Identification of Drugs for the Treatment of Spinocerebellar Ataxia Type 2

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What's ahead…

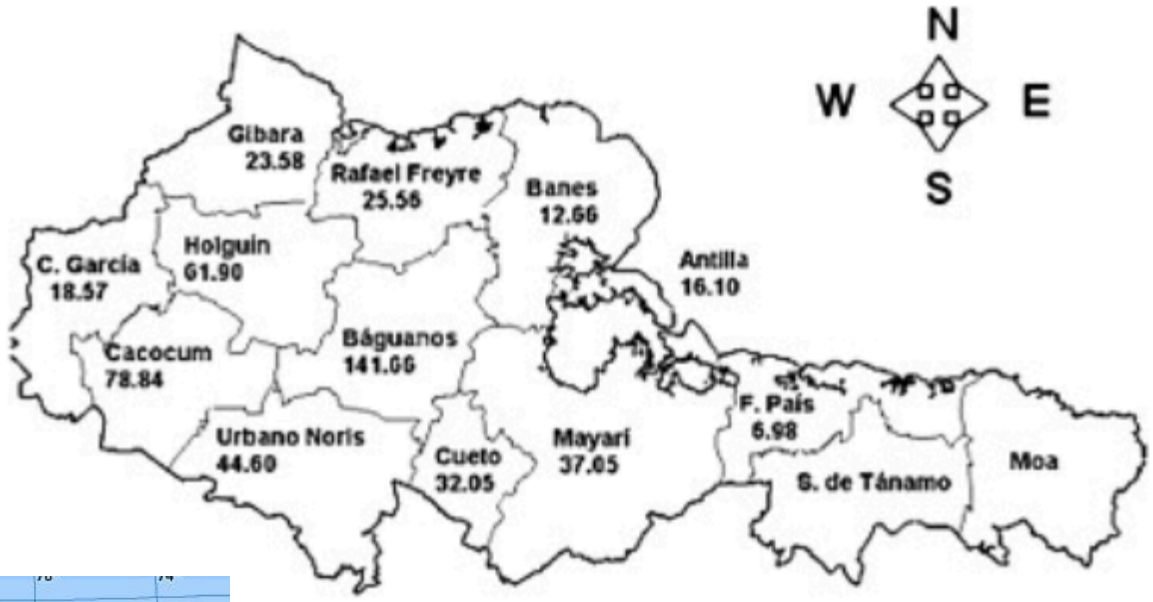
- Introduction to SCA2
  - Clinical Features, Epidemology & Molecular Biology
- Compound screening
  - Secondary screens
  - Biological relevance tests
    - *In vitro* tests
      - Endogenous ataxin-2 expression
      - *In vitro* functional model
    - *Promoter & 3'-UTR characterization*
      - Transcription and translation control
    - *Mouse models*
      - Reporter mouse
        - » Bioavailability, expression & localization
      - Transgenic mouse
        - » Phenotype

SCA2: A brief introduction to its clinical features, epidemiology and molecular biology.

Spinocerebellar Ataxia Type 2 (SCA2)

- Patients are characterized by gait ataxia, frontal executive dysfunction, slow saccades, and DOPA-responsive Parkinsonism.
- SCA2 is a polyglutamine disorder.
- Age of onset varies with CAG length (anticipation).
- Rare (~$1/10^6$) but common in Cuba (3/2000).
- Disease of the cerebellum (SCA2 accounts for 13% of ADCA patients).
- Characterized by Purkinje cell death.

Prevalence rate in Eastern Cuba (per 100,000 people)

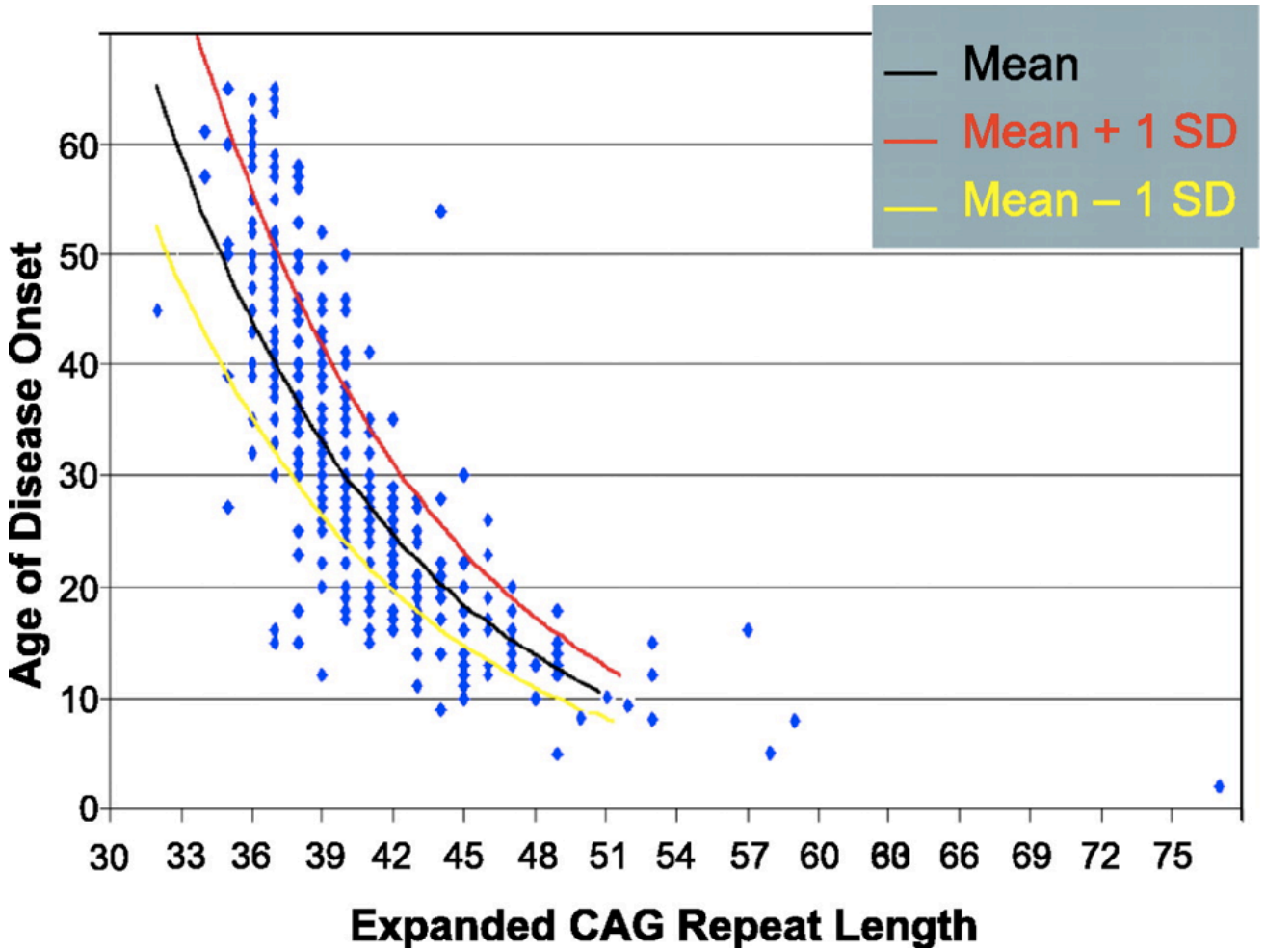


Velazquez Perez et al. Neurosci. Lett. 454:157-60; 2009.

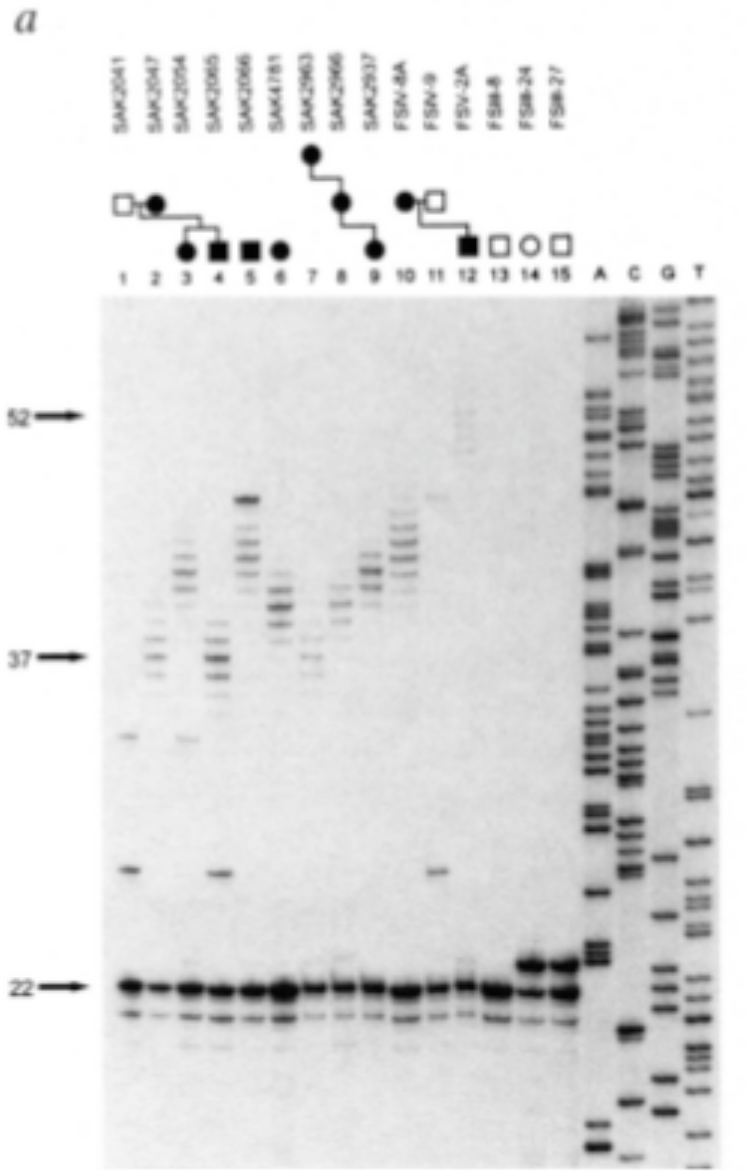
Molecular Genetics of SCA2

- Caused by CAG expansion in the *ATXN2* gene coding region encoding a polyglutamine.
- CAG <32 is normal, >32 is disease-causing.
- In patients the CAG lengthens with each generation.
- Longer CAG tracts are associated with earlier age of onset and greater severity of disease.

Anticipation



Pulst et al. Brain 2005



PAGE

Most common normal allele

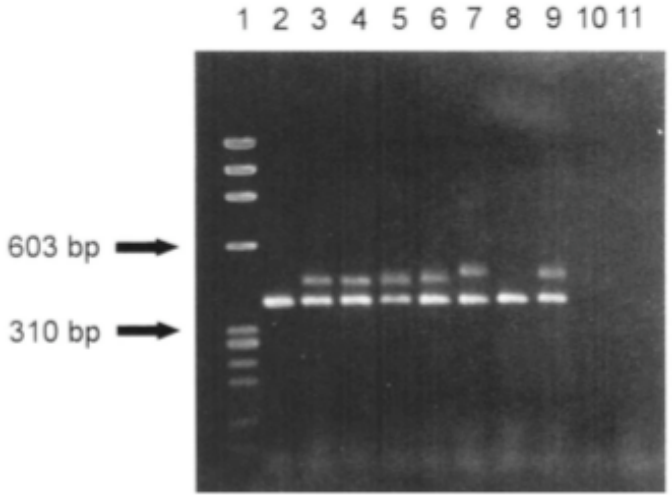
$(CAG)_8CAA(CAG)_4CAA(CAG)_8$

Also observed frequently in the normal population

$(CAG)_{14}CAA(CAG)_7$

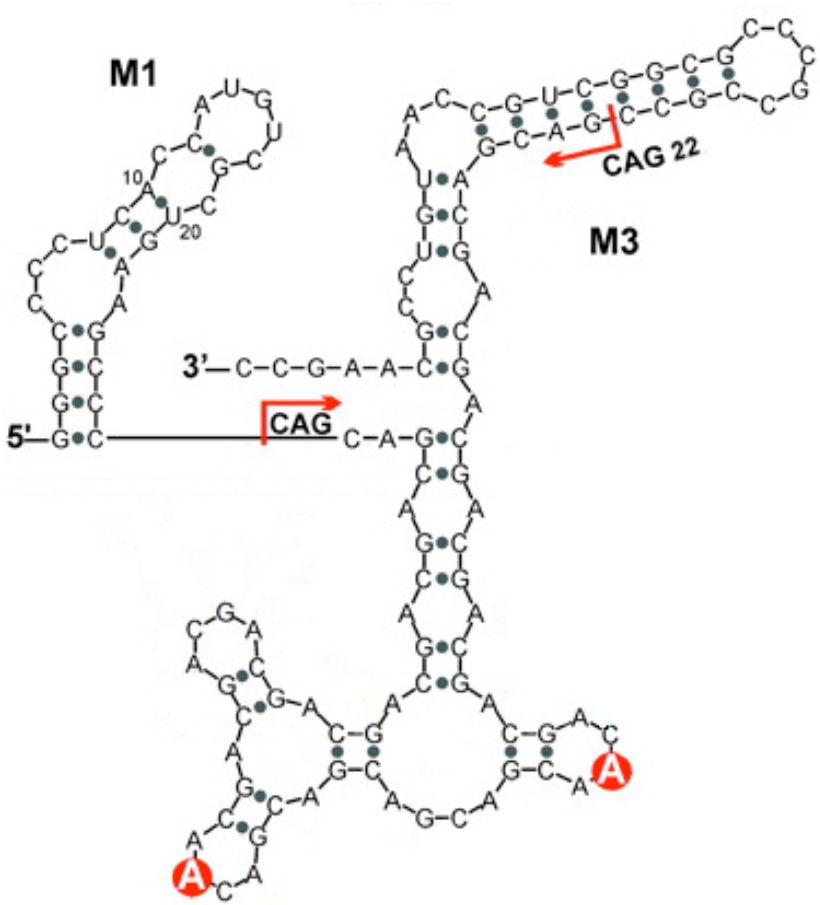
Disease allele

$(CAG)_{>32}$



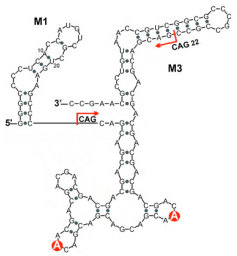
AGE

Secondary RNA structure of the most common normal allele $(CAG)_8CAA(CAG)_4CAA(CAG)_8$

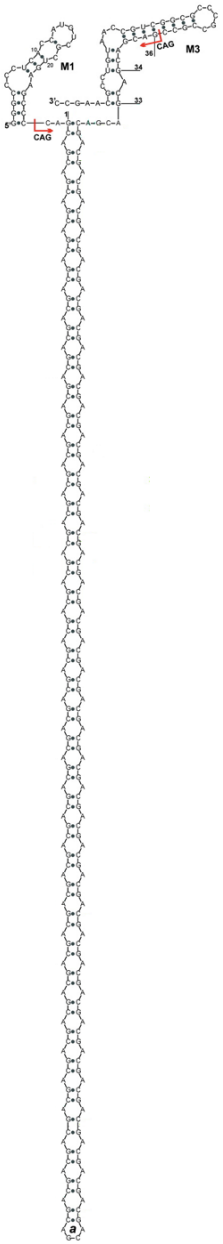


Sobczak, K. et al. J. Biol. Chem. 2005;280:3898-3910

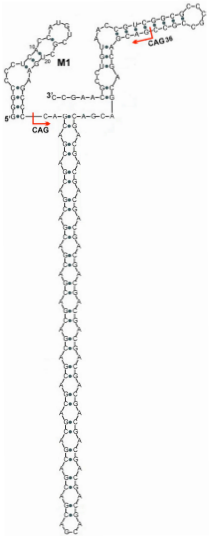
CAG22



CAG110



CAG36

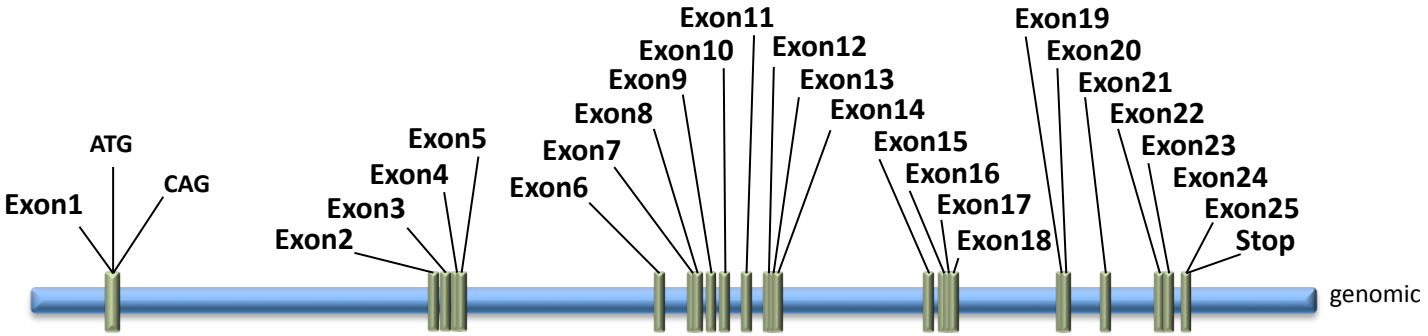


What would this feature do to…

Expression?

Toxicity?

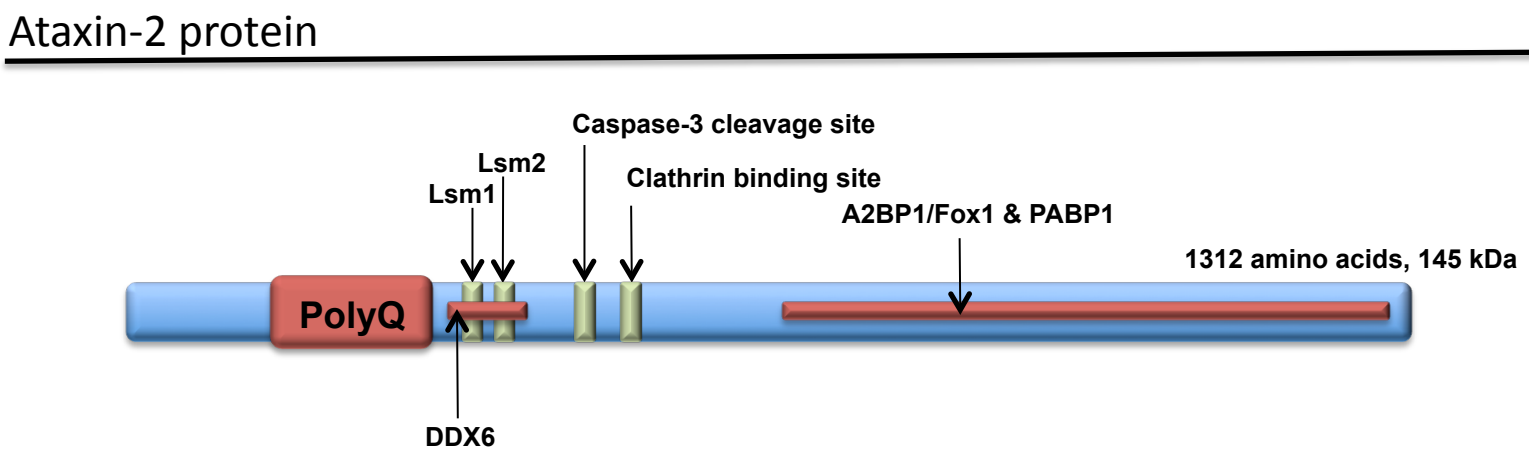
The *ATXN2* gene



150 Kb
25 exons
CHR 12q24.1
3,939 bp cDNA

Ataxin-2 protein & function

- Ataxin-2 appears to control RNA metabolism or RNA expression through its interactions with polyA binding protein (PABP1), RNA splicing factor A2BP1/Fox1, and polyribosomes.
- Ataxin-2 localizes to Golgi, stress granules and p-bodies.
- Overexpression of ataxin-2 induces apoptosis and is toxic.



SCA2 problem

- We have known the *ATXN2* gene causes SCA2 since 1996 but we do not know enough about its function to devise functional therapeutics.

- With the lack of knowledge on its function we decided that the best approach would be to devise therapeutics to knock down its expression.

Hypothesis

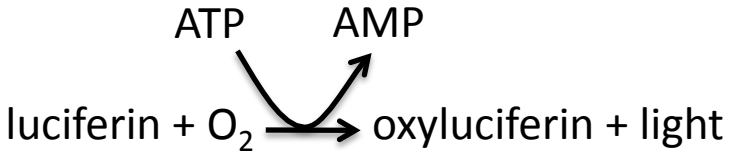
Compounds that lower ataxin-2 expression will be therapeutic for SCA2

- SCA2 pathology is related to a gain of normal function or gain of toxic *ATXN2* mutant function.
- In humans outcome worsens with increased mutant *ATXN2* dose, and this is supported by studies of transgenic mice (1,2).
- *ATXN2* knockout mice have only mild phenotypes including obesity and reduced fertility (3).
- Reversibility of SCA1&3 transgenic mouse phenotype (4).

1. Ragothaman et al., 2004 & 2008
2. Huynh et al., 2000, 2009
3. Kiehl et al., 2006, Lastres-Becker 2008
4. Zu et al., 2004; Boy et al., 2009.

Towards compound screening: building a valid system.

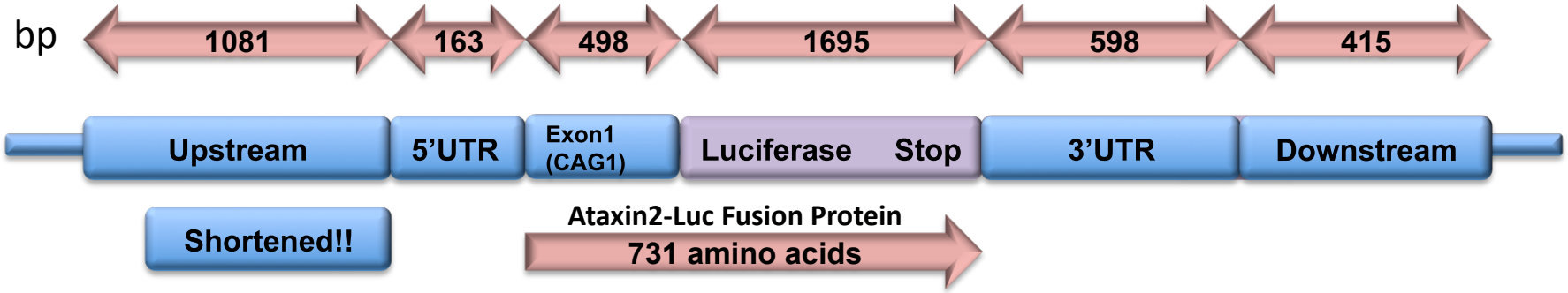
Firefly (*Photinus pyralis*) Luciferase



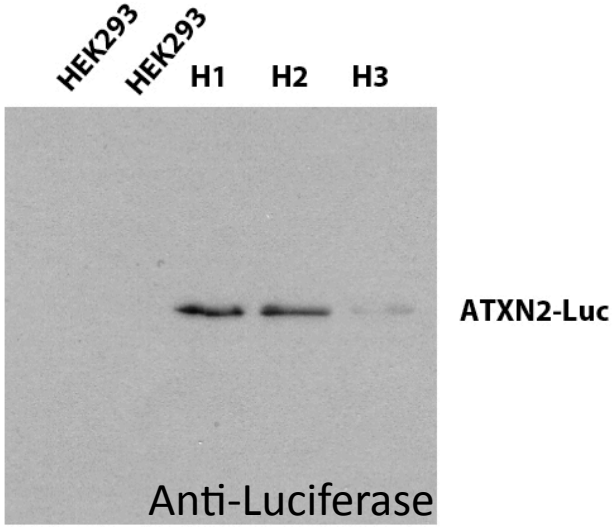
Sea Pansy (*Renilla reniformis*) Luciferase

pGL2-ATXN2-Luc

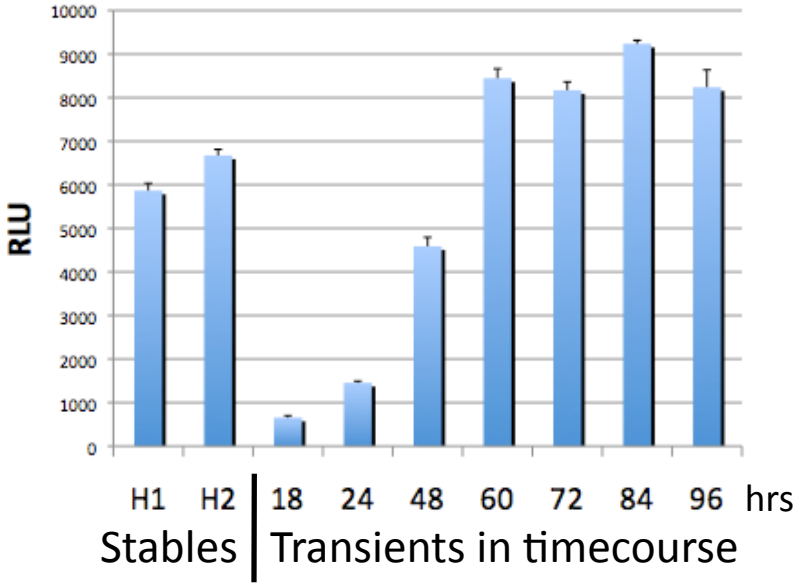
ATXN2-Luciferase Expression Construct



Western Blotting



Luciferase Assays



Which luciferase to use

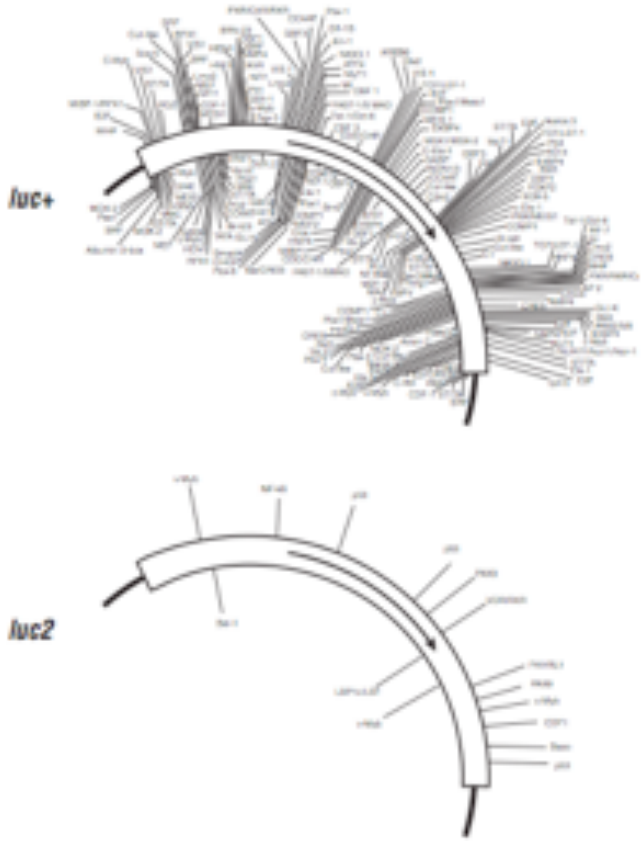
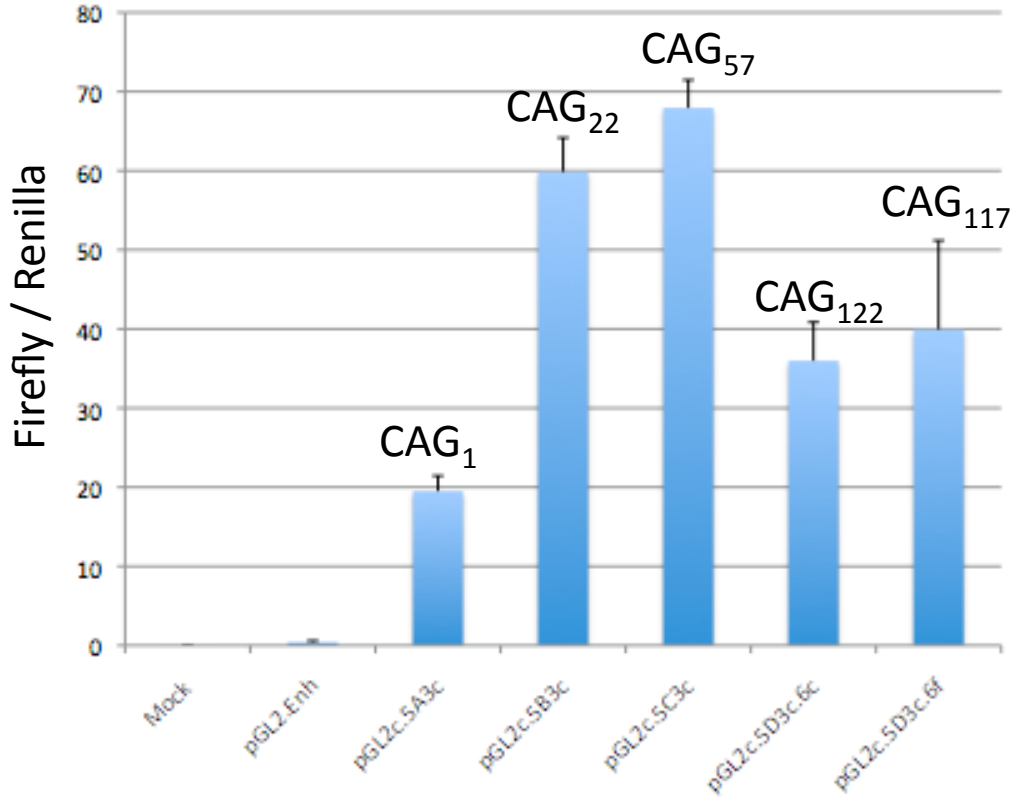


Fig 11. Comparison of consensus transcription factor binding sites in luc+ and luc2 genes (*Promega Notes* 89).

Comparison of constructs with different CAG repeats.



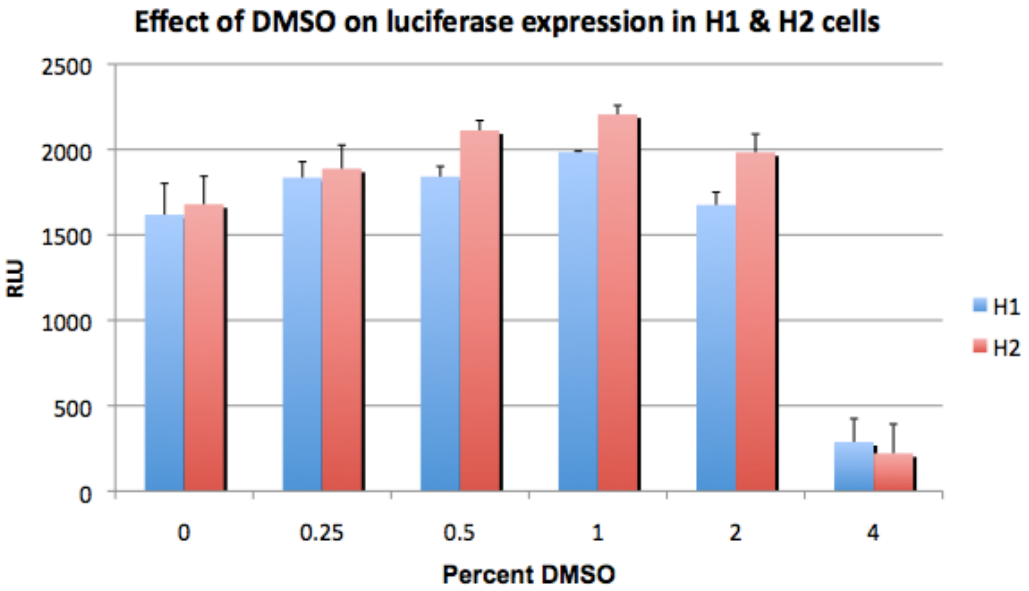
3/4/09

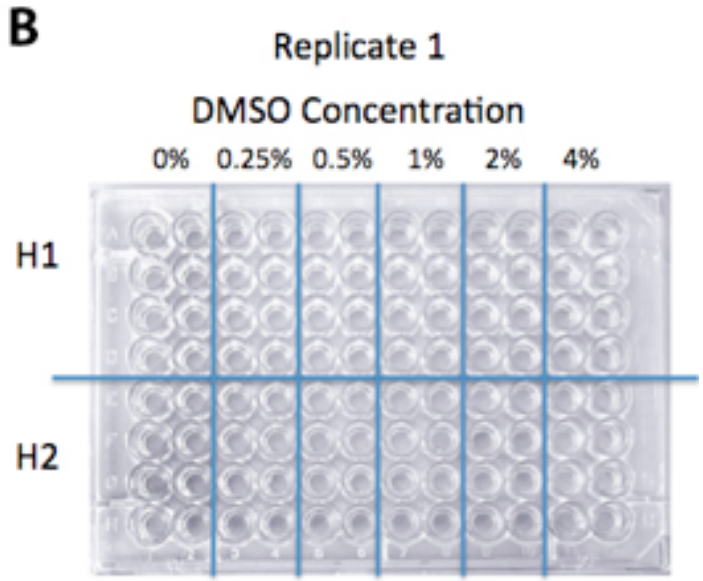
Optimizations before screening

Optimizations

- Effect of DMSO on proliferation.
- Numbers of cells to plate.
- Method of drug addition.
- Length of time to incubate cells on drugs.
- Linear signal & SD in multi-well format.
- Positive control compounds.

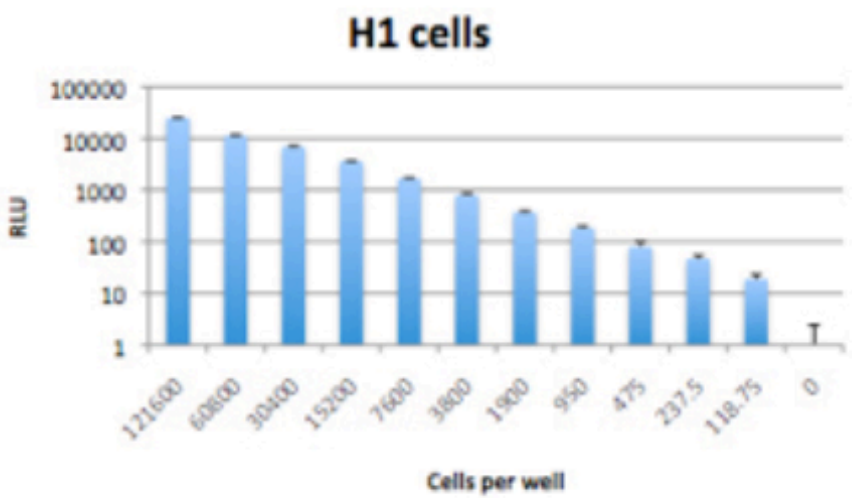
Effect of DMSO on proliferation

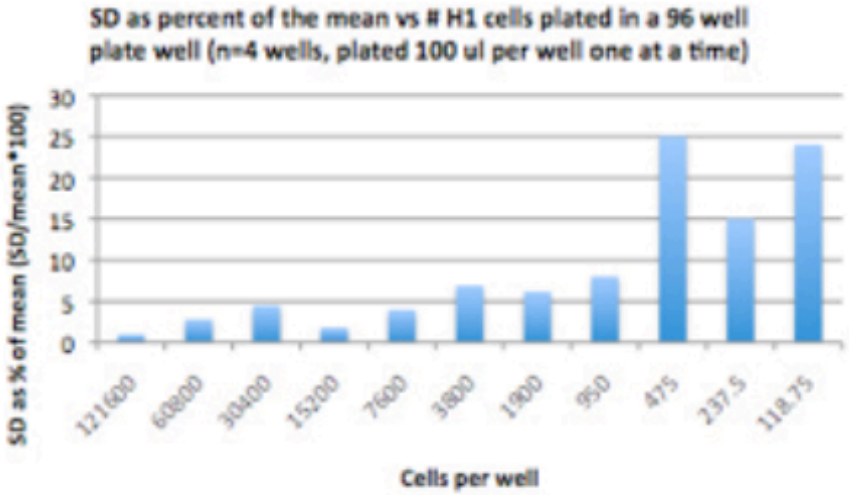




How fast does the SD increase with reduced number of cells plated

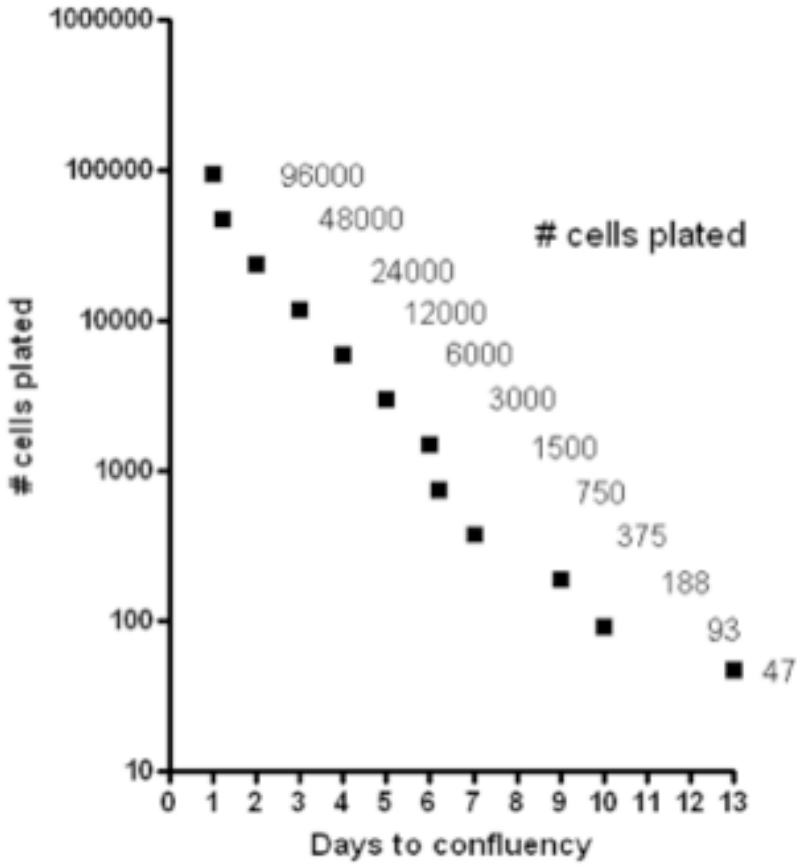
A



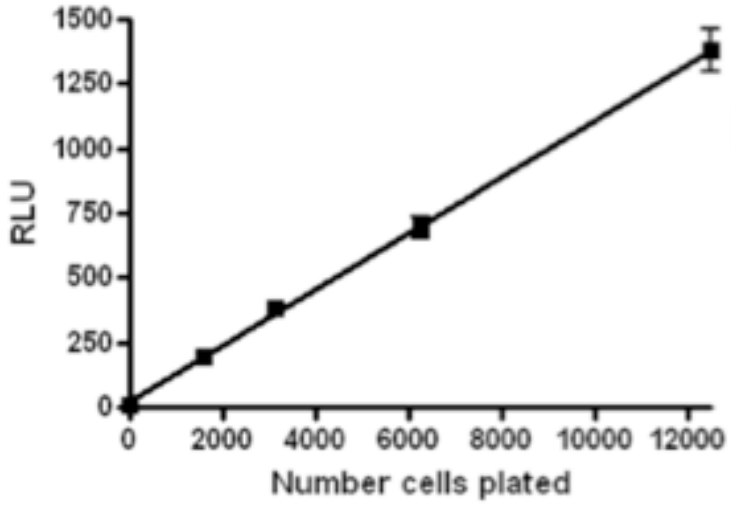


How fast do your cells come to confluency

# cells plated	Hours to confluency
96000	24
48000	24
24000	48
12000	72
6000	96
3000	120
1500	144
750	144
375	168
188	216
93	240
47	312



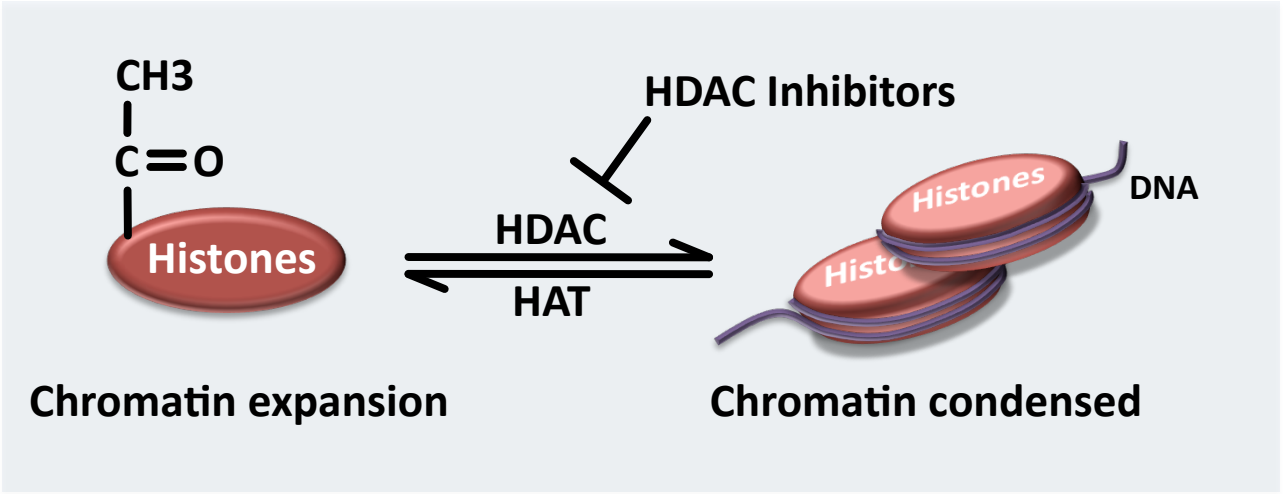
Linearity tested in 384 well plate format



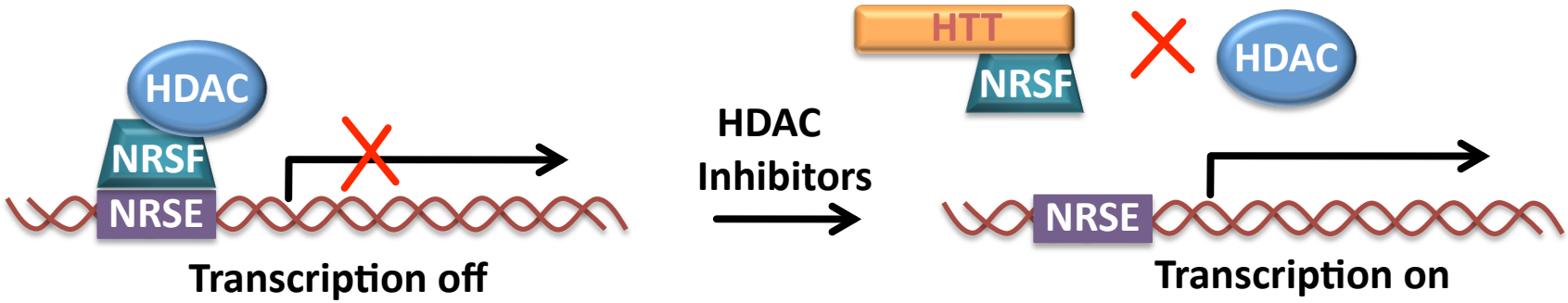
Cells per well	Mean ± SD
12500	1382 ± 82.2
6250	696.2 ± 40.4
3125	380.4 ± 18.7
1563	199.1 ± 16.4
0	7.000 ± 4.78

HDAC inhibitors as therapeutics for polyglutamine disorders

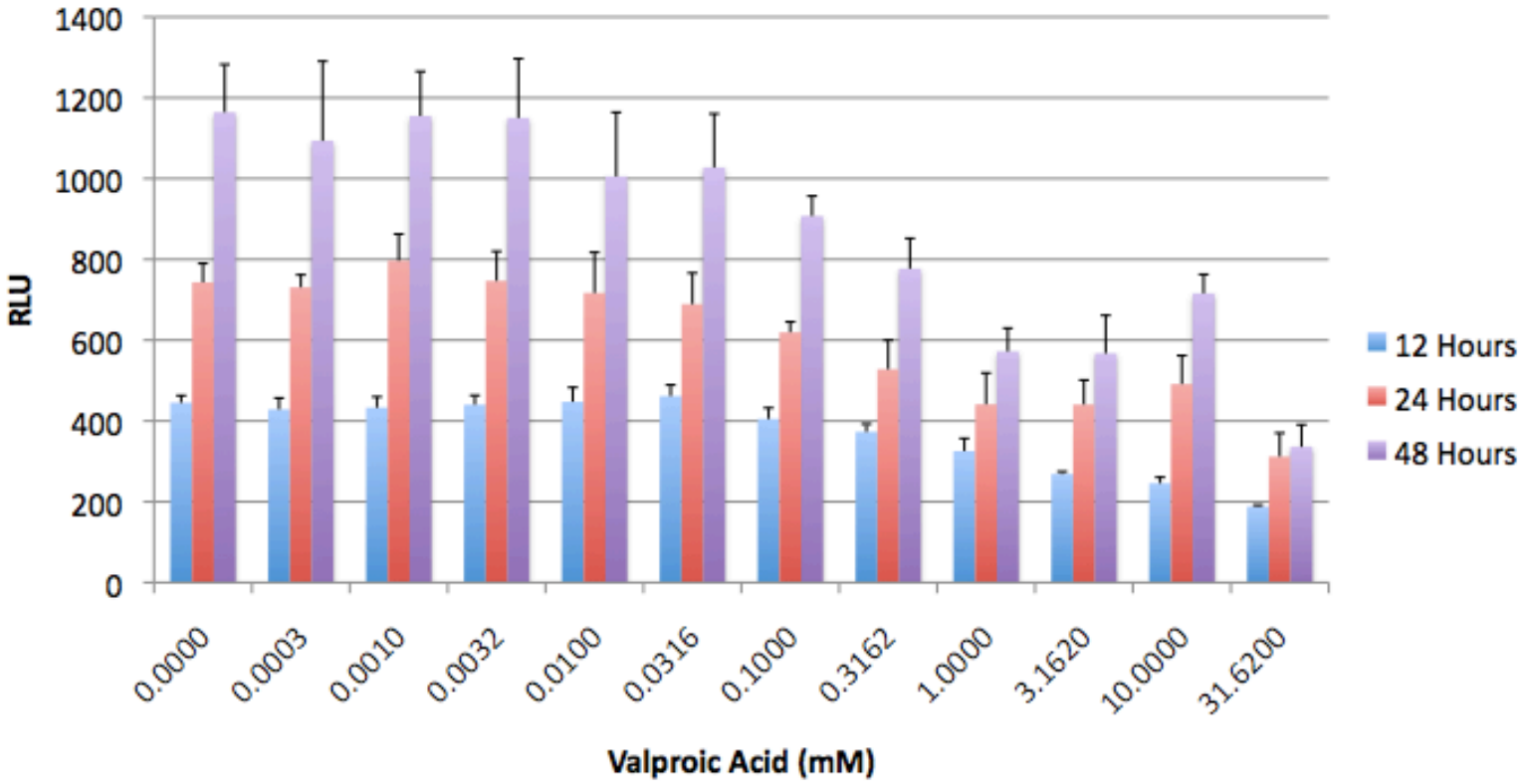
Butler & Bates, Nat Rev Neurosci 7, 2006.



Neuron restrictive silencer factor & Huntingtin

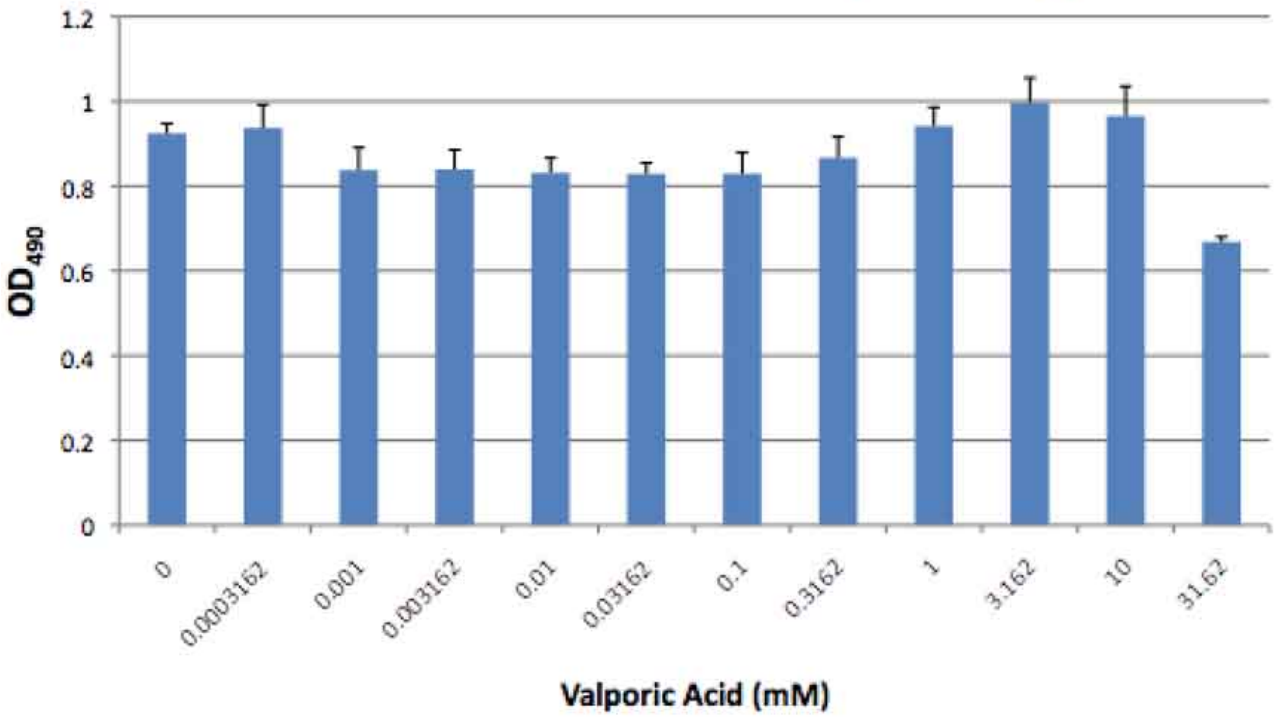


Valproic acid inhibits ATXN2-Luc



The level of VPA that is achievable in patient serum is ~0.3 mM

VPA does not inhibit cell proliferation at the physiological dose



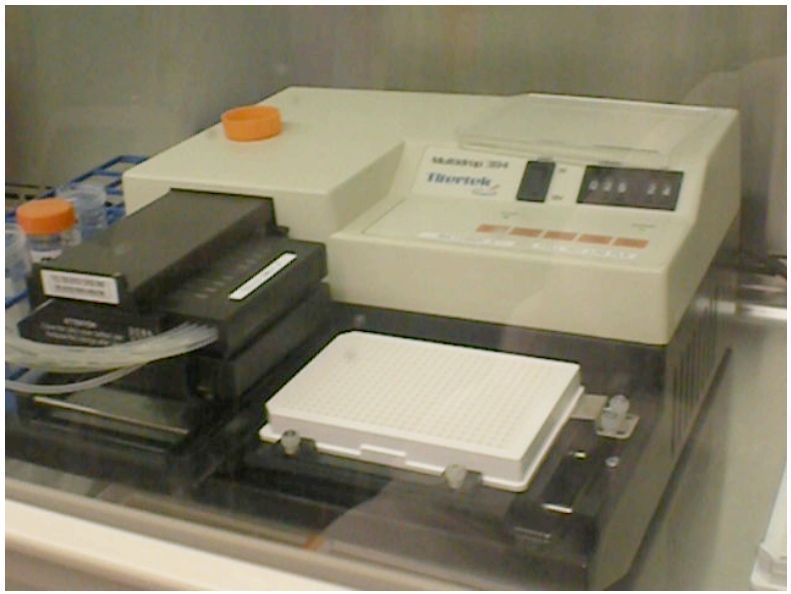
Compound Screening

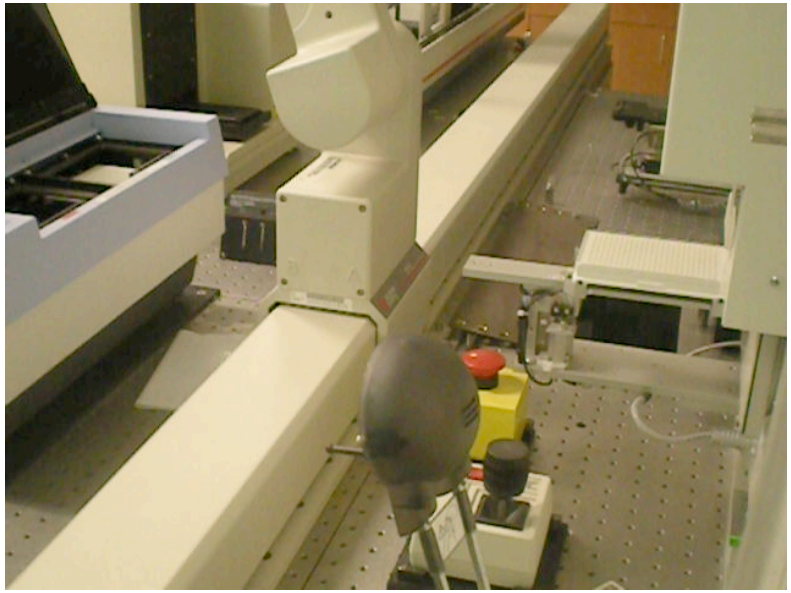
Libraries Screened

Library	Source	Plates	Compounds	Hits (3SD)
Enzymes	Biomol International	1	300	7
Lipids	Biomol International	1	204	0
PW	Prestwick Chemical (FDA approved)	4	1120	27
MS	Microsource Spectrum	7	2000	41
TAR	Asinex (Targeted Library)	27	8505	19
NIH	BioFocus DPI (NIH Clinical Collection)	2	607	15
ES	Unpublished	4	1408	7
S	Unpublished	4	1408	2
DL	Asinex (Platinum Collection)	53	19,570	74
UCLA	ChemBridge Corp.	94	30,000	171

Total number of 384 well plates = 197
Total number of compounds = 65,122
Total hits(3SD down)=363
Hit rate=363/65,122 x 100 = 0.5%

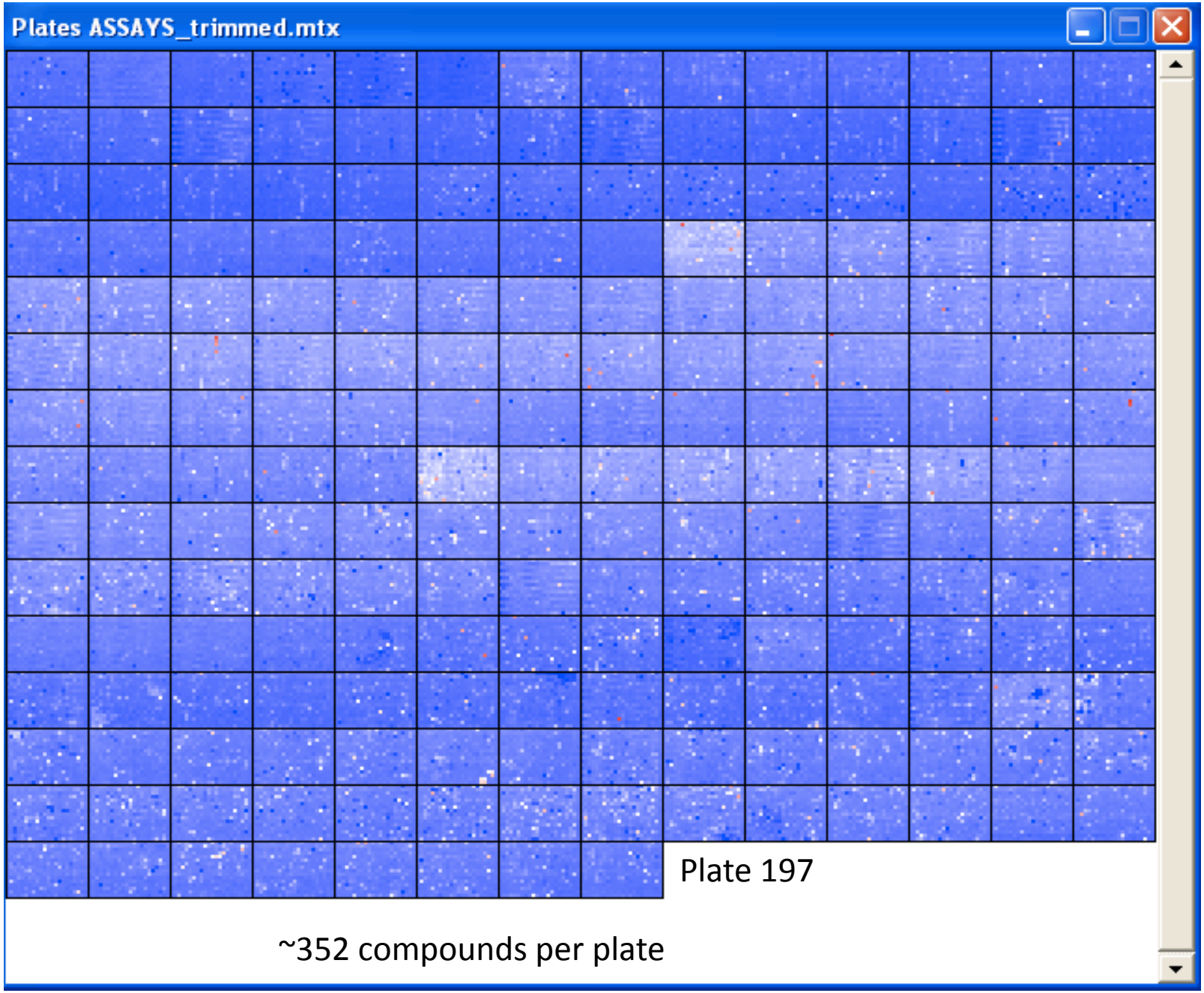
Conducting the screen





Uncorrected RLU Values

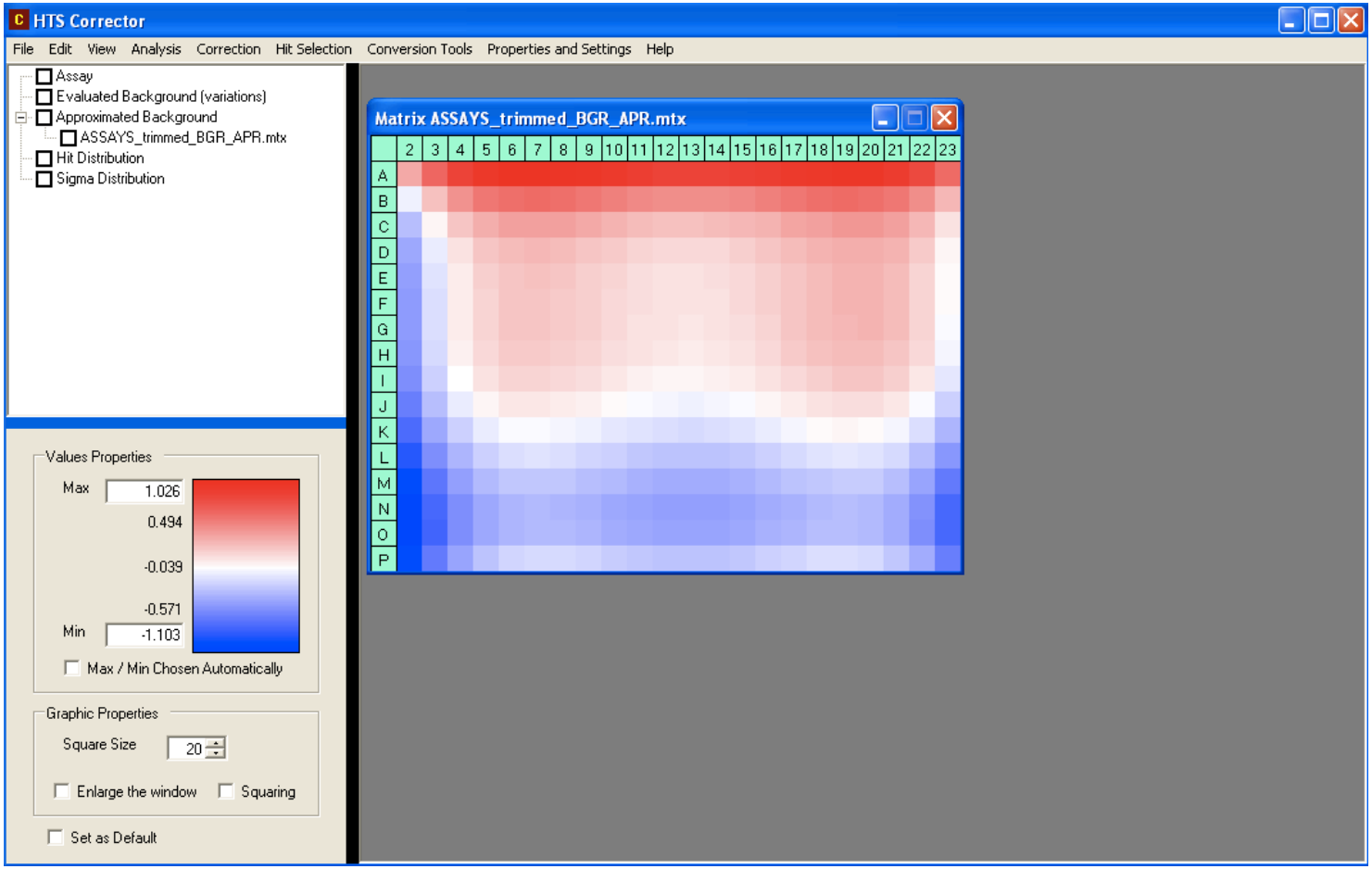
Plate 1



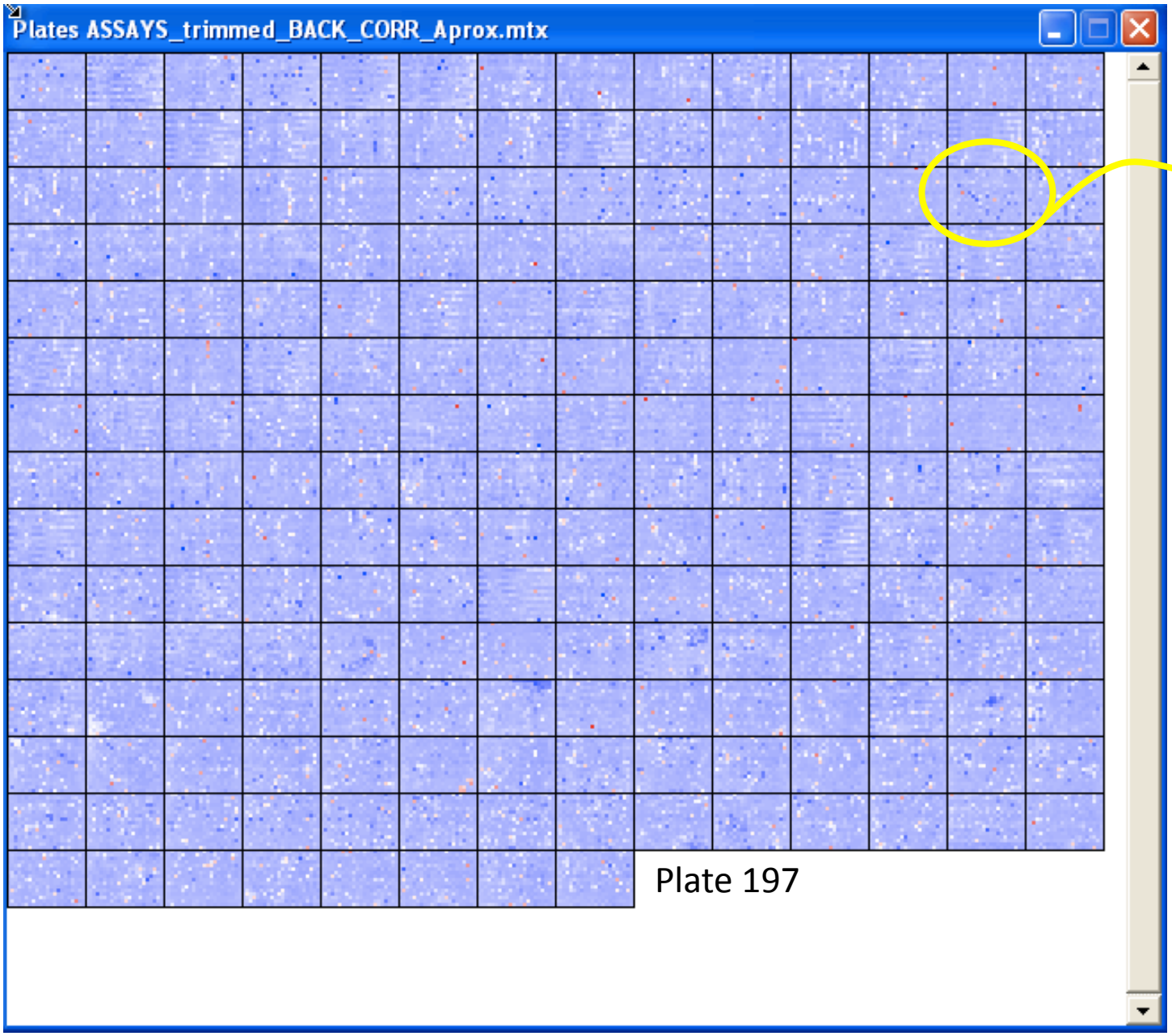
First all values are normalized to a mean of zero and standard deviation of one…

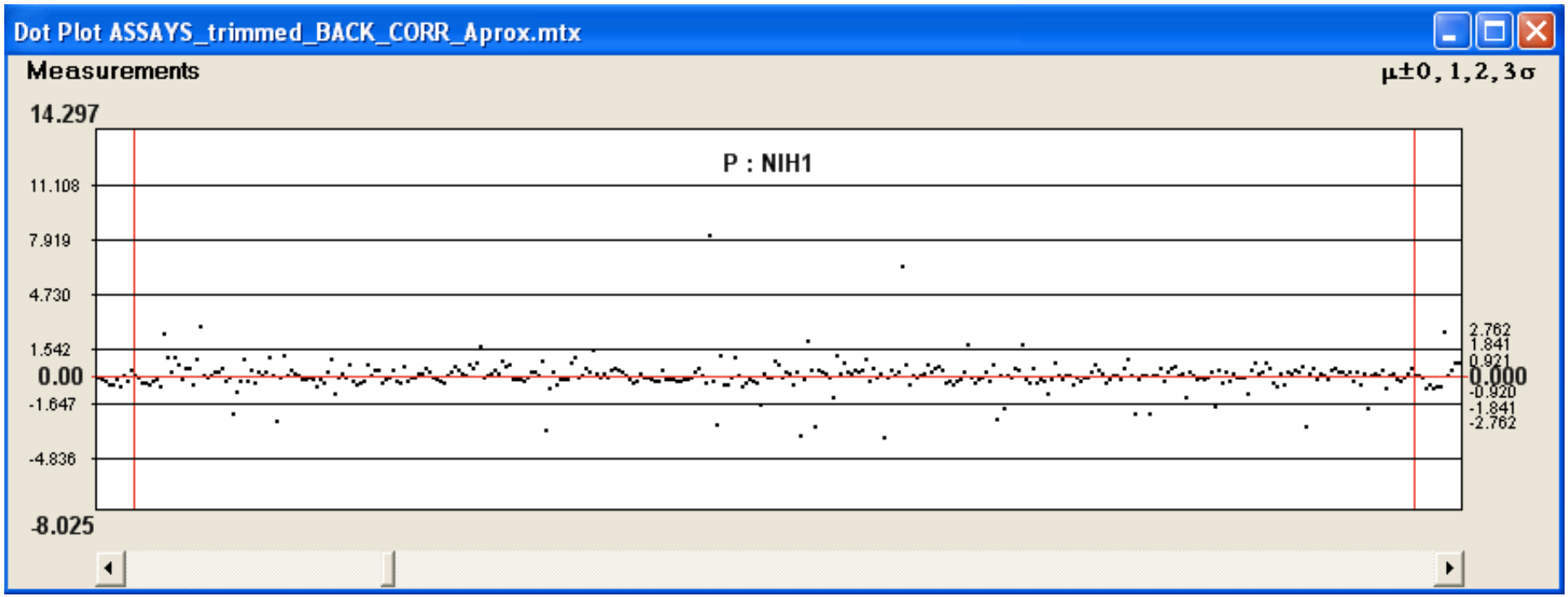
$$x_i' = \frac{x_i - \mu}{\sigma}$$

Then the background is calculated and subtracted

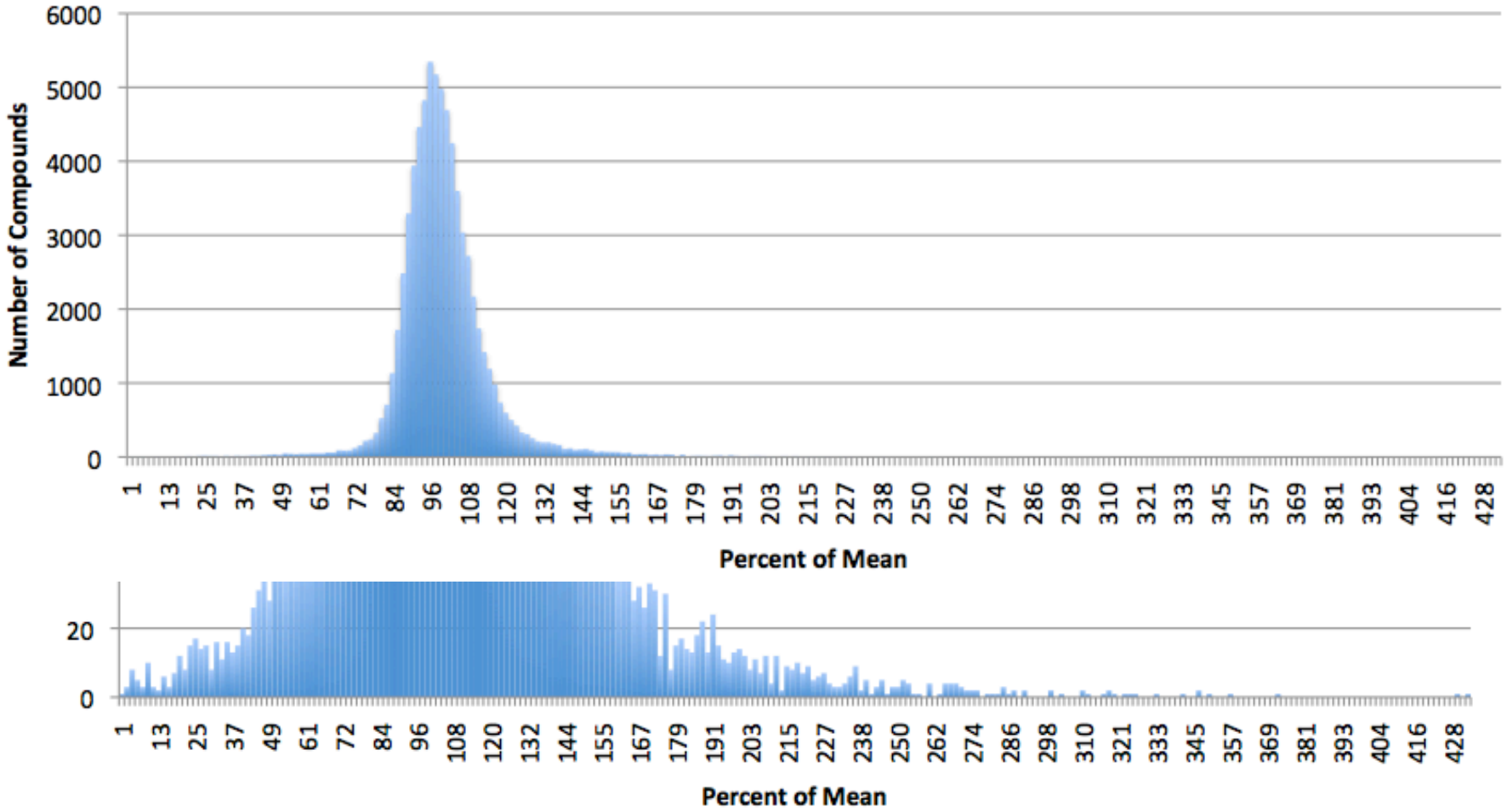


Corrected RLU Scores




Dot Plot ASSAYS_trimmed_BACK_CORR_Aprox.mtx
Measurements
μ±0,1,2,3σ
14.297
P : NIH1
11.108
7.919
4.730
1.542
0.00
-1.647
-4.836
-8.025
2.762
1.841
0.921
0.000
-0.920
-1.841
-2.762

Frequency distribution
(freq of compounds vs effect on expression)



The Hits

Antagonist | Agonist

20 NaK ATPases

Turn up *ATXN2*-Luc ("uppers")

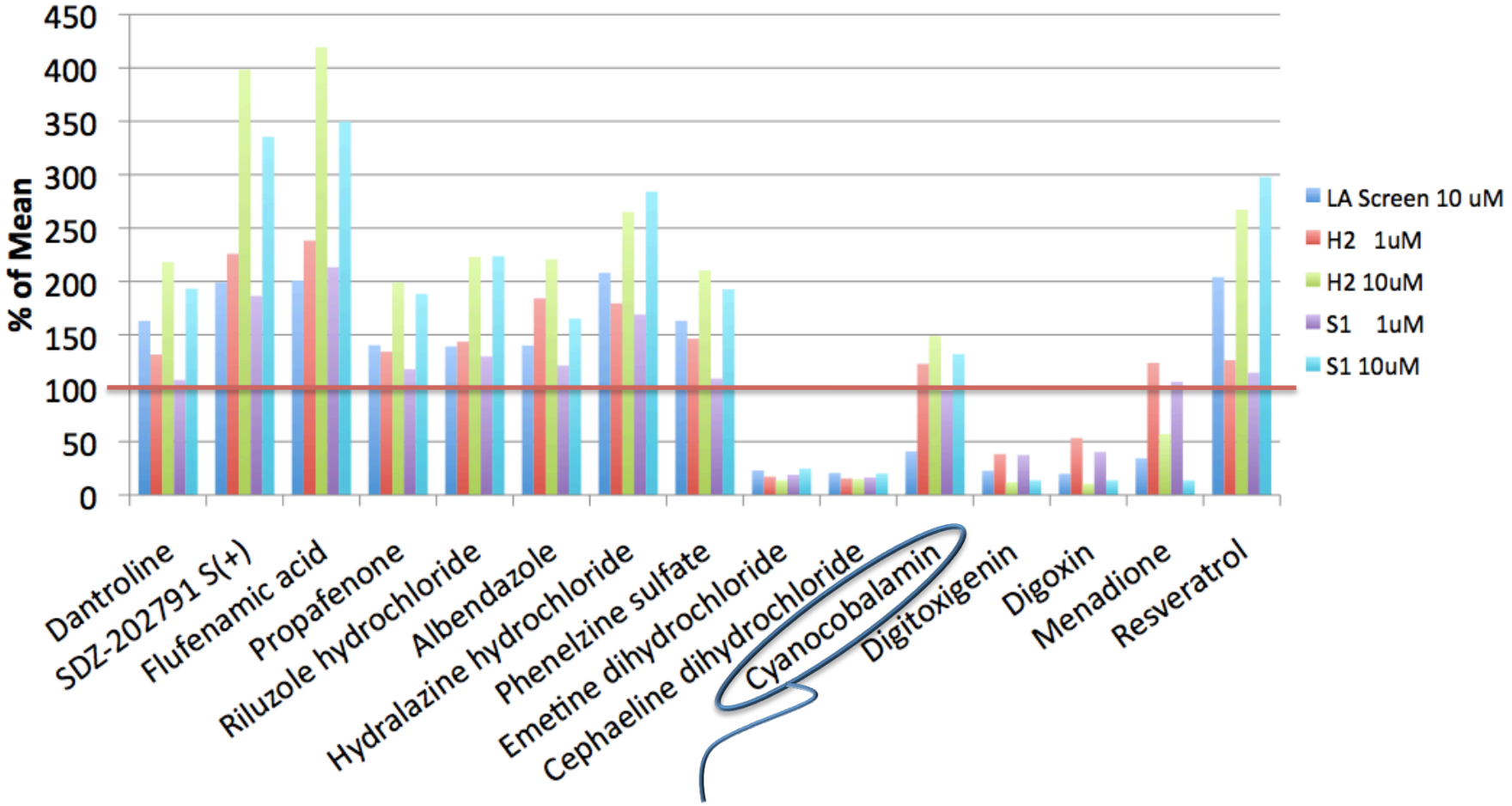
- Albendazole
- Dantroline
- Diphenoxylate
- Flufenamic acid
- Hydralazine hydrochloride
- Ipriflavone
- Kaempferol
- Nabumetone
- Phenelzine sulfate
- Piperine
- Propafenone
- Resveratrol
- Riluzole hydrochloride
- SDZ-202791 S(+)

Turn Down *ATXN2*-Luc ("downers")

- 2-amino-9,10-phenanthrenedione
- 5-Nonyloxytryptamine
- Aklavine hydrochloride
- Cephaeline dihydrochloride heptahydrate
- Cobalamine
- Convallatoxin
- Cyanocobalamin
- Cymarin
- Derrubone
- Digitoxigenin
- Digitoxin
- Digoxigenin
- Digoxin
- Emetine dihydrochloride
- Emicymarin
- Gitoxigenin diacetate
- Gitoxin
- Helveticoside
- Indatraline
- Isopropamide iodide
- L-694,247
- Lanatoside C
- Lomerizine DiHCl
- Menadione
- Neriifolin
- Nicardipine
- Ouabain
- Periplocymarin
- Peruvoside
- Pinacidil
- Proscillaridin A
- Resorcinol monoacetate
- Ryanodine
- Sanguinarine
- Sarmentogenin
- SKF-96365
- Strophanthidin
- Strophanthidinic acid lactone acetate
- SU1498
- Tegaserod maleate
- TMB-8
- Tramadol
- Trifluoperazine hydrochloride
- Trifluridine
- U-37883A
- U-50488

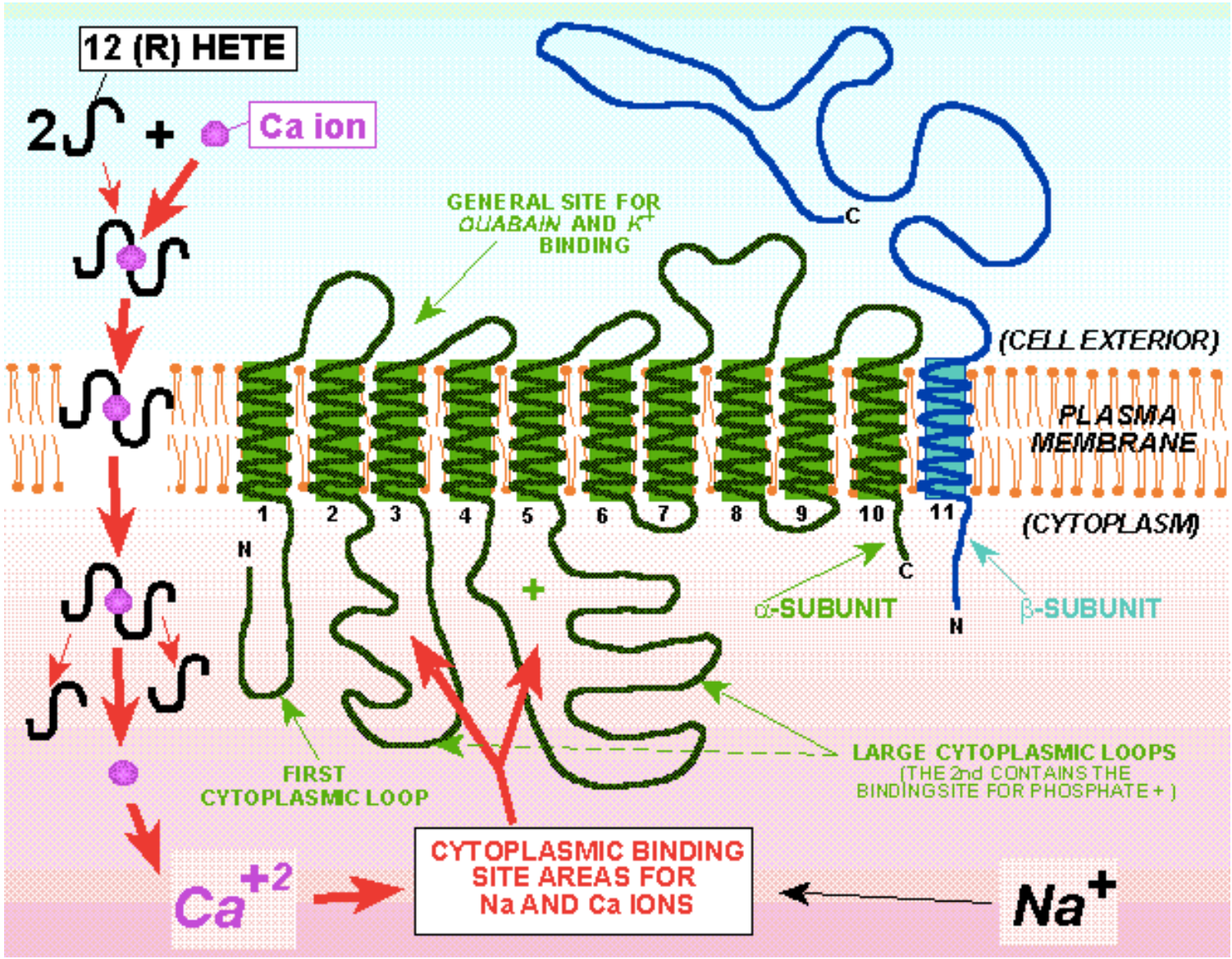
Validation Assays

Validation assay using HEK293 & SH-SY5Y
(showing only 15 compounds)



No effect in validation assay
Only three out of 225 were like this one

Cardiac glycosides alkylate the active site of NaK-ATPases

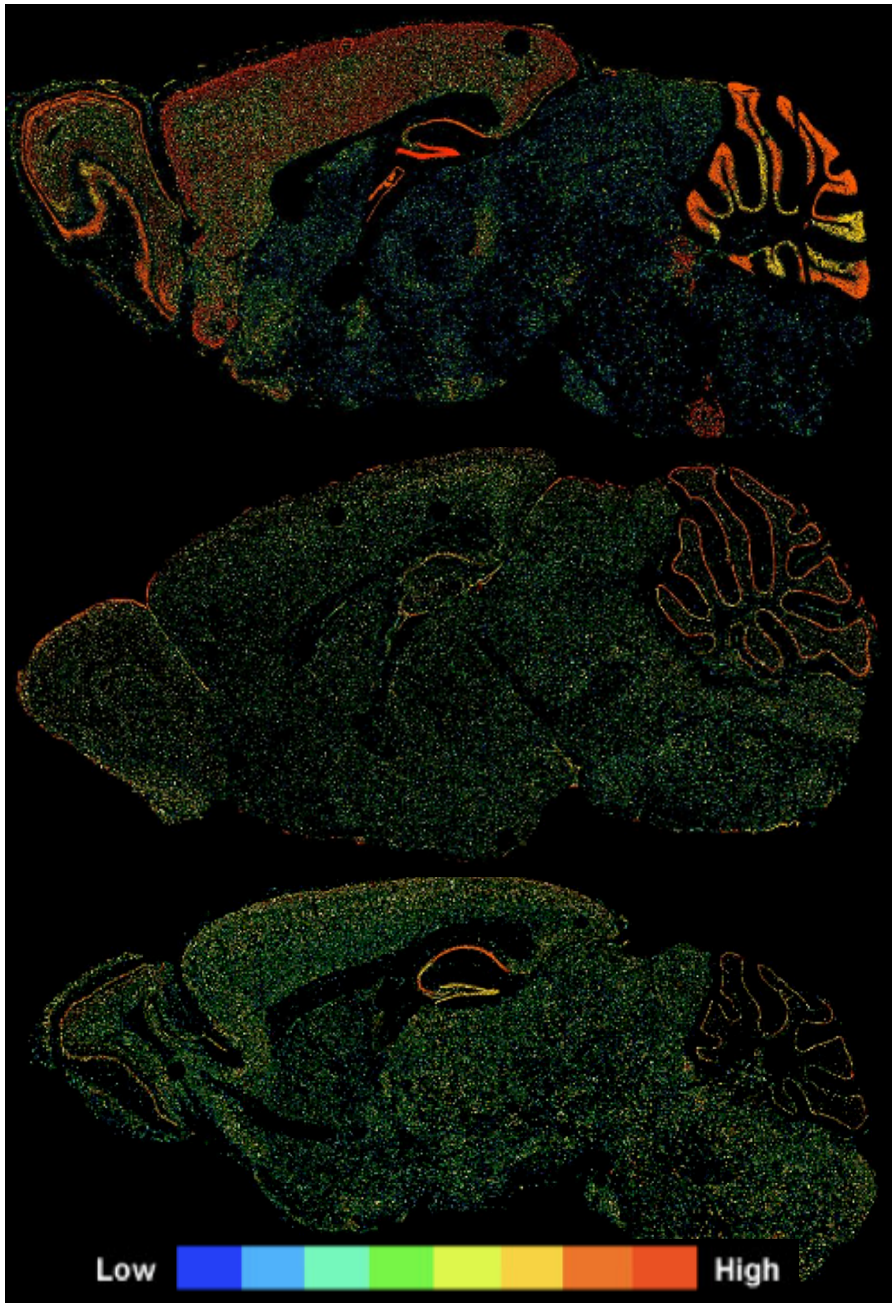


Expression of Na/K ATPases in brain

ATP1α1
ATP1α2
ATP1α3

ATP1α4

ATXN2

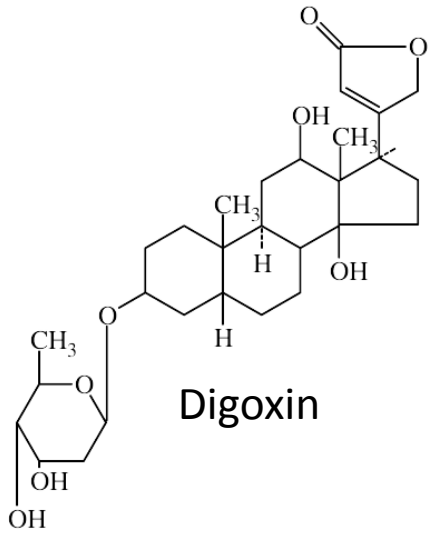


Down-regulation of *ATP1α3* in Purkinje cells by the ATPase inhibitor ouabain (and others) is neuroprotective (Ghoumari et al., FASEB J. 20, 2006)

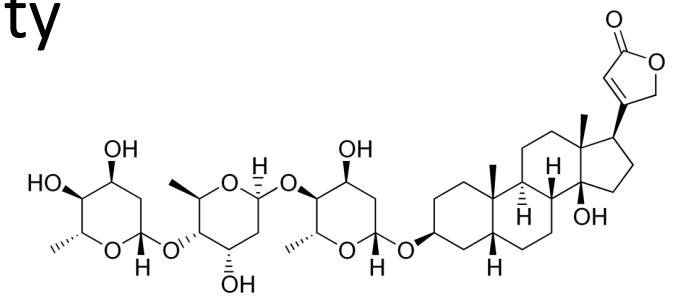
Foxglove (*Digitalis purpurea*)

Digitalis because they fit on your fingers.
Foxglove because the fairies gave them to foxes to warm their feet.
Also called “dead man’s bells” and “witches gloves”

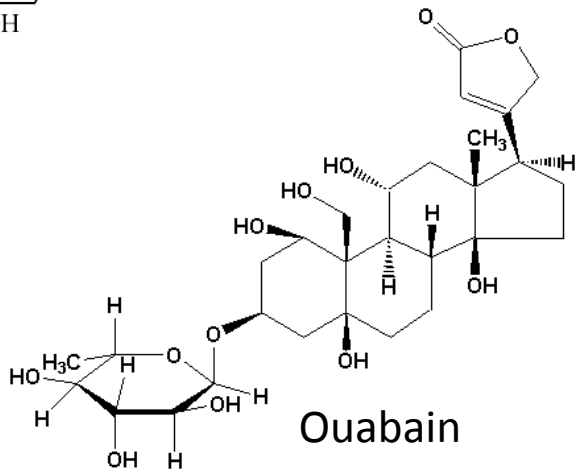
Structurally, they have a common steroid feature with a unique R (alkyl) group that determines toxicity



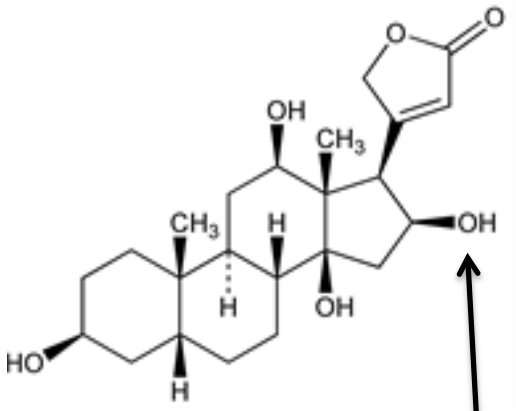
Digoxin



Digitoxin



Ouabain

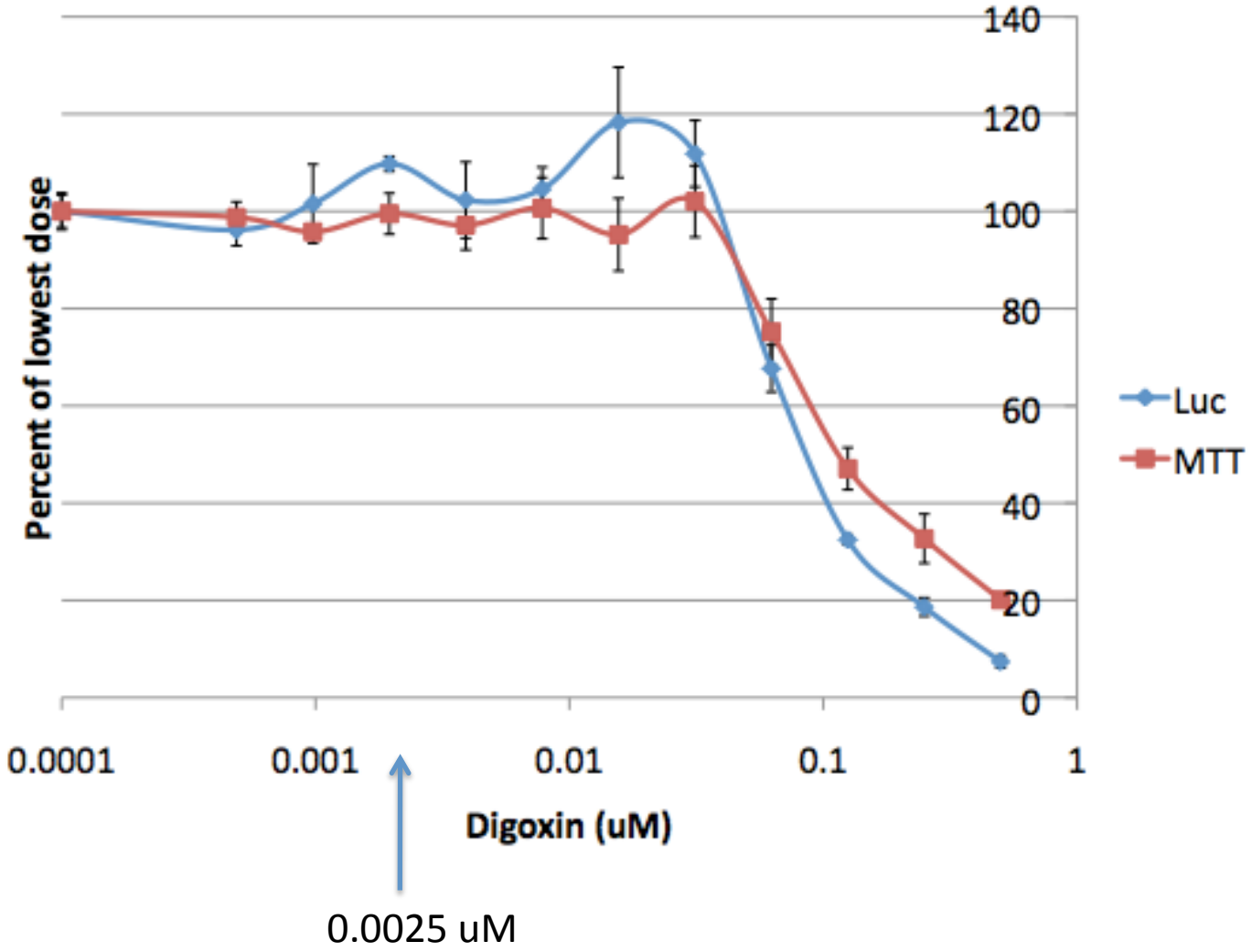


Gitoxigenin

Digitoxigenin (lacks –OH)

Digoxin

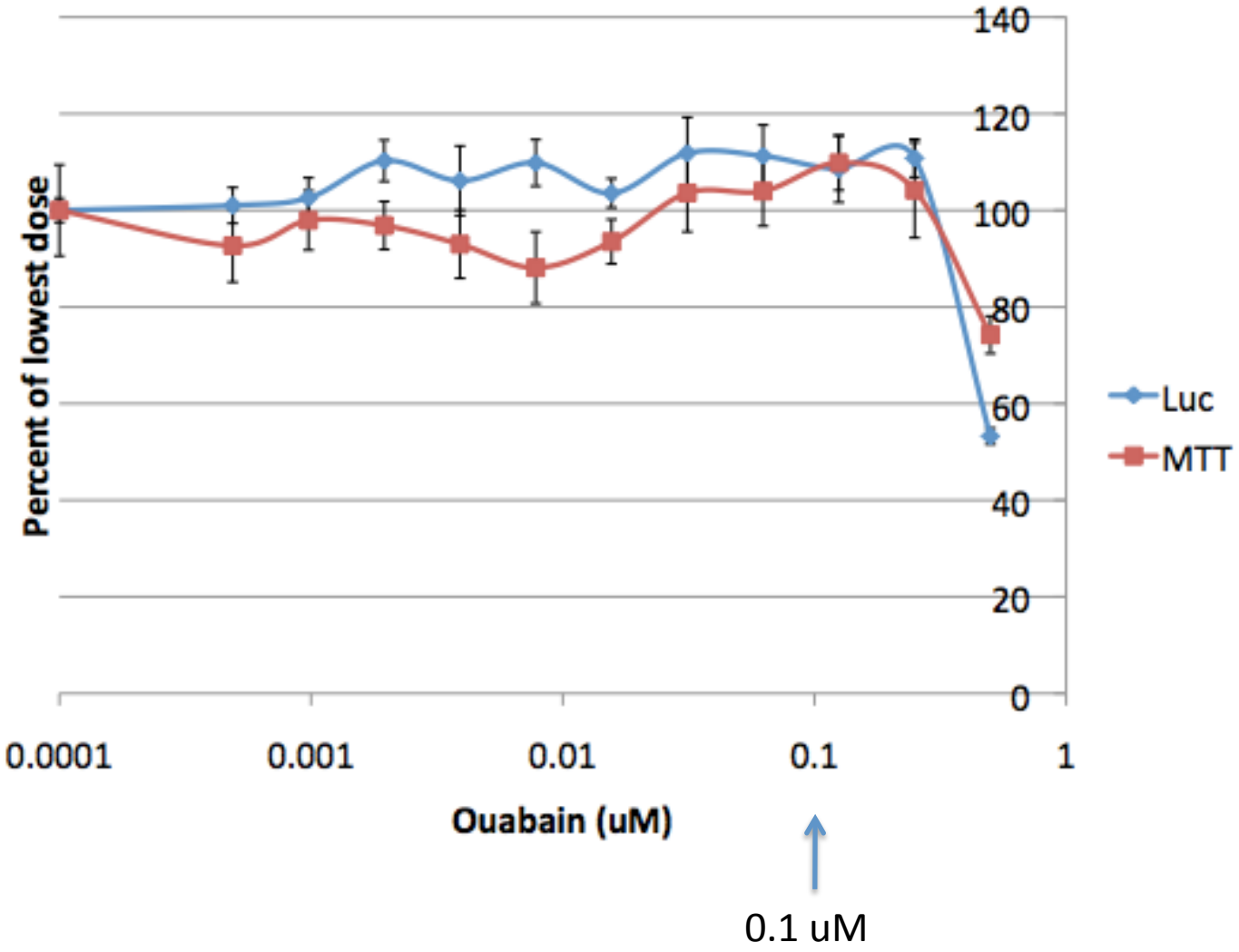
SH-SY5Y cells, 48 hours



Ouabain

NaK ATPase inhibitor
Translation inhibitor

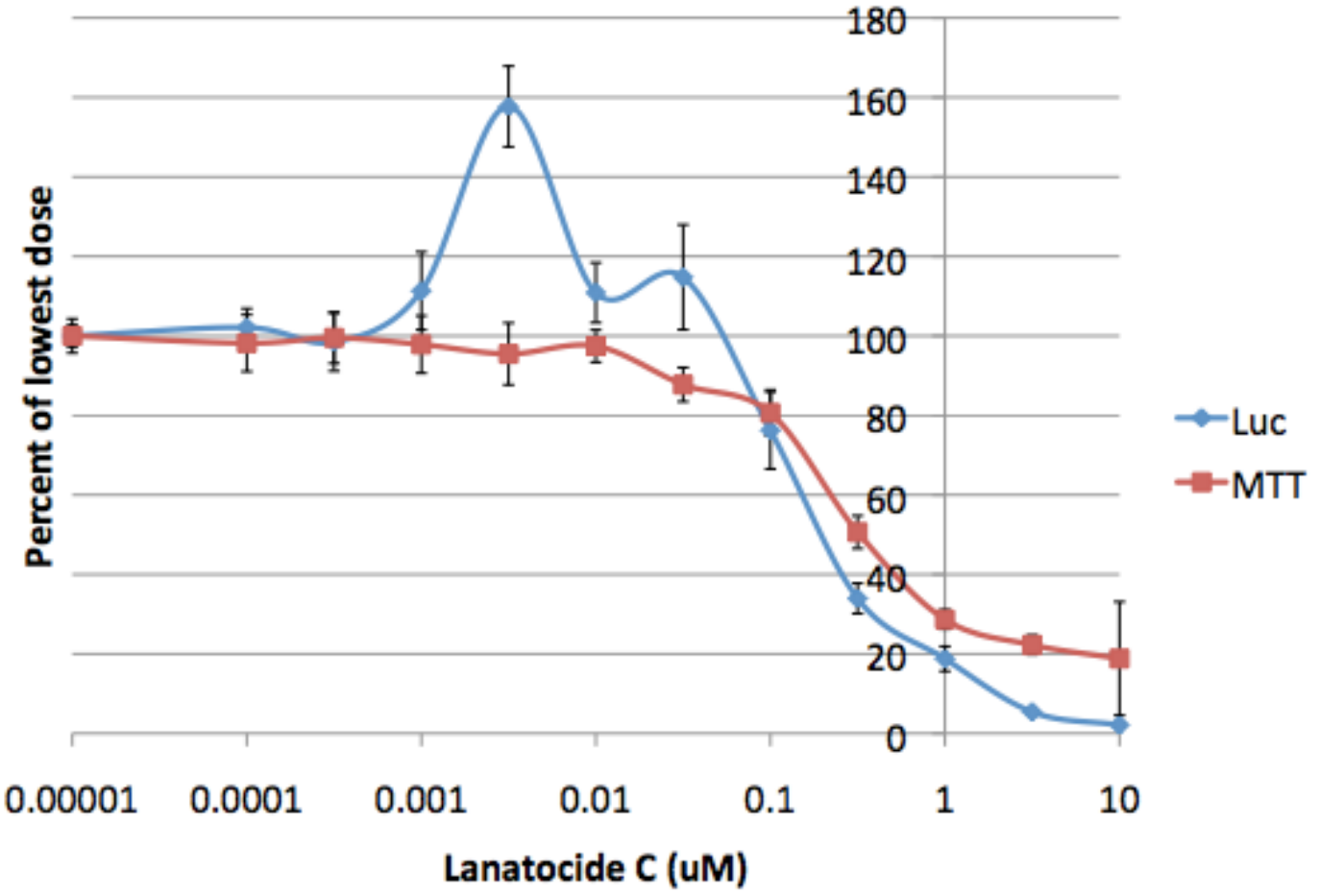
SH-SY5Y cells, 48 hours



Lanatocide C

NaK ATPase inhibitor

SH-SY5Y cells, 48 hours

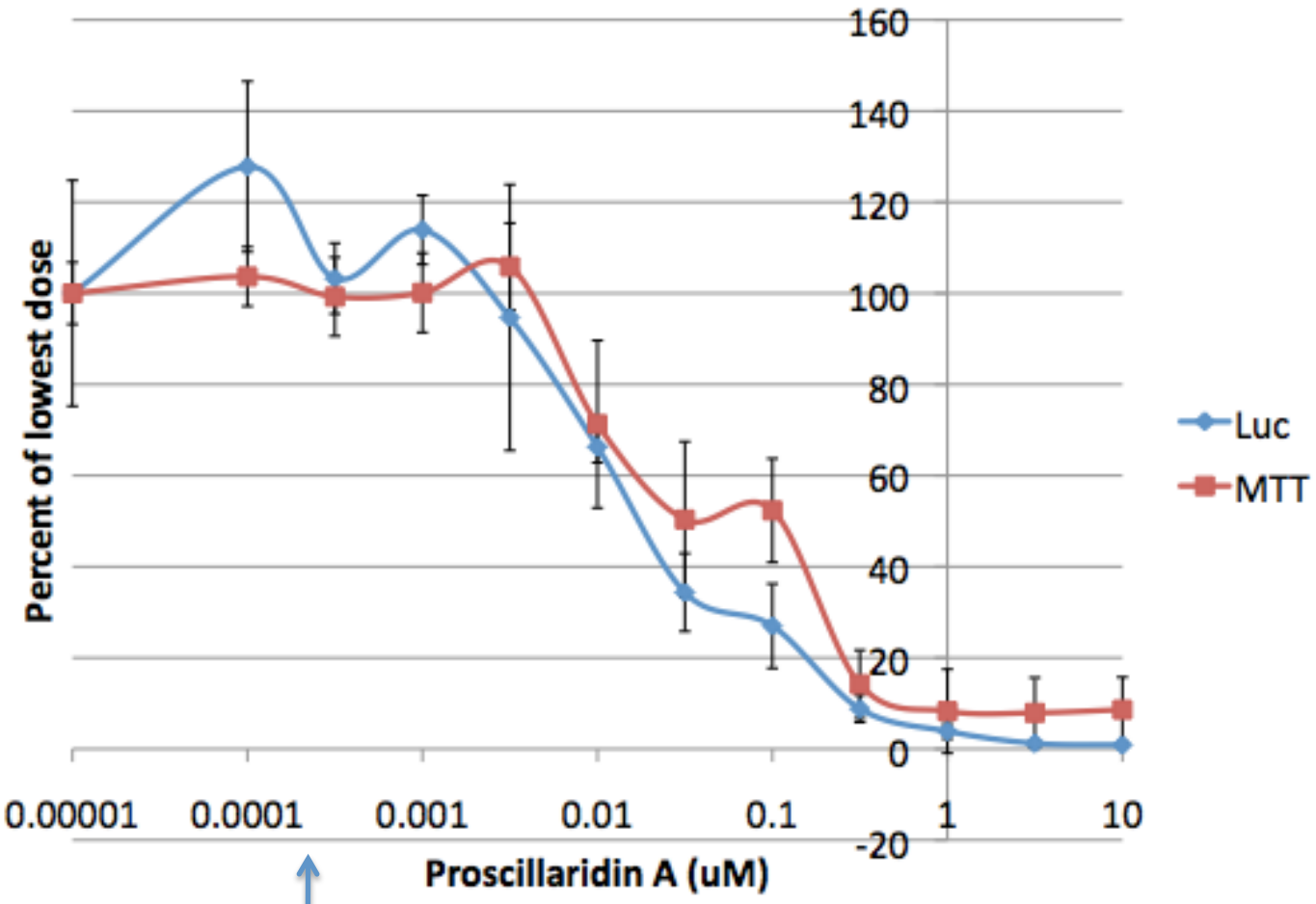


0.002 uM

Proscillaridin A

NaK ATPase inhibitor

SH-SY5Y cells, 48 hours

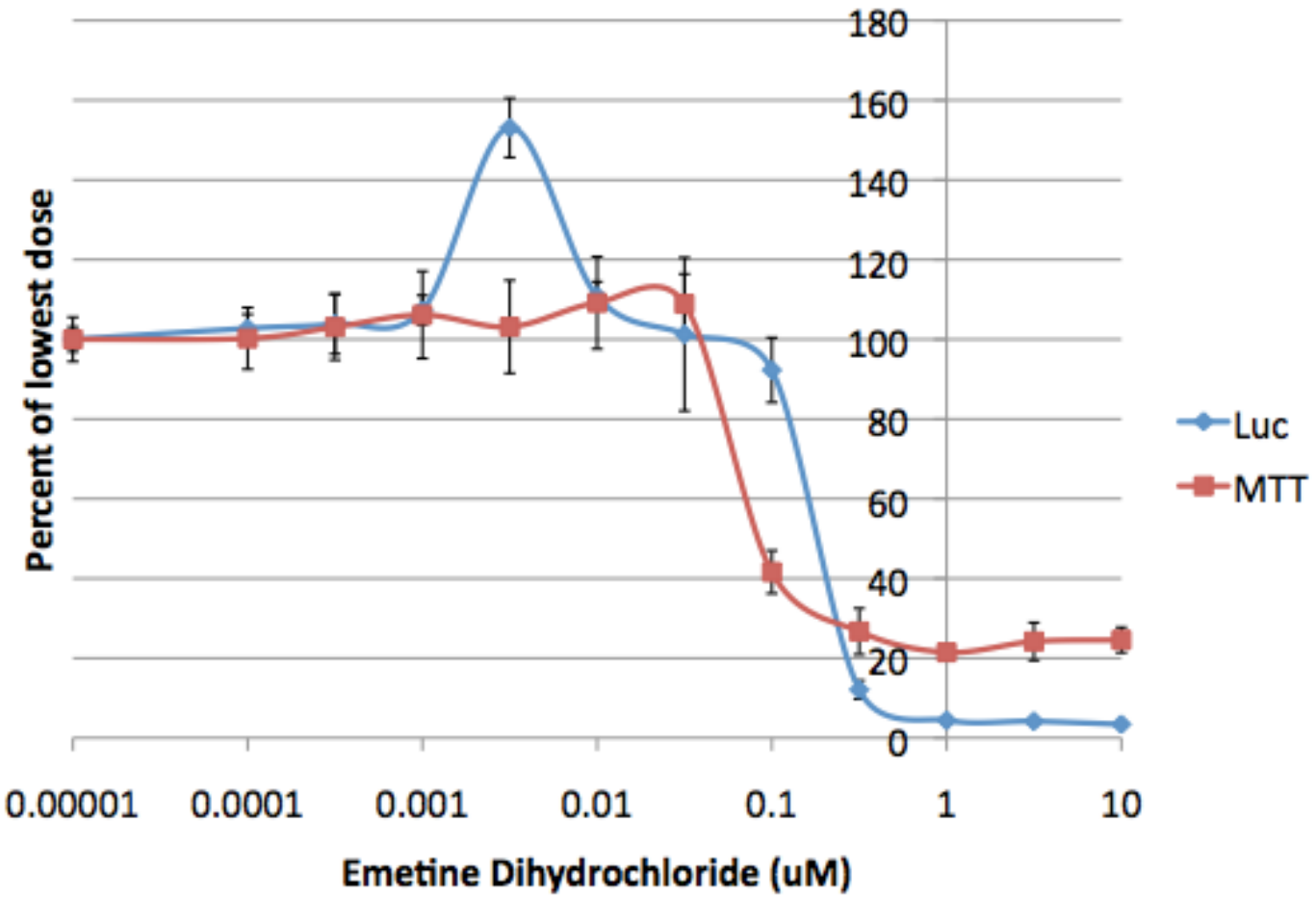


0.0008 uM

Emetine Dihydrochloride

SH-SY5Y cells, 48 hours

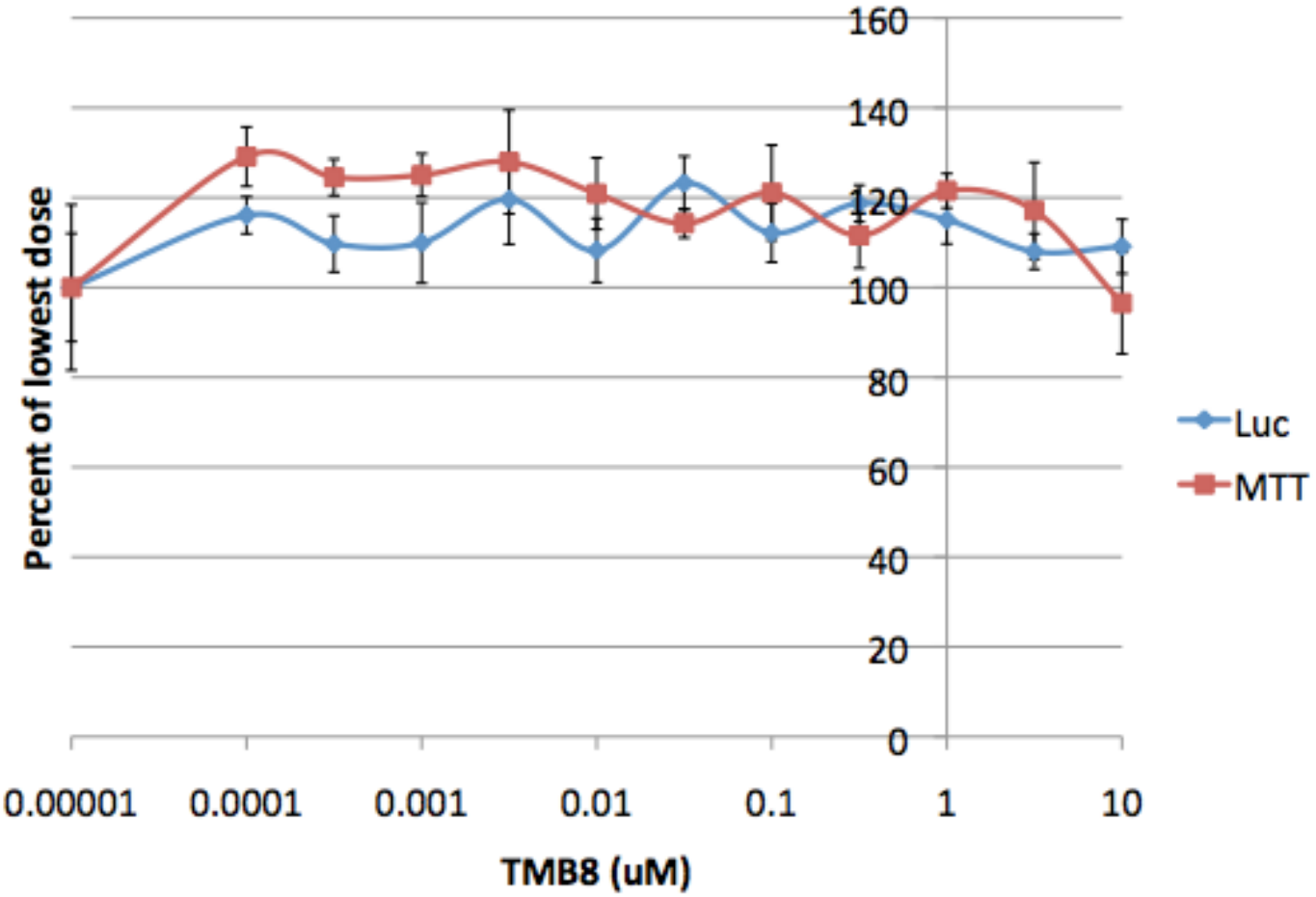
Calcium Channel Blocker



TMB8

Calcium Channel Blocker

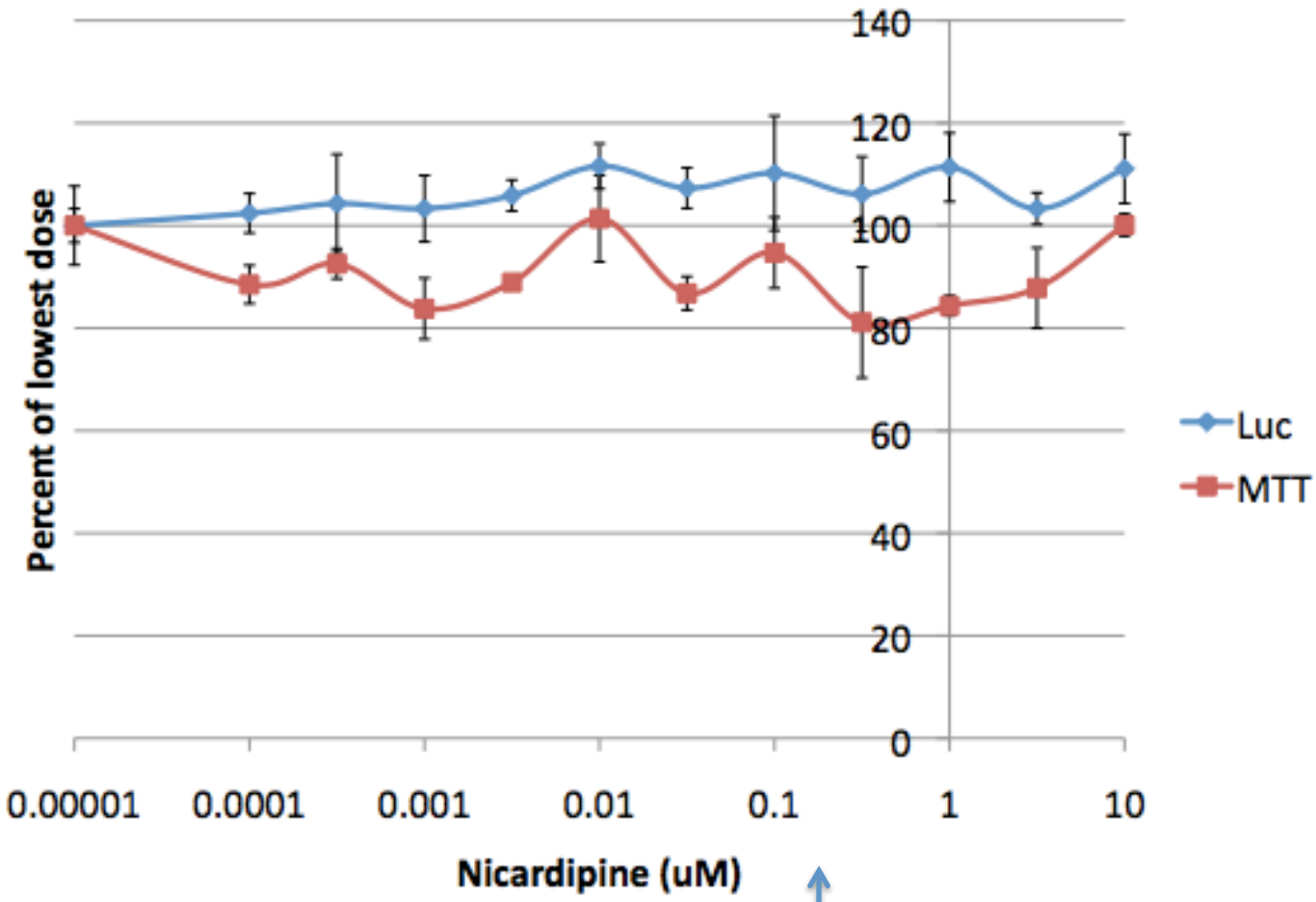
SH-SY5Y cells, 48 hours



Nicardipine

Calcium Channel Blocker

SH-SY5Y cells, 48 hours

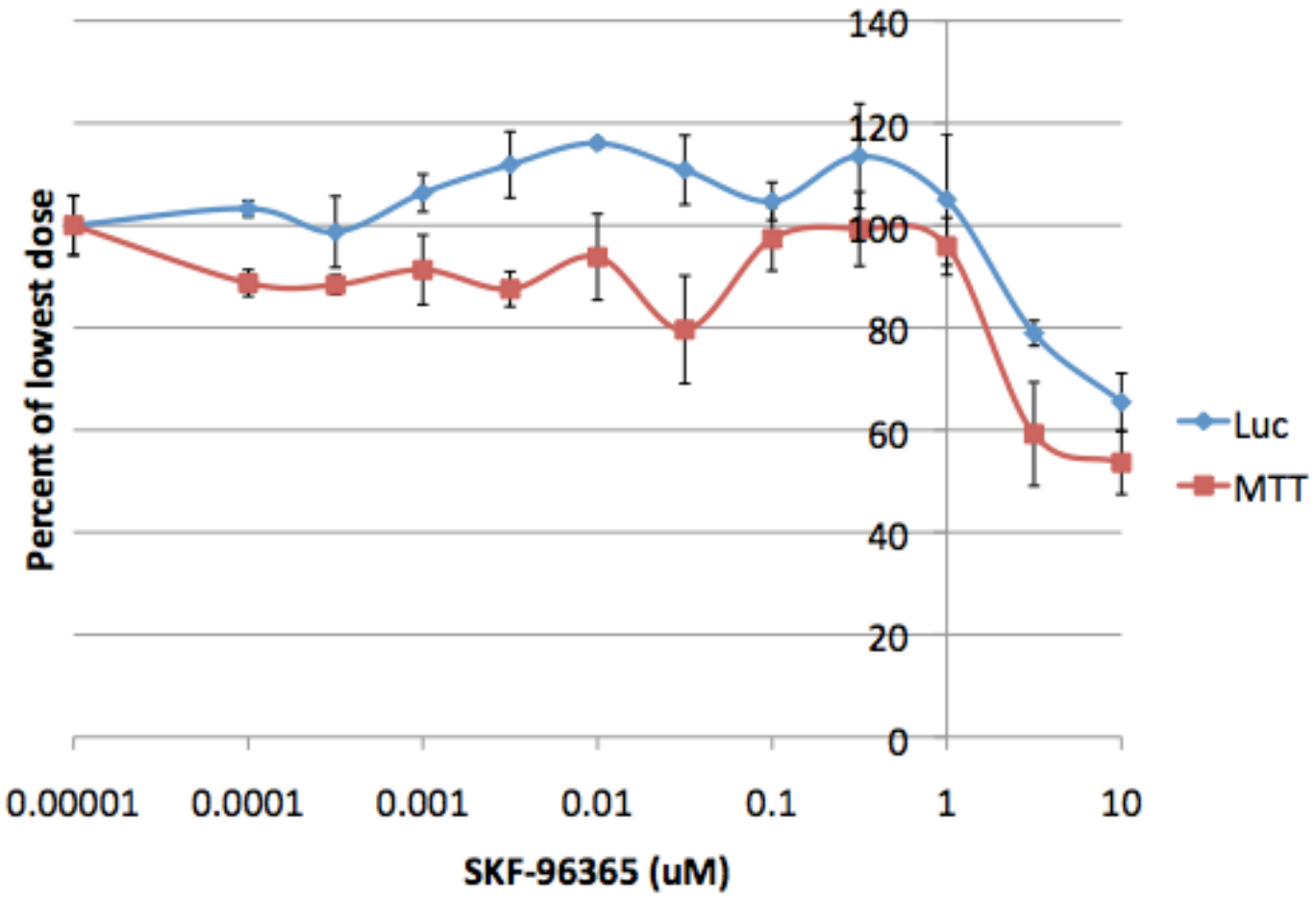


0.16 uM

SKF-96365

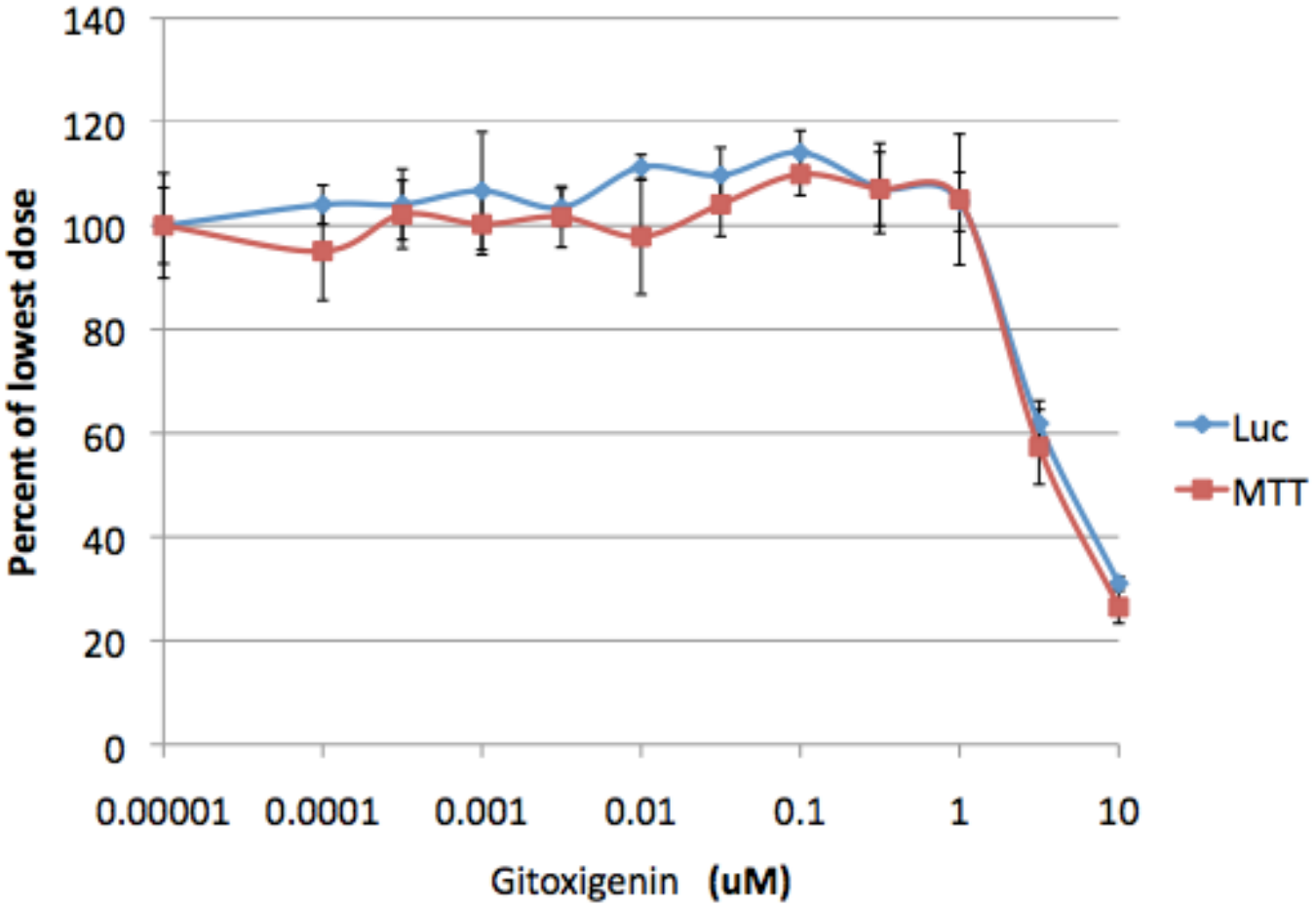
Calcium Channel Blocker

SH-SY5Y cells, 48 hours



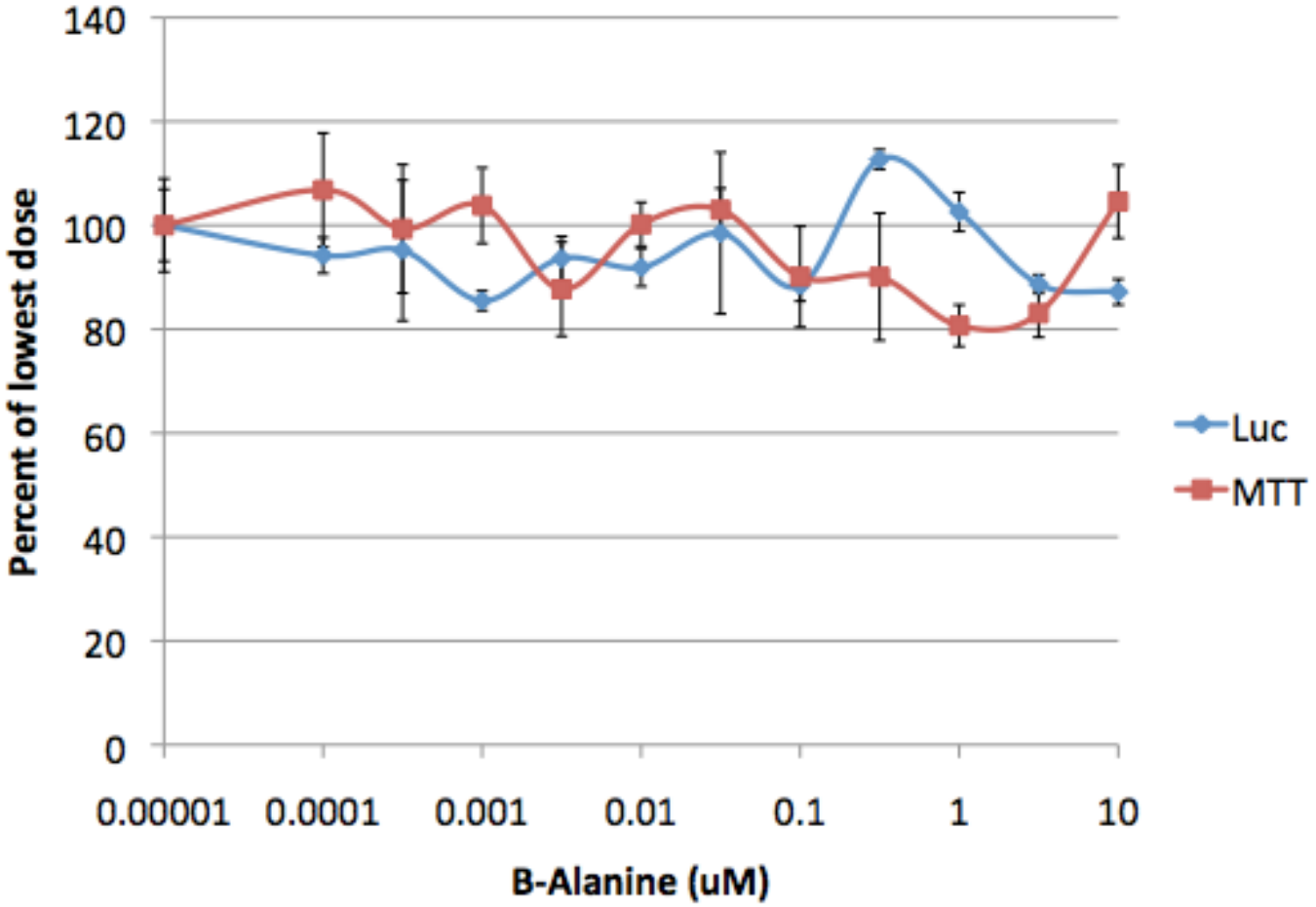
Gitoxigenin

SH-SY5Y cells, 48 hours



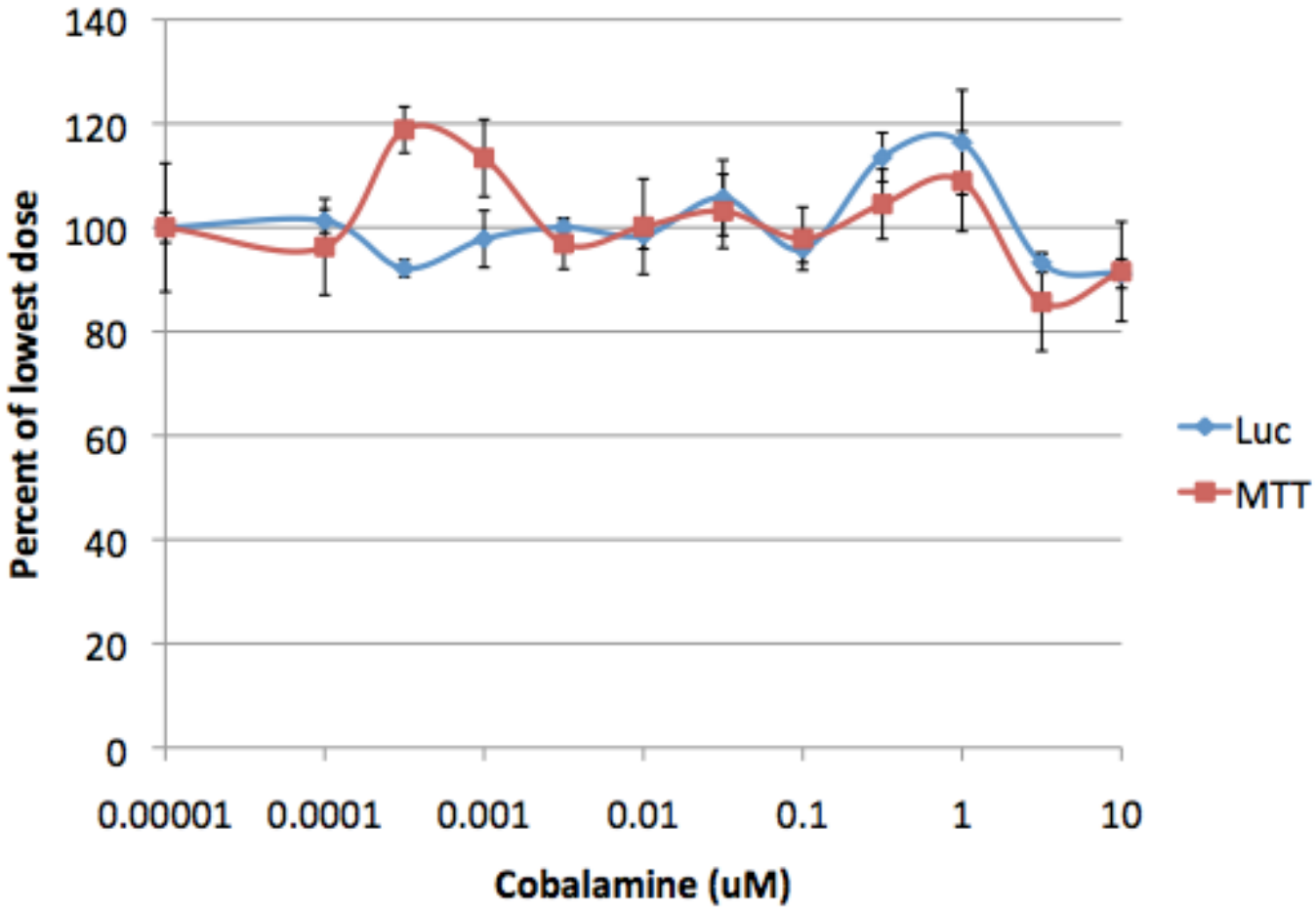
Beta-Alanine

SH-SY5Y cells, 48 hours



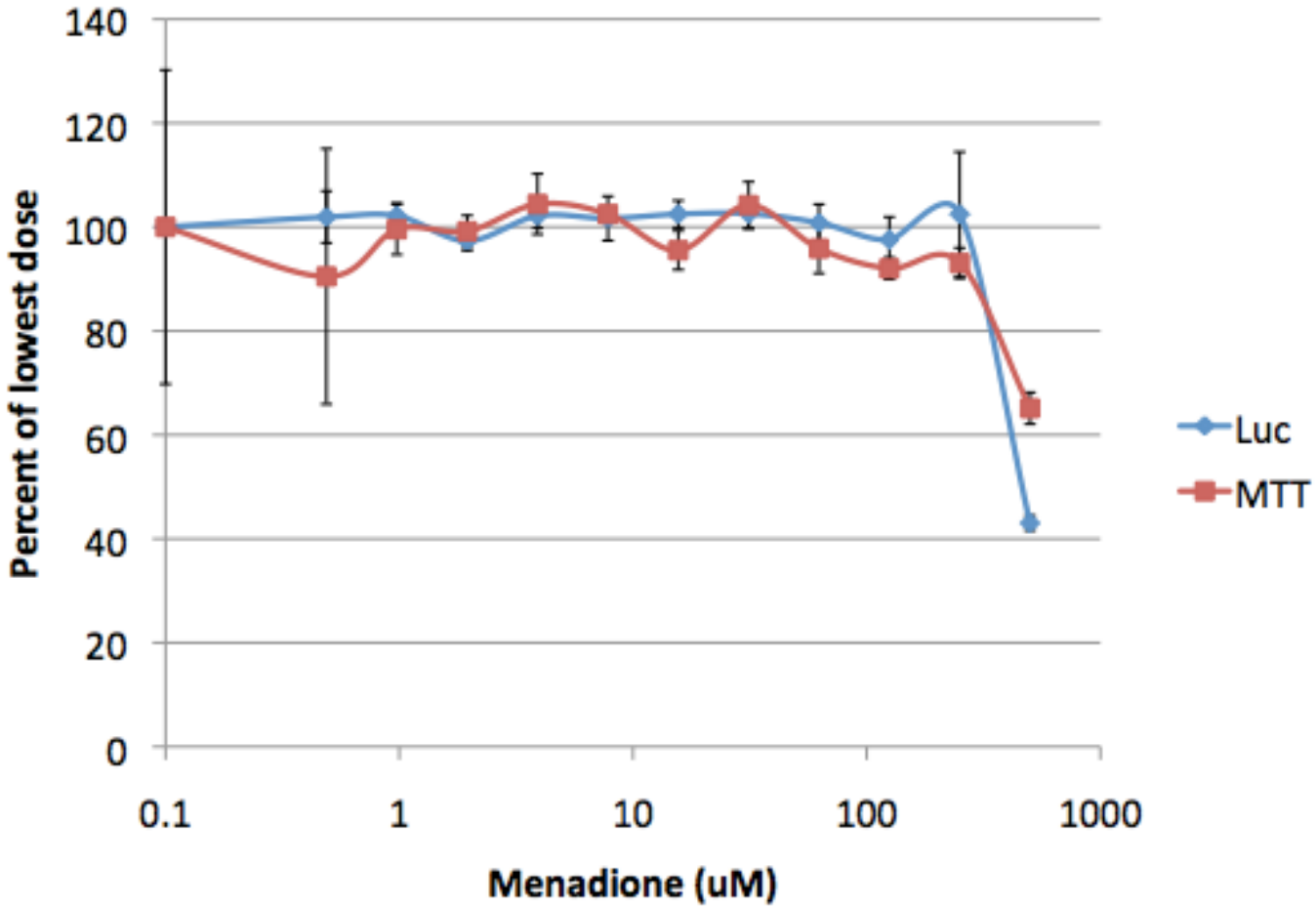
Cyanocobalamine

SH-SY5Y cells, 48 hours



Menadione

SH-SY5Y cells, 48 hours

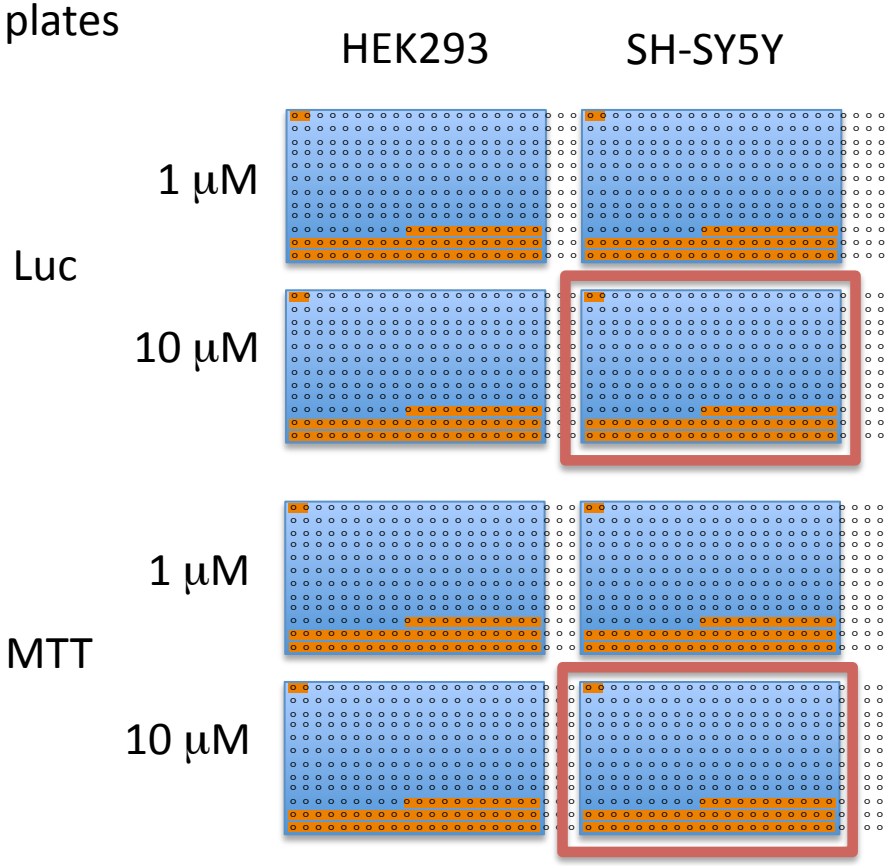


Orthogonal Screen

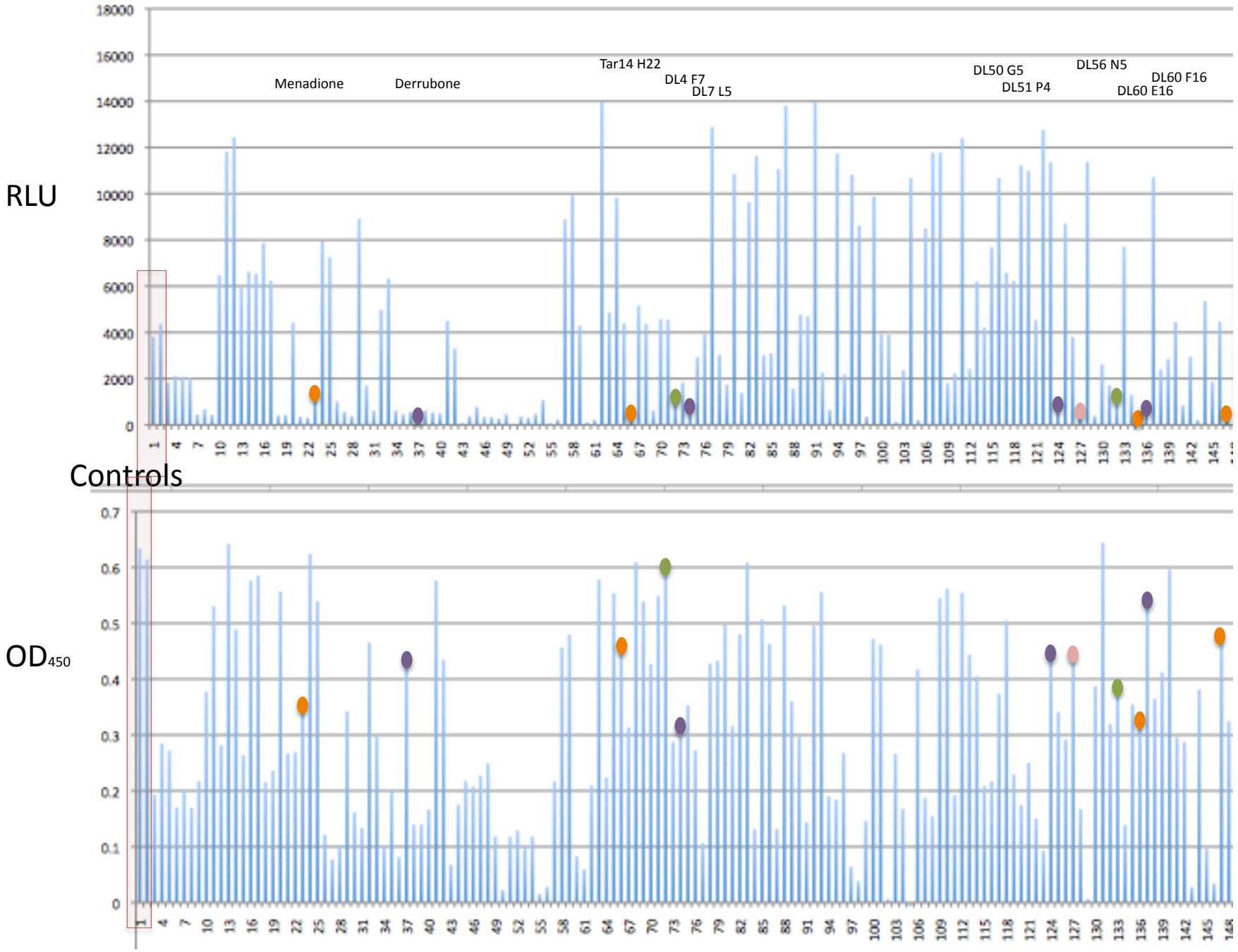
HEK293 or SH-SY5Y cells stably expressing ATXN2-Luc

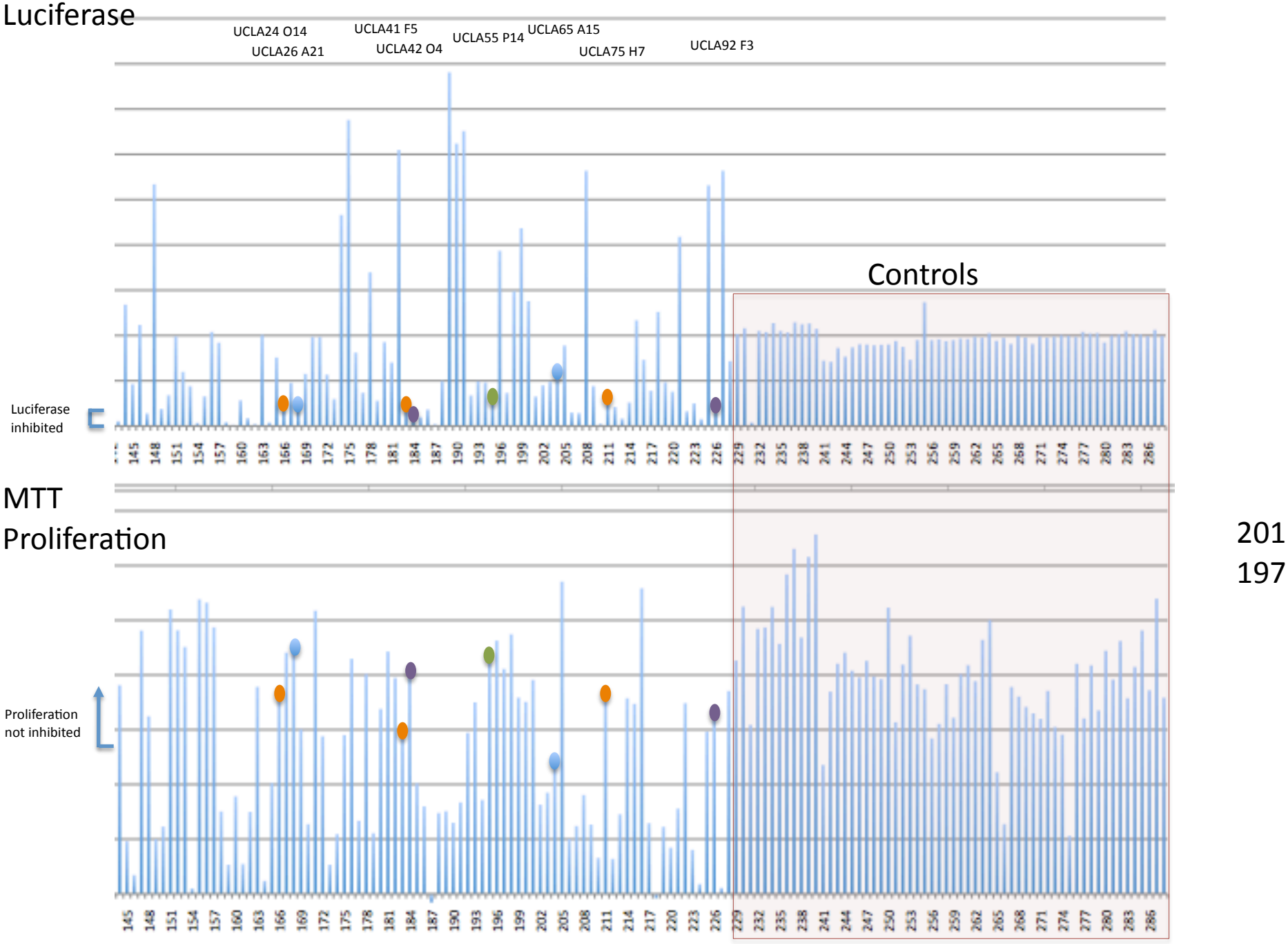
Conducted paired MTT and Luciferase assays for 1 μM and 10 μM compounds

384 well plates

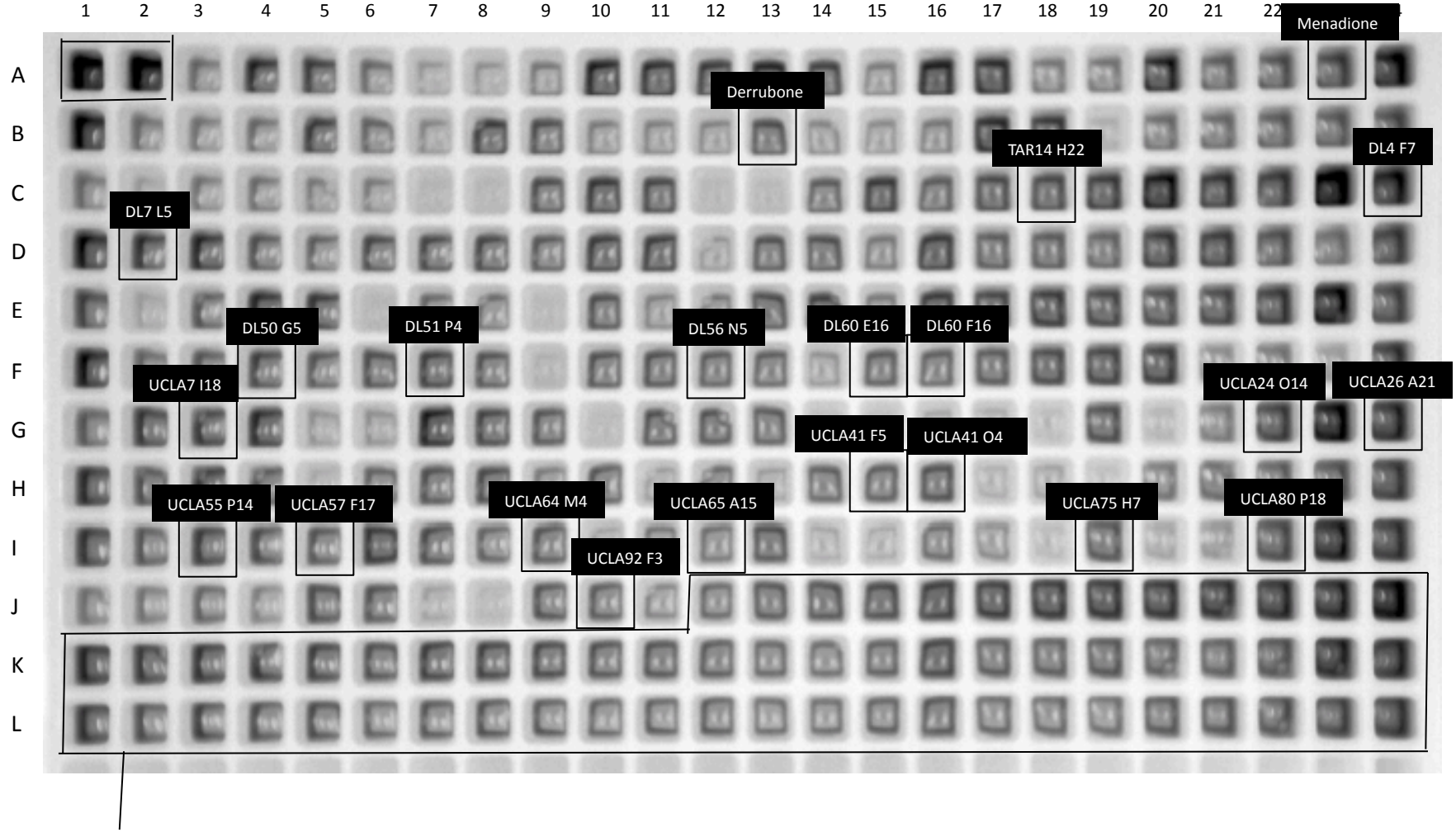


Control DMSO only wells in orange


RLU
Controls
OD450
Menadione
Derrubone
Tar14 H22
DL4 F7
DL7 L5
DL50 G5
DL51 P4
DL56 N5
DL60 E16
DL60 F16

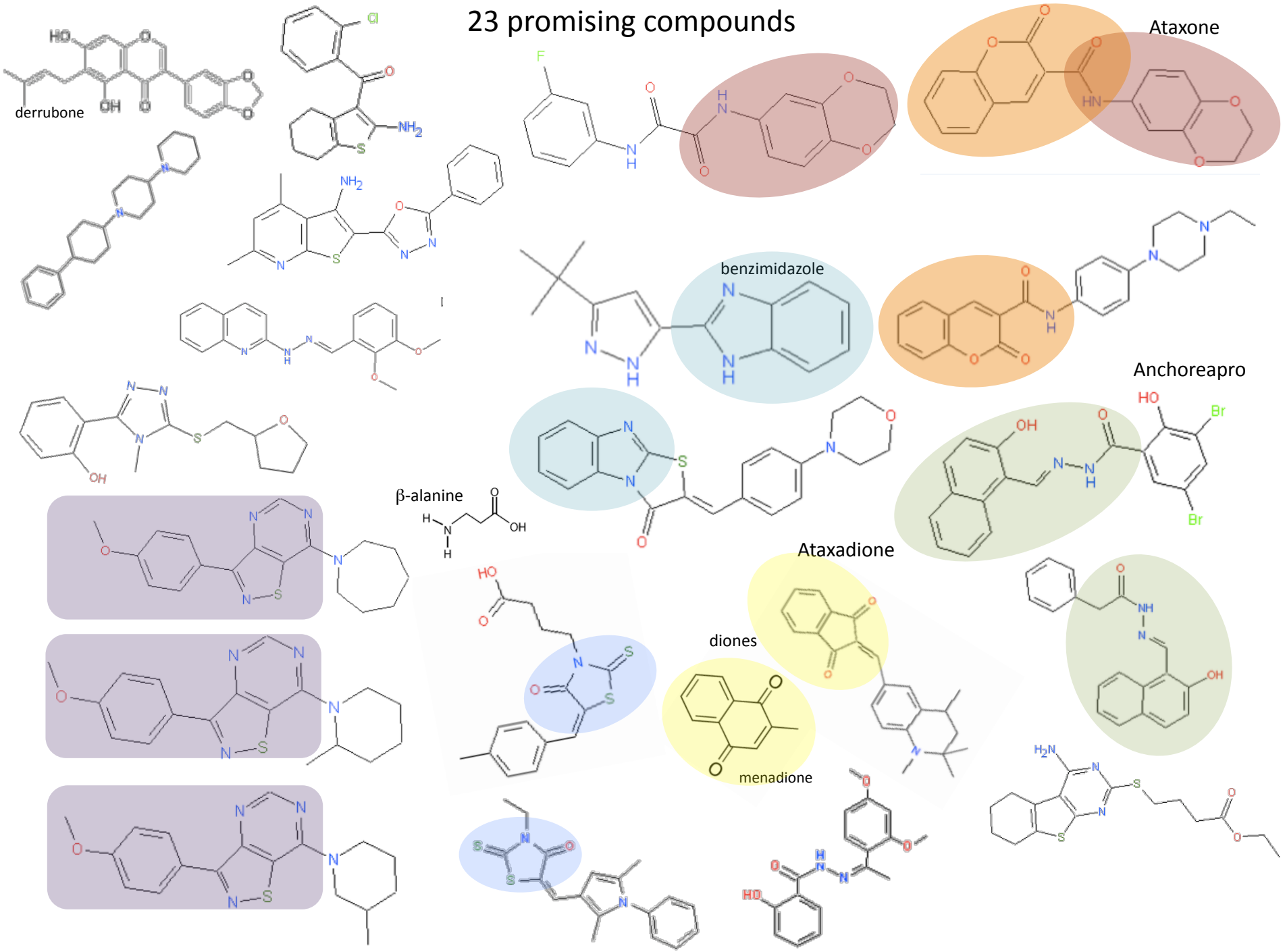

Luciferase
UCLA24 O14
UCLA26 A21
UCLA41 F5
UCLA42 O4
UCLA55 P14
UCLA65 A15
UCLA75 H7
UCLA92 F3
Controls
Luciferase inhibited
MTT Proliferation
Proliferation not inhibited
201
197

SH-SY5Y cells
MTT proliferation assay
10 uM compounds



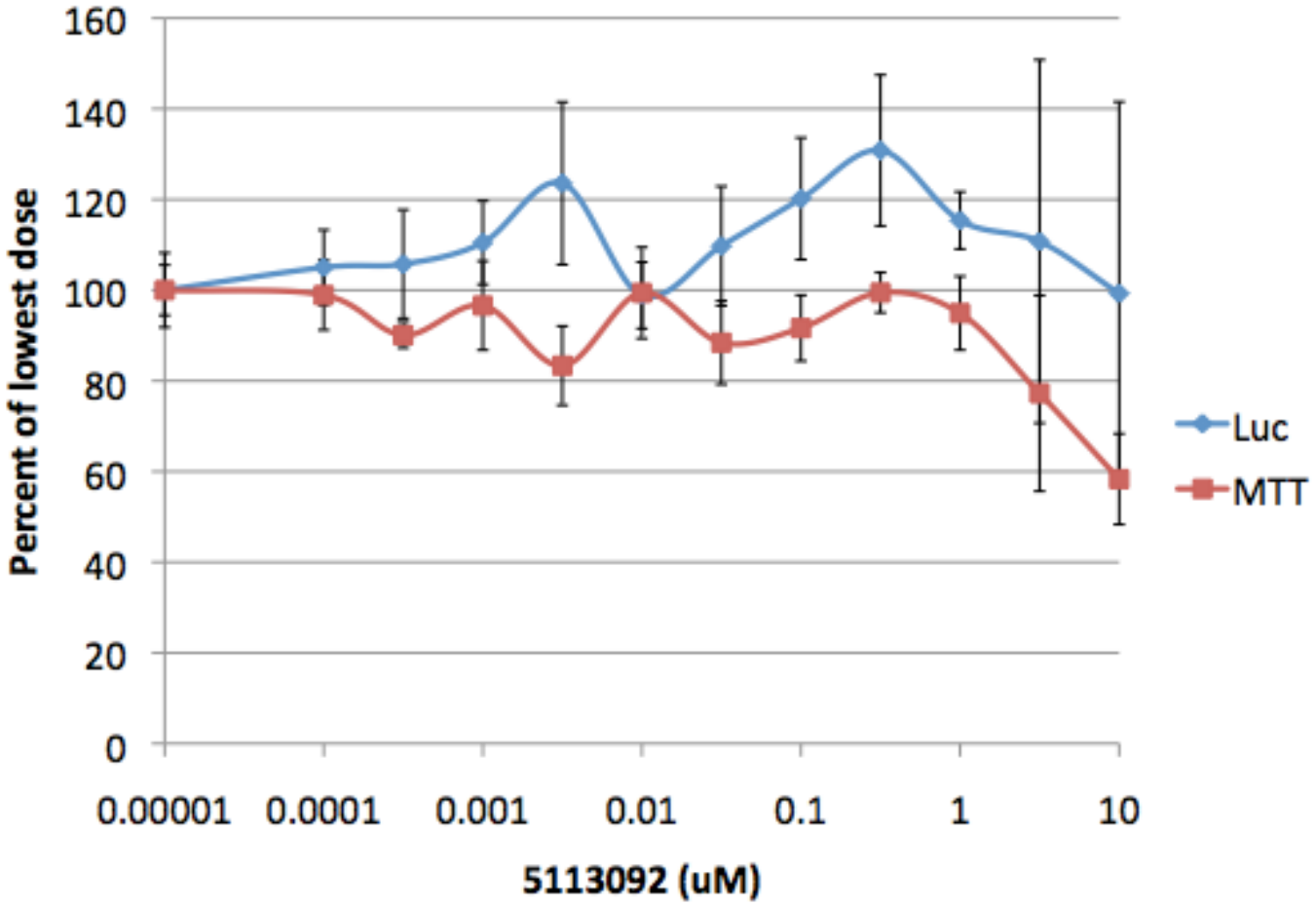
Controls

23 promising compounds



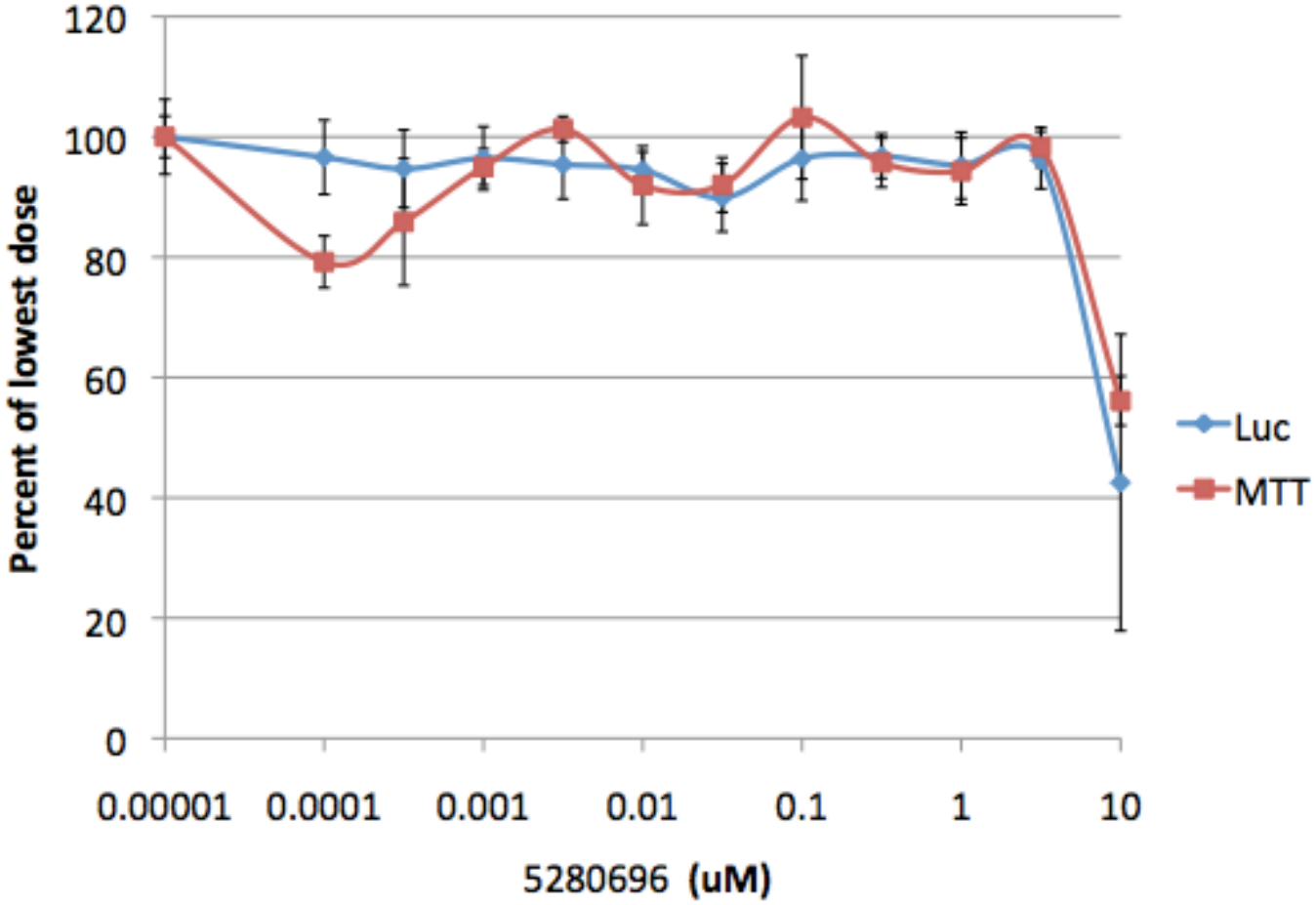
5113092

SH-SY5Y cells, 48 hours



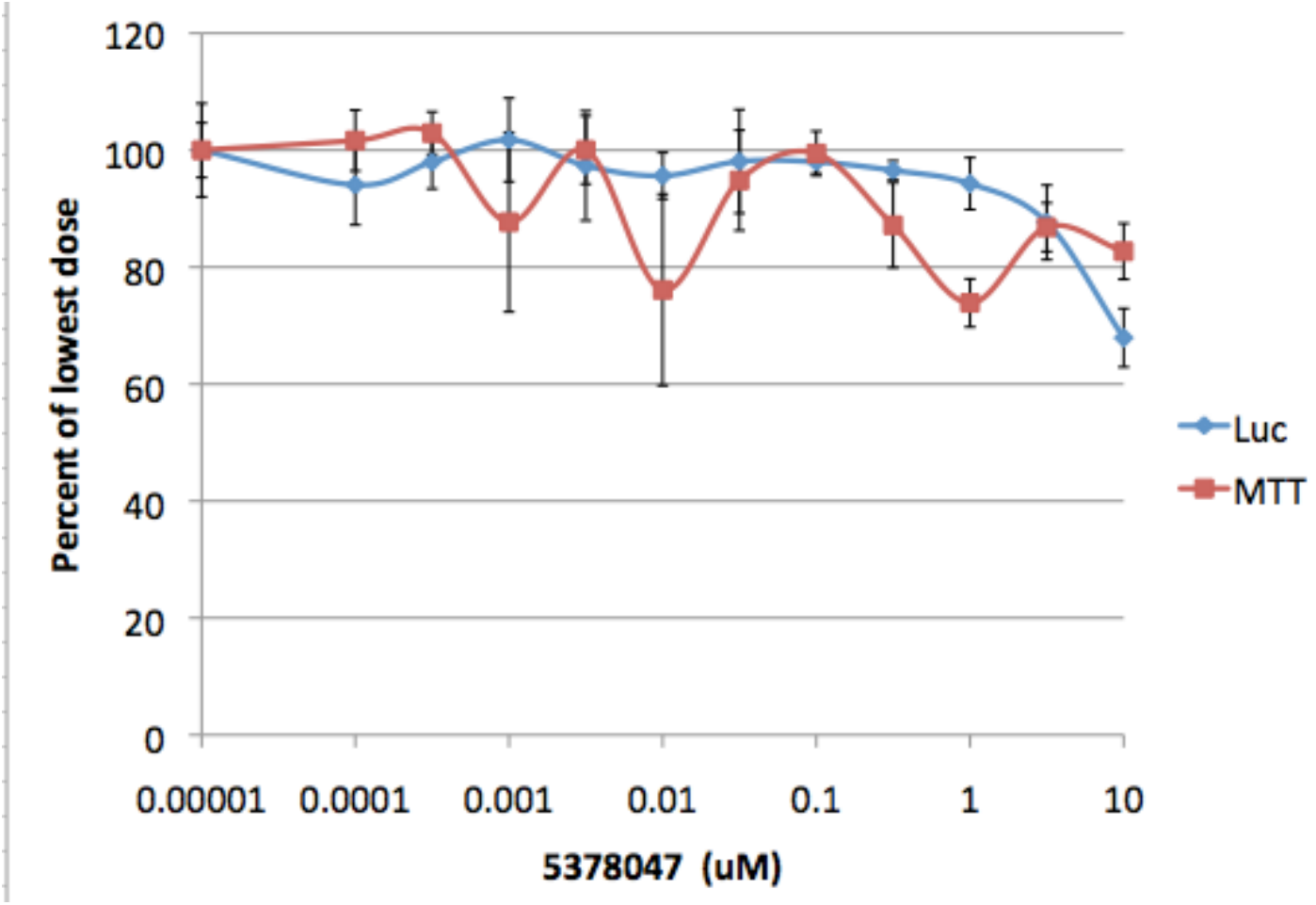
5280696

SH-SY5Y cells, 48 hours



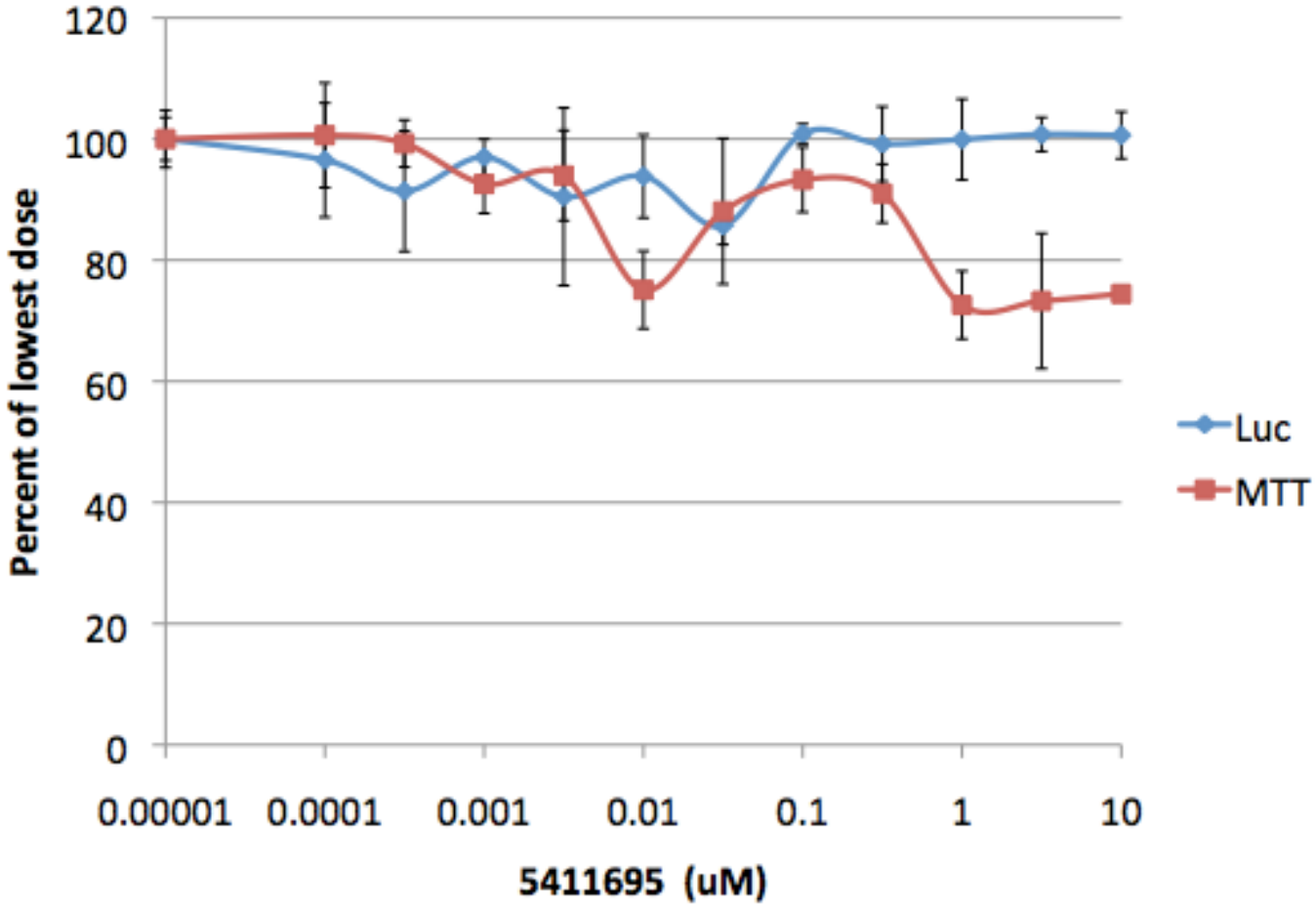
5378047

SH-SY5Y cells, 48 hours



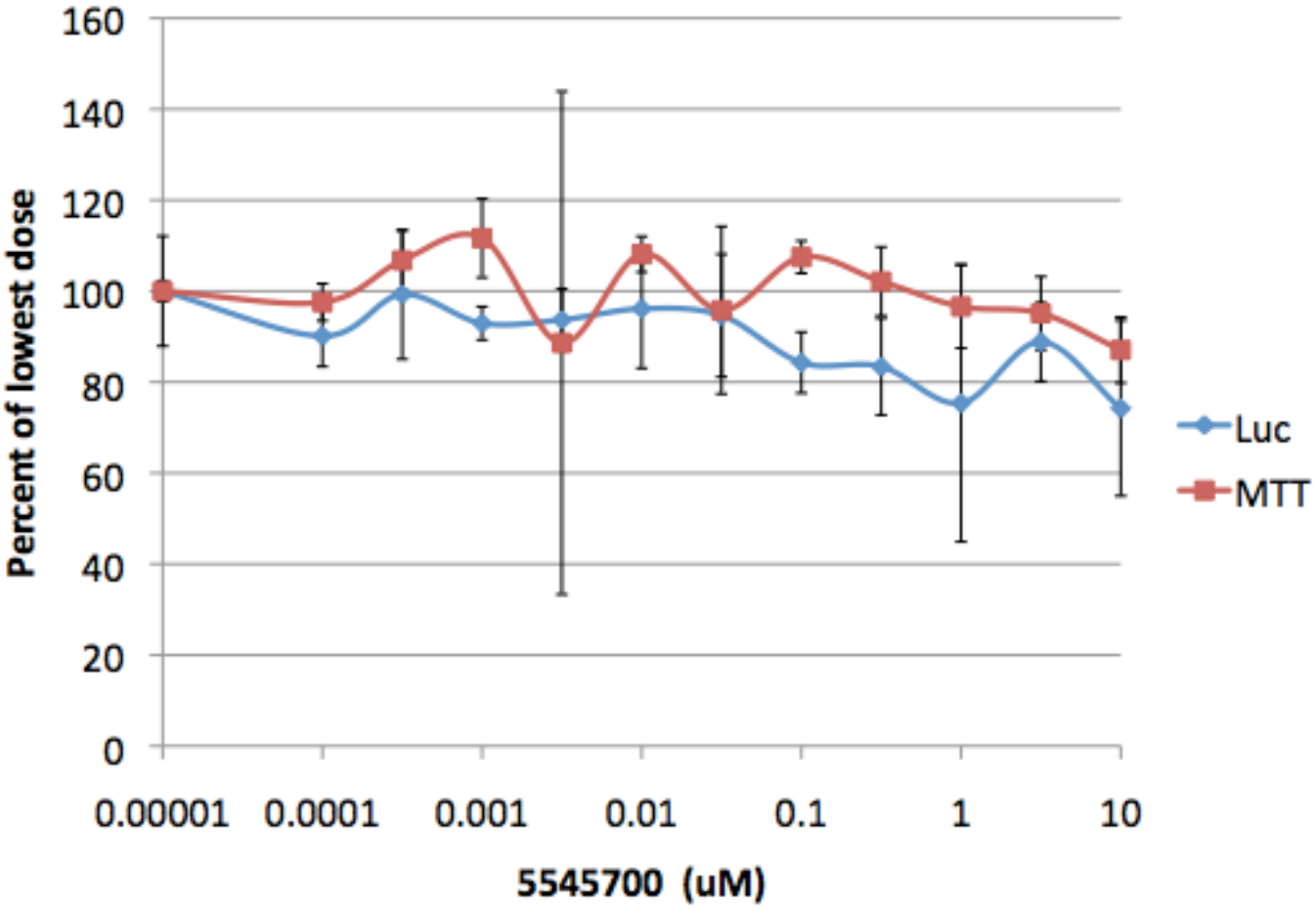
5411695

SH-SY5Y cells, 48 hours



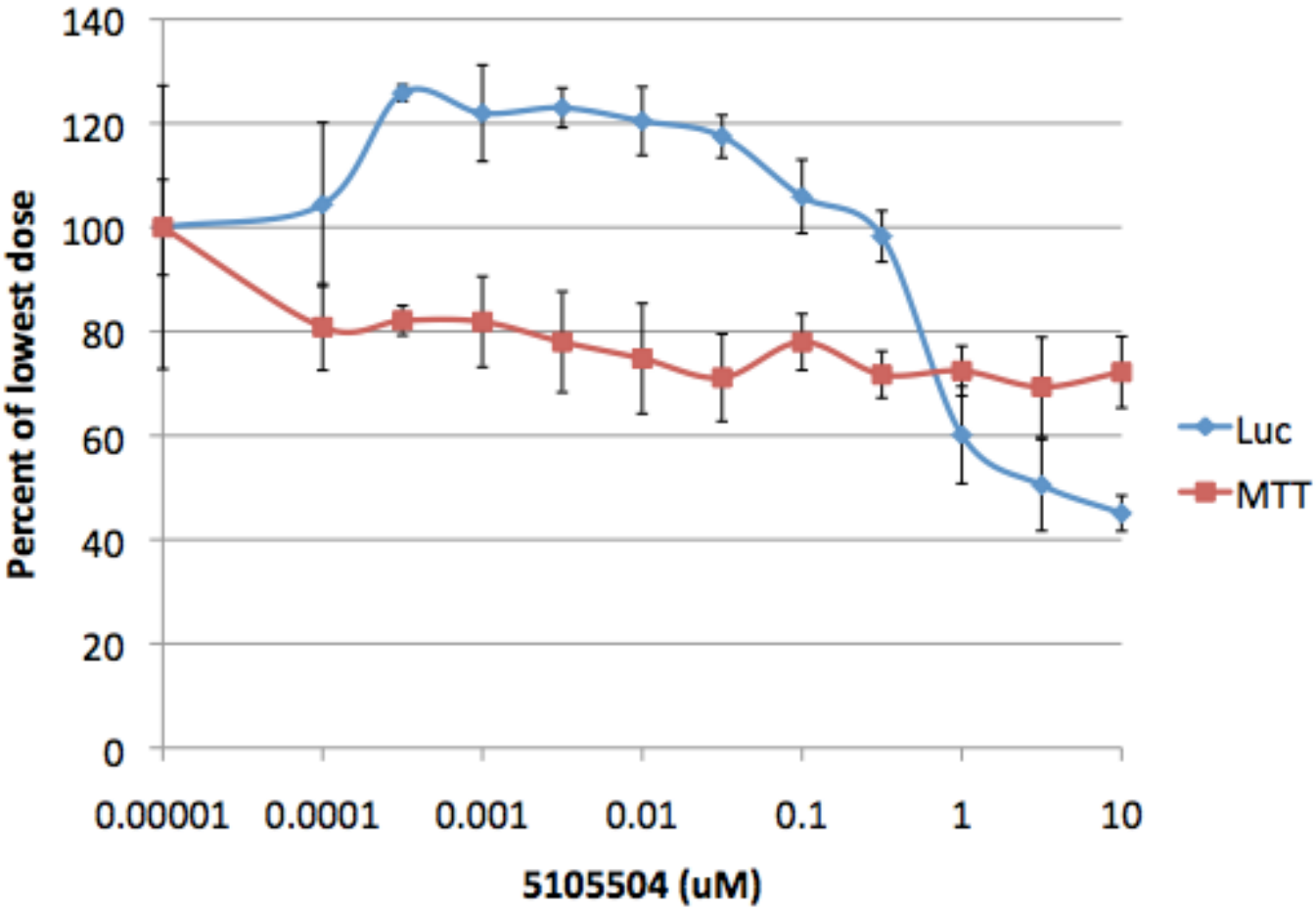
5545700

SH-SY5Y cells, 48 hours



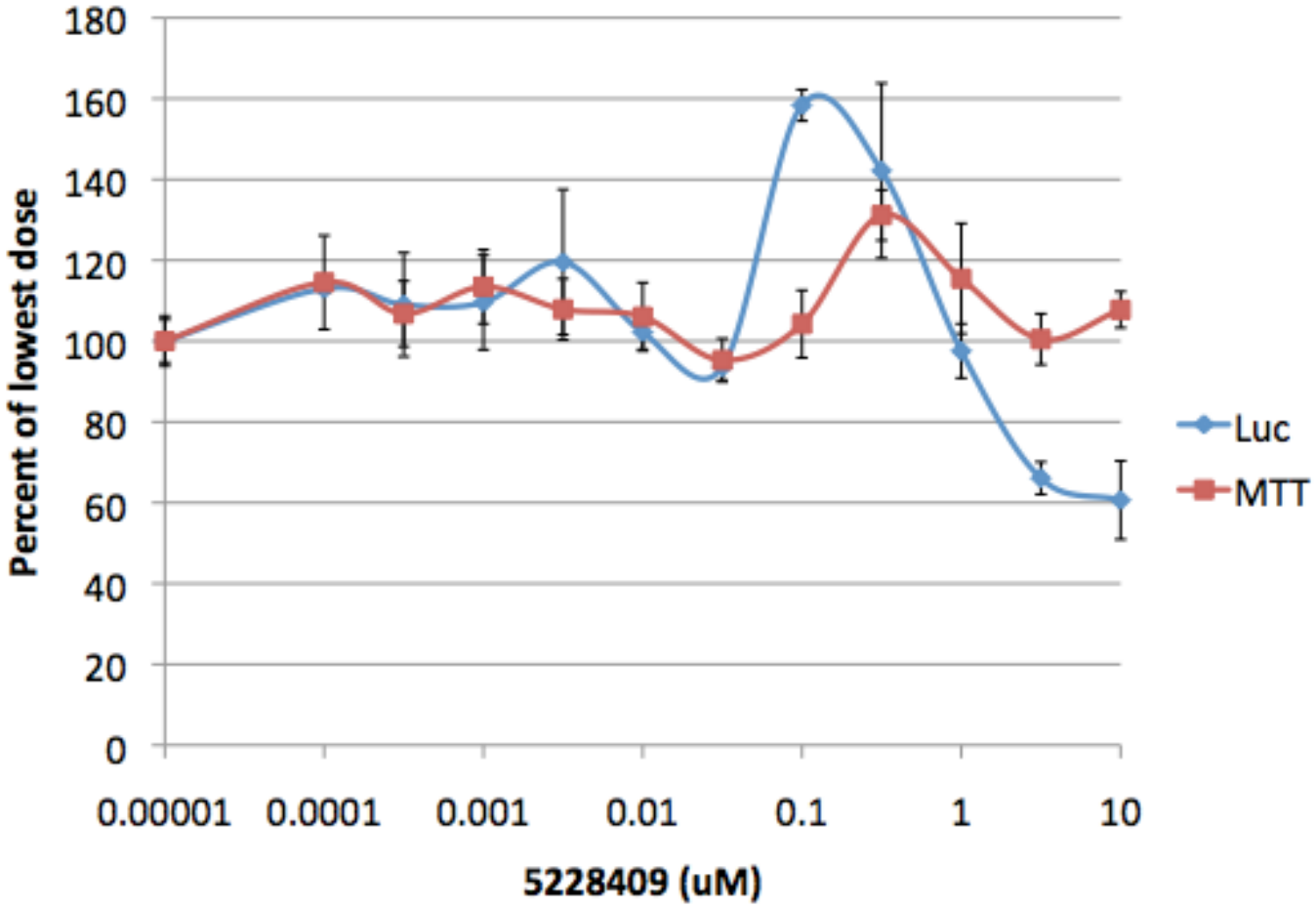
5105504

SH-SY5Y cells, 48 hours



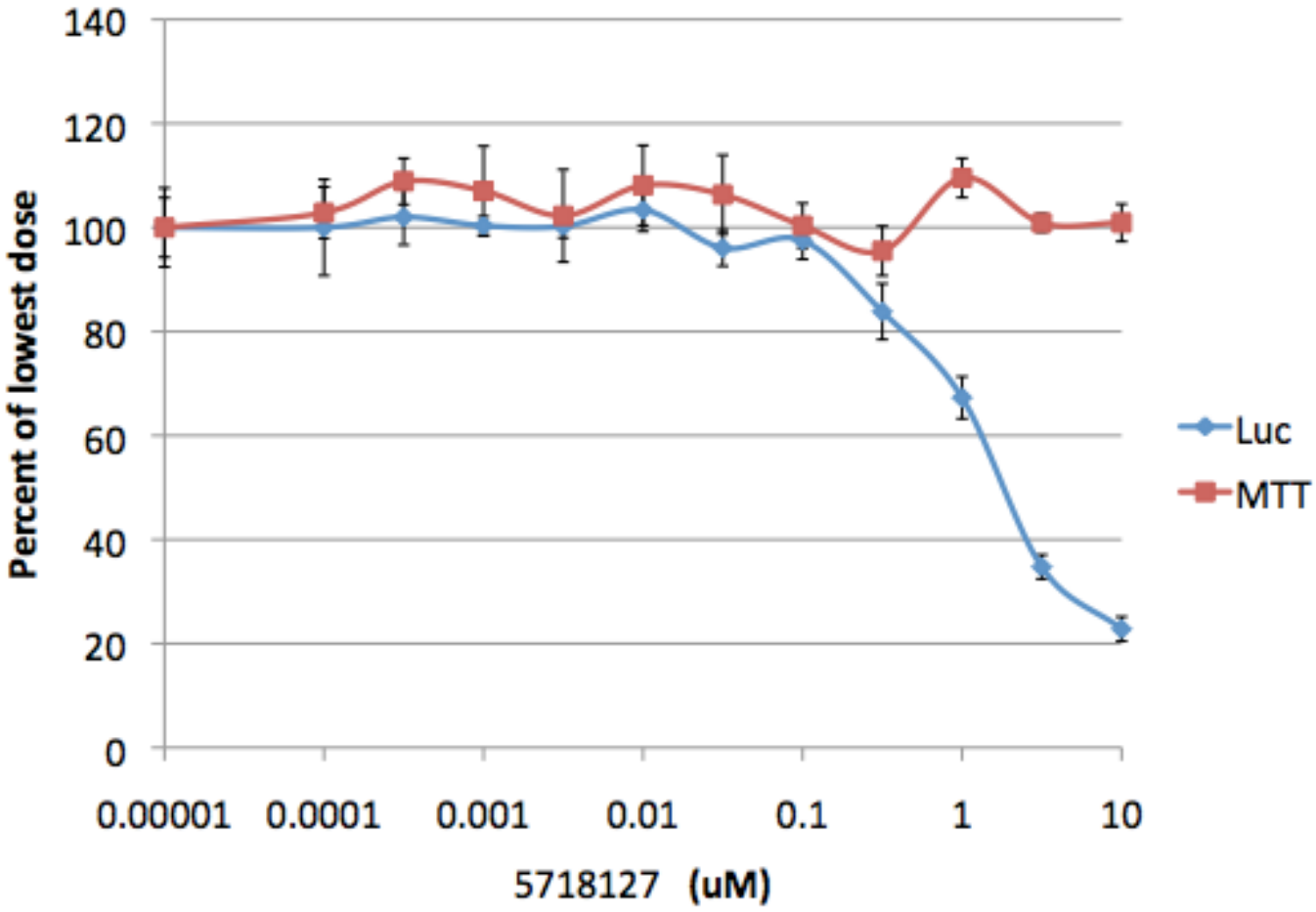
5228409

SH-SY5Y cells, 48 hours



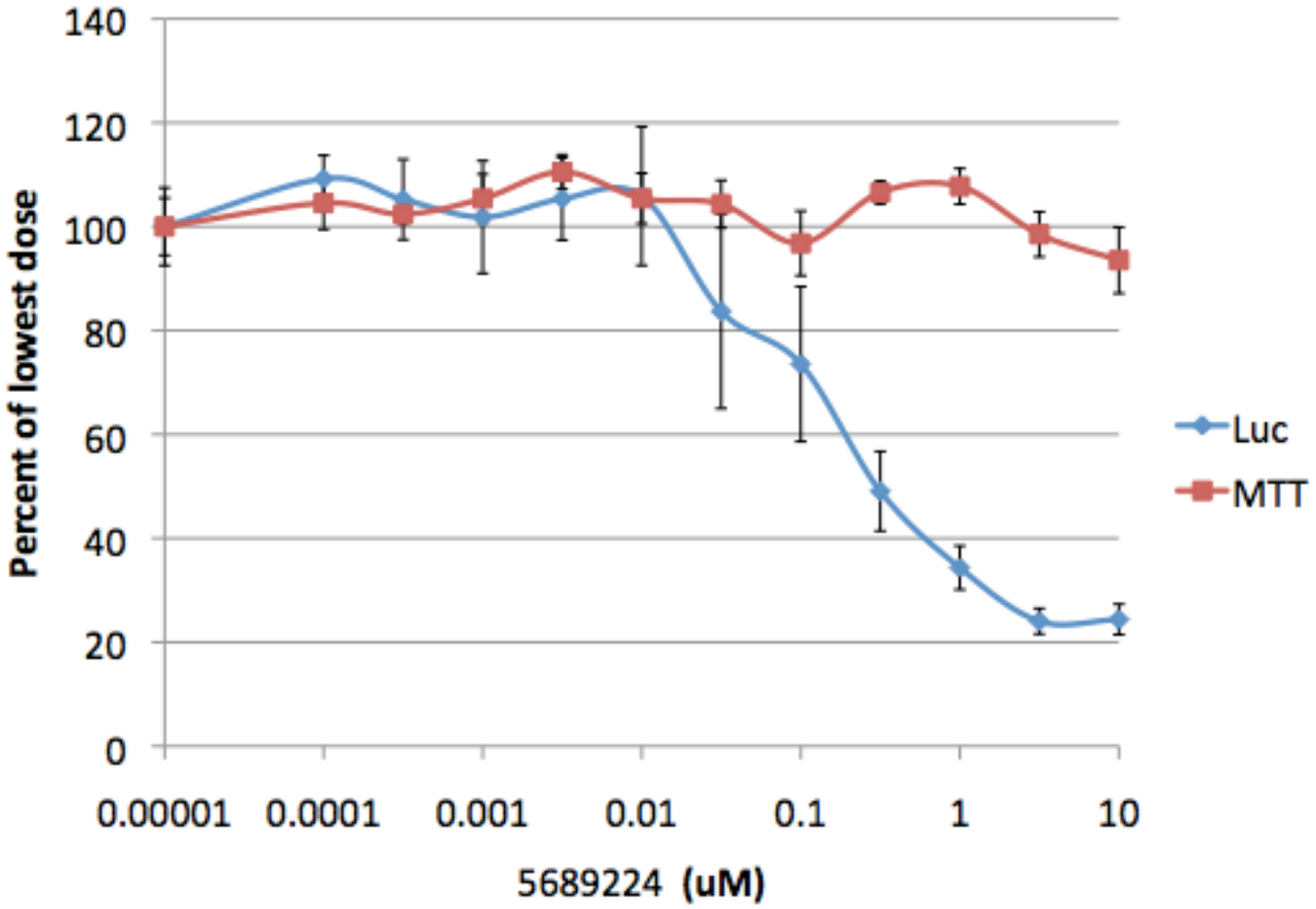
5718127

SH-SY5Y cells, 48 hours



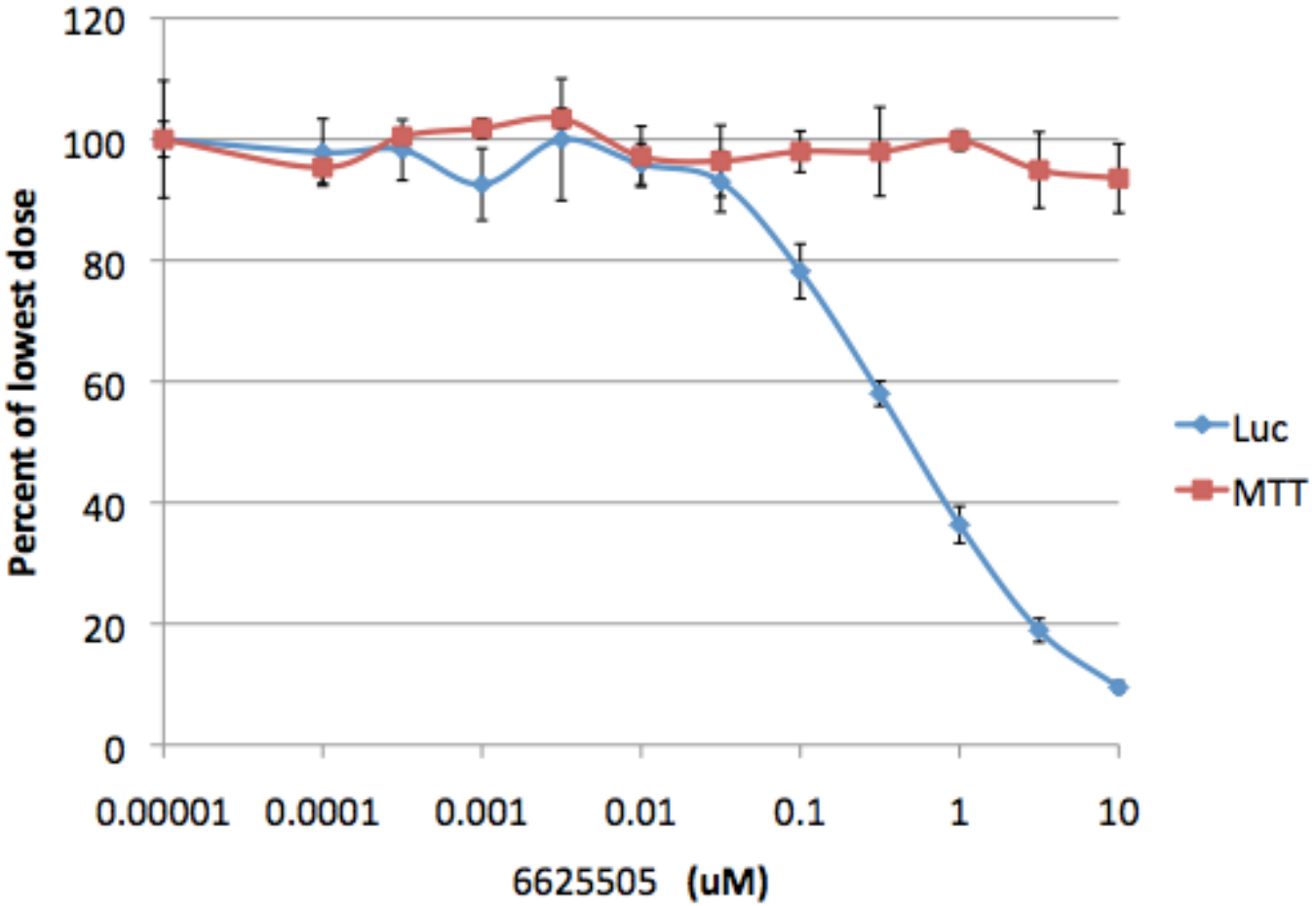
5689224

SH-SY5Y cells, 48 hours



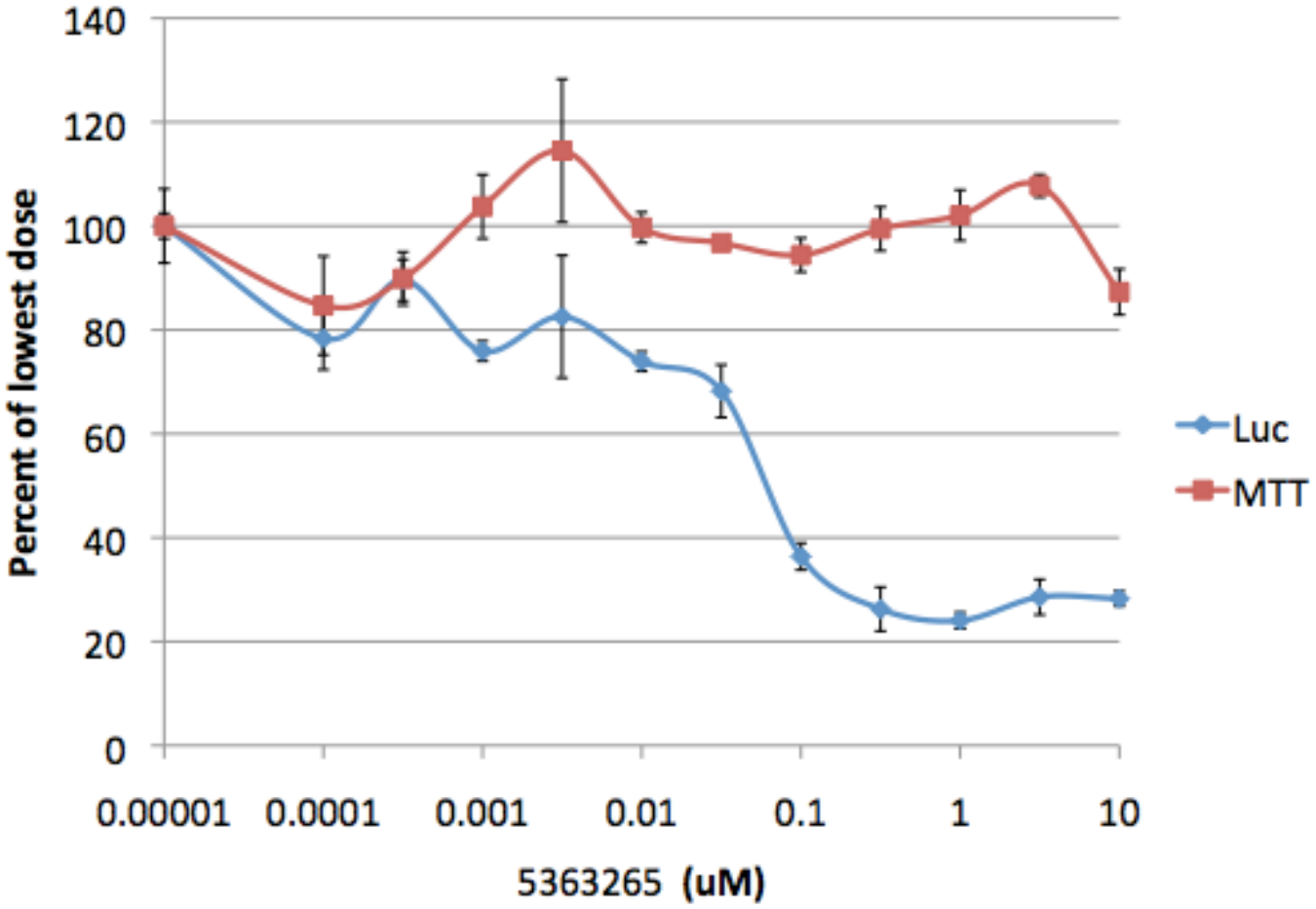
6625505

SH-SY5Y cells, 48 hours



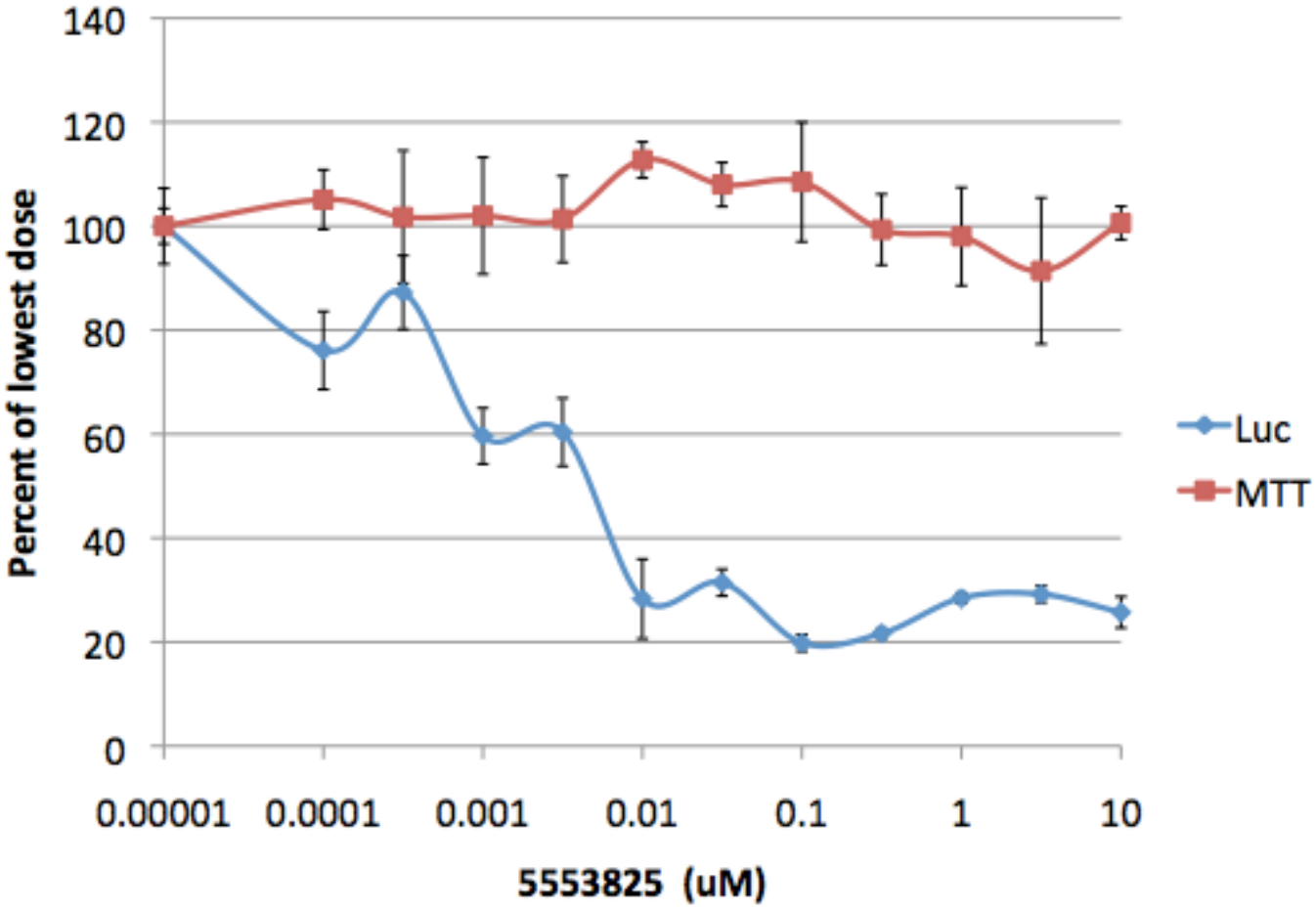
5363265

SH-SY5Y cells, 48 hours



5553825

SH-SY5Y cells, 48 hours



Orthogonal Assays

Other controlling constructs

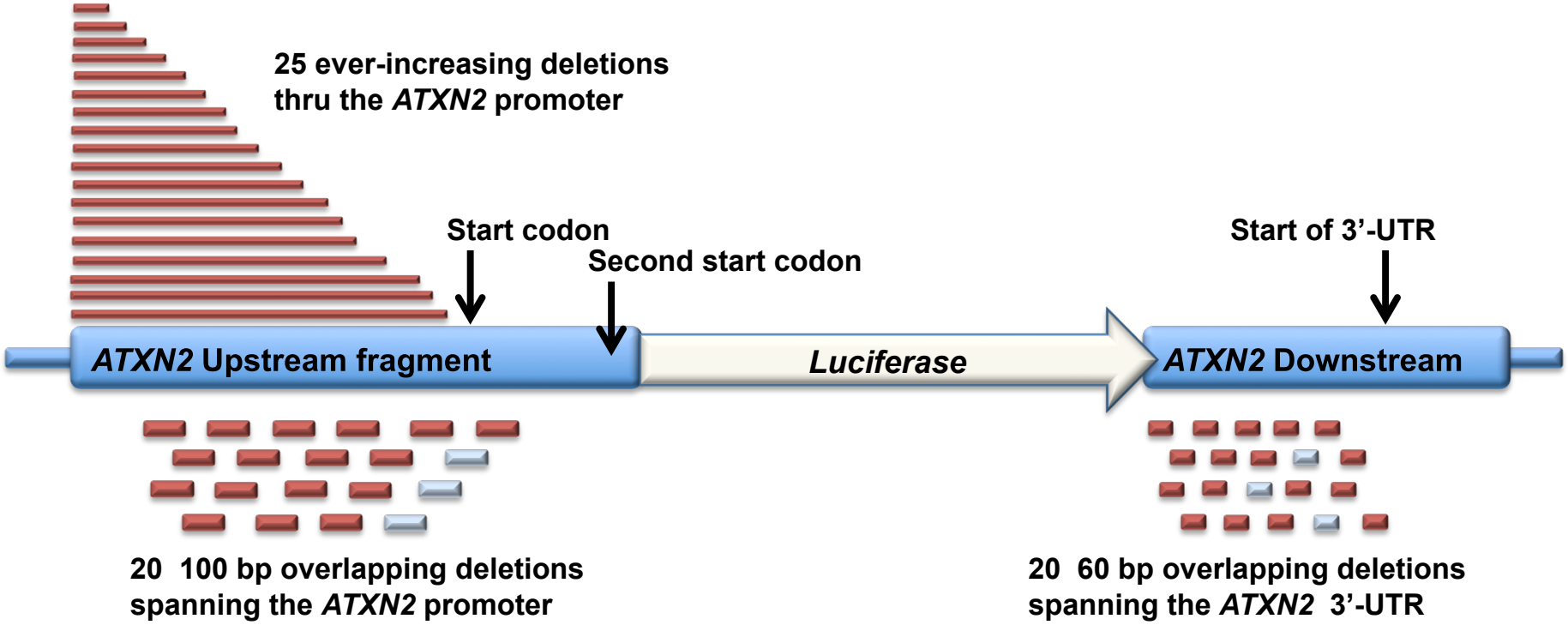
Validation Screen:

Orthoganol Screen:

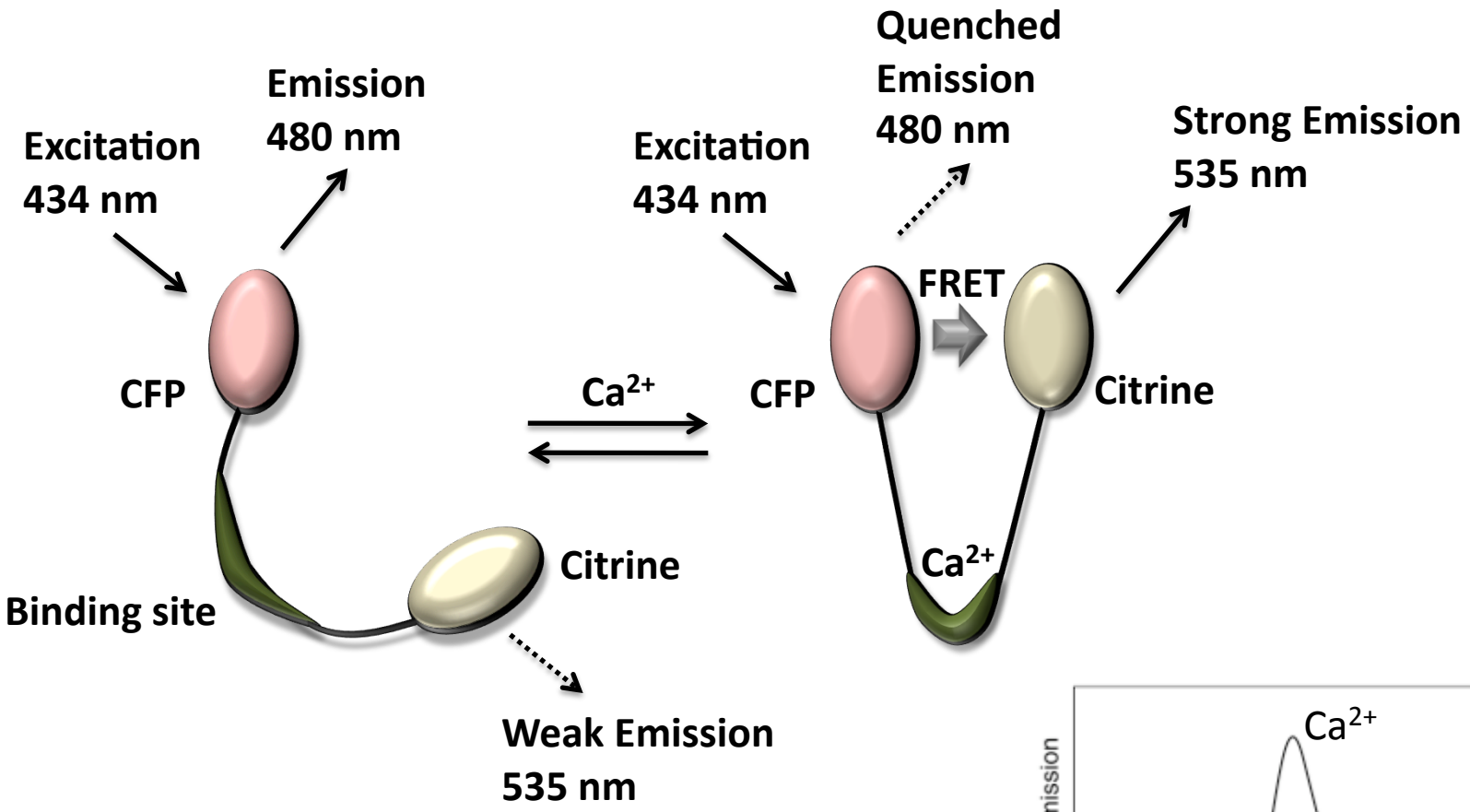
Counter Screen:

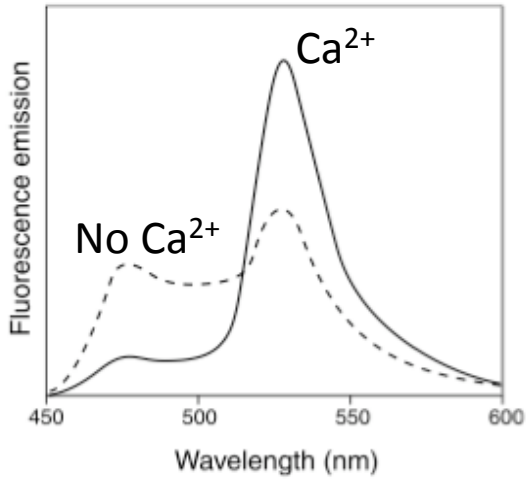
Compound Function

Deletion constructs



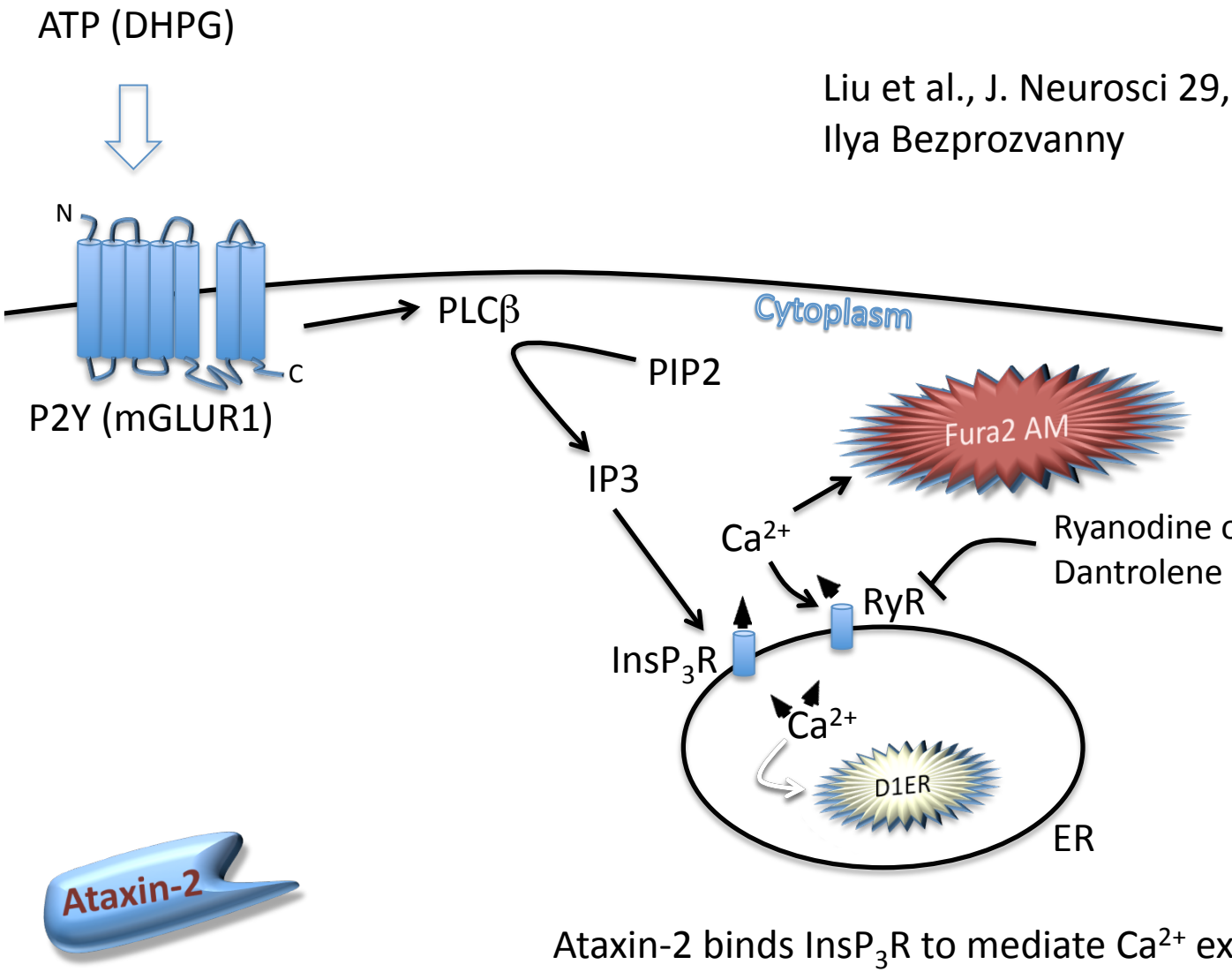
D1ER Cameleon





Targeted to the endoplasmic reticulum
From Roger Tsien's lab

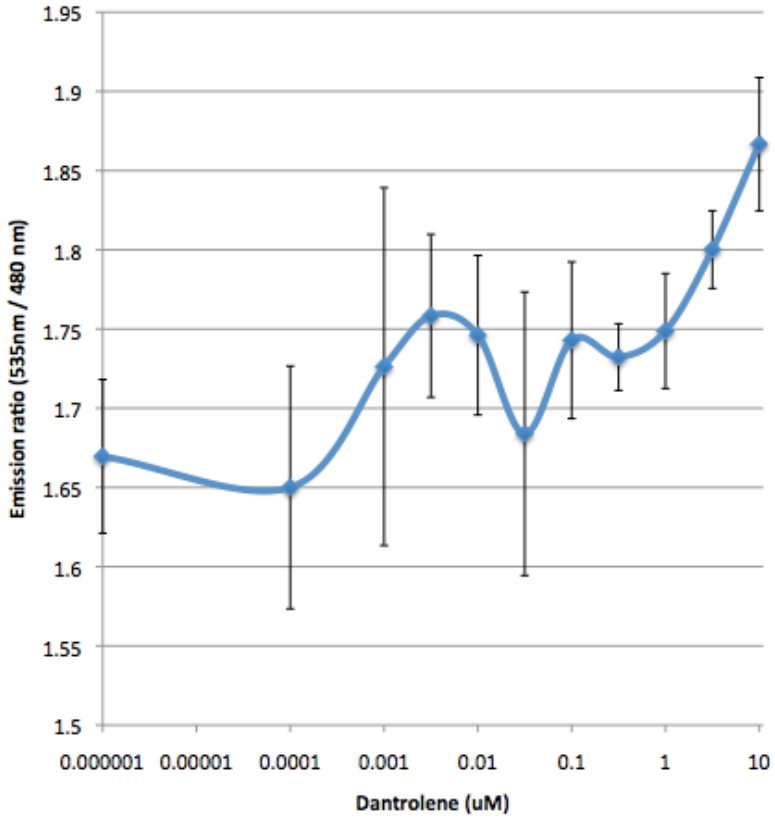
Cell line model for measuring Ataxin-2 effect on Ca^{2+} movement

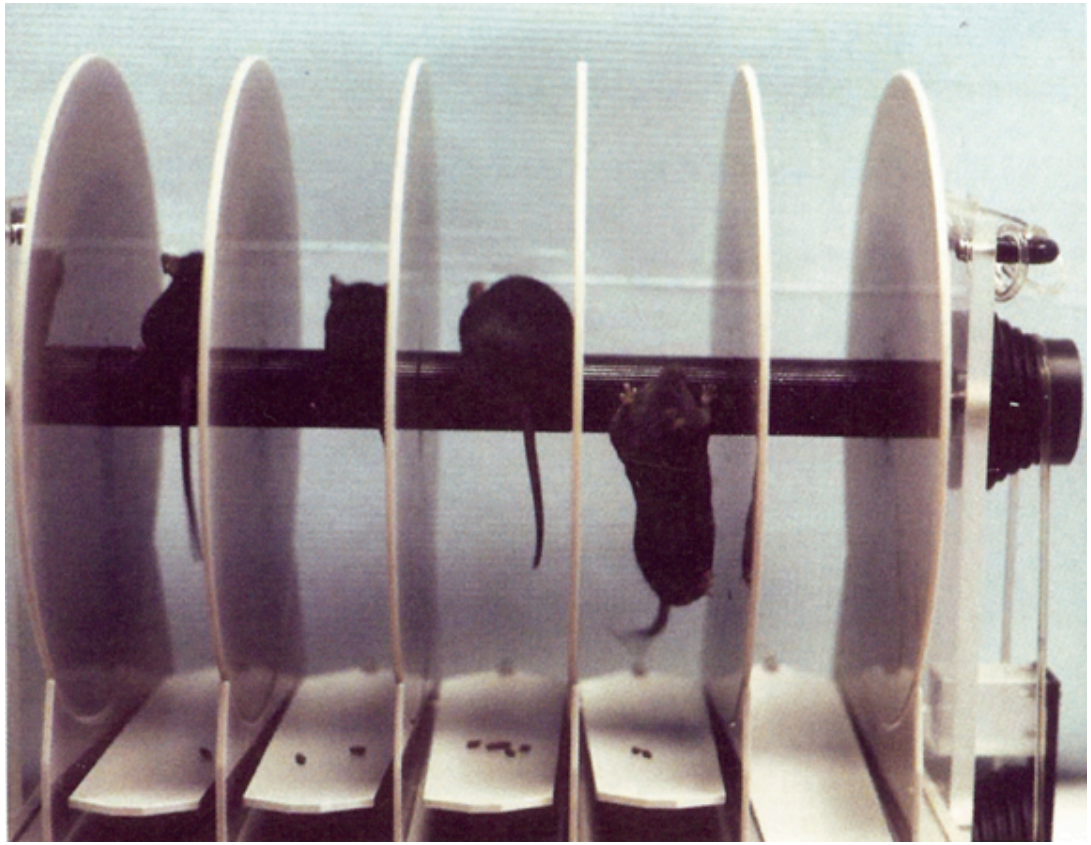


Liu et al., J. Neurosci 29,2009.
Ilya Bezprozvanny

Ataxin-2 binds $InsP_3R$ to mediate Ca^{2+} exit from ER stronger when mutated

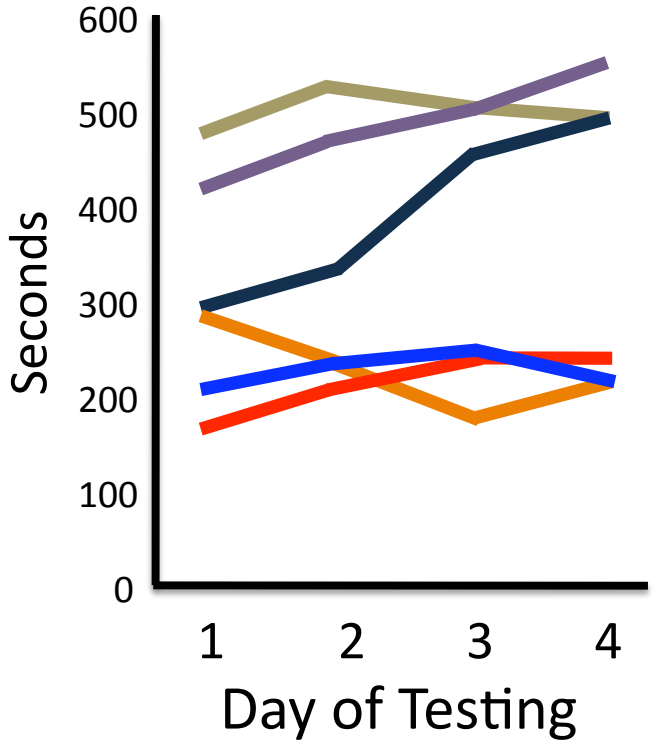
First test of dantrolene treated HEK293-D1ER cells in full serum regular Ca2+ media





Mouse Models

Rotorod performance for SCA2 Q58 transgenic mice

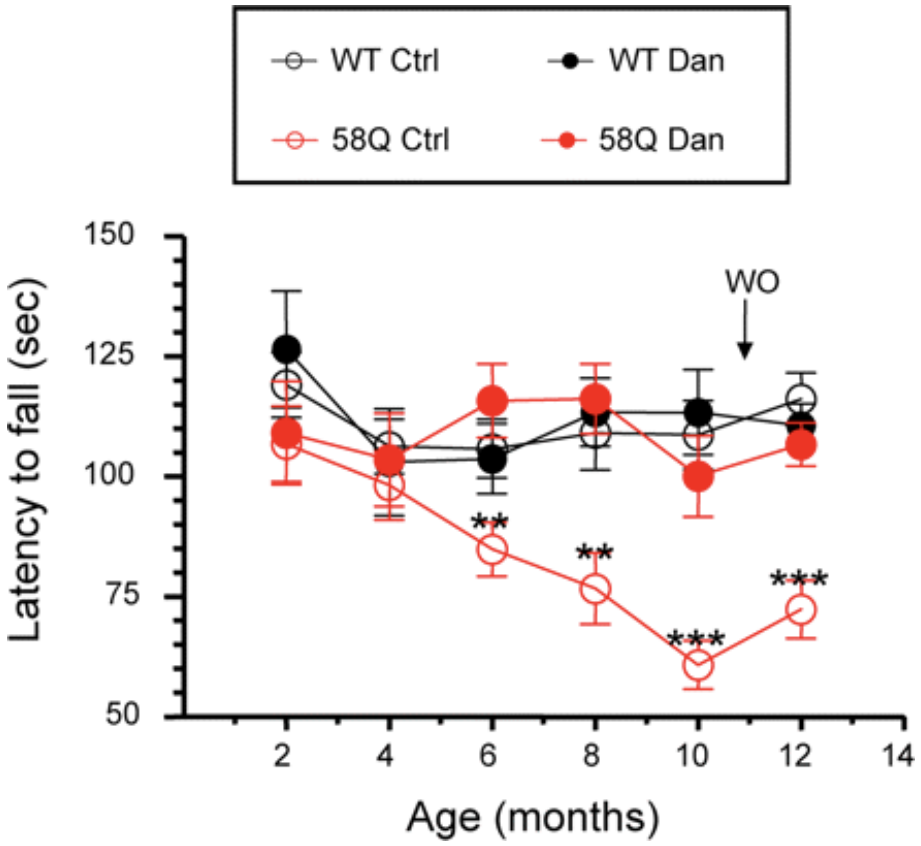


+/+	14 wks (n=10)
+/+	26 wks (n=8)
+/Q58	14 wks (n=8)
+/Q58	16 wks (n=12)
Q58/Q58	26 wks (n=9)
Q58/Q58	26 wks (n=9)

4 to 40 rpm for 10 min

Huynh et al., *Nature Genetics 26, 44 - 50 (2000)*

Rotorod analysis used to measure dantroline effect on SCA2 Q58 transgenic mice

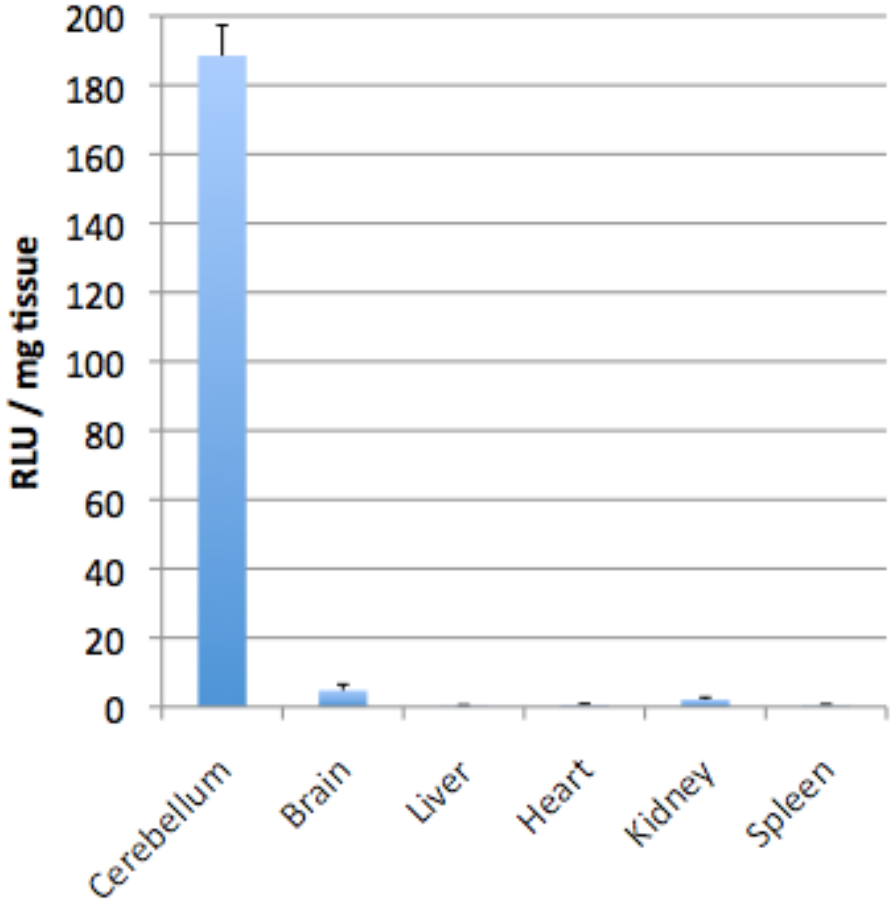


PCP2 promoter

N=9-12 mice per group
0 to 40 rpm over 200 s
Fed 5 mg/kg dantrolene orally twice/wk

Liu et al., J. Neurosci 29,2009.
Ilya Bezprozvanny

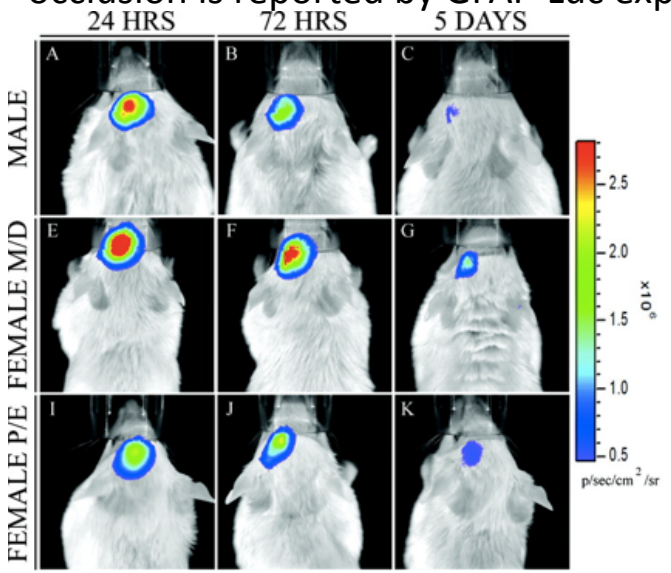
Tissue-specific expression of SCA2-Luciferase in *ATXN2*-Luc transgenic mice



Reporter Mouse

- Atxn2-Luc transgenic mice
  - Histology
  - Xenogen IVIS 200 imaging
    - Extracted brains or live animals

Stroke model
Reactive gliosis induced by middle cerebral artery occlusion is reported by GFAP-Luc expression.



Cordeau et al. Stroke 39:935, 2008.

Summary Slide

Several compounds that inhibit *ATXN2*-luciferase expression

Validation tests → Drugs for SCA2

A functional element: We conducted a screen that was not based on *ATXN2* function that resulted in targets of common function.

Modern methods of drug discovery

Acknowledgments

Seth Andrew Christensen

Lance Pflieger

Steven Hansen

Stefan Pulst & the lab

Pilot Grant from the Neurodegenerative Disease Center