

GPCR-stimulated cAMP signaling kinetics

Mock report for demonstration purposes

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Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

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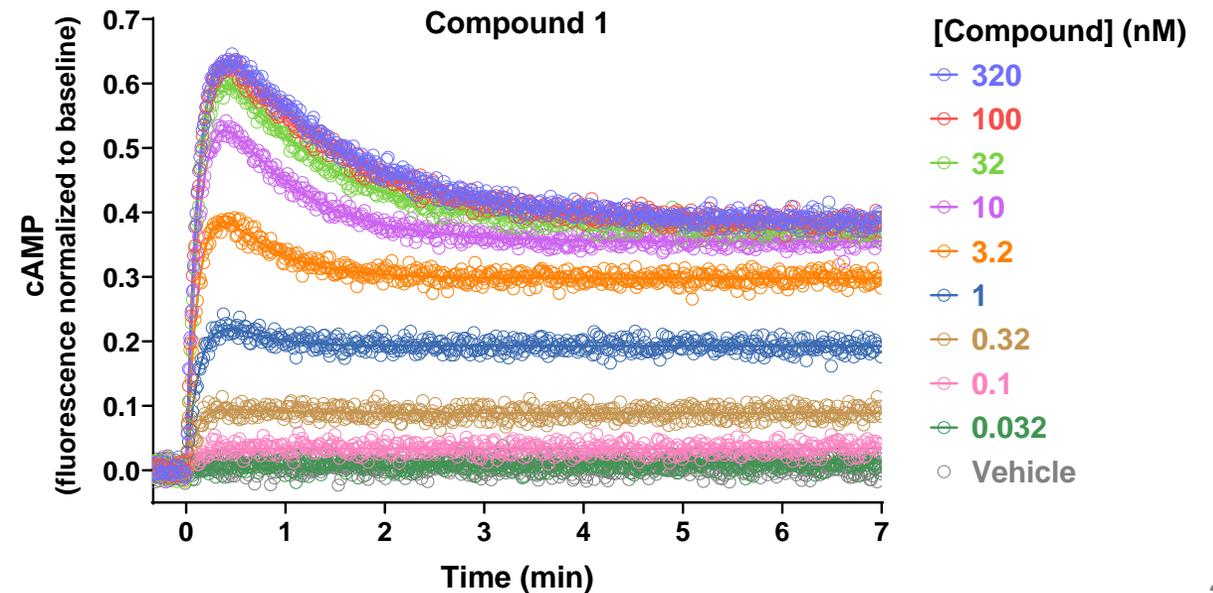
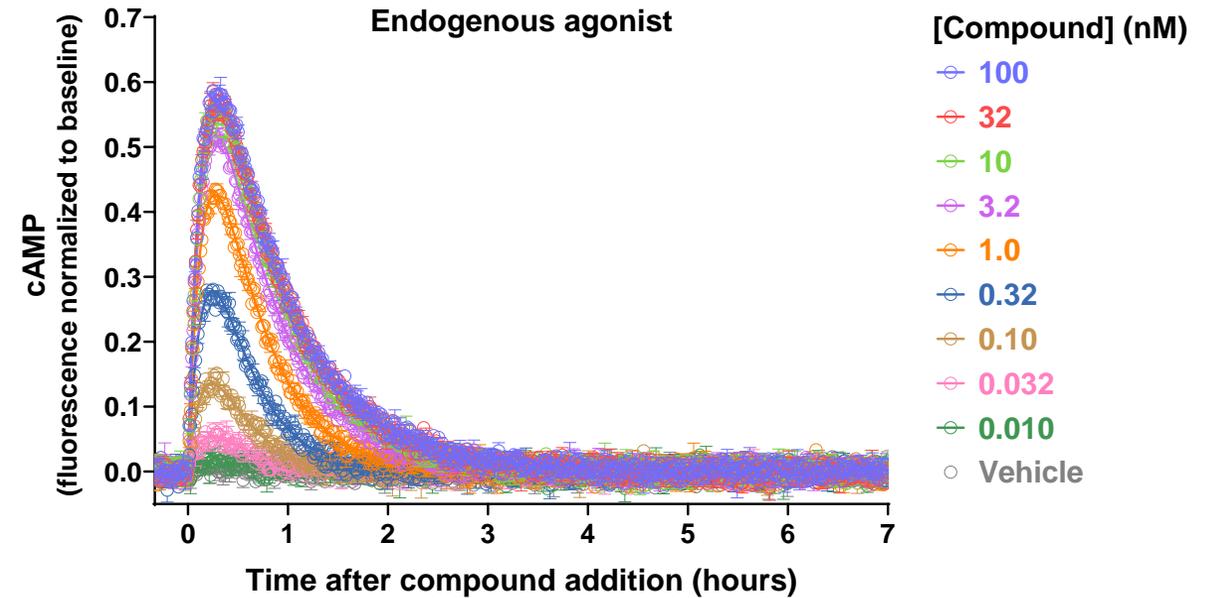
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Summary

- cAMP generation stimulated by a GPCR was measured using the cADDis fluorescent cAMP biosensor in live cells for seven hours.
- The response to the endogenous peptide agonist and three small molecule agonists was measured.
- The response to the peptide agonist became fully desensitized, the response returning to baseline.
- By contrast the response to small molecule agonists was sustained, the signal persisting up to seven hours.
- Compound potency (EC_{50}) and E_{max} was quantified for various kinetic parameters. Compound 3 was a partial agonist.

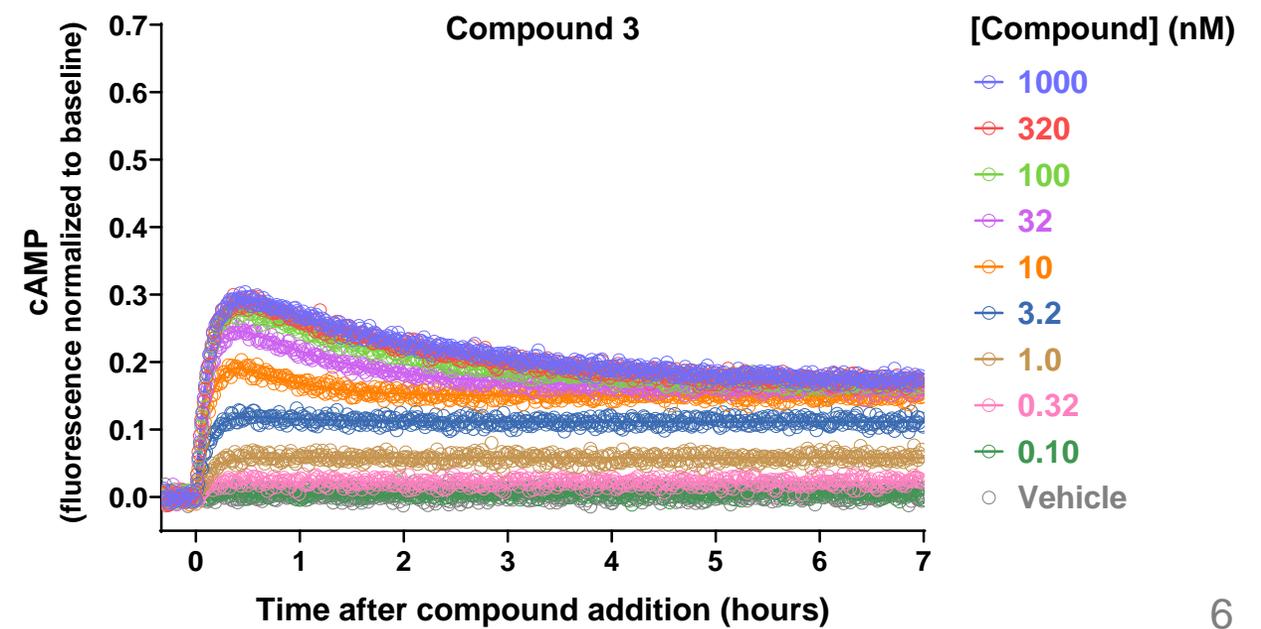
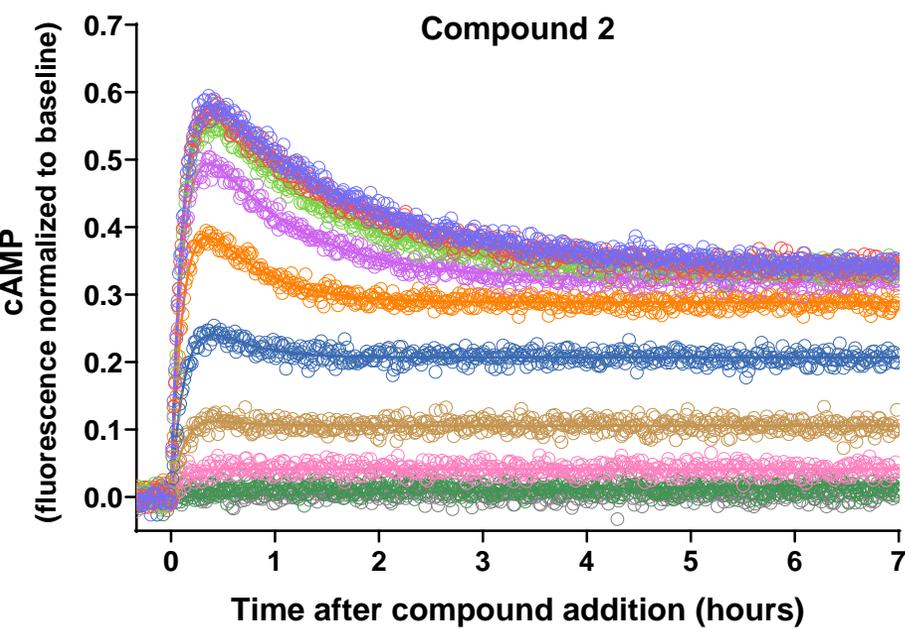
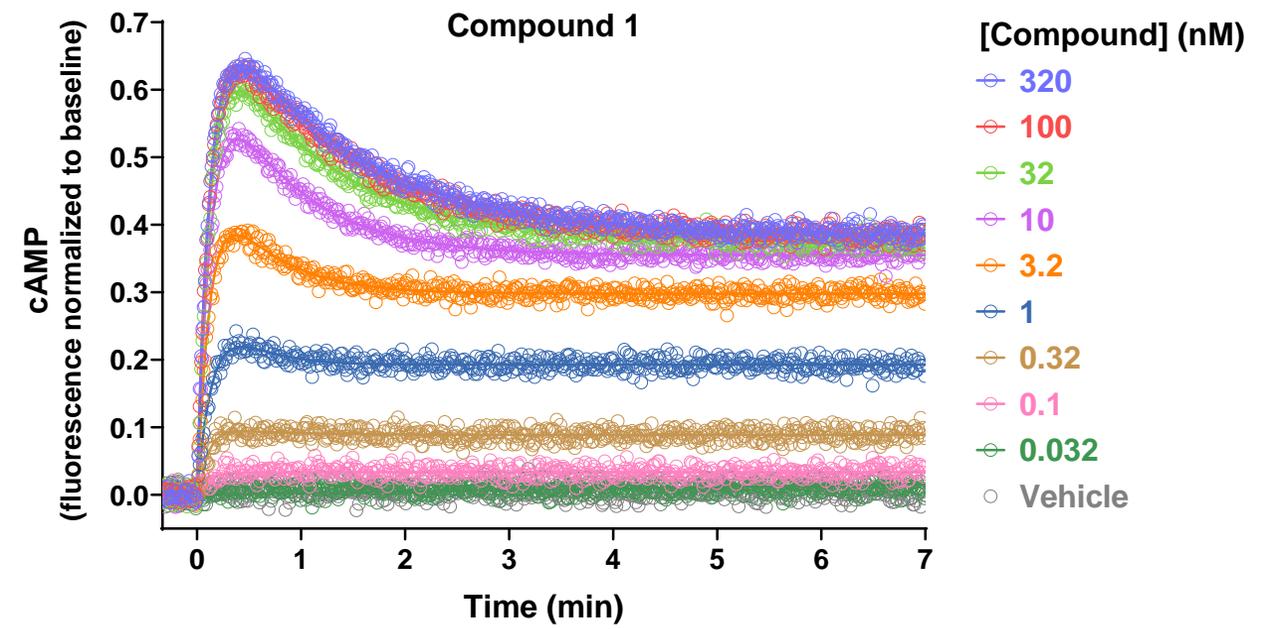
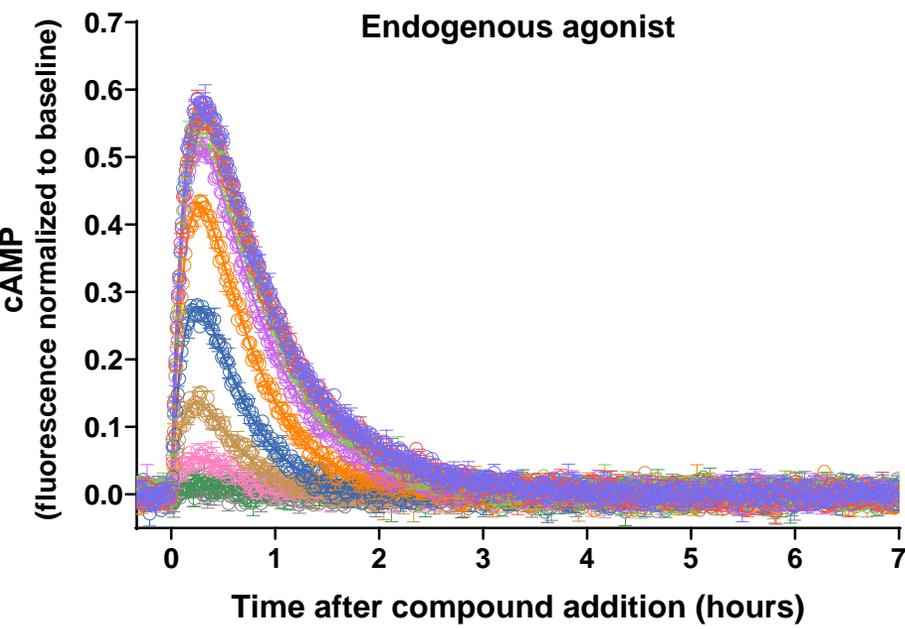
[Note this report employs simulated data]



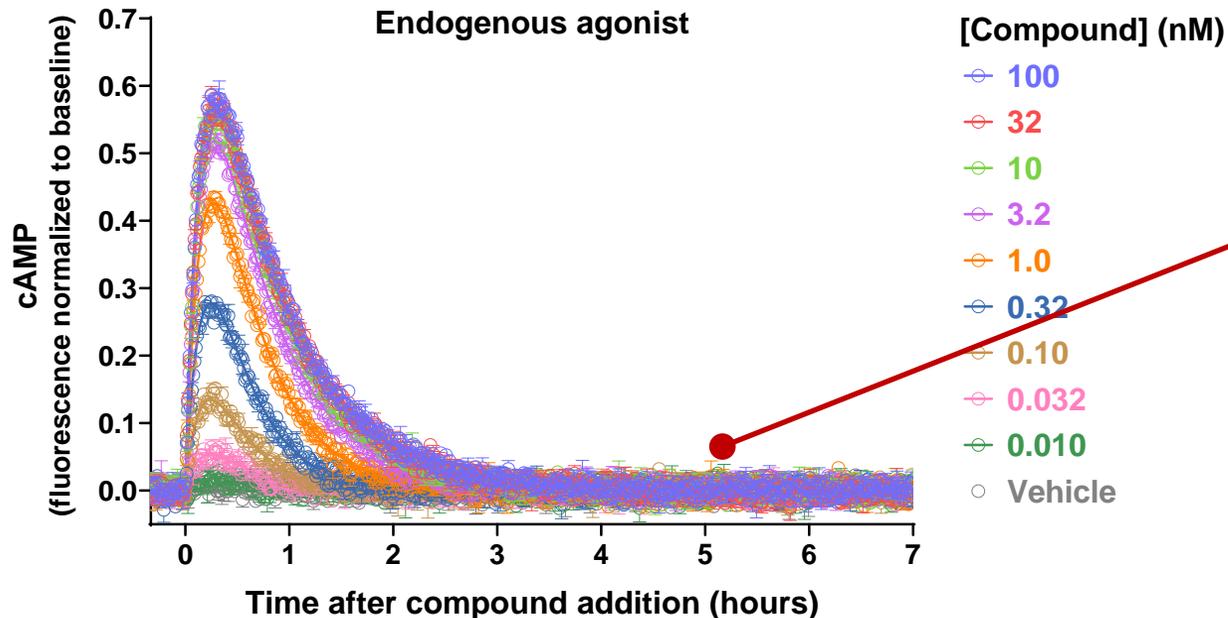
- ❑ HEK293T cells ([ATCC](#)) transduced with the GPCR and [Green Upward cADDIs biosensor](#).
- ❑ cAMP: Fluorescence measured on a [BioTek Neo2](#) and agonist injection performed on [Integra Viaflo 384](#). Baseline measured for 15 min at 45 sec intervals, agonist added, and fluorescence measured for another 7 hr.
- ❑ Compounds and controls:
 - Compounds serially-diluted in 100% DMSO in a low-binding plate, 1/2 log dilution factor.
 - Compounds diluted in DPBS then transferred to assay plate (0.3% DMSO on assay plate).
 - Negative control: Vehicle-only treated samples.
- ❑ Time course data [normalized](#) to baseline fluorescence and vehicle [subtracted](#).
- ❑ Time course data analyzed with [kinetic equations](#) and dose response of fitted parameters determined.
- ❑ Time course data shown as mean \pm SEM from 2 technical replicates.



Time course data



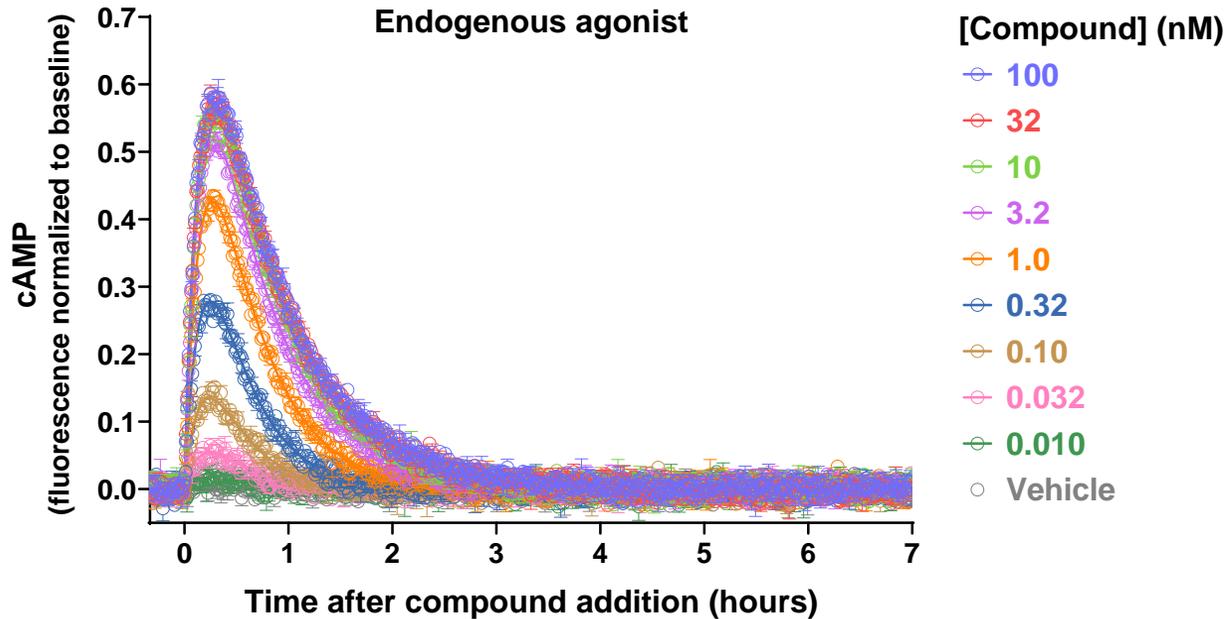
Time course curve shape – endogenous agonist



- cAMP rises to a peak in response to endogenous agonist, then declines.
- Response declines completely back down to baseline level.
- This decline is probably due to receptor desensitization ^{1,2}.

1. [Front Cell Neurosci 2022, 15:814547](#)
2. [Sci Report 2020 10: 12263](#)

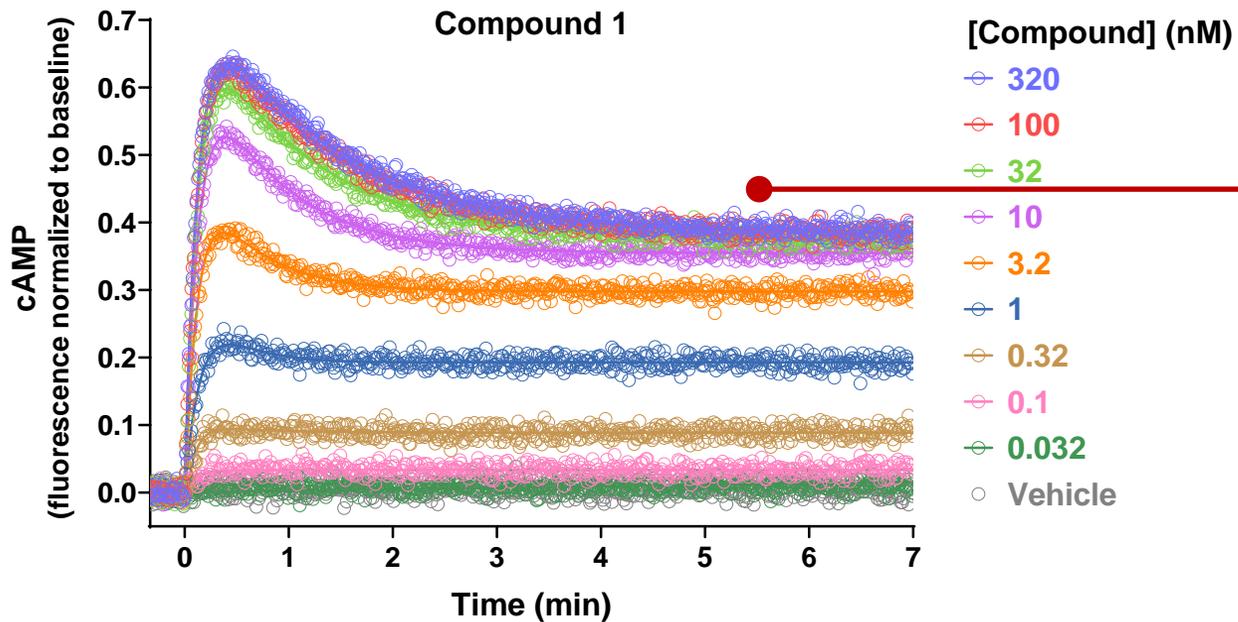
Curve fitting – endogenous agonist



- Data for active concentrations fit to a rise and fall to baseline curve.
- Data for inactive concentrations fit to a straight line curve.
- Parameters quantified include peak cAMP, cAMP generation rate (initial rate), and decline rate.
- Data analysis was performed using GraphPad Prism, utilizing the Parmechnics plug in of time course equations ¹⁻⁴ . See [here](#) for details of curve fitting procedure.

1. [Front Cell Neurosci 2022, 15:814547](#)
2. [Sci Report 2020 10: 12263](#)
3. www.pharmechnics.com/time-course-tool-pack
4. https://youtu.be/_Pb7Sq6lZlY

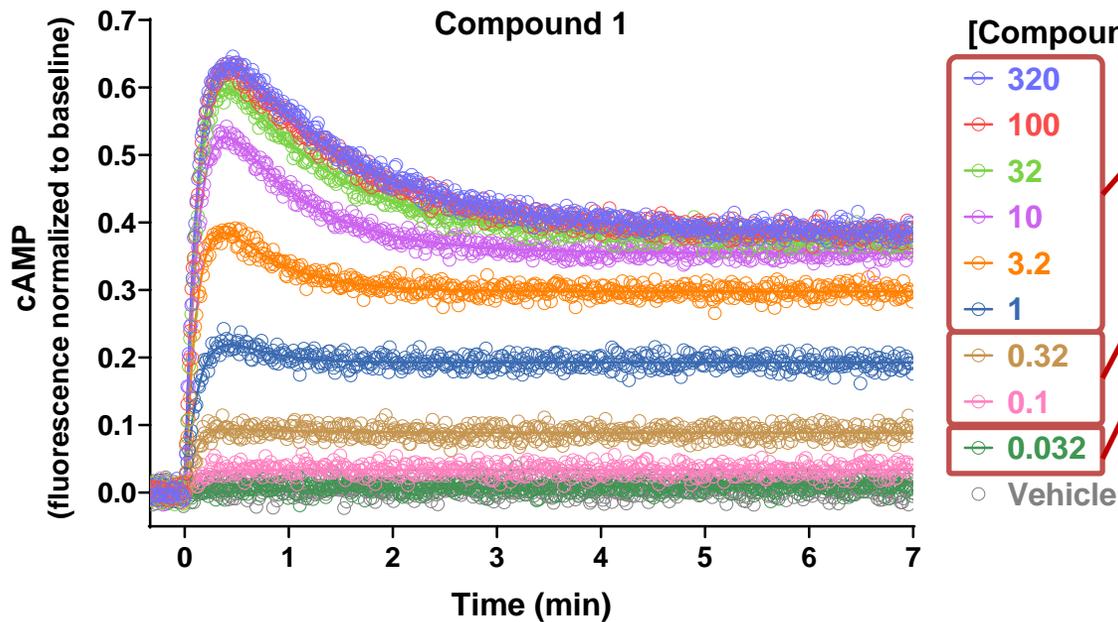
Time course curve shape – test compounds



- cAMP rises to a peak in response to endogenous agonist, then declines.
- Response declines down to a level that is above baseline, indicating persistent signaling.
- This persistent signaling could be due to resensitization of the receptor, or signaling by internalized receptors ^{1,2}.

1. [Front Cell Neurosci 2022, 15:814547](#)
2. [Sci Report 2020 10: 12263](#)

Curve fitting – endogenous agonist



Three time course shapes were observed, dependent on the agonist concentration.

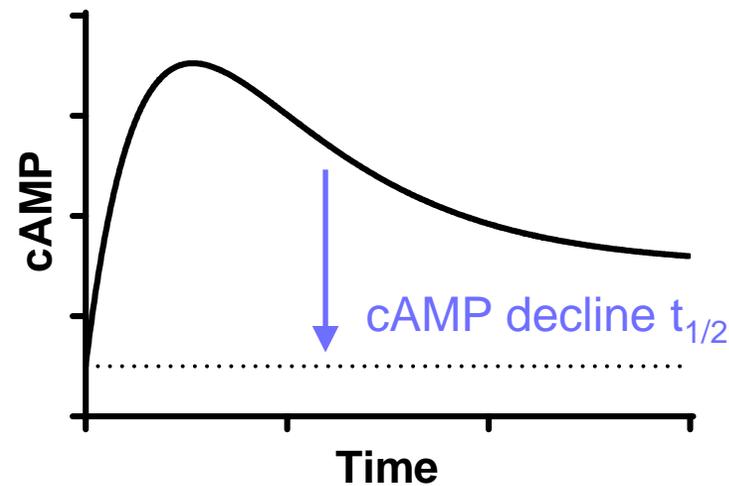
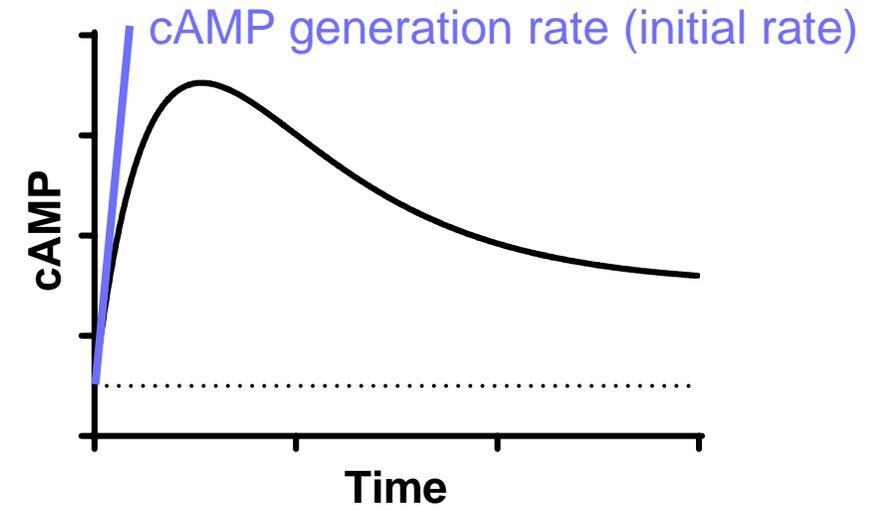
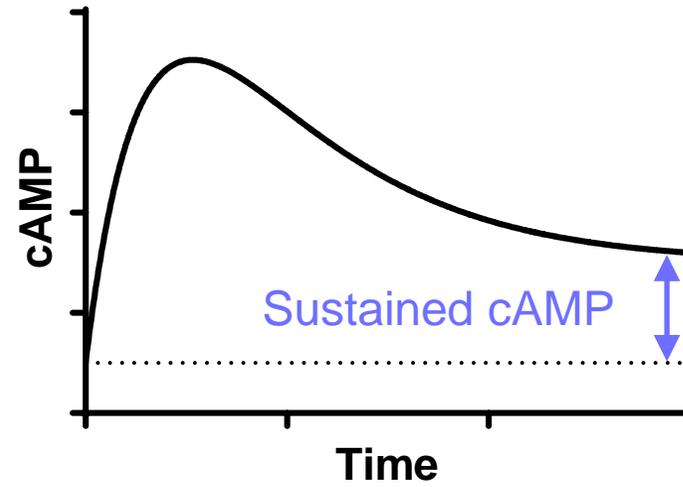
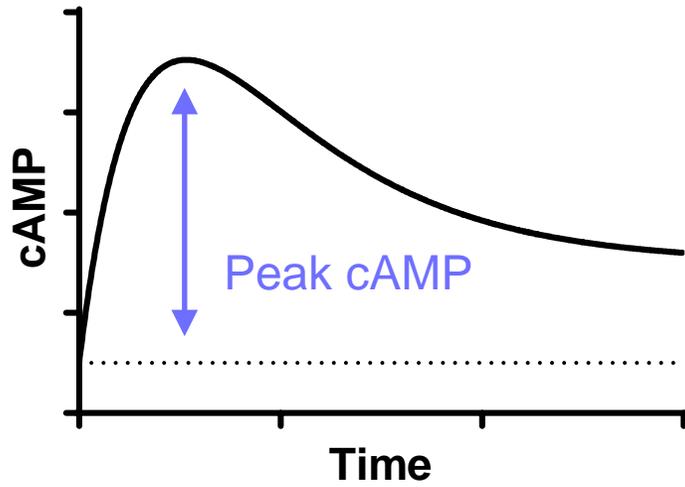
1. Rise and fall to steady-state curve (highest concs.)
2. Rise to steady-state curve (intermediate concs.)
3. Straight line (lowest, inactive concs.)

For active concentrations, data were fit to both the rise and fall to steady-state curve and rise to steady-state curve. The preferred fit was then determined using a partial F-test – see [here](#).

Data analysis was performed using GraphPad Prism, utilizing the Pharmedics plug in of time course equations ¹⁻⁴. See [here](#) for details of curve fitting procedure.

1. [Front Cell Neurosci 2022, 15:814547](#)
2. [Sci Report 2020 10: 12263](#)
3. [www.pharmedics.com/time-course-tool-pack](#)
4. <https://youtu.be/Pb7Sq6lZlY>

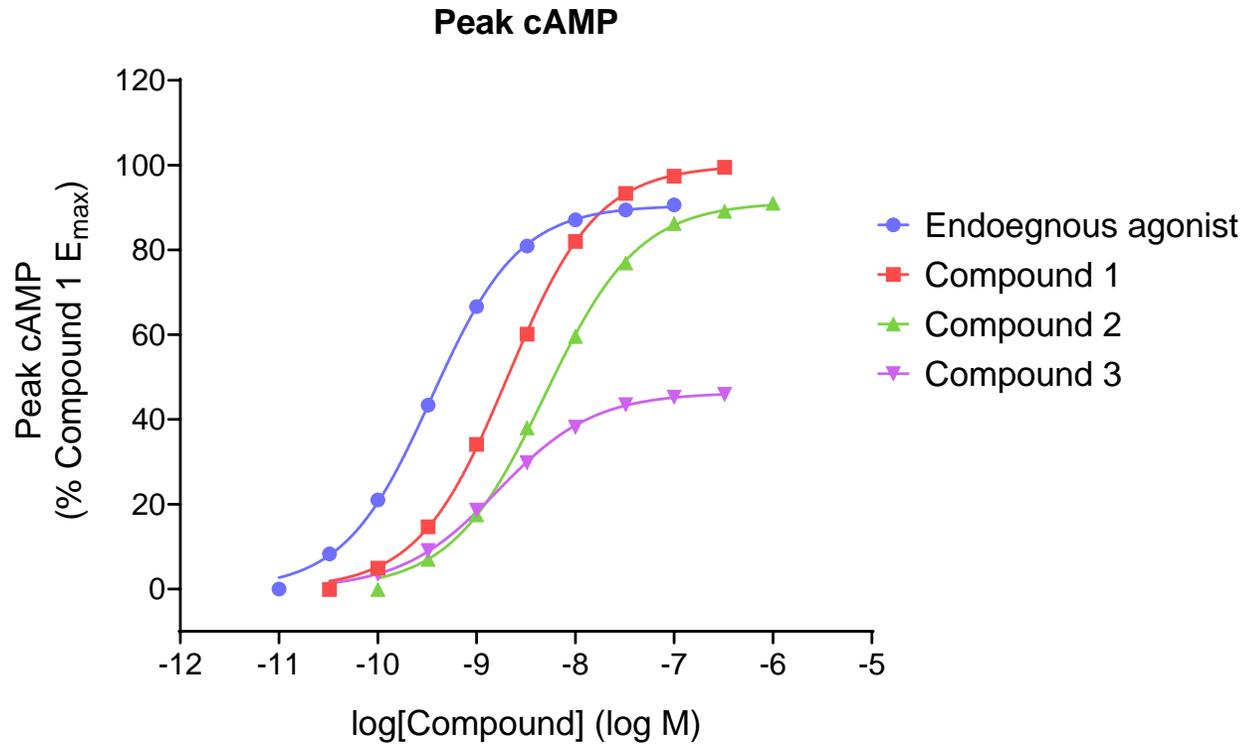
Parameters quantified





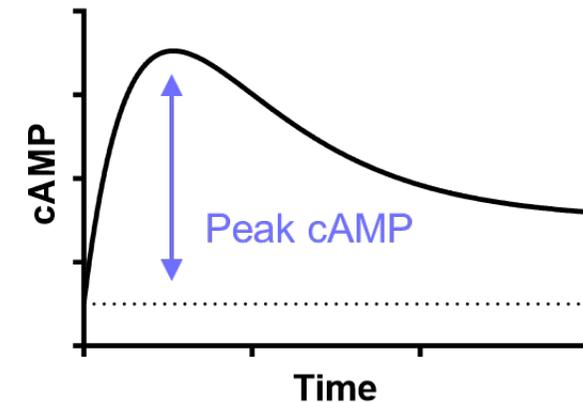
Concentration response data

Peak cAMP concentration response

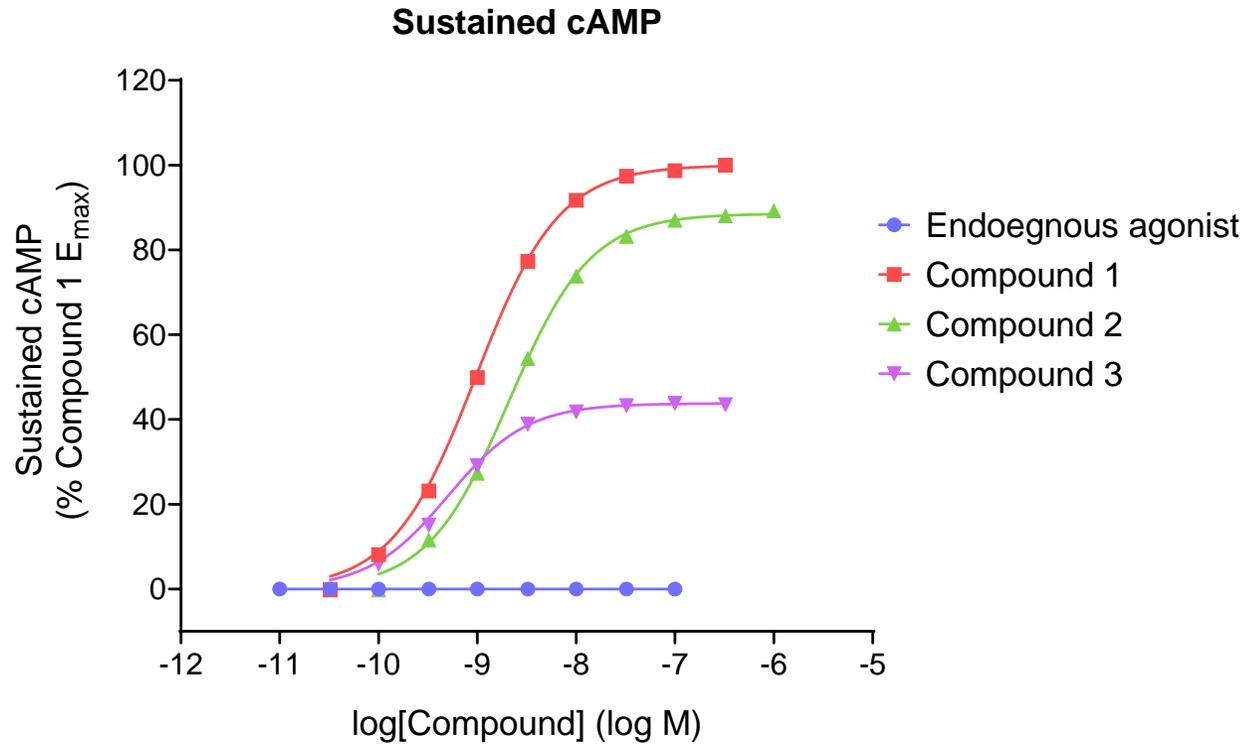


- The endogenous agonist is the most potent ligand (EC_{50} 0.35 nM).
- Compounds 1 and 3 are the most potent small molecules (EC_{50} 2.1 and 1.6 nM).
- Compound 3 is a partial agonist.

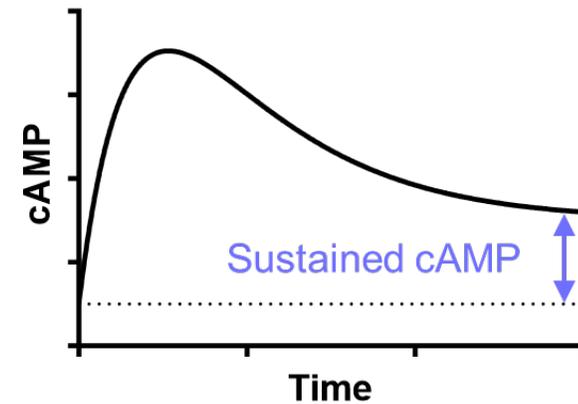
Compound	EC_{50} (nM)	E_{max} (% Cmpd 1 E_{max} ^A)
Endogenous agonist	0.35	91
Compound 1	2.1	100
Compound 2	4.9	91
Compound 3	1.6	46



Sustained cAMP concentration response

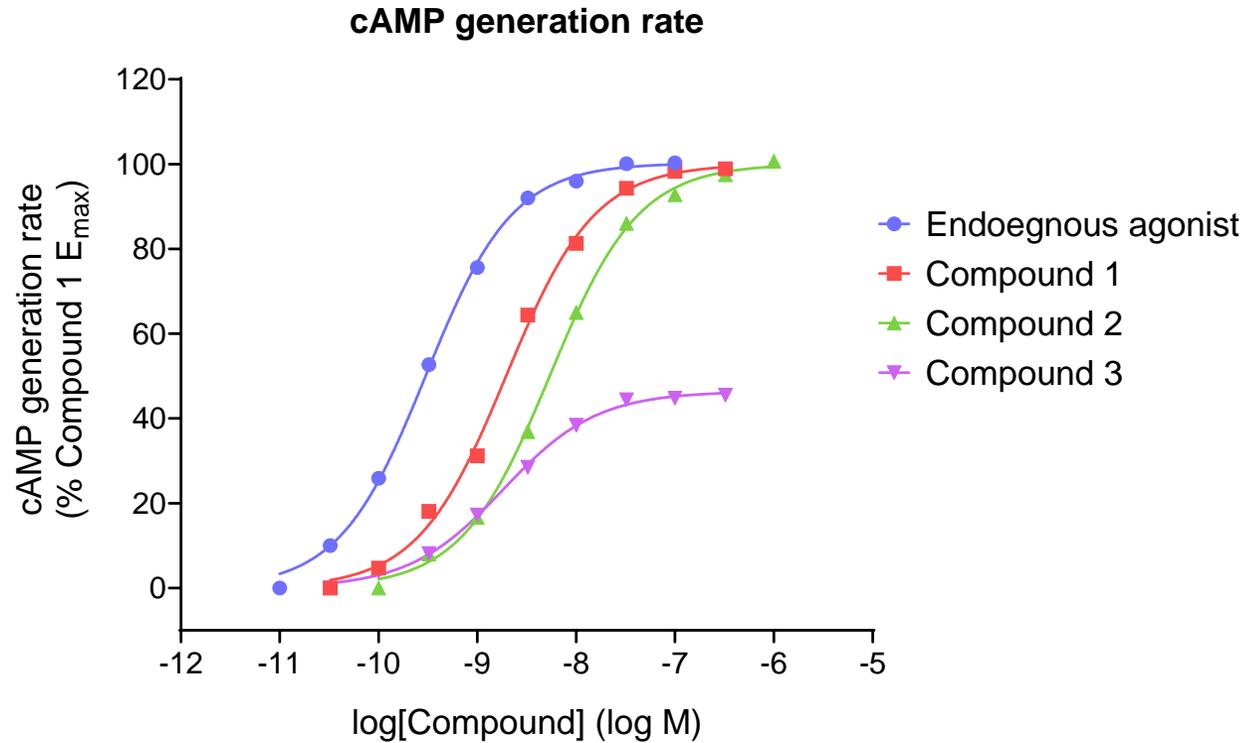


- No sustained signaling detected for the endogenous agonist.
- Compound potency slightly higher than for peak cAMP (compare with previous page).
- Compound 3 is a partial agonist.



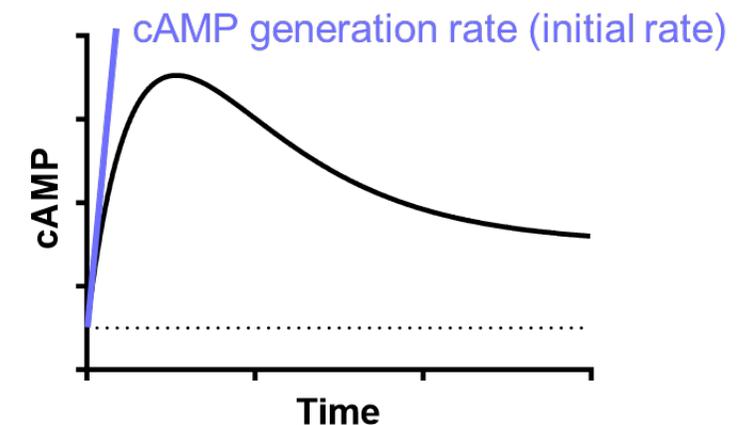
Compound	EC ₅₀ (nM)	E _{max} (% Cmpd 1 E _{max} ^A)
Endogenous agonist	Not detected	Not detected
Compound 1	0.96	100
Compound 2	2.1	89
Compound 3	0.50	44

cAMP signal generation rate (initial rate)



- Maximum cAMP generation rate the same for endogenous agonist, Compound 1 and Compound 2.
- For Compound 3, maximum cAMP generation rate is lower, indicating partial agonism involves a reduced cAMP generation rate by the Compound 3-bound receptor.

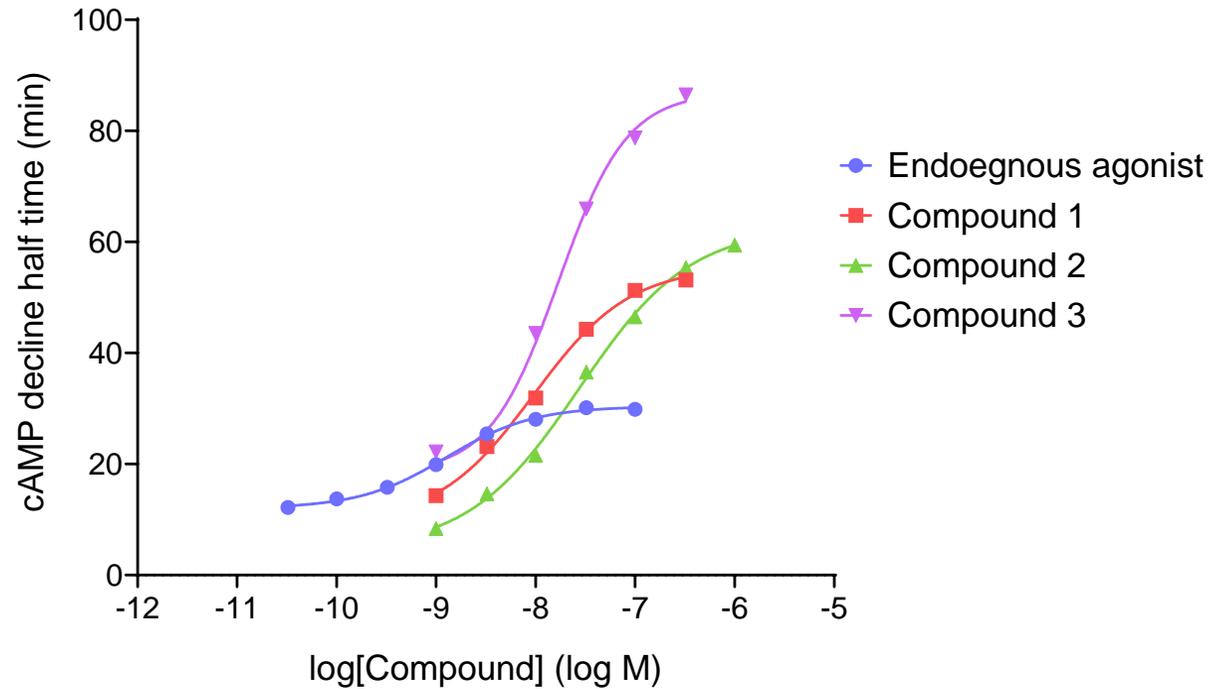
Compound	EC ₅₀ (nM)	E _{max} (% Cmpd 1 E _{max} ^A)
Endogenous agonist	0.30	100
Compound 1	2.0	100
Compound 2	5.4	100
Compound 3	1.8	46



A. E_{max} for Cmpd 1 is 0.10 normalized fluorescence units per min

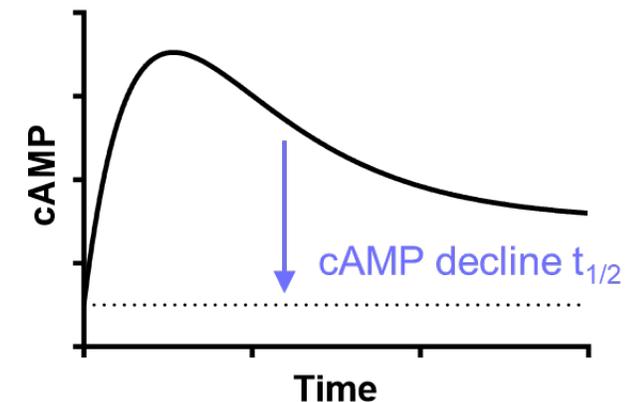
cAMP decline half time

cAMP decline half time



- Decline for small molecules slower than decline for endogenous ligand (higher maximum half time).
- Decline for partial agonist Compound 3 slightly slower than that for full agonists Compounds 1 and 2.

Compound	EC ₅₀ (nM)	E _{max} (min)
Endogenous agonist	1.3	30
Compound 1	9.5	56
Compound 2	27	63
Compound 3	17	87

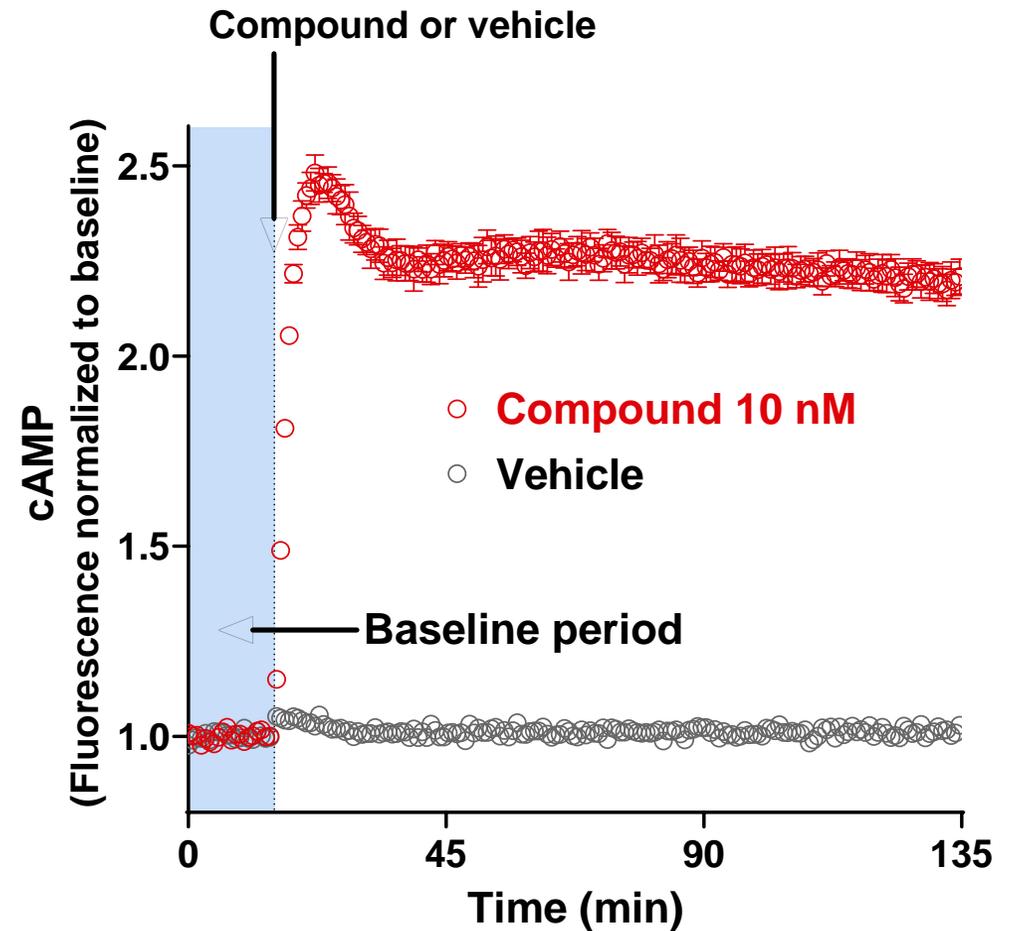
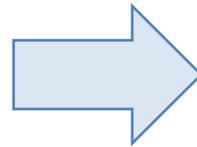
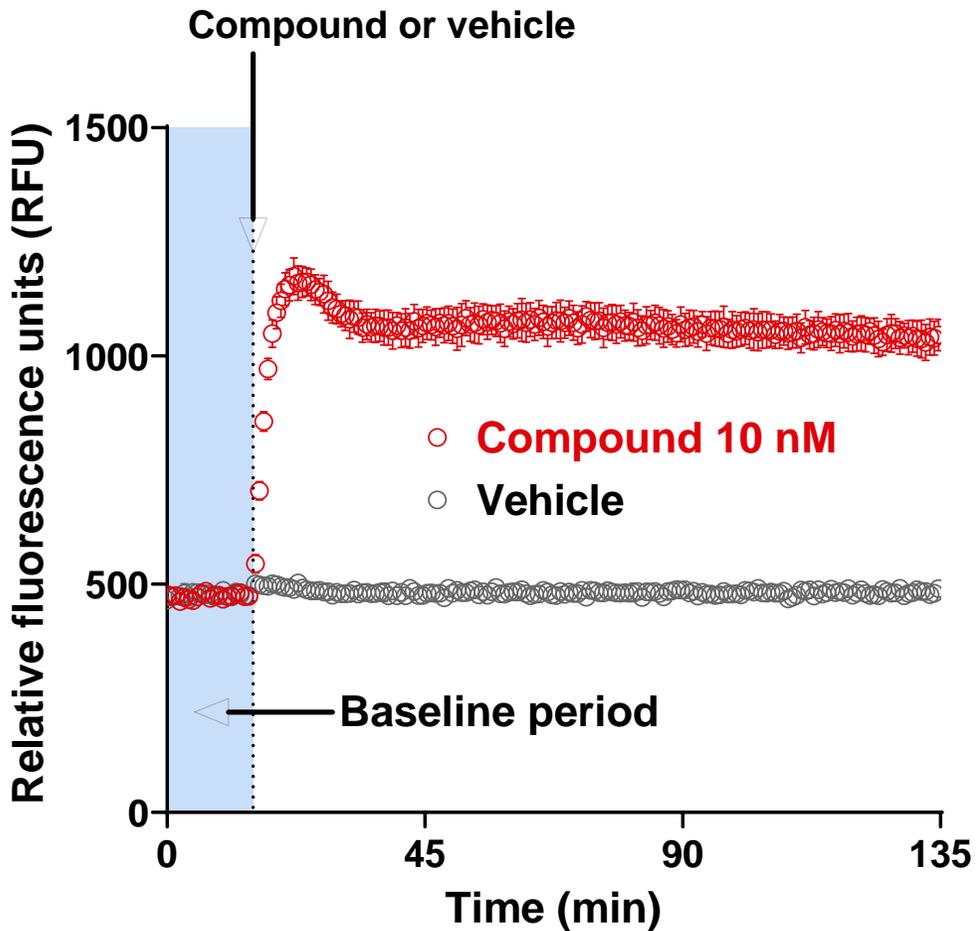




Data handling and normalization

Normalizing to baseline

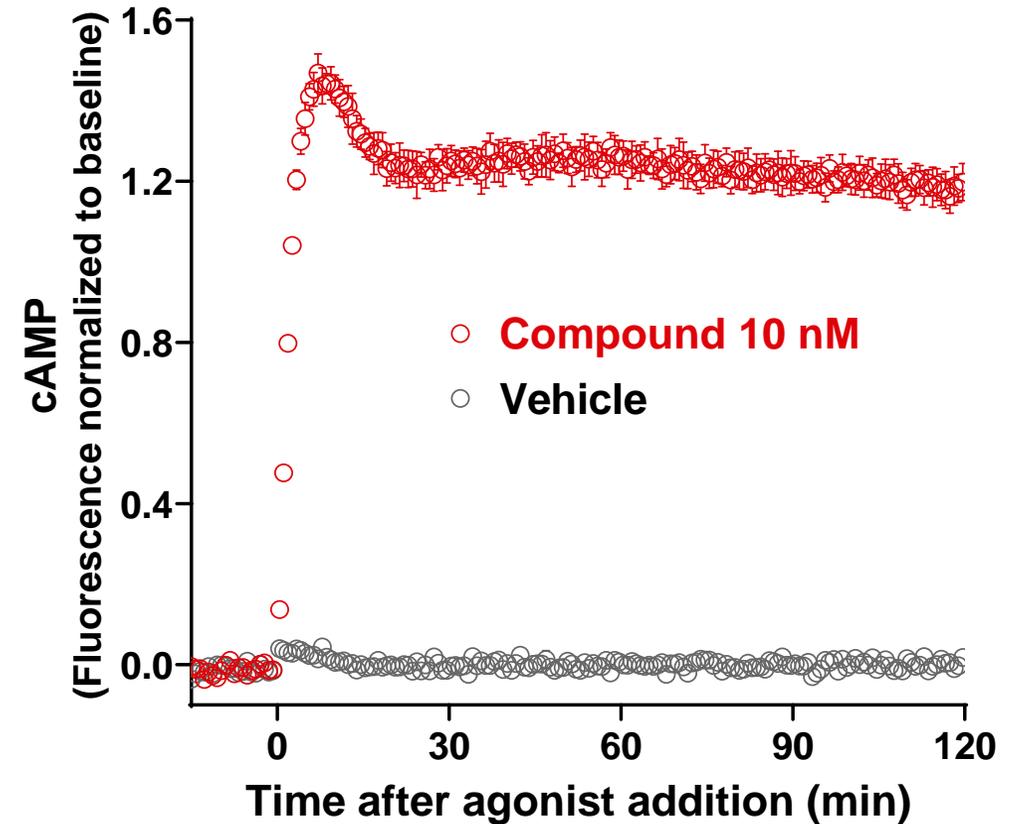
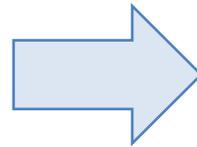
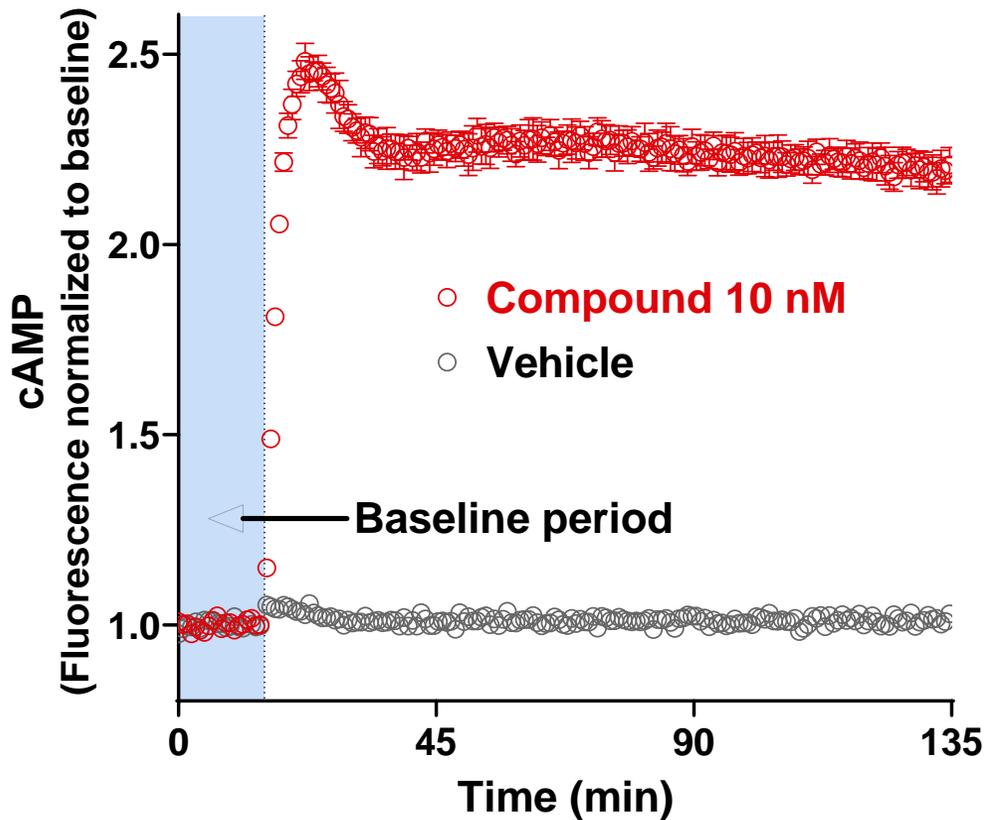
$$\text{Fluorescence normalized to baseline} = \frac{\text{RFU}}{\text{RFU average of baseline period}}$$



Subtracting vehicle

Linear regression performed for vehicle-treated cells.
 Calculated vehicle Y value from linear regression for each time point subtracted.

X axis adjusted to time after agonist addition.



Prism steps for data normalization



Normalizing to baseline

Parameters: Remove Baseline and Column Math

Definition of baseline

Selected column(s) Every other data set (column): 2nd, 4th, 6th, ...

Assume the baseline is linear with X, so use values predicted from the regression line.

Selected row(s)

First row

Last row

Mean of first 20 rows and last 0 rows.

Remove baseline(s) from the results

Calculation

Difference: Value - Baseline

Sum: Value + Baseline

Product: Value * Baseline

Ratio: Value/Baseline

Percent: $100 * \text{Value} / \text{Baseline}$

Fractional difference: $(\text{Value} - \text{Baseline}) / \text{Baseline}$

Percentage difference: $100 * (\text{Value} - \text{Baseline}) / \text{Baseline}$

Subcolumns

Repeated measures. When computing results for the Y2 subcolumn, only consider baseline values in the Y2 subcolumn.

Replicates. No matching. Average the baseline replicates and do calculations with the average.

Ignore subcolumns. Average all replicates, and only do calculations with the mean values.

How to label the results columns

Column shortcuts (A - B)

New graph

Create a new graph of the results

Learn Cancel OK

Subtracting vehicle response

Parameters: Remove Baseline and Column Math

Definition of baseline

Selected column(s) Data Set M

Assume the baseline is linear with X, so use values predicted from the regression line.

Selected row(s)

First row

Last row

Mean of first 3 rows and last 3 rows.

Remove baseline(s) from the results

Calculation

Difference: Value - Baseline

Sum: Value + Baseline

Product: Value * Baseline

Ratio: Value/Baseline

Percent: $100 * \text{Value} / \text{Baseline}$

Fractional difference: $(\text{Value} - \text{Baseline}) / \text{Baseline}$

Percentage difference: $100 * (\text{Value} - \text{Baseline}) / \text{Baseline}$

Subcolumns

Repeated measures. When computing results for the Y2 subcolumn, only consider baseline values in the Y2 subcolumn.

Replicates. No matching. Average the baseline replicates and do calculations with the average.

Ignore subcolumns. Average all replicates, and only do calculations with the mean values.

How to label the results columns

Value column title only

New graph

Create a new graph of the results

Learn Cancel OK



Curve fitting details

Prism analysis for endogenous agonist

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Choose an equation

- Enzyme kinetics - Velocity as a function of substrate
 - Exponential
 - Lines
 - Polynomial
 - Gaussian
 - Sine waves
 - Growth curves
 - Linear quadratic curves
 - Classic equations from prior versions of Prism
 - [Pharmacokinetics] Fall to steady state equations
 - [Pharmacokinetics] Fall-and-rise equations
 - [Pharmacokinetics] Rise to steady state equations
 - [Pharmacokinetics] Rise-and-fall equations
 - [Pharmacokinetics] Rise-and-fall to baseline time course
 - [Pharmacokinetics] Rise-and-fall to steady state time course
 - [Pharmacokinetics] Baseline then rise-and-fall to baseline time course
 - [Pharmacokinetics] Baseline then rise-and-fall to steady state time course
 - [Pharmacokinetics] Baseline then rise-and-fall to baseline with drift
 - [Pharmacokinetics] Baseline then rise-and-fall to steady state with drift

Use for time course experiment in which effect is initiated after a baseline period

Initial values might need manual entry
 K1, 1/t-half rise phase
 K2, 1/t-half fall phase
 [Pharmacokinetics] Baseline then rise-and-fall to baseline time course
 Numerical derivatives

Interpolate

Interpolate unknowns from standard curve. Confidence interval: None

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Outliers

No special handling of outliers

Detect and eliminate outliers $Q = 1$ % Create a table of clean data (with outliers removed)

Report the presence of outliers

Fitting method

Least squares regression. Used most commonly.

Robust regression. Outliers have little impact.

Poisson regression. Y values are counts of objects or events.

Don't fit the curve. Instead plot the curve defined by the initial values of the parameters.

Convergence criteria

How strict: Medium Automatically switch to strict convergence when needed

Maximum number of iterations: 1000

Weighting method

No weighting. Minimize the sum-of-squares of the distances of the points from the curve. Choose when you expect the average distance between points and curve to be unrelated to the value of Y.

Weight by $1/Y^2$. Minimize the sum of the squares of the relative distance of the points from the curve. Choose when you expect the average distance between points and curve to be proportional to Y.

Weight by: $1/Y$ $K = 2$

Replicates

Consider each replicate Y value as an individual point

Only consider the mean Y value of each point

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Parameter Name	Constraint Type	Value	Hook
X0	Must be greater than	20	
Baseline	No constraint		
Initial_rate	Must be greater than	0	
K1	No constraint		
K2	Must be greater than	0	

Constrain one parameter relative to another

K1 must be greater than 1 times K2

must be greater than 1 times

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Select Data Set

- Endogenous agonist:A:100
- Endogenous agonist:B:32
- Endogenous agonist:C:10
- Endogenous agonist:D:3.2
- Endogenous agonist:E:1.0
- Endogenous agonist:F:0.32

To select several data sets, press Control or Shift while selecting.

Parameter Name	Choose Automatically	Initial Value	Hook
X0	<input type="checkbox"/>	20	
Baseline	<input checked="" type="checkbox"/>	-0.01973980454	
Initial_rate	<input checked="" type="checkbox"/>	0.013777052517	
K1	<input checked="" type="checkbox"/>	0.045413260672	
K2	<input checked="" type="checkbox"/>	0.001362397820	

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Confidence intervals (CI) of parameters

Calculate CI of parameters

Confidence level: 95%

Output Format: Range ("1.23 to 4.56")

Asymmetrical (profile-likelihood) CI
Recommended because they are more accurate. Can be slow.

Compute even when the fit is ambiguous or unstable and the CIs would be difficult to interpret.

Symmetrical (asymptotic) approximate CI
Less accurate so not recommended. Matches Prism 1-6 and most programs. Faster to calculate.

Show SE of parameters

Confidence or prediction bands

Plot confidence/prediction bands

Confidence level: 95%

Confidence bands
Confidence bands show you the likely location of the TRUE curve.

Prediction bands
Prediction bands show you the likely location of additional data points.

Unstable parameters and ambiguous fits

Identify "unstable" parameters (recommended).

Identify "ambiguous" fits. Matches Prism 8.1 and earlier.

Neither. Just show the best-fit values even when the fit is problematic.

Make these choices the default for future fits.

Learn Cancel OK

Prism analysis for Compounds 1-3

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Choose an equation

- Binding - Kinetics
- Enzyme kinetics - Inhibition
- Enzyme kinetics - Velocity as a function of substrate
- Exponential
- Lines
- Polynomial
- Gaussian
- Sine waves
- Growth curves
- Linear quadratic curves
- Classic equations from prior versions of Prism
- [Pharmacokinetics] Fall to steady state equations
- [Pharmacokinetics] Fall-and-rise equations
- [Pharmacokinetics] Rise to steady state equations
 - [Pharmacokinetics] Rise to steady state time course
 - [Pharmacokinetics] Baseline then rise to steady state with drift
- [Pharmacokinetics] Rise-and-fall equations
- [Pharmacokinetics] Straight line equations

Use for time course experiment in which effect is initiated after a baseline period.
 X0 initial value might need to be entered manually.
 [Pharmacokinetics] Baseline then rise to steady state time course
 Numerical derivatives

Interpolate

Interpolate unknowns from standard curve. Confidence interval: None

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Outliers

No special handling of outliers
 Detect and eliminate outliers
 Report the presence of outliers

Fitting method

Least squares regression. Used most commonly.
 Robust regression. Outliers have little impact.
 Poisson regression. Y values are counts of objects or events.
 Don't fit the curve. Instead plot the curve defined by the initial values of the parameters.

Convergence criteria

How strict: Medium
 Automatically switch to strict convergence when needed
 Maximum number of iterations: 1000

Weighting method

No weighting. Minimize the sum-of-squares of the distances of the points from the curve.
 Weight by $1/Y^2$. Minimize the sum of the squares of the relative distance of the points from the curve.
 Weight by $1/Y$. Minimize the average distance between points and curve to be proportional to Y.

Replicates

Consider each replicate Y value as an individual point
 Only consider the mean Y value of each point

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

What question are you asking?

No comparison
 For each data set, which of two equations (models) fits best?
 Do the best-fit values of selected unshared parameters differ between data sets?
 For each data set, does the best-fit value of a parameter differ from a hypothetical value?
 Does one curve adequately fit all the data sets?

Comparison method

Akaike's Information Criterion (AICc). Select the model that is most likely to have generated the data.
 Extra sum-of-squares F test. Select the simpler model unless the P value is less than 0.05.

Choose the second equation

- [Pharmacokinetics] Fall-and-rise equations
- [Pharmacokinetics] Rise to steady state equations
- [Pharmacokinetics] Rise-and-fall equations
 - [Pharmacokinetics] Rise-and-fall to baseline time course
 - [Pharmacokinetics] Rise-and-fall to steady state time course
 - [Pharmacokinetics] Baseline then rise-and-fall to baseline time course
 - [Pharmacokinetics] Baseline then rise-and-fall to steady state time course
 - [Pharmacokinetics] Baseline then rise-and-fall to baseline with drift
 - [Pharmacokinetics] Baseline then rise-and-fall to steady state with drift

For each data set, compare the fit of "[Pharmacokinetics] Baseline then rise to steady state time course" (chosen on the Model tab) with the fit of a second model (which you choose above).
 Prism will fit both models to your data and compare them. Note that if you choose to compare with the extra sum-of-squares F test, the models must be nested - one model must be a special case of the other. If your models are not nested, choose the AICc comparison.

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Equation 1: [Pharmacokinetics] Baseline then rise to steady state time course

Parameter Name	Constraint Type	Value	Hook
X0	Must be greater than	20	
Baseline	No constraint		
SteadyState	Must be greater than	0	
K	Must be greater than	0	

Constrain one parameter relative to another

must be greater than 1 times
 must be greater than 1 times

Equation 2: [Pharmacokinetics] Baseline then rise-and-fall to steady state time course

Parameter Name	Constraint Type	Value	Hook
X0	Must be greater than	20	
Baseline	No constraint		
SteadyState	Must be greater than	0	
D	Must be greater than	0	

Constrain one parameter relative to another

K1 must be greater than 1 times K2
 must be greater than 1 times

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Select Data Set

- Compound 1:A:320
- Compound 1:B:100
- Compound 1:C:32
- Compound 1:D:10
- Compound 1:E:3.2
- Compound 1:F:1

To select several data sets, press Control or Shift while selecting.

Equation 1: [Pharmacokinetics] Baseline then rise to steady state time course

Parameter Name	Choose Automatically	Initial Value	Hook
X0	<input checked="" type="checkbox"/>	44.04	
Baseline	<input checked="" type="checkbox"/>	-0.01151020247	
SteadyState	<input checked="" type="checkbox"/>	0.657595211551	
K	<input checked="" type="checkbox"/>	0.122508456982	

Equation 2: [Pharmacokinetics] Baseline then rise-and-fall to steady state time course

Parameter Name	Choose Automatically	Initial Value	Hook
X0	<input checked="" type="checkbox"/>	44.04	
Baseline	<input checked="" type="checkbox"/>	-0.01151020247	
SteadyState	<input checked="" type="checkbox"/>	0.328797605775	
D	<input checked="" type="checkbox"/>	3	

Learn Cancel OK

Parameters: Nonlinear Regression

Model Method Compare Constrain Initial values Range Output Confidence Diagnostics Flag

Confidence intervals (CI) of parameters

Calculate CI of parameters
 Confidence level: 95%
 Output Format: Range ("1.23 to 4.56")

Asymmetrical (profile-likelihood) CI
 Recommended because they are more accurate. Can be slow.
 Compute even when the fit is ambiguous or unstable and the CIs would be difficult to interpret.

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Plot confidence/prediction bands
 Confidence level: 95%
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 Confidence bands show you the likely location of the TRUE curve.
 Prediction bands
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Identify "unstable" parameters (recommended).
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 Neither. Just show the best-fit values even when the fit is problematic.

Make these choices the default for future fits.

Learn Cancel OK