# 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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# Summary

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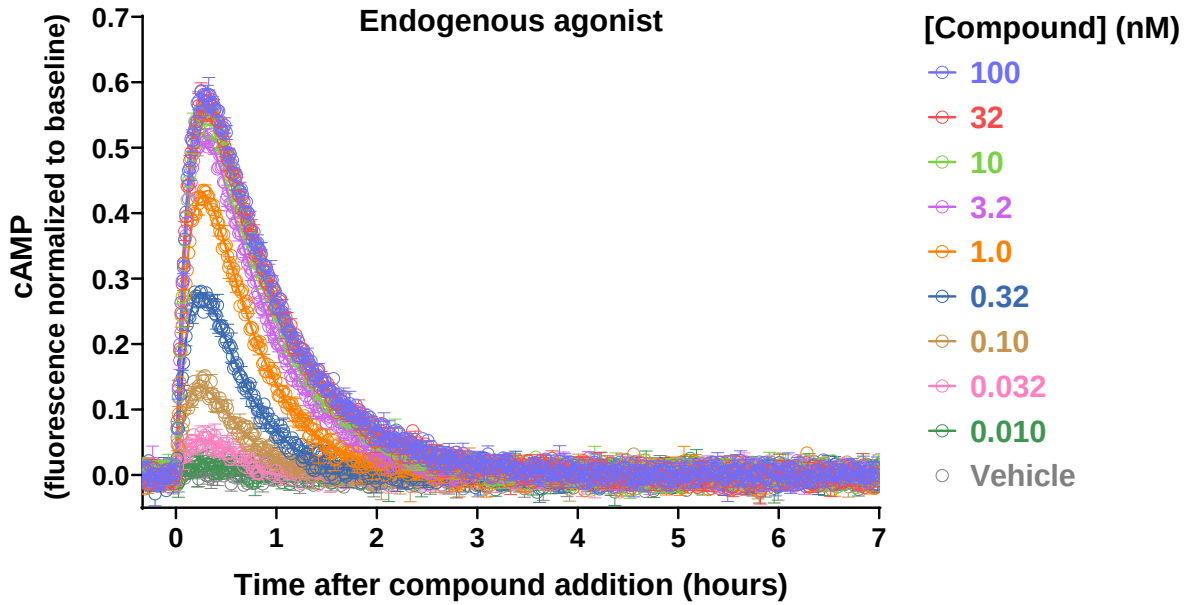
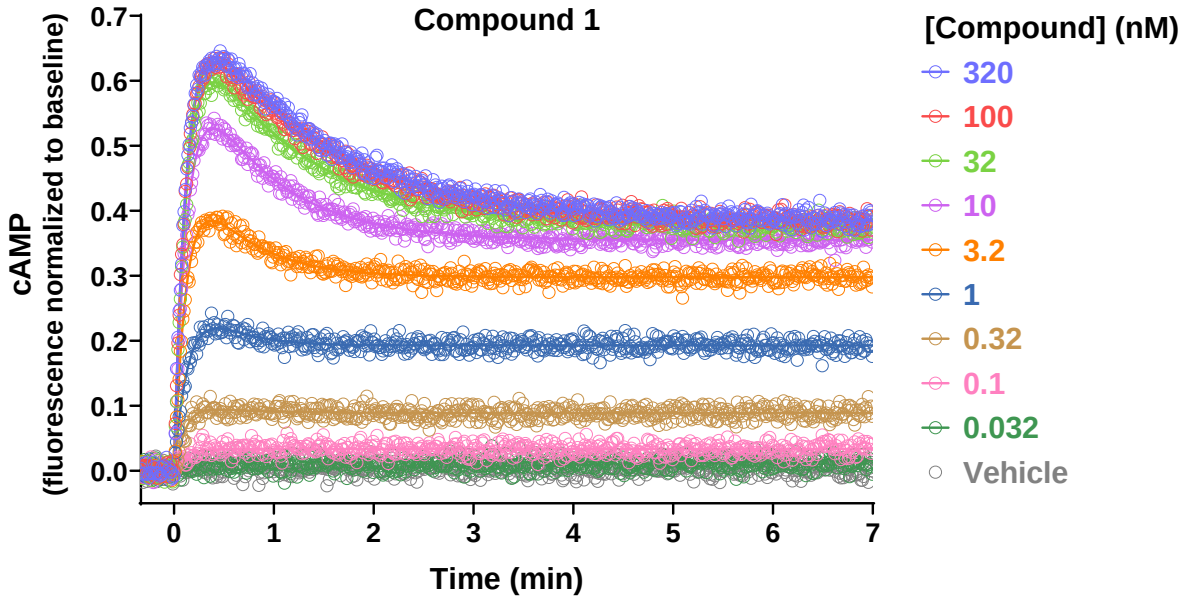
- cAMP generation stimulated by a GPCR was measured using the cADDis fluorescent cAMP biosensor in live cells for seven hours.
- The response to the endogenous peptide agonist and three small molecule agonists was measured.
- The response to the peptide agonist became fully desensitized, the response 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]



# Methods

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- 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 ± SEM from 2 technical replicates.

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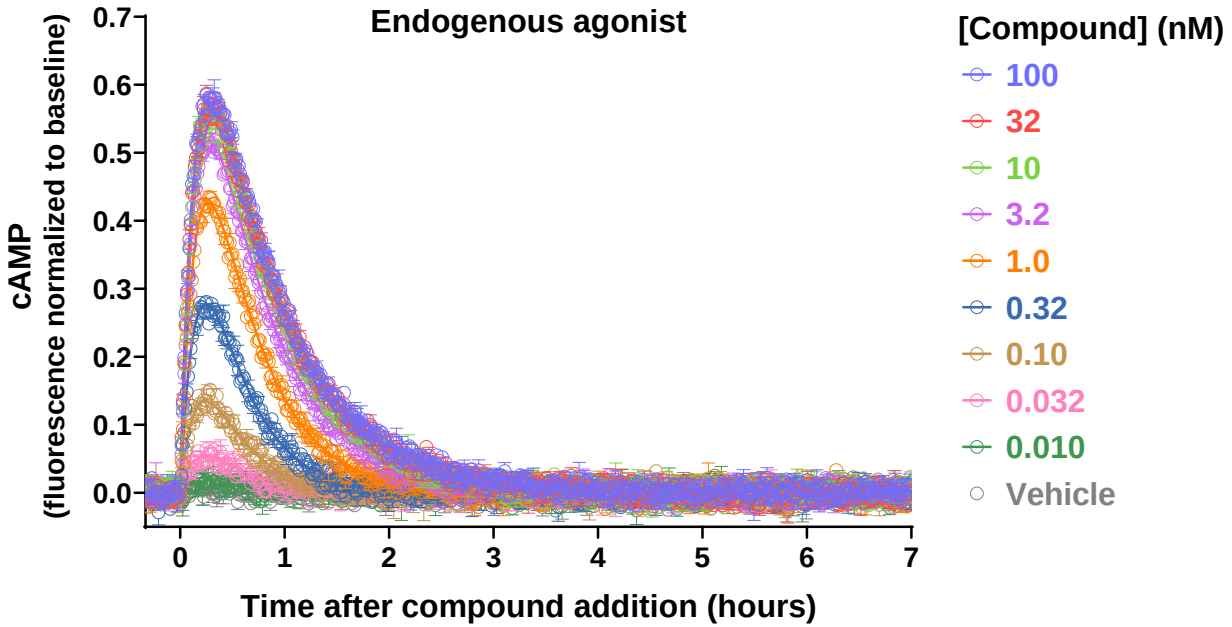
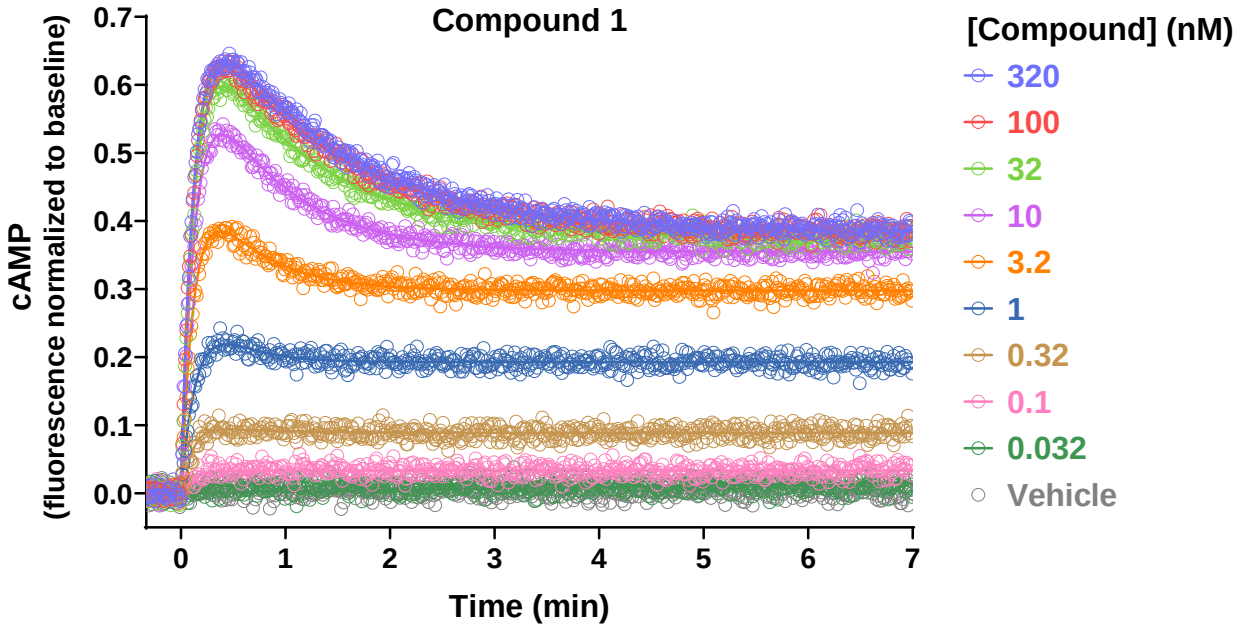
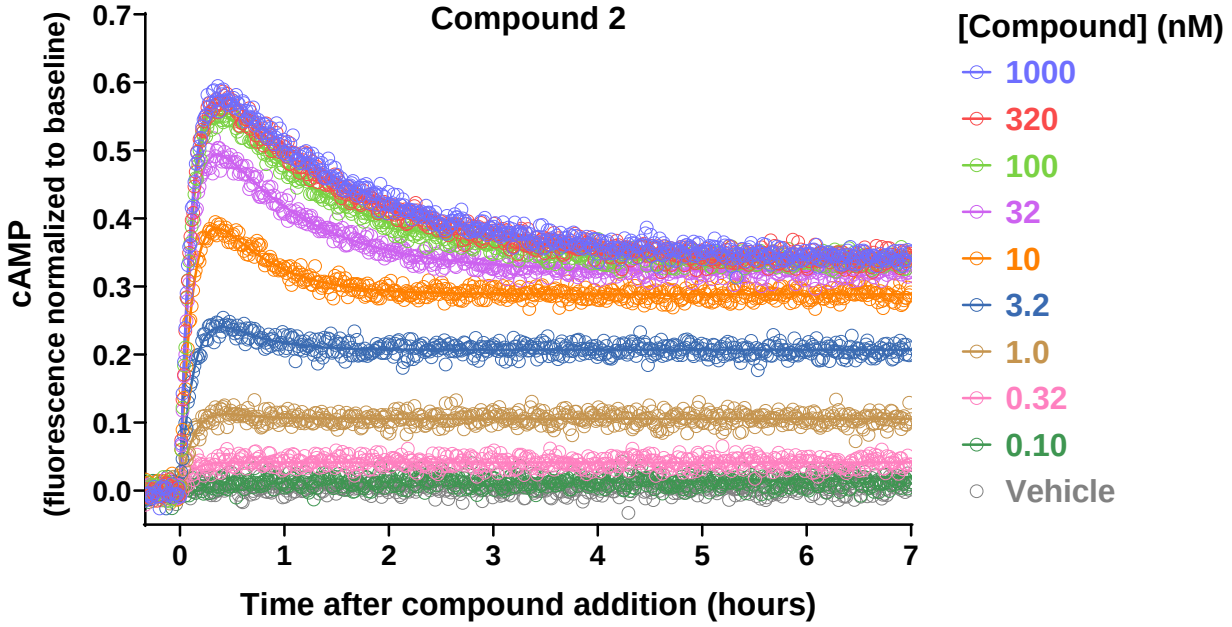
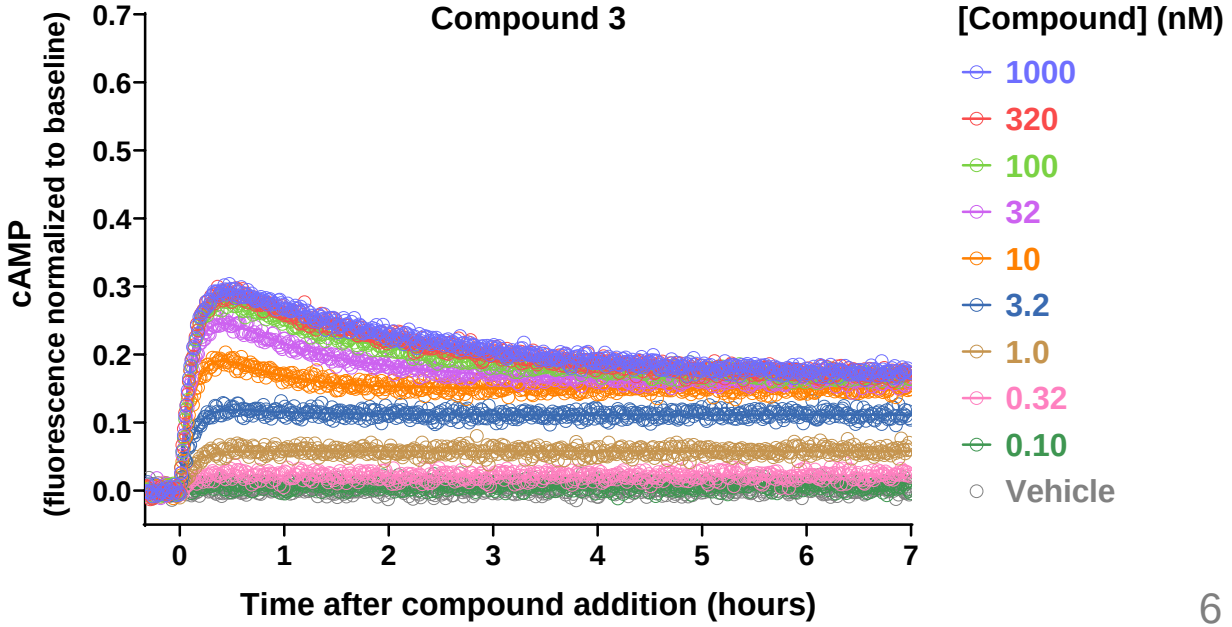
# Time course data

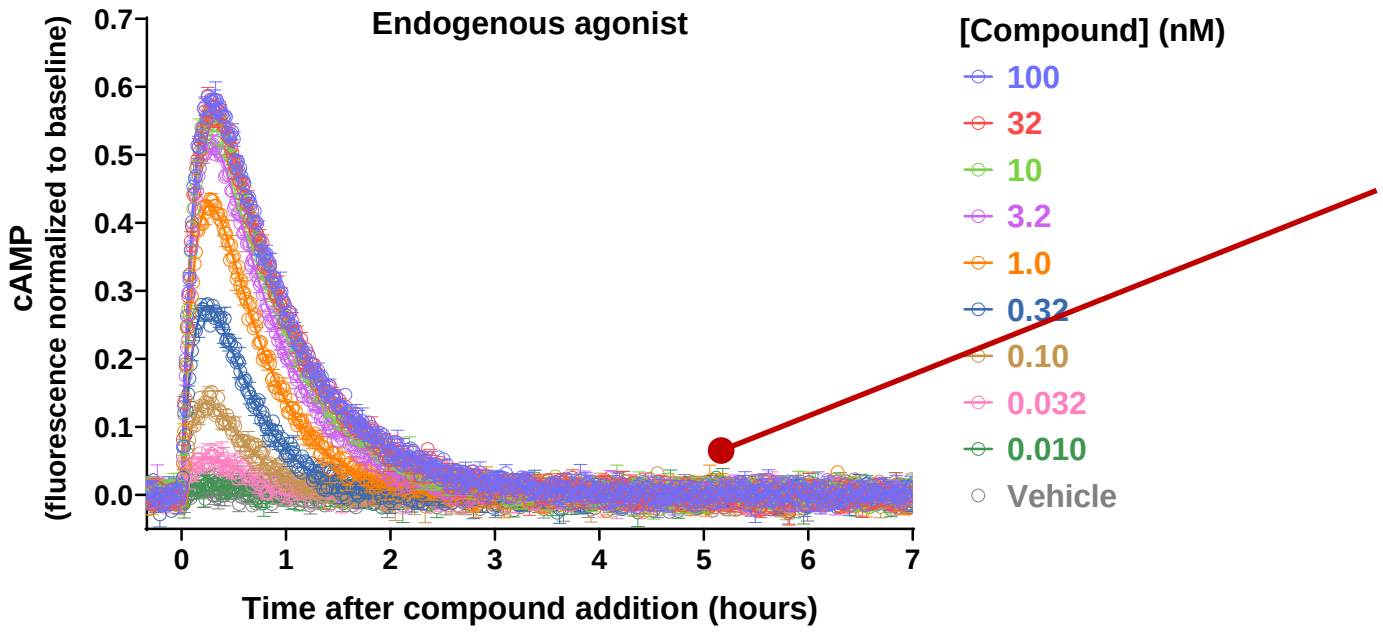

Endogenous agonist
cAMP
(fluorescence normalized to baseline)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Time after compound addition (hours)
[Compound] (nM)
100
32
10
3.2
1.0
0.32
0.10
0.032
0.010
Vehicle


Compound 1
cAMP
(fluorescence normalized to baseline)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Time (min)
[Compound] (nM)
320
100
32
10
3.2
1
0.32
0.1
0.032
Vehicle


Compound 2
cAMP
(fluorescence normalized to baseline)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Time after compound addition (hours)
[Compound] (nM)
1000
320
100
32
10
3.2
1.0
0.32
0.10
Vehicle


Compound 3
cAMP
(fluorescence normalized to baseline)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Time after compound addition (hours)
[Compound] (nM)
1000
320
100
32
10
3.2
1.0
0.32
0.10
Vehicle

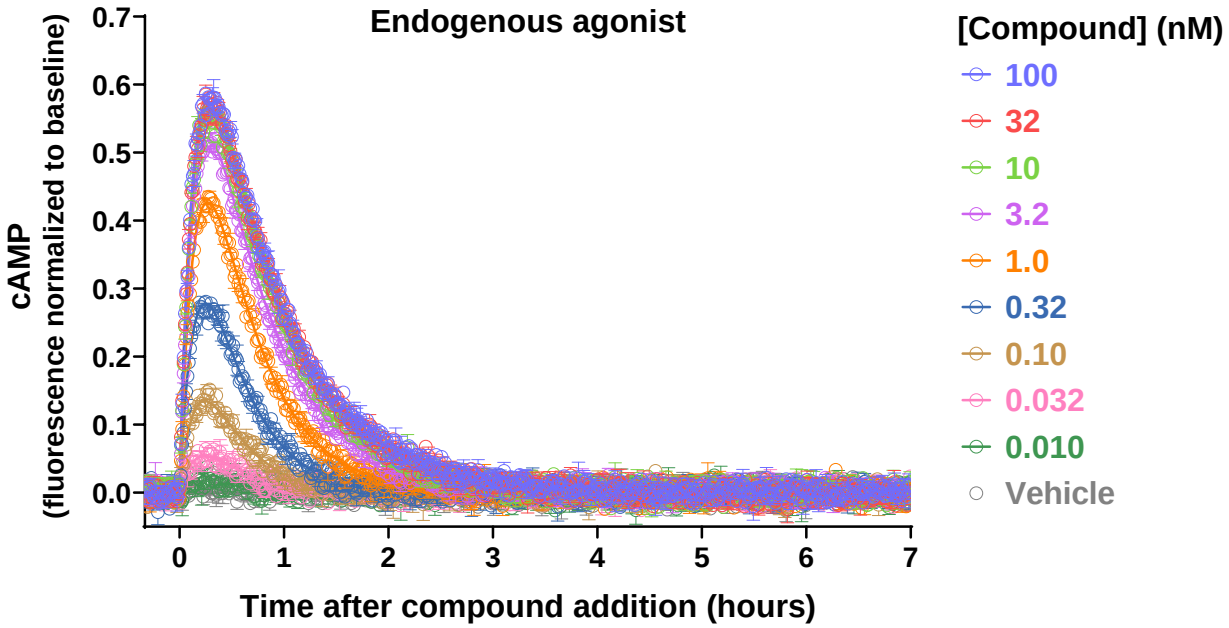
# Time course curve shape – endogenous agonist




- 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

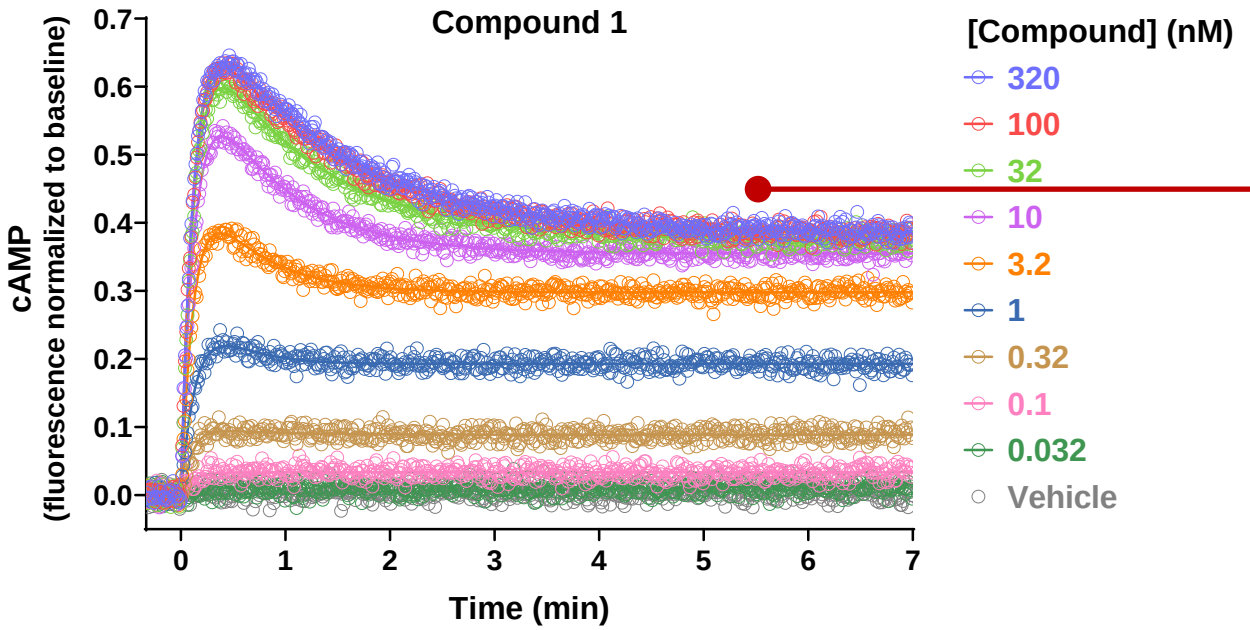
# Curve fitting – endogenous agonist




- 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 Pharmechanics 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.pharmechanics.com/time-course-tool-pack
4. https://youtu.be/_Pb7Sq6lZIY

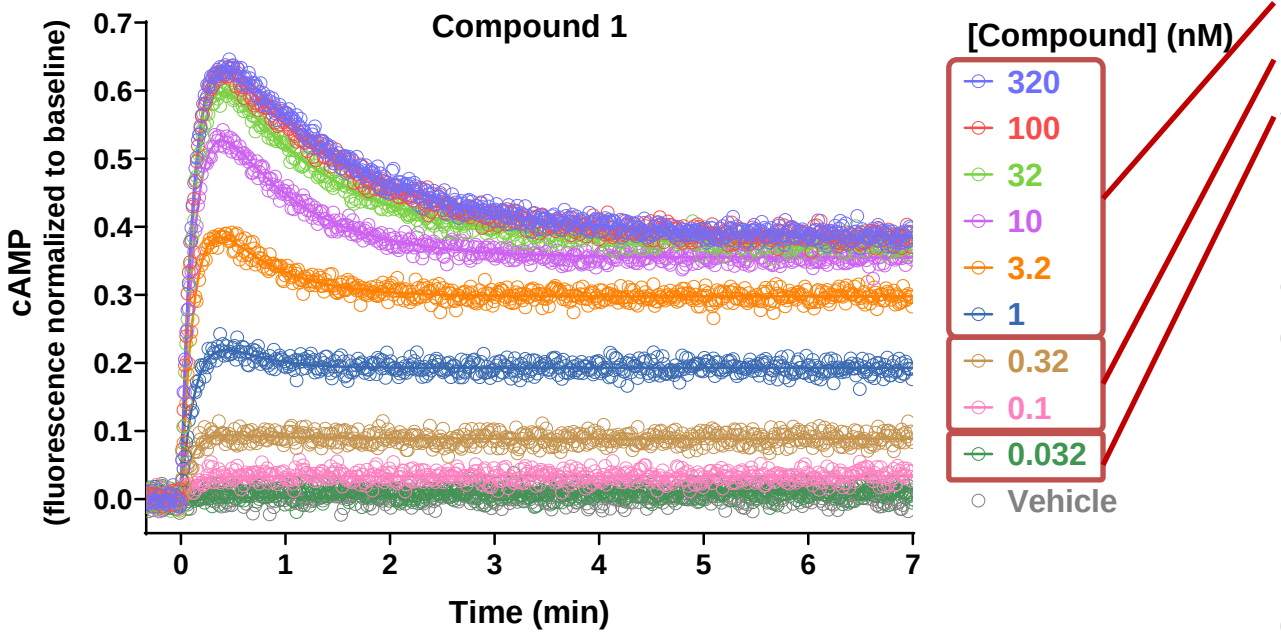
# Time course curve shape – test compounds




- 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 resesensitization 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 Pharmechanics 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.pharmechanics.com/time-course-tool-pack
4. https://youtu.be/_Pb7Sq6lZIY

# Parameters quantified

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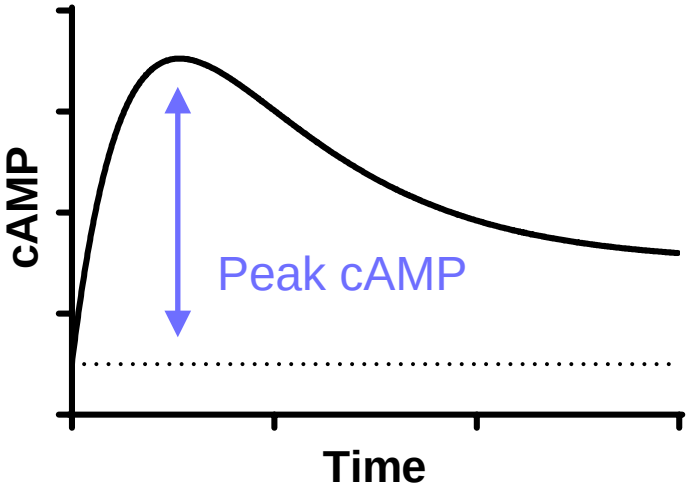


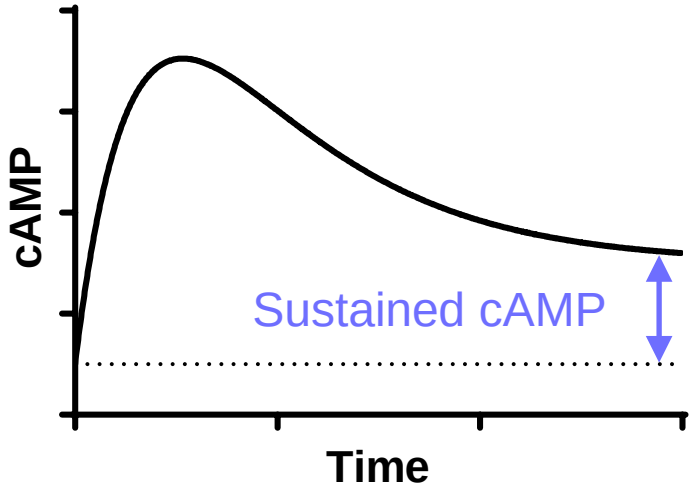


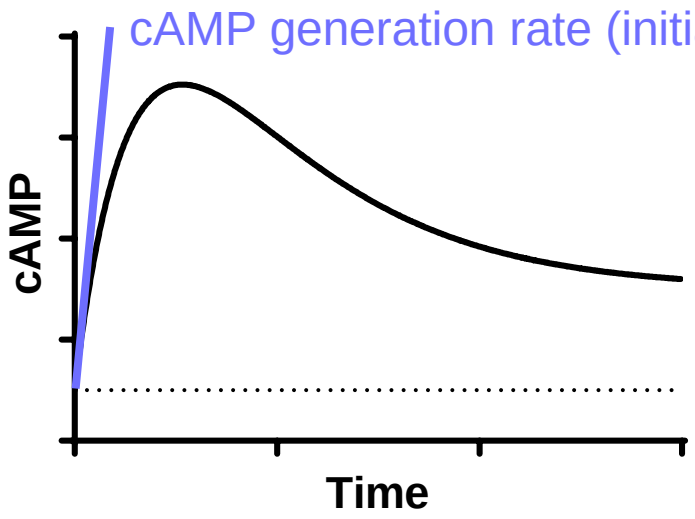


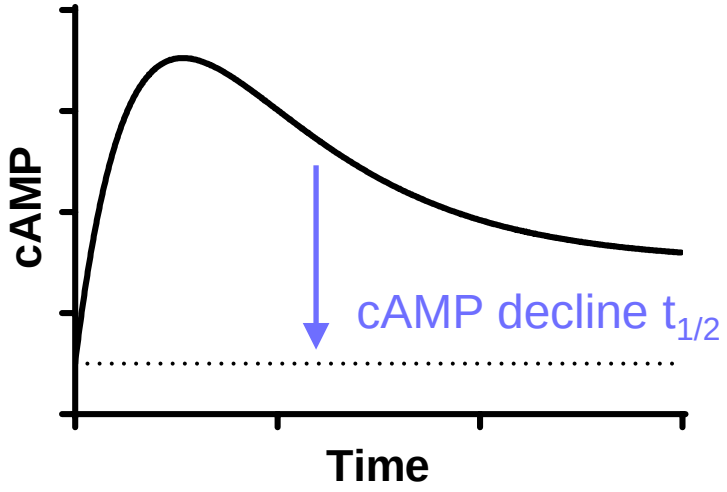

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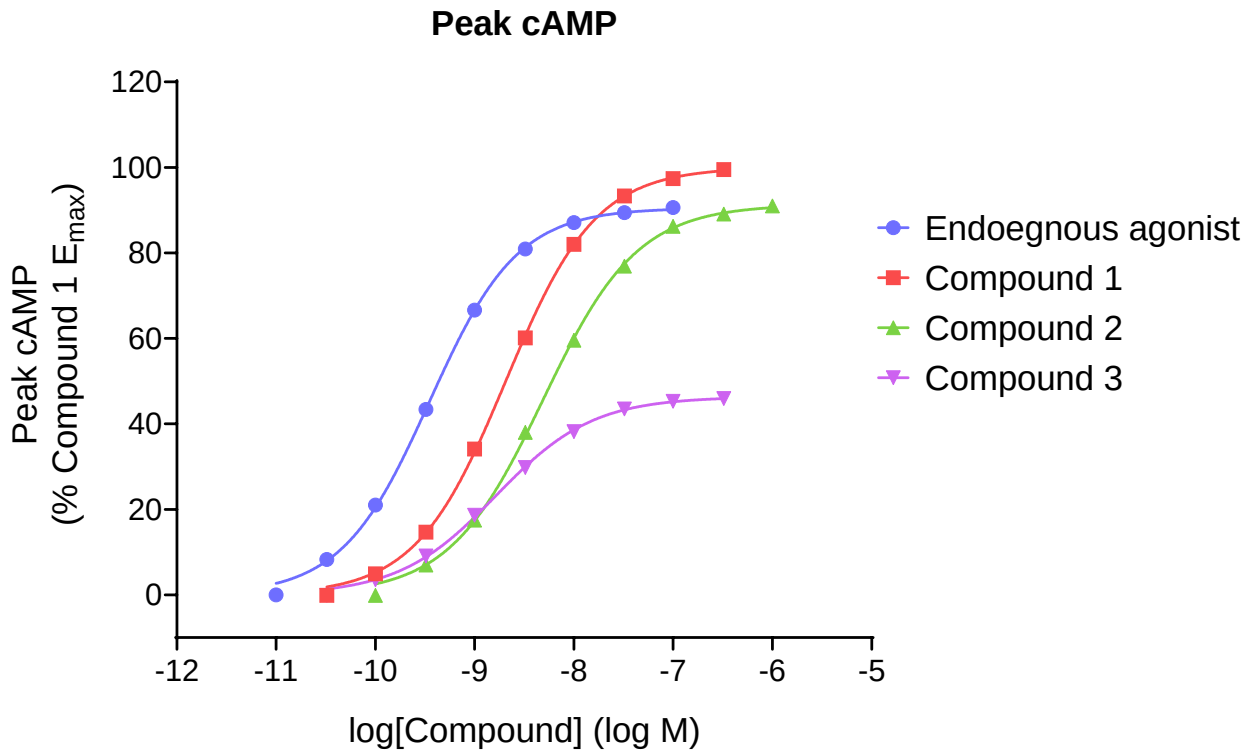
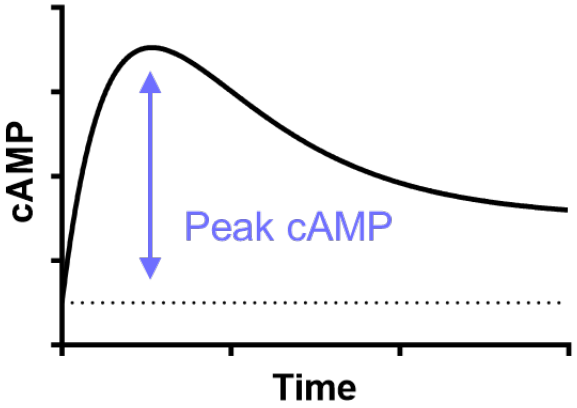
# Concentration response data

# Peak cAMP concentration response



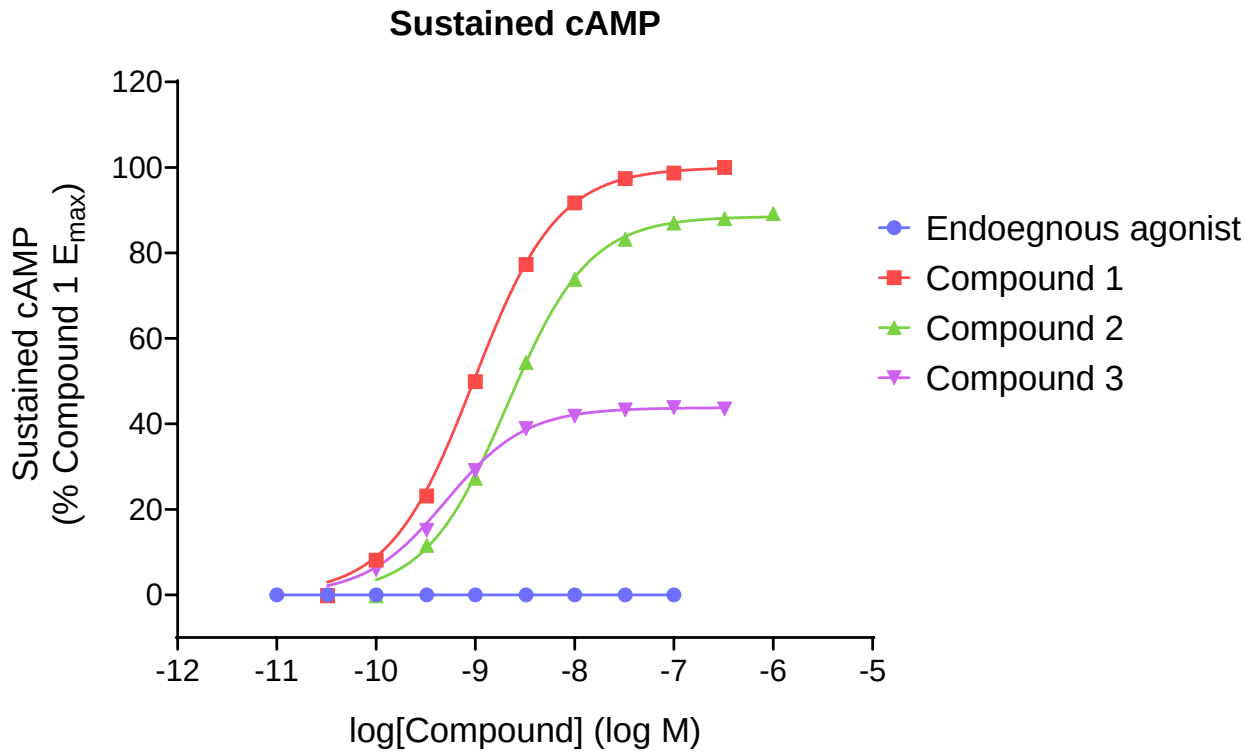

| Compound | $EC_{50}$ (nM) | $E_{max}$ (% Cmpd 1 $E_{max}$ [A]) |
|---|---|---|
| Endogenous agonist | 0.35 | 91 |
| Compound 1 | 2.1 | 100 |
| Compound 2 | 4.9 | 91 |
| Compound 3 | 1.6 | 46 |

- 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.



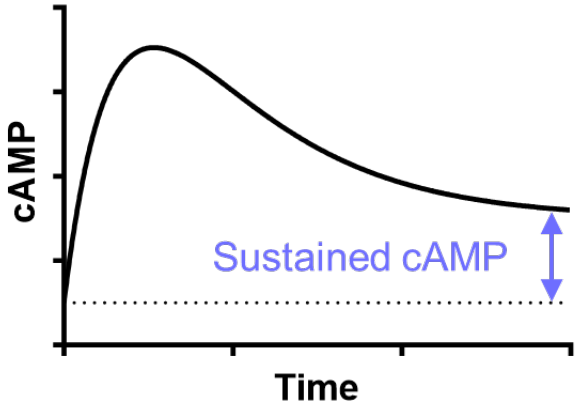

A. $E_{max}$ for Cmpd 1 is 0.64 normalized fluorescence units

# Sustained cAMP concentration response



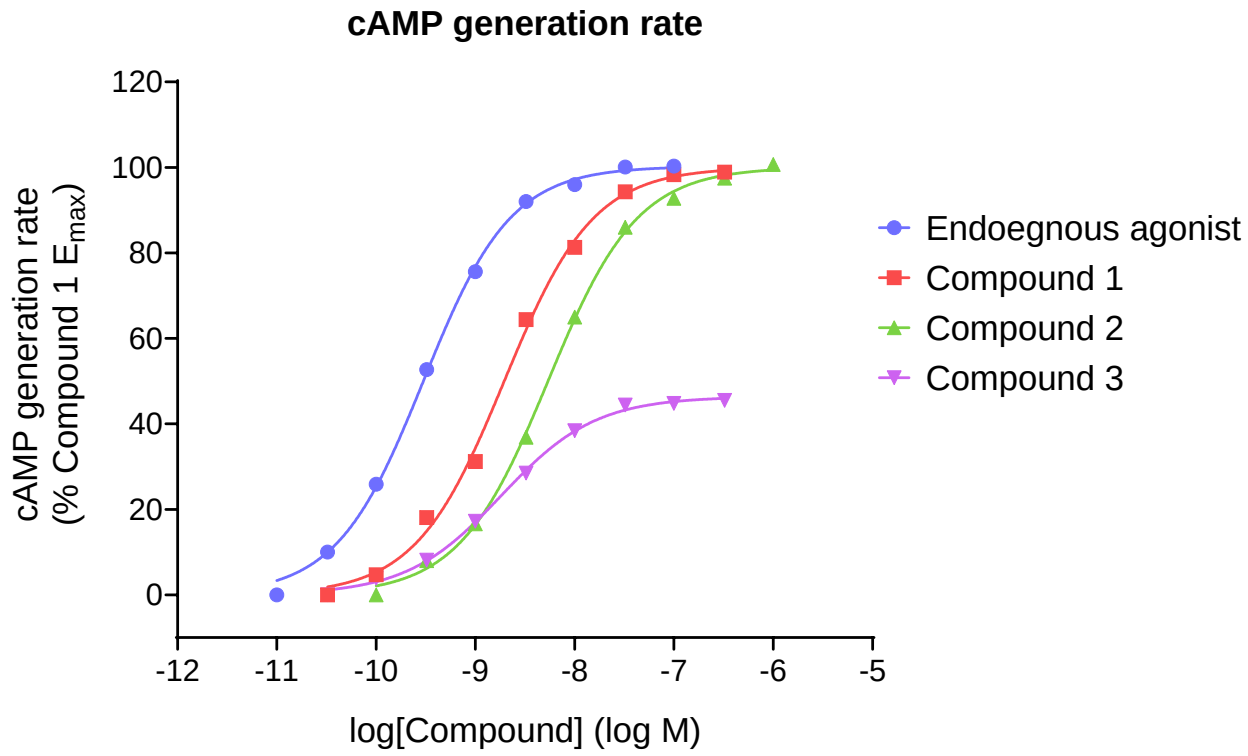

| Compound | $EC_{50}$ (nM) | $E_{max}$ (% Cmpd 1 $E_{max}$ [A]) |
|---|---|---|
| Endogenous agonist | Not detected | Not detected |
| Compound 1 | 0.96 | 100 |
| Compound 2 | 2.1 | 89 |
| Compound 3 | 0.50 | 44 |

- 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.



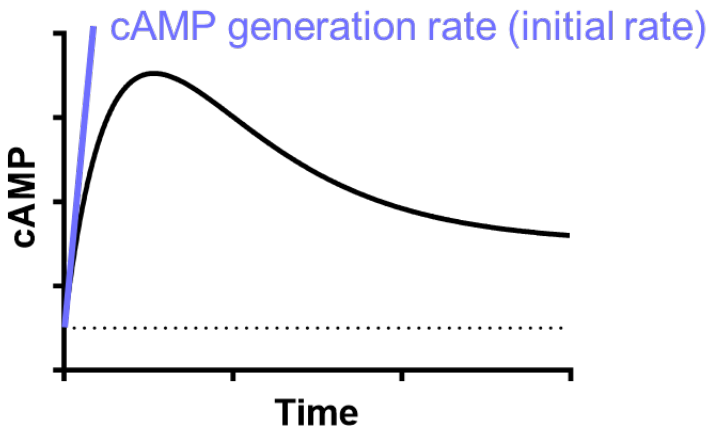

A. $E_{max}$ for Cmpd 1 is 0.64 normalized fluorescence units

# cAMP signal generation rate (initial rate)



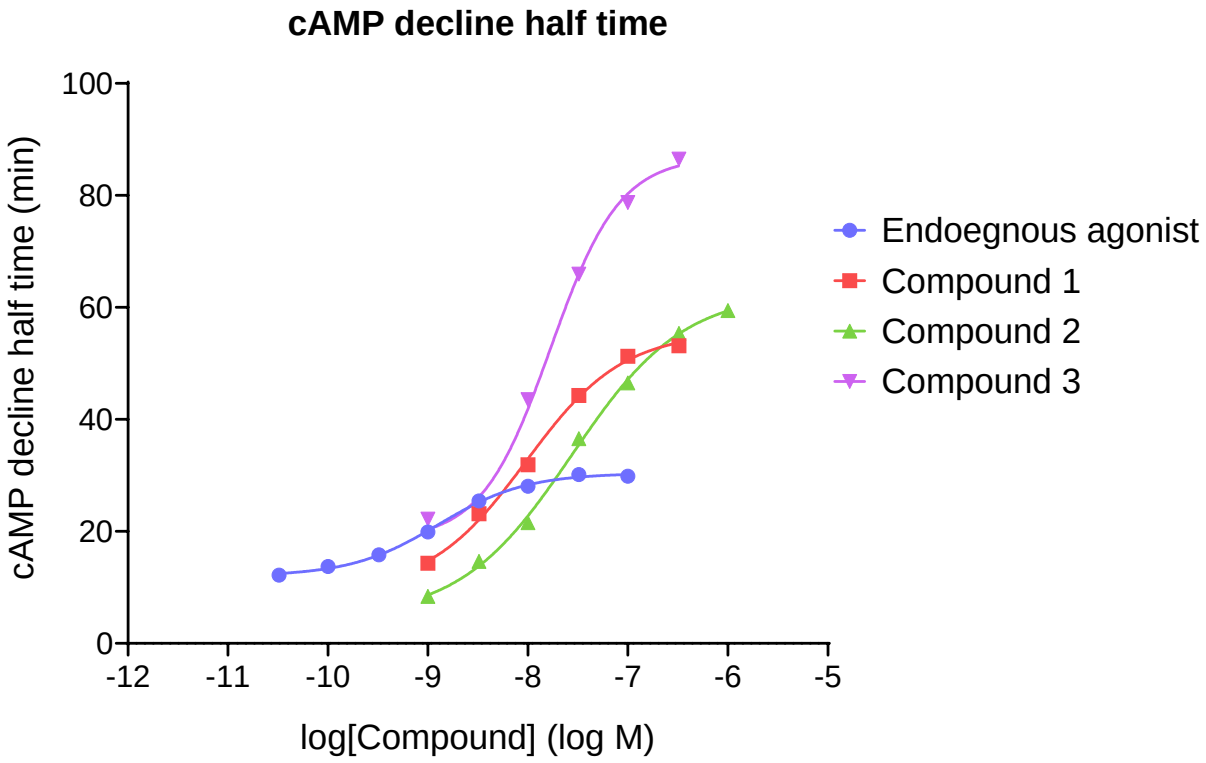

| Compound | $EC_{50}$ (nM) | $E_{max}$ (% Cmpd 1 $E_{max}$ [A]) |
|---|---|---|
| Endogenous agonist | 0.30 | 100 |
| Compound 1 | 2.0 | 100 |
| Compound 2 | 5.4 | 100 |
| Compound 3 | 1.8 | 46 |

- 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.



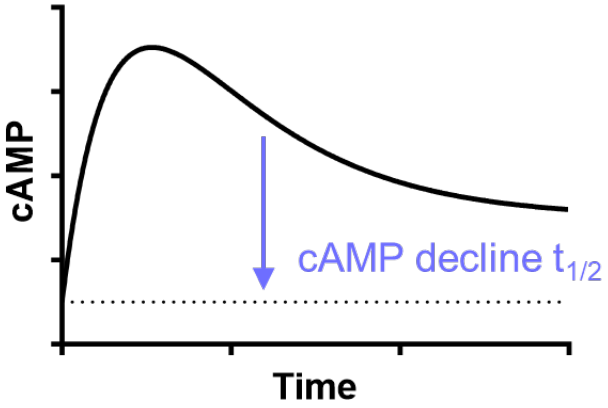

A. $E_{max}$ for Cmpd 1 is 0.10 normalized fluorescence units per min

# cAMP decline half time




| 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 |

- 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.



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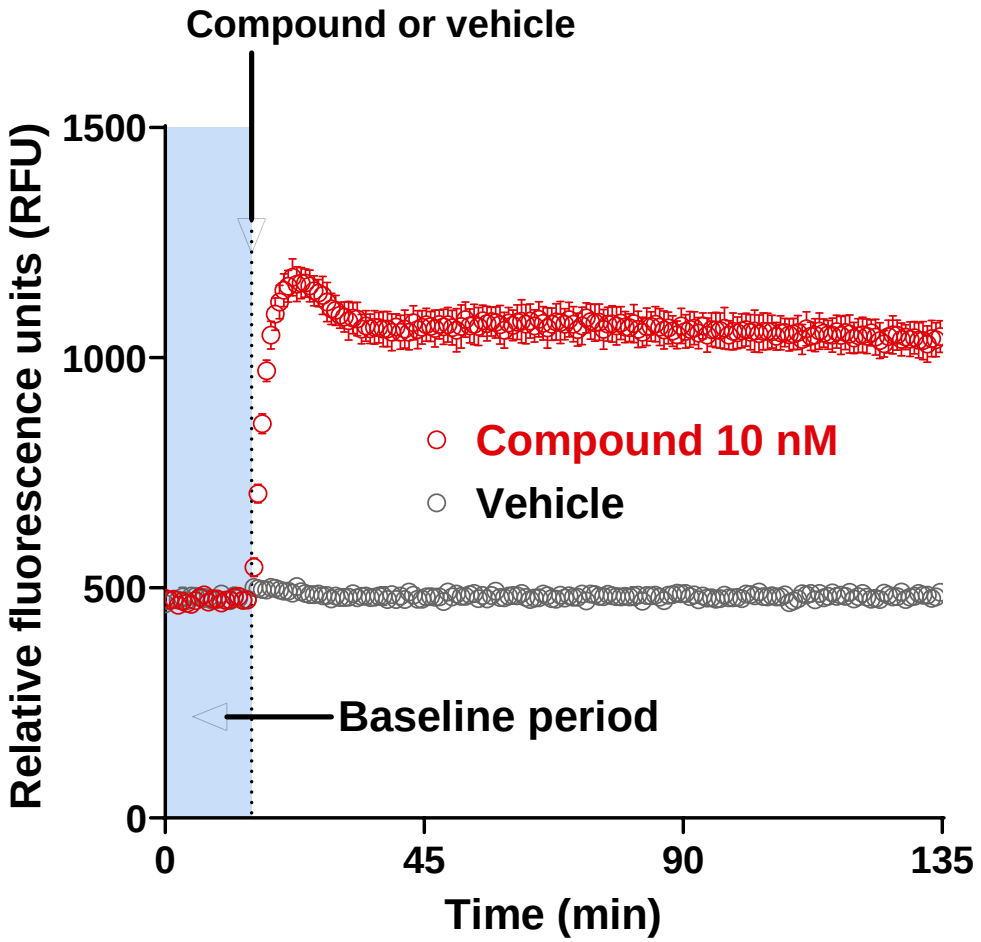
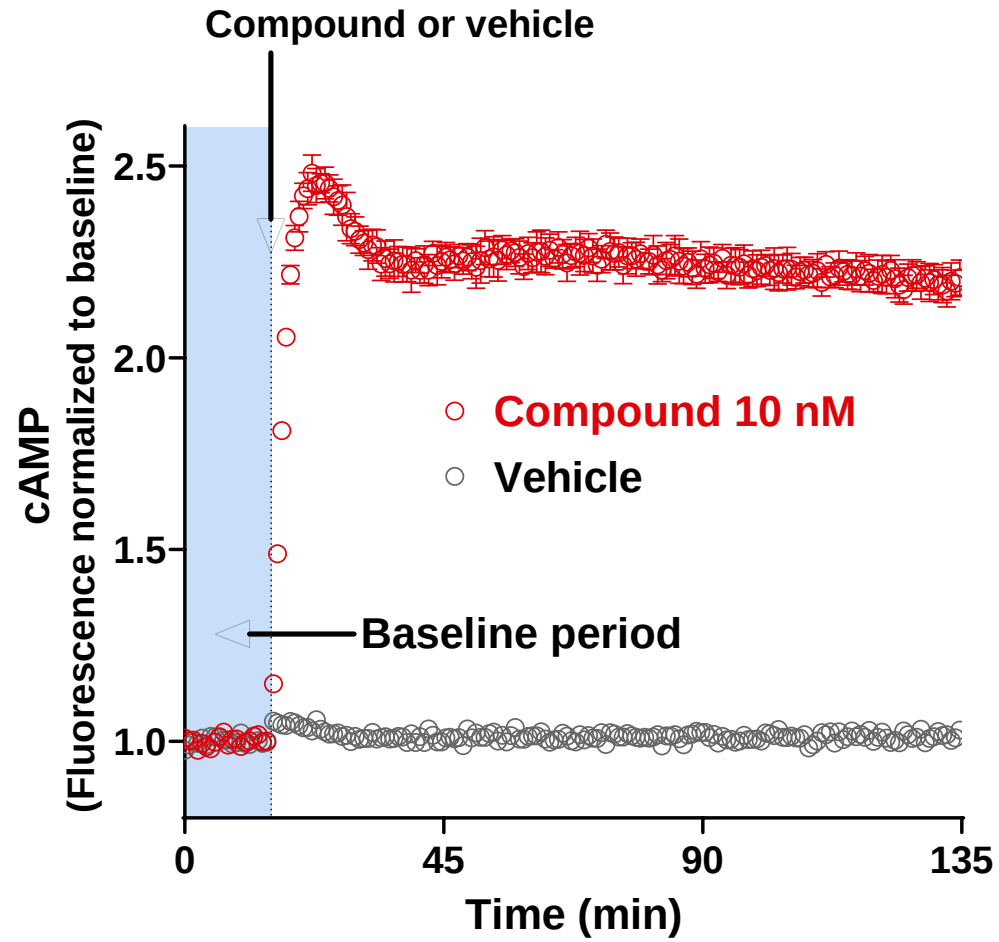
# Data handling and normalization

# Normalizing to baseline

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



# Subtracting vehicle

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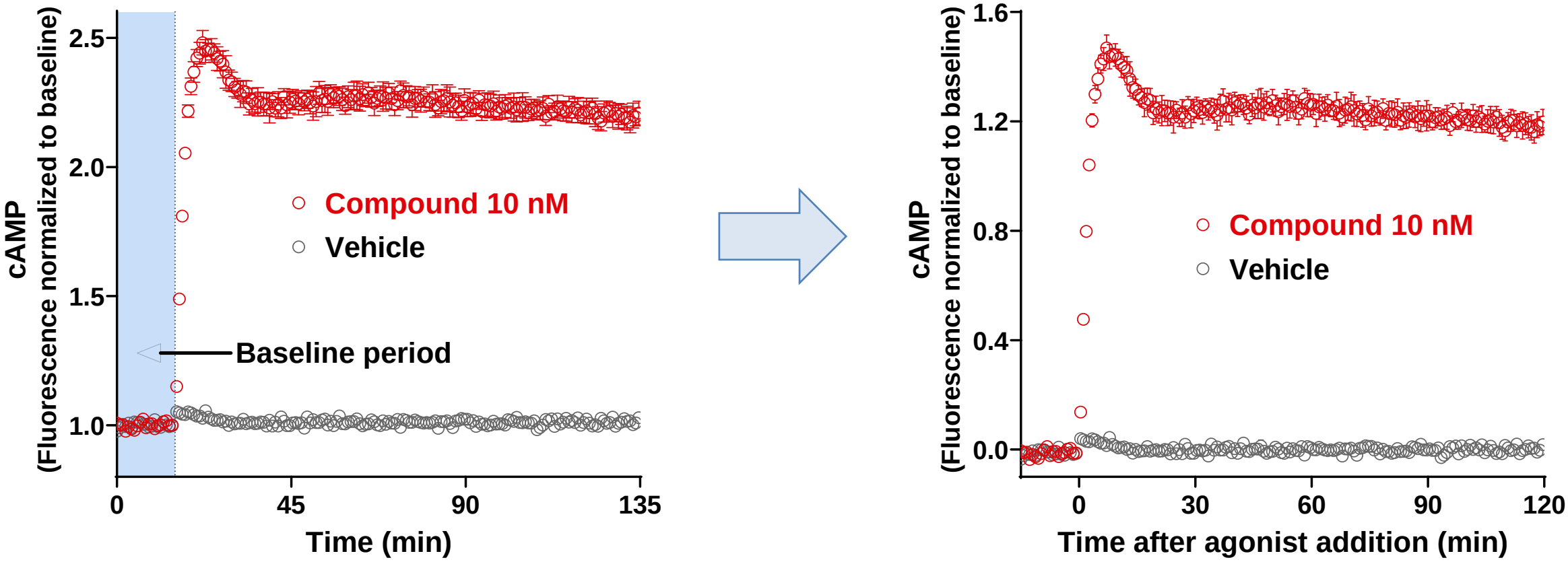
Linear regression performed for vehicle-treated cells.
Calculated vehicle Y value from linear regression for each time point subtracted.

X axis adjusted to time after agonist addition.



# Prism steps for data normalization

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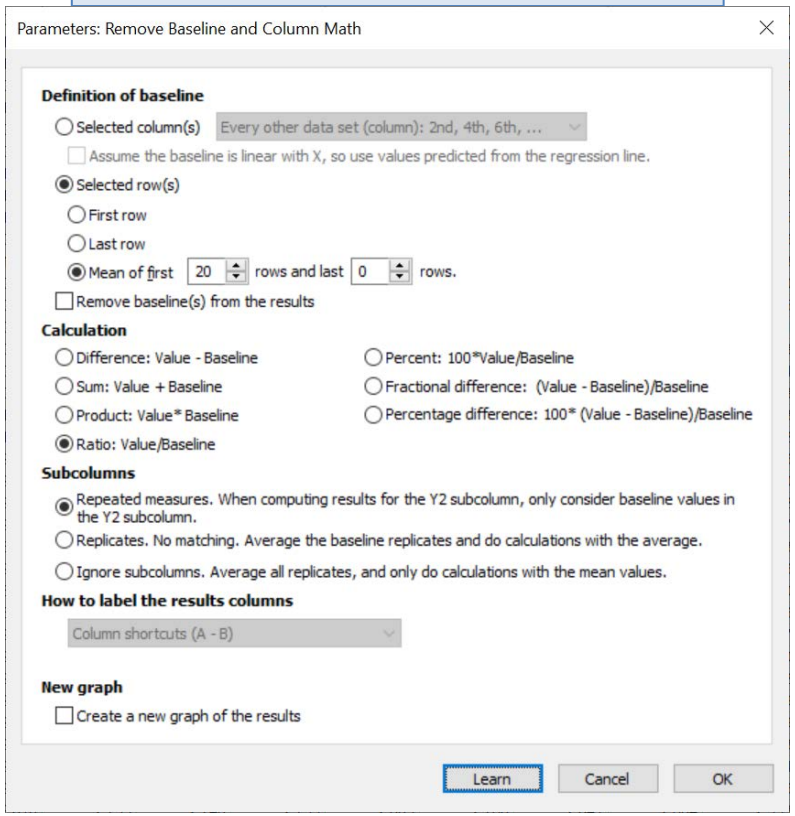
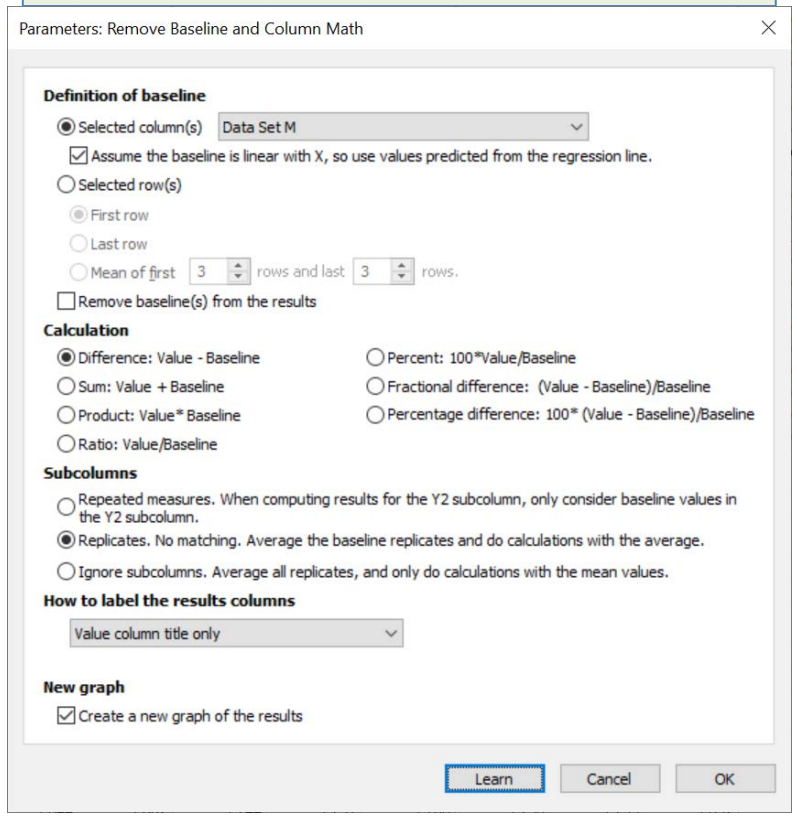
## Normalizing to baseline




## Subtracting vehicle response



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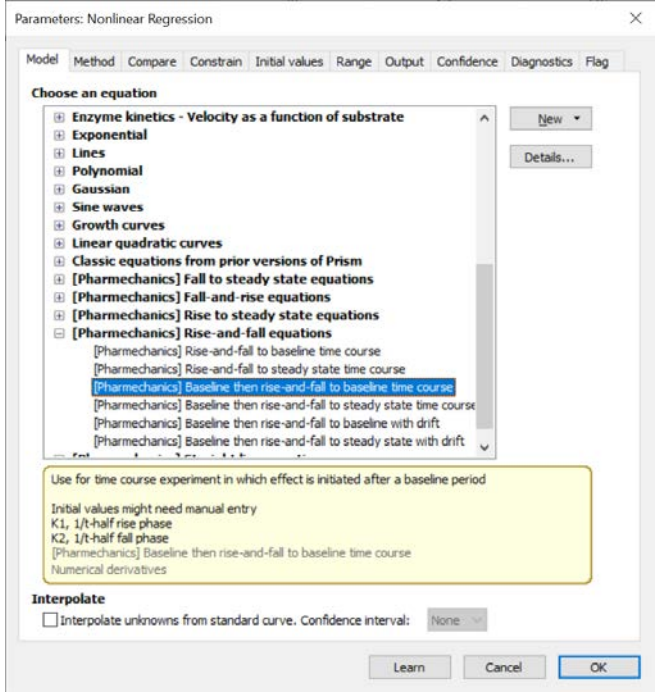
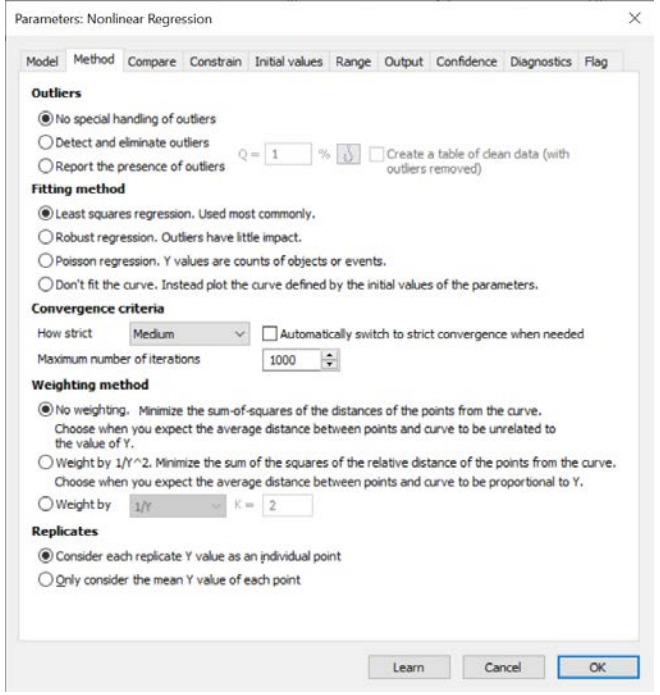
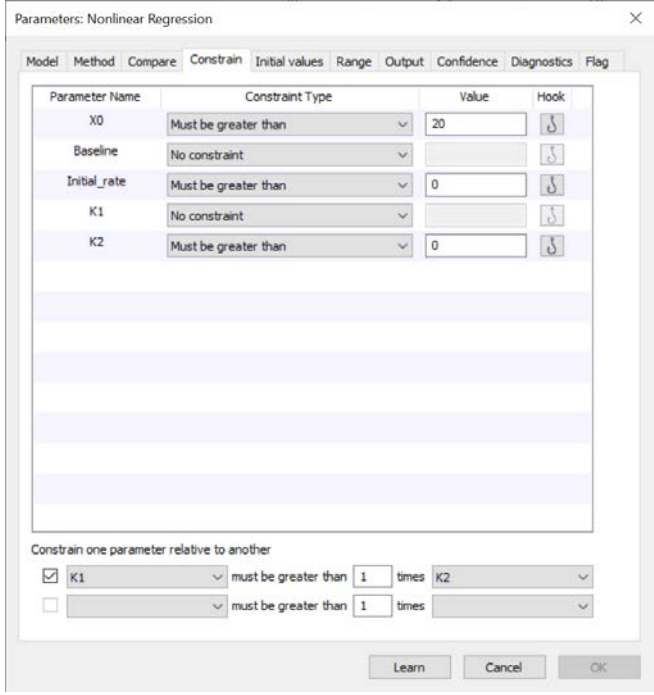
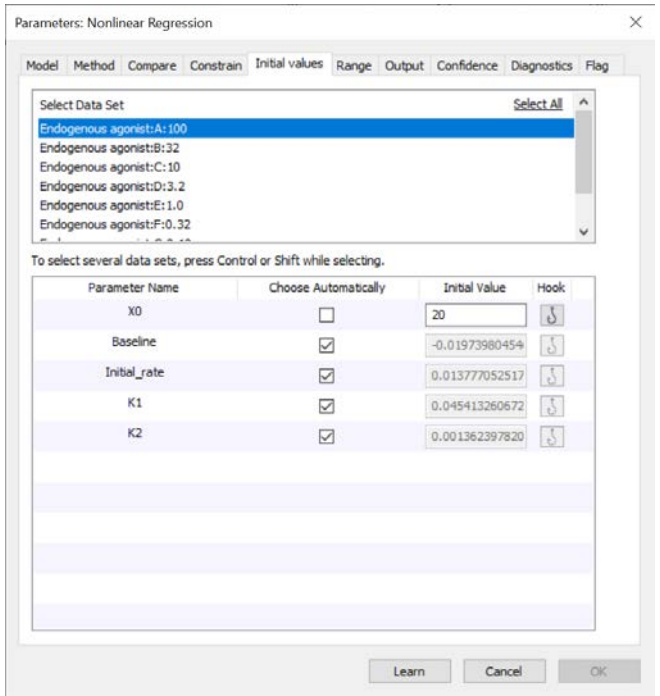
# Curve fitting details

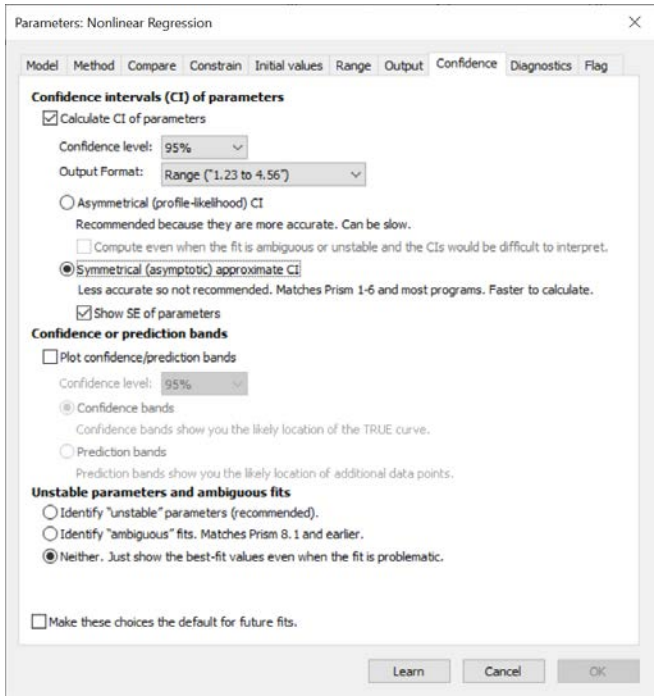
# Prism analysis for endogenous agonist



# Prism analysis for Compounds 1-3