Appendix 2

Evaluate inhibition pattern for compound 95 and 96 using Cleland's program

Cis amide

Data

Velocity*1E6	sub conc*1E6	cis amide conc*1E6
1.81200	25.5700	0.00000n1
1.86800	25.5700	0.00000
4.19300	62.3300	0.00000
4.35400	62.3300	0.00000
7.36800	124.7000	0.00000
6.83600	124.7000	0.00000
10.58000	249.3000	0.00000
10.06000	249.3000	0.00000
12.45000	498.6000	0.00000
12.46000	498.6000	0.00000
1.07000	25.5700	1.51000
1.04500	25.5700	1.51000
2.55100	62.3300	1.51000
2.79700	62.3300	1.51000
4.72300	124.7000	1.51000
4.34900	124.7000	1.51000
7.20600	249.3000	1.51000
7.43000	249.3000	1.51000
10.21000	498.6000	1.51000
0.67560	25.5700	3.02000
0.75390	25.5700	3.02000
1.63600	62.3300	3.02000
1.99600	62.3300	3.02000

3.33500	124.7000	3.02000
3.60800	124.7000	3.02000
5.66700	249.3000	3.02000
6.16700	249.3000	3.02000
8.53500	498.6000	3.02000
0.45130	25.5700	6.04000
0.48730	25.5700	6.04000
1.30600	62.3300	6.04000
1.28900	62.3300	6.04000
2.39200	124.7000	6.04000
2.15600	124.7000	6.04000
3.83300	249.3000	6.04000
4.33400	249.3000	6.04000
6.57600	498.6000	6.04000

Competitive hyperbolic fit

FIT TO Y = V*A/(K (1+I/KI) + A)

K = 185.010178   S.E. (K)   = 7.634134   W = 0.17159E-01

V = 17.212734   S.E. (V)   = 0.301141   W = 0.11027E+02

KIS= 1.797789   S.E. (KIS) = 0.070553   W = 0.20090E+03

1/KIS = 0.556239   C.V. (KIS) = 0.039244   WL = 1/CVKIS**2 = 0.64931E+03

SIGMA = 0.182715402

VARIANCE = 0.33385E-01

Competitive linear fit

FIT TO LOG (V) = LOG (VMAX*A/K (1+I/KI) + A))

K = 234.516288   S.E. (K)   = 18.902564   W = 0.27987E-02

V = 19.520768   S.E. (V)   = 1.021601   W = 0.95816E+00

KI = 1.923905   S.E. (KI) = 0.108867   W = 0.84373E+02

VARIANCE = 0.46118E-02   SIGMA = 0.0679103

Non-competitive hyperbolic fit

FIT TO   Y = V*A/(K (1+I/KIS) + A (1+I/KII))

V = 17.180101   S.E. (V)   = 0.335273   W = 0.88962E+01

K = 184.222650   S.E. (K)   = 8.426834   W = 0.14082E-01

KIS= 1.775674   S.E. (KIS) = 0.115439   W = 0.75040E+02

KII= inf   S.E. (KII) = inf   W = 0.00000E+00

CPT= inf   S.E. (CPT) = nan   W = nan

CKII/CKIS = inf   S.E. (CKII/CKIS) = inf

SIGMA = 0.186228334

VARIANCE = 0.34681E-01

Non-competitive linear fit

FIT TO LOG Y = LOG (V*A/(K (1+I/KIS)+A (1+I/KII)))

CPT = K*KII/KIS

K = 225.022234   S.E. (K)   = 21.303159   W = 0.22035E-02

V = 18.976965   S.E. (V)   = 1.172075   W = 0.72793E+00

KIS= 1.853622   S.E. (KIS) = 0.134460   W = 0.55311E+02

KII=   -40.818959   S.E. (KII) = 48.297840   W = 0.42869E-03

1/KIS = 0.539484   C.V. (KIS) = 0.072539   WL = 1/CVKIS**2 = 0.19004E+03

1/KII =   -0.024498   C.V. (KII) =   -1.183221   WL = 1/CVKII**2 = 0.71428E+00

CPT= -4955.256541   S.E. (CPT) = *********** W = 0.28874E-07

SIGMA = 0.068223555

VARIANCE = 0.46545E-02

Uncompetitive hyperbolic fit

FIT TO Y = V*A/(K + A (1+I/KI))

K = 373.720543   S.E. (K)   = 73.140554   W = 0.18693E-03

V = 23.335224   S.E. (V)   = 2.649899   W = 0.14241E+00

KII = 2.139885   S.E. (KII) = 0.366368   W = 0.74502E+01

1/KII = 0.467315   C.V. (KII) = 0.171209   WL = 1/CVKII**2 = 0.34115E+02

SIGMA = 0.856051711

VARIANCE = 0.73282E+00

Uncompetitive linear fit

FIT TO LOG Y = V*A/(K+A (1+I/KI))

K = 1575.579772   S.E. (K)   = 1464.483376   W = 0.46626E-06

V = 70.480765   S.E. (V)   = 61.641889   W = 0.26318E-03

KII = 0.500892   S.E. (KII) = 0.474976   W = 0.44326E+01

1/KII = 1.996436   C.V. (KII) = 0.948260   WL = 1/CVKII**2 = 0.11121E+01

SIGMA = 0.312440689

VARIANCE = 0.97619E-01

Trans amide

Data

Velocity*1E6	sub conc*1E6	trans amide conc*1E6
1.81200	25.5700	0.00000
1.86800	25.5700	0.00000
4.19300	62.3300	0.00000
4.35400	62.3300	0.00000
7.36800	124.7000	0.00000
6.83600	124.7000	0.00000
10.58000	249.3000	0.00000

10.06000	249.3000	0.00000
12.45000	498.6000	0.00000
12.46000	498.6000	0.00000
0.53030	25.5700	118.400
0.53800	25.5700	118.400
1.19000	62.3300	118.400
1.14200	62.3300	118.400
2.50200	124.7000	118.400
2.36300	124.7000	118.400
4.04700	249.3000	118.400
4.38200	249.3000	118.400
6.77800	498.6000	118.400
0.77580	25.5700	59.200
0.80870	25.5700	59.200
2.25800	62.3300	59.200
2.17200	62.3300	59.200
3.65800	124.7000	59.200
3.99900	124.7000	59.200
6.80100	249.3000	59.200
6.56700	249.3000	59.200
9.18500	498.6000	59.200

Competitive hyperbolic fit

FIT TO Y = V*A/(K (1+I/KI) + A)

K = 180.403068   S.E. (K)   = 11.823758   W = 0.71530E-02

V = 17.281529   S.E. (V)   = 0.478636   W = 0.43651E+01

KIS= 39.760282   S.E. (KIS) = 2.374018   W = 0.17743E+00

1/KIS = 0.025151   C.V. (KIS) = 0.059708   WL = 1/CVKIS**2 = 0.28050E+03

SIGMA = 0.277612420

VARIANCE = 0.77069E-01

Competitive linear fit

FIT TO LOG (V) = LOG (VMAX*A/K (1+I/KI) + A))

K = 231.513671   S.E. (K)   = 22.092132   W = 0.20489E-02

V = 19.657951   S.E. (V)   = 1.220443   W = 0.67137E+00

KI = 41.404828   S.E. (KI) = 2.634119   W = 0.14412E+00

VARIANCE = 0.52922E-02   SIGMA = 0.0727474

Non-competitive hyperbolic fit

FIT TO   Y = V*A/(K (1+I/KIS) + A (1+I/KII))

CPT = K*KII/KIS

V = 17.322021   S.E. (V)   = 0.515173   W = 0.37679E+01

K = 181.335228   S.E. (K)   = 12.659081   W = 0.62402E-02

KIS= 40.599144   S.E. (KIS) = 4.163691   W = 0.57682E-01

KII= 3335.637745   S.E. (KII) = ***********   W = 0.58965E-08

CPT= 14898.556257   S.E. (CPT) = ***********   W = 0.92723E-08

CKII/CKIS = 82.160297   S.E. (CKII/CKIS) = 327.568095

SIGMA = 0.282926313

VARIANCE = 0.80047E-01

Non-competitive linear fit

FIT TO LOG Y = LOG (V*A/(K (1+I/KIS)+A(1+I/KII)))

CPT = K*KII/KIS

K = 224.167927   S.E. (K) = 24.317137   W = 0.16911E-02

V = 19.225051   S.E. (V) = 1.364606   W = 0.53701E+00

KIS= 40.107880   S.E. (KIS) = 3.252180   W = 0.94548E-01

KII= -977.356207 S.E. (KII) = 1484.196610 W = 0.45396E-06

1/KIS = 0.024933 C.V. (KIS) = 0.081086 WL = 1/CVKIS**2 = 0.15209E+03

1/KII = -0.001023 C.V. (KII) = -1.518583 WL = 1/CVKII**2 = 0.43363E+00

CPT= -5462.565323 S.E. (CPT) = *********** W = 0.14521E-07

SIGMA = 0.073594352

VARIANCE = 0.54161E-02

Uncompetitive hyperbolic fit

FIT TO Y = V*A/(K + A (1+I/KI))

K = 319.693973 S.E. (K) = 73.183961 W = 0.18671E-03

V = 21.728596 S.E. (V) = 2.684360 W = 0.13878E+00

KII = 50.252416 S.E. (KII) = 9.587739 W = 0.10878E-01

1/KII = 0.019900 C.V. (KII) = 0.190792 WL = 1/CVKII**2 = 0.27471E+02

SIGMA = 0.948418806

VARIANCE = 0.89950E+00

Uncompetitive linear fit

FIT TO LOG Y = V*A/(K+A (1+I/KI))

K = 1282.867746 S.E. (K) = 1220.300958 W = 0.67153E-06

V = 59.374434 S.E. (V) = 52.094329 W = 0.36848E-03

KII = 12.759498 S.E. (KII) = 12.139670 W = 0.67856E-02

1/KII = 0.078373 C.V. (KII) = 0.951422 WL = 1/CVKII**2 = 0.11047E+01

SIGMA = 0.344043842

VARIANCE = 0.11837E+00

Protocol for Pin1 Inhibition Assay IC_{50}

By Xiaodong J. Wang

Feb 7, 2004

IC_{50} measurement

1. Prepare solutions/buffers

Assay buffer: 35 mM HEPES, pH 7.8

Pin1 buffer: 20 mM Tris-HCl, pH 7.8

Substrate solution: in a flame-dried glass vial with a septum, cooled under dry N_2, prepare 400 μL ~ 6 mM substrate (Suc-Ala-Glu-Pro-Phe-pNA) solution in properly dried TFE (distilled from sodium before use) containing 0.5 M LiCl dried under vacuum at 150 °C for 24 hr.

Determine the concentration of substrate (Suc-Ala-Glu-Pro-Phe-pNA). Hydrolyze ~ 0.2 mg substrate in 0.2 mL of 6 M HCl in a sealed glass reactor at 110°C for 24 hr. Dilute the hydrolysate in 2 mL 1:1 H_2O:CH_3CN. Prepare a standard solution of p-nitroaniline and dilute to concentrations of: 0.5 mM, 0.75 mM, 1.0 mM, 1.25 mM, 1.5mM, 1.75 mM, 2.0 mM. Inject each standard 20 μL on 100 × 046 C18 column HPLC and monitor at 220 nm to create a standard curve. Inject the substrate solution 3 times (20 μL) and determine the concentration by linear fit to the standard curve.

α-Chymotrypsin solution: 60mg (51unit/mg)/ mL 0.001 M HCl

Inhibitor solutions: stocks of 2000 μM, 200 μM, 20 μM in 1:3 DMSO: H_2O (for IC_{50} ~1 μM, if the inhibition is poorer, use higher inhibitor concentrations). Determine the concentration of inhibitor in a similar fashion as above.

2. Choose final concentration of Pin1 (67 nM, concentration measured by Bradford assay) to give $k_{obs} \approx 0.07\ s^{-1}$ without inhibitors.
3. To a 1.5 mL polystyrene cuvette (application range 280 nm - 800 nm), add
    a) 1050 μL assay buffer (HEPES, 35 mL, pH 7.8)
    b) 10 μL Pin1 stock solution in 20 mM Tris-HCl, pH 7.8
    c) 10 μL of inhibitor stock solution
4. Incubate at 4 °C for 10 min
5. Assay samples:

    To the same cuvette, add

    a) 120 μL 4°C α-chymotrypsin solution
    b) 10 μL substrate solution

    Mix by inversion 3 times and start scan, read every 0.5 s at 390 nM for 90 s.
6. Fit the data to a first order exponential equation $A=A_0+A_1 * \exp(-k_{obs} \cdot t)$ to obtain $k_{obs.}$ (We use Tablecurve3.2 program)
7. Calculate percent inhibition vs. inhibitor concentration, thermal isomerization rate constant $k_3 = 0.00257\ s^{-1}$ determined independently for each day.
8. Fit the percent inhibition vs. inhibitor concentration to a hyperbolic equation. IC_{50} is given by curve fitting Eq. $y = \frac{a \cdot x}{b + x}$. (We use Tablecurve3.2 program, for those data that would not fit in above equation, fit them to equation #8013 in Tablecurve)

Protocol for Pin1 Inhibition Assay K_i

By Xiaodong J. Wang

Feb 7, 2004

K_i measurement

9. Choose final concentration of Pin1 to give $k_{obs} \approx 0.07\ s^{-1}$ without inhibitors.
10. Measure IC_{50} first so that the range of inhibitors concentration to use can be determined.
11. To a 15 mL Falcon tube, add
    a) 12.6 mL assay buffer (HEPES, 35 mM, pH 7.8)
    b) 120 μL Pin1 solution (proper concentration in 20 mM Tris-HCl, pH 7.8)
    c) 120 μL of inhibitor solution (stock of 720 μM in 1:3 DMSO: H_2O, final concentration 6 μM [a])
12. Incubate at 4°C for 10 min.
13. Assay samples:

    To a polystyrene cuvette (capacity 1.5 mL, application range 280 nM—800 nM) add:

    a) 1070 μL of the above solution from the Falcon tube.
    b) 120 μL 4°C α-chymotrypsin solution (60 mg/mL in 0.001 HCl, 51unit/mg).
    c) 10 μL of Suc-Ala-Glu-Pro-Phe-*p*NA solution (stocks of 6.2 mM in 0.48 M LiCl in TFE, final concentration 52 μM, final cis substrate concentration ~26 μM[b]).

Mix by inversion 3 times and start scan, read every 0.5 s at 390 nM for 90 s.

14. Fit the data to a first order exponential equation $A=A_0+A_1* \exp(-k_{obs}\bullet t)$ or equation $a \ln\left(1-\frac{A-d}{c+b+a}\right)+b\cdot\ln\left(1-\frac{A-d}{c}\right)+k_3\cdot t\cdot(a+b)=0$ to obtain parameters a, b, c, and d. (We use Tablecurve3.2 program)
15. Calculate initial velocity by equation $v_{0,\, enzyme}=\frac{a\cdot k_3}{b+c}\cdot[cis]_0$, thermal isomerization rate constant k_3 = 0.00257 s^{-1}.
16. Repeat steps 5, 6, 7 using different Suc-Ala-Glu-Pro-Phe-*p*NA concentration (stocks of 15.5 mM, 31 mM, 62 mM, 124 mM, final concentration 129 μM, 258 μM, 517 μM, final cis substrate concentration ~52 μM, ~129 μM, ~258 μM[b]) assay twice for each concentration.
17. Repeat steps 3, 4, 5, 6, 7, 8, using different inhibitor concentration (stocks of 360 μM, 180 μM and 0 in 1:3 DMSO: H_2O, final concentration 3 μM, 1.5 μM and 0[a]).
18. Run Cleland Program on Indigo to get the inhibition pattern (competitive, non-competitive or uncompetitive), K_i value and errors.

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a. Those concentrations are only good for Suc-Ala-Glu-Pro-Phe-*p*NA, K_m=180 μM, for other substrate, change those concentrations according to its K_m value.

b. Those inhibitors concentrations are only good for an inhibitor with IC_{50} value of around 1 μM. Increase those concentrations for poorer inhibitor.