRVY HYRINTASDG KLYVSSESRF NTLAELVHHH STVADGLITT LHYPAPKRNK


PTVY
```

Mw: 20566.80

CUT C-TERM

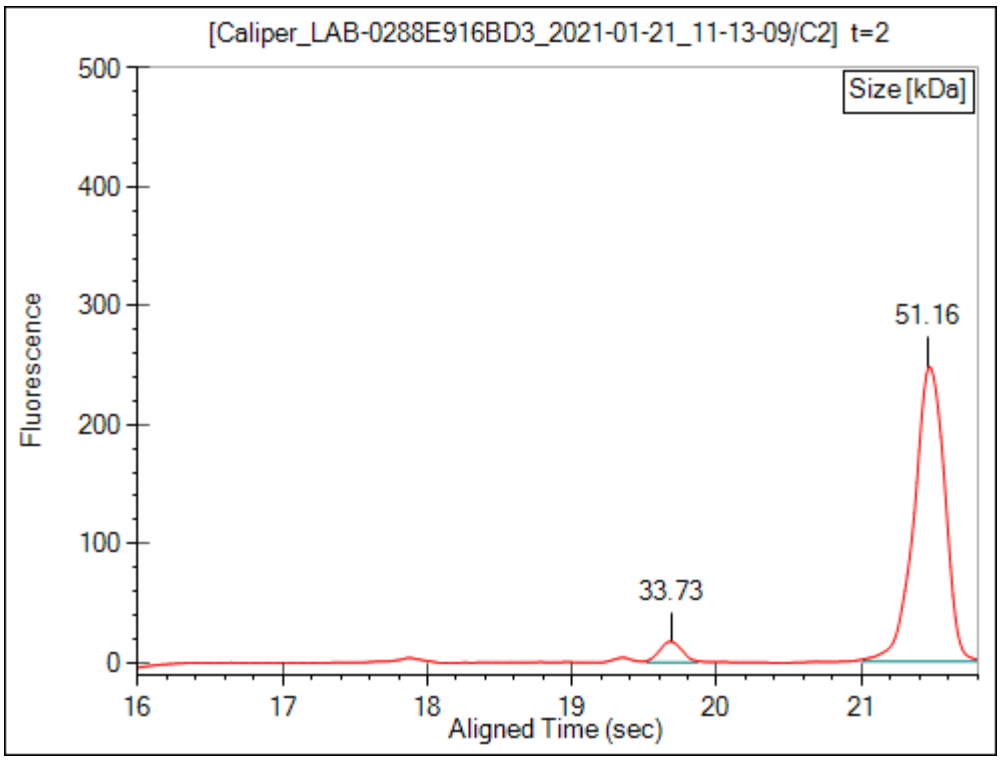
Theoretical pI/Mw (average) for the user-entered sequence:

```
        10         20         30         40         50         60
VSPNYDKWEM ERTDITMKHK LGGGQYGEVY EGVWKKYSLT VAVKTLKEDT MEVEEFLKEA

        70         80         90        100        110        120
AVMKEIKHPN LVQLLGVCTR EPPFYIITEF MTYGNLLDYL RECNRQEVNA VVLLYMATQI

       130        140        150        160        170        180
SSAMEYLEKK NFIHRDLAAR NCLVGENHLV KVADFGLSRL MTGDTYTAHA GAKFPIKWTA

       190        200        210        220        230        240
PESLAYNKFS IKSDVWAFGV LLWEIATYGM SPYPGIDLSQ VYELLEKDYR MERPEGCPEK

       250        260        270        280
VYELMRACWQ WNPSDRPSFA EIHQAFETMF QESSISDEVE KELGKQGV
```

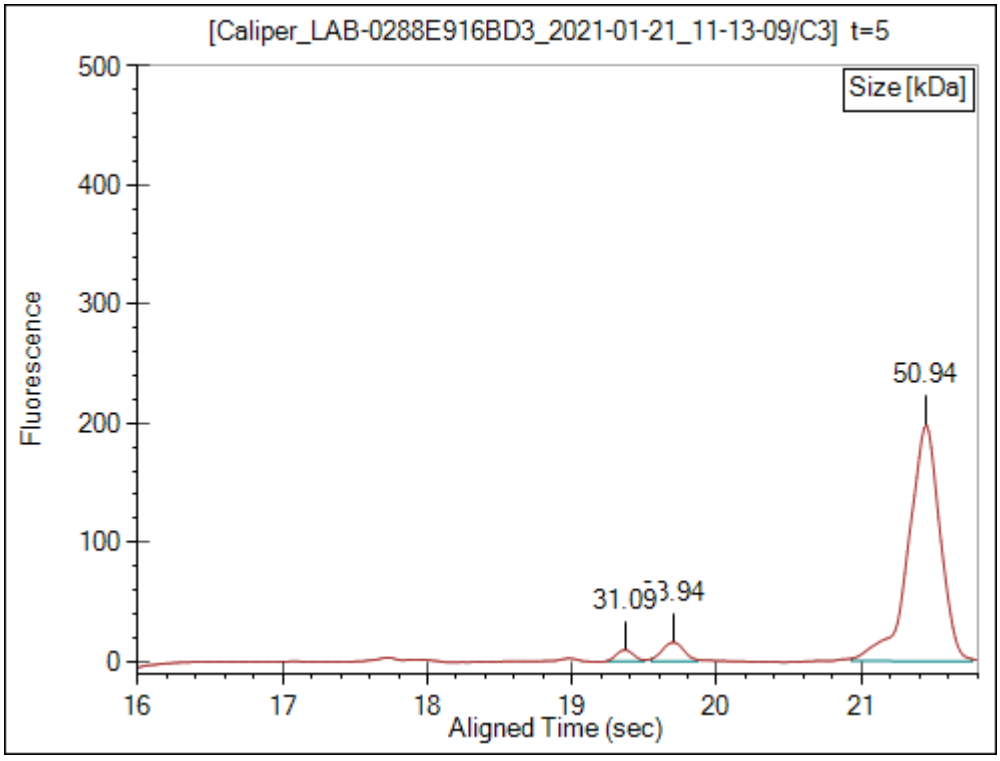
Mw: 33242.07

Cleavage of Abl by thermolysin over time. Peak at 33.7 is an internal control.

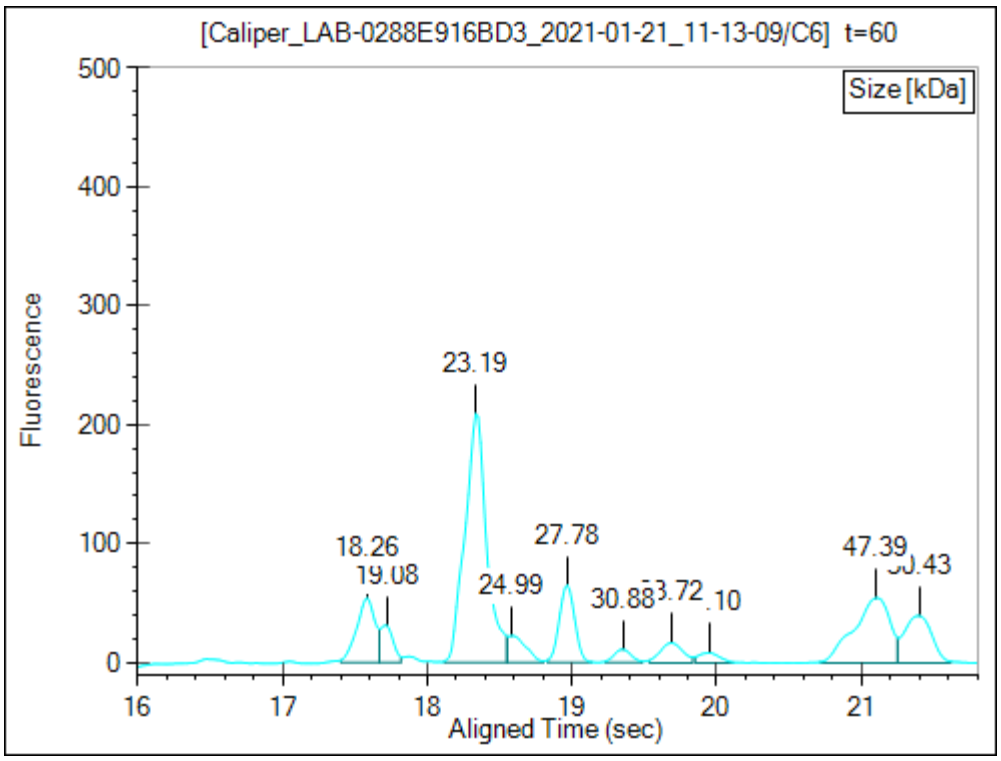
t = 2 min:



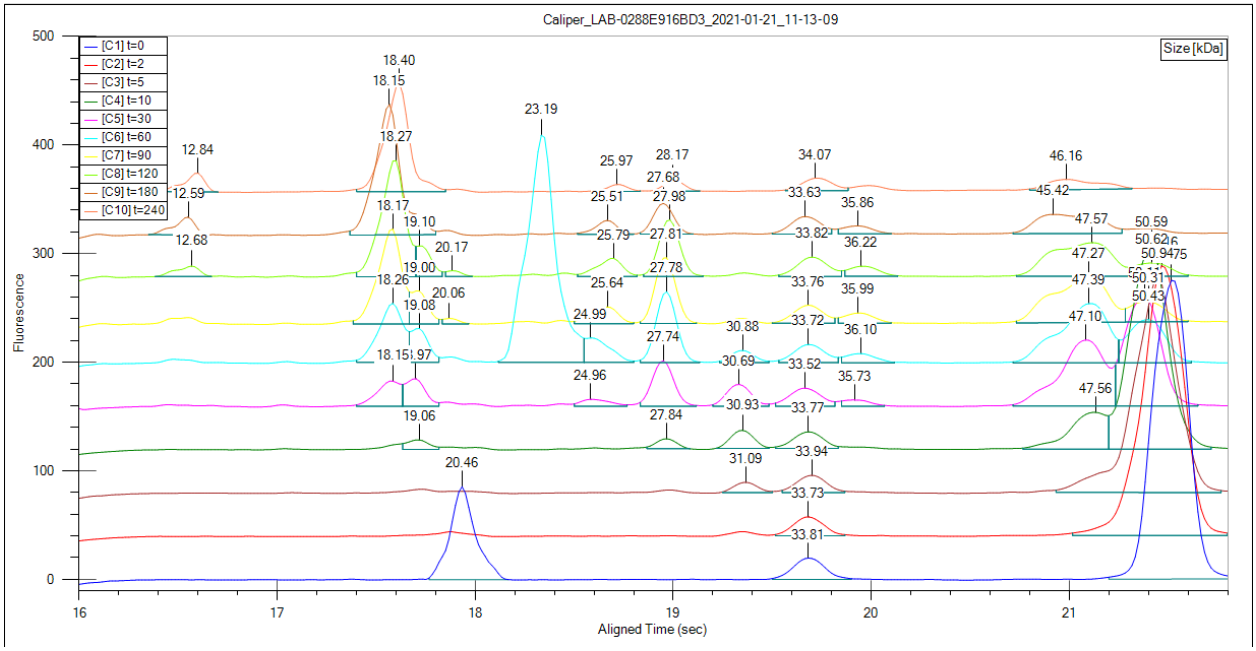
t = 5 min:



t = 60 min:

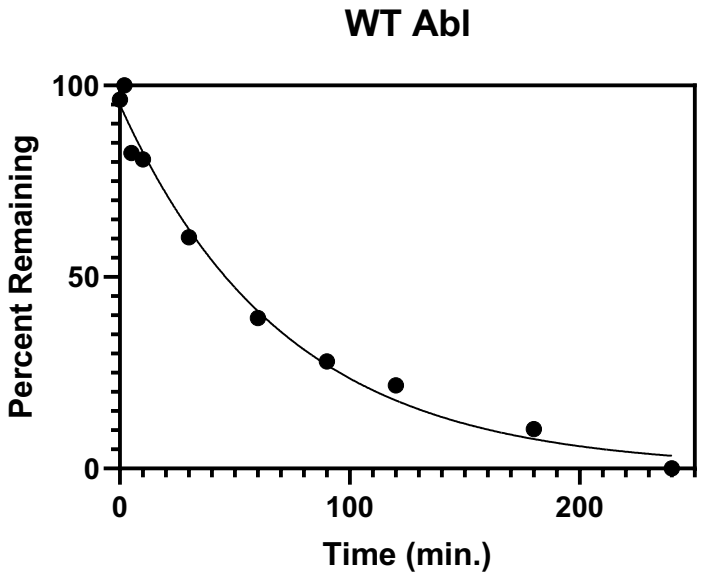


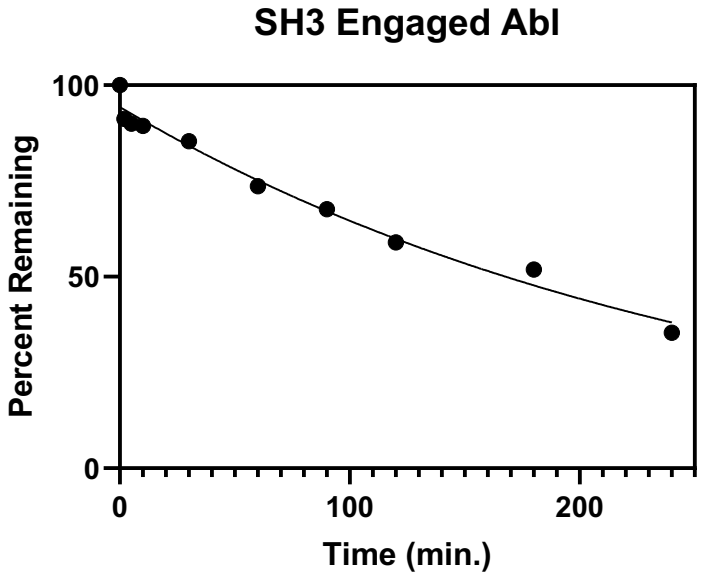
Full timecourse:

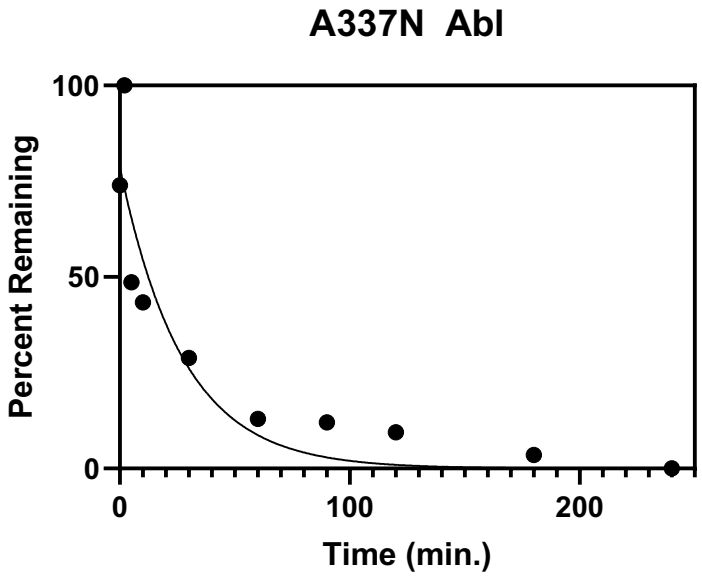


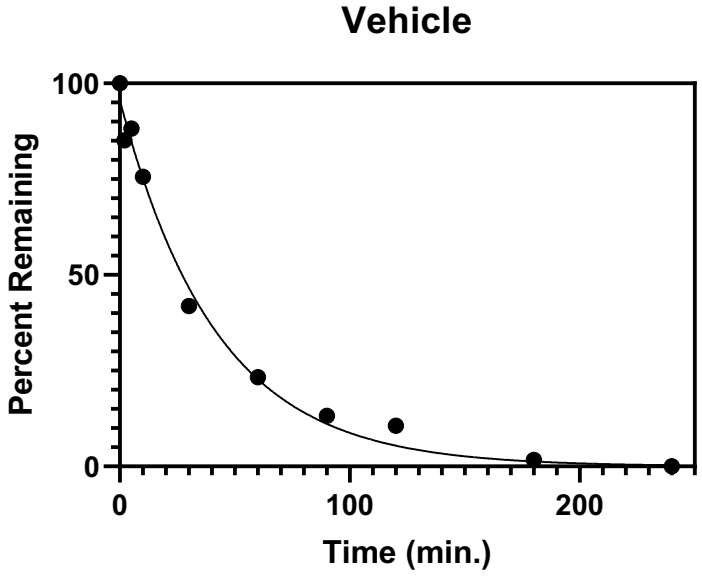
VI. Analytical data for Protein Half Lives as Determined *via* Proteolysis Assay.

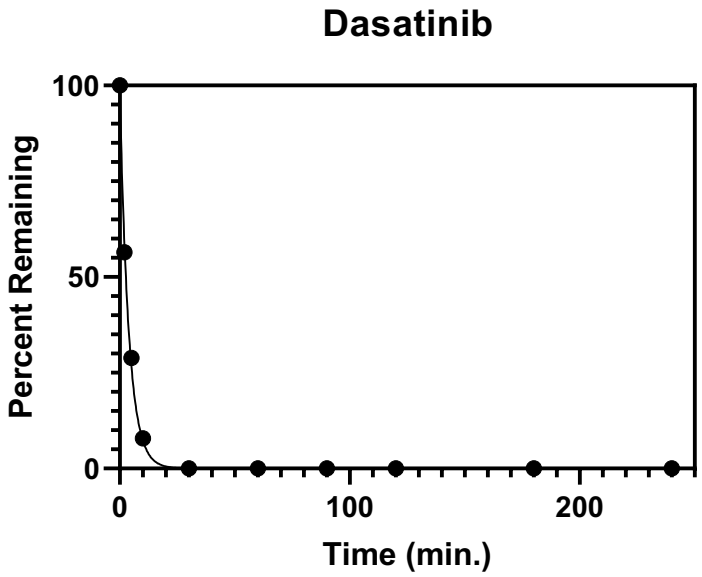
	Half Life (min)	$\frac{T_{1/2}}{\text{WT Abl } T_{1/2}}$	log(Relative Half Life)
WT Abl	43.8 ± 5.7	1	0
SH3 Engaged Abl	169.8±20.5	3.87	0.59
A337N Abl	17.0±6.3	0.39	-0.41
Vehicle	29.3 ± 4.1	1	0
Dasatinib	2.7 ± 0.1	0.09	-1.05
Imatinib	2.3 ± 0.2	0.08	-1.1
Nilotinib	2.5 ± 0.1	0.09	-1.05
Ponatinib	2.3 ± 0.3	0.08	-1.1
GNF-2	367.5 ± 59.1	12.54	1.1
Asciminib	282.5 ± 17.9	9.64	0.98
Das-DFGO-II	2.2 ± 0.7	0.07	-1.15
Das-CHO-II	47.2 ± 7.9	1.61	0.21
Vehicle	25.4 ± 2.6	0.87	-0.06
GNF-2	330.7 ± 11	11.28	1.05
Asciminib	297 ± 24	10.13	1.01
GFN-2+Das-DFGO-II	22.5 ± 1.7	0.77	-0.11
GNF-2+Das-CHO-II	393.7 ± 12	13.43	1.13
Asciminib+Das-DFGO-II	19.9 ± 3	0.68	-0.17
Asciminib+Das-CHO-II	512.6 ± 22.7	17.49	1.24

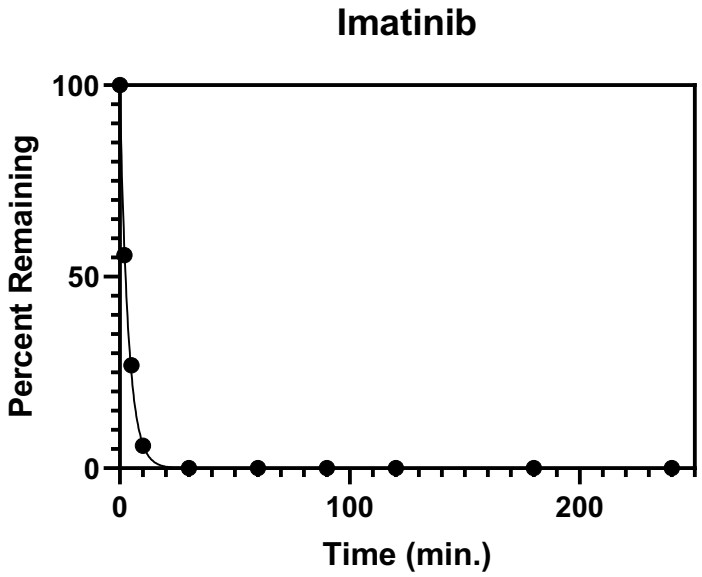

WT Abl
Percent Remaining
100
50
0
0
100
200
Time (min.)

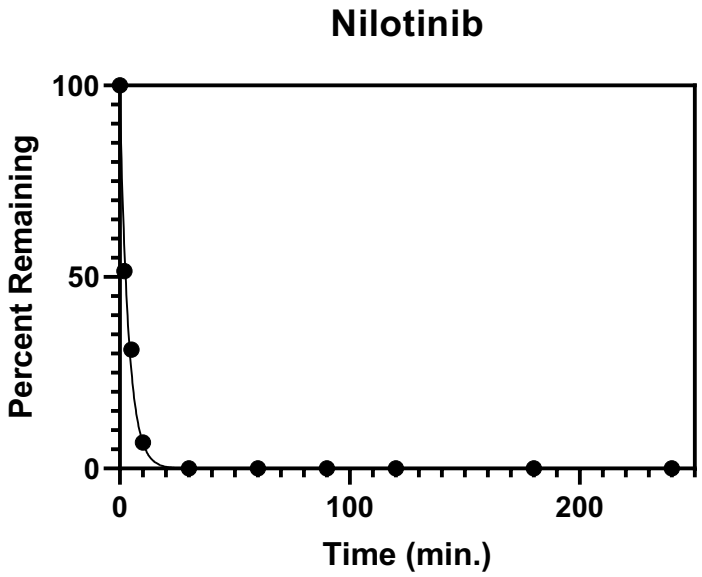

SH3 Engaged Abl
Percent Remaining
100
50
0
0
100
200
Time (min.)

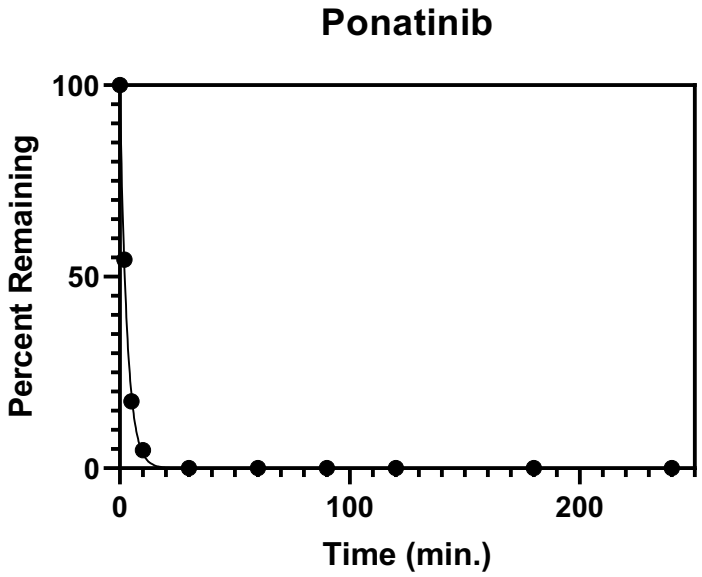

A337N Abl
Percent Remaining
100
50
0
0
100
200
Time (min.)

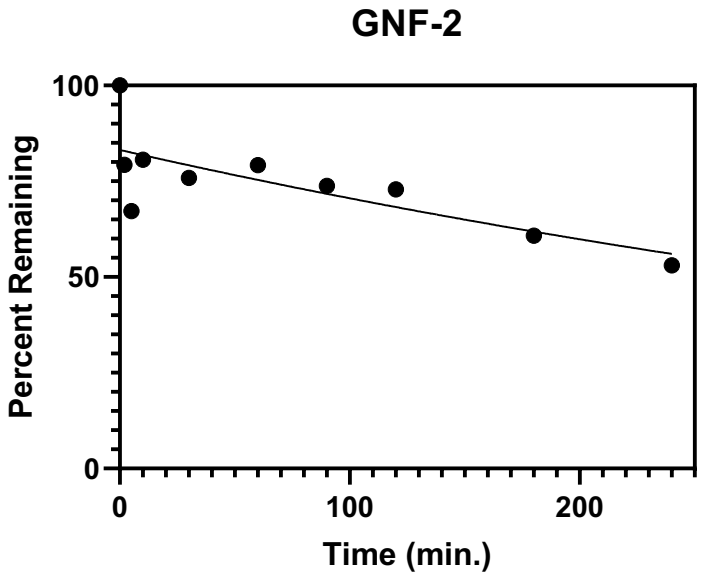

Vehicle
Percent Remaining
100
50
0
0
100
200
Time (min.)

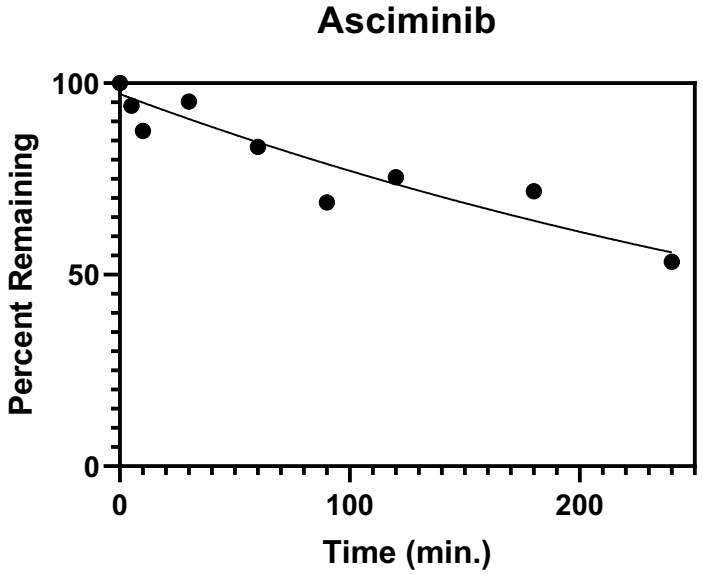

Dasatinib
Percent Remaining
100
50
0
0
100
200
Time (min.)

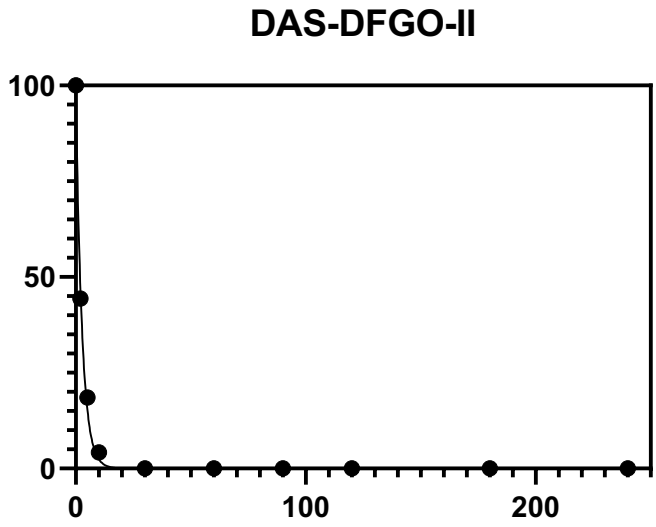

Imatinib
Percent Remaining
100
50
0
0
100
200
Time (min.)

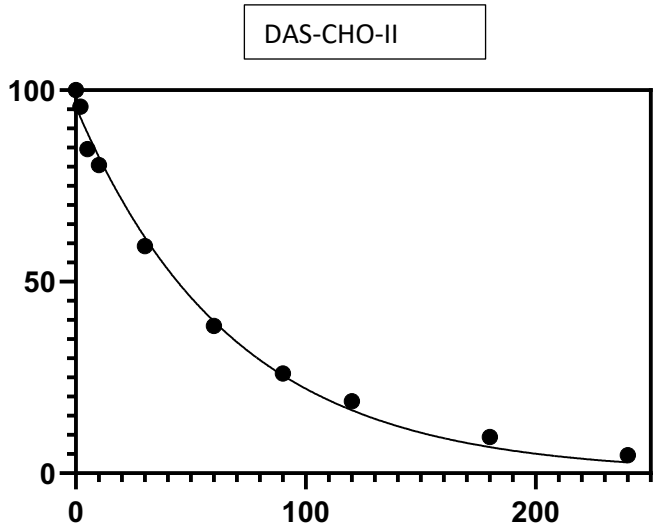

Nilotinib
Percent Remaining
100
50
0
0
100
200
Time (min.)

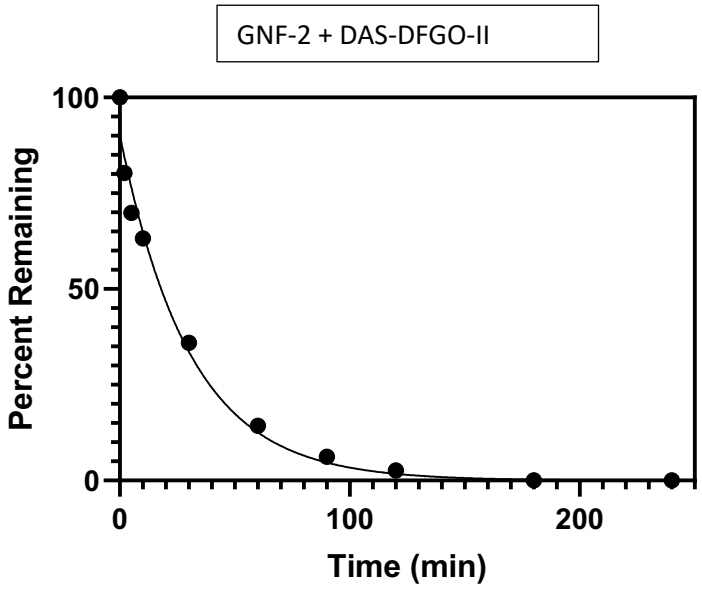

Ponatinib
Percent Remaining
100
50
0
0
100
200
Time (min.)

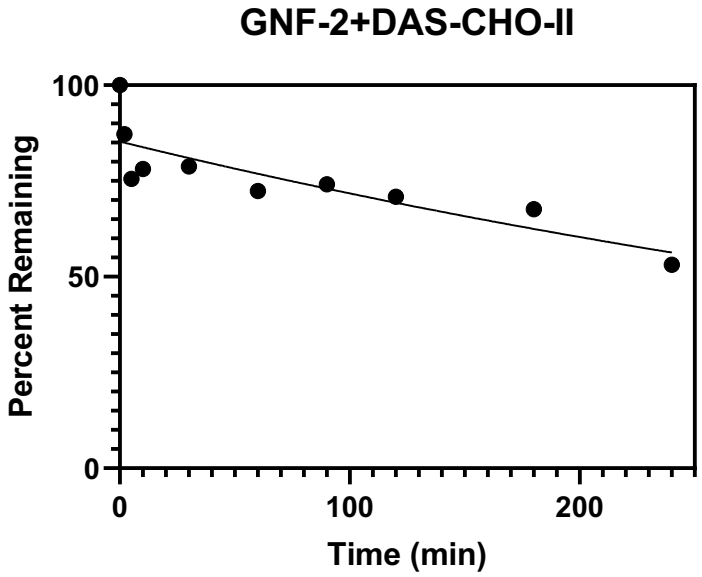

GNF-2
Percent Remaining
100
50
0
0
100
200
Time (min.)

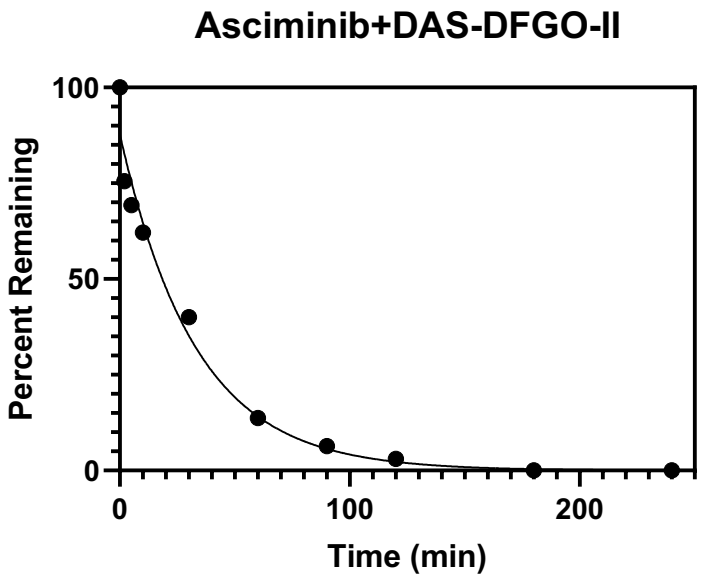

Asciminib
Percent Remaining
100
50
0
0
100
200
Time (min.)

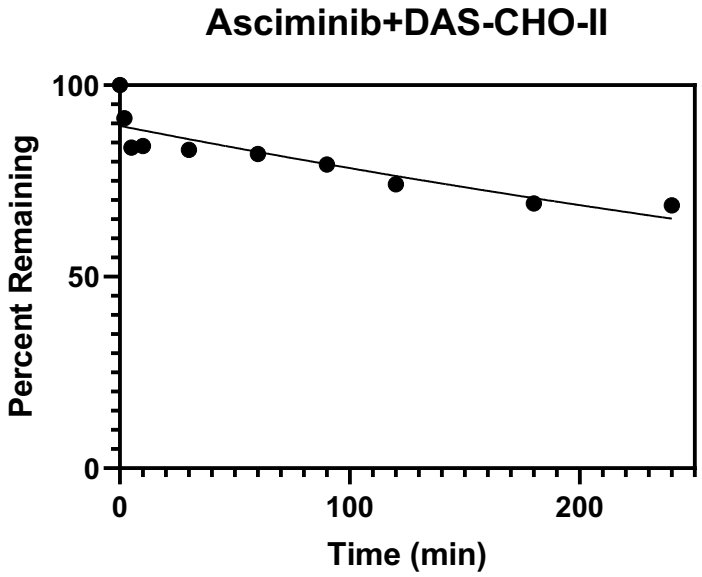

DAS-DFGO-II
100
50
0
0
100
200


DAS-CHO-II
100
50
0
0
100
200


GNF-2 + DAS-DFGO-II
Percent Remaining
100
50
0
0
100
200
Time (min)


GNF-2+DAS-CHO-II
Percent Remaining
100
50
0
0
100
200
Time (min)


Asciminib+DAS-DFGO-II
Percent Remaining
100
50
0
0
100
200
Time (min)


Asciminib+DAS-CHO-II
Percent Remaining
100
50
0
0
100
200
Time (min)

VII. References

(1) Chou, T.-C.; Talalay, P. Quantitative Analysis of Dose-Effect Relationships: The Combined Effects of Multiple Drugs or Enzyme Inhibitors. *Adv. Enzym. Regul.* **1984**, *22*, 27–55.