A Quantitative High-throughput Screening Assay using Krabbe Disease Patient Cells

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SUPPLEMENTARY MATERIAL

LEGENDS TO SUPPLEMENTAL FIGURES

Figure S1. Three-plate uniformity-assessment of HTS assay of GALC. The SV40T fibroblasts from control (CTRL) and a GLD patient (GALC-G270D/G270D) were cultured in three 384-well plates. Cells were seeded in three interleaved 384-well plates on different days using freshly prepared reagents. CTRL, GLD patient cells and wells without cells (only medium) were displayed in interleaved plate format (in different column-positions of each plate). Individual scatter plots from plate 1 (**A**), plate 2 (**B**) and plate 3 (**C**) are shown with response plotted against well number, ordered firstly by column and then by row.

Figure S2. Scatter plot of GLD patient cellular HTS assay of GALC against LOPAC. Panels represent GLD patient SV40T (GALC-G270D) and control (GALC-WT). GLD patient fibroblasts were exposed to the 1,280 compounds from LOPAC in different concentrations (panels **A-E**) in 1,536-well plates. Each color represents 32 different columns of each high-dense plate used in the HTS. The fluorescence signals generated by the GALC activity from GLD patient fibroblasts treated with LOPAC are located in columns 5-44. Columns 1 and 2 represent fluorescence signals from wells containing only medium and no fibroblasts. SV40T control fibroblasts are located in columns 3 and 4. GLD patient fibroblasts treated only with DMSO (solvent of LOPAC compounds) were located in columns 45-48. Scatter plot results LOPAC treatment concentrations of 9.2 μM (**A**), 23 μM (**B**), 46 μM (**C**) and 115 μM (**D**) are shown. The scatter plot results from highest (230 μM) and lowest (4.6 μM) concentrations along with plates with cells treated only with DMSO are shown in figure 5 (main text).

Figure S3. Other concentration-response curve analysis derived from quantitative HTS assay for GALC against LOPAC library. In the cell-based HTS for GALC using LOPAC concentrations ranging from 4.6 to 230 μM, 19 small molecules (SM) showed active concentration-response curves (CRCs) of class 1 to 3. Guanabenz acetate **(**SM-5; **A)**, 4-Cyclohexylmethoxy-2,6-diamino-5-nitrosopyrimidine or NU6027 **(**SM-6; **B)**, isoproterenol

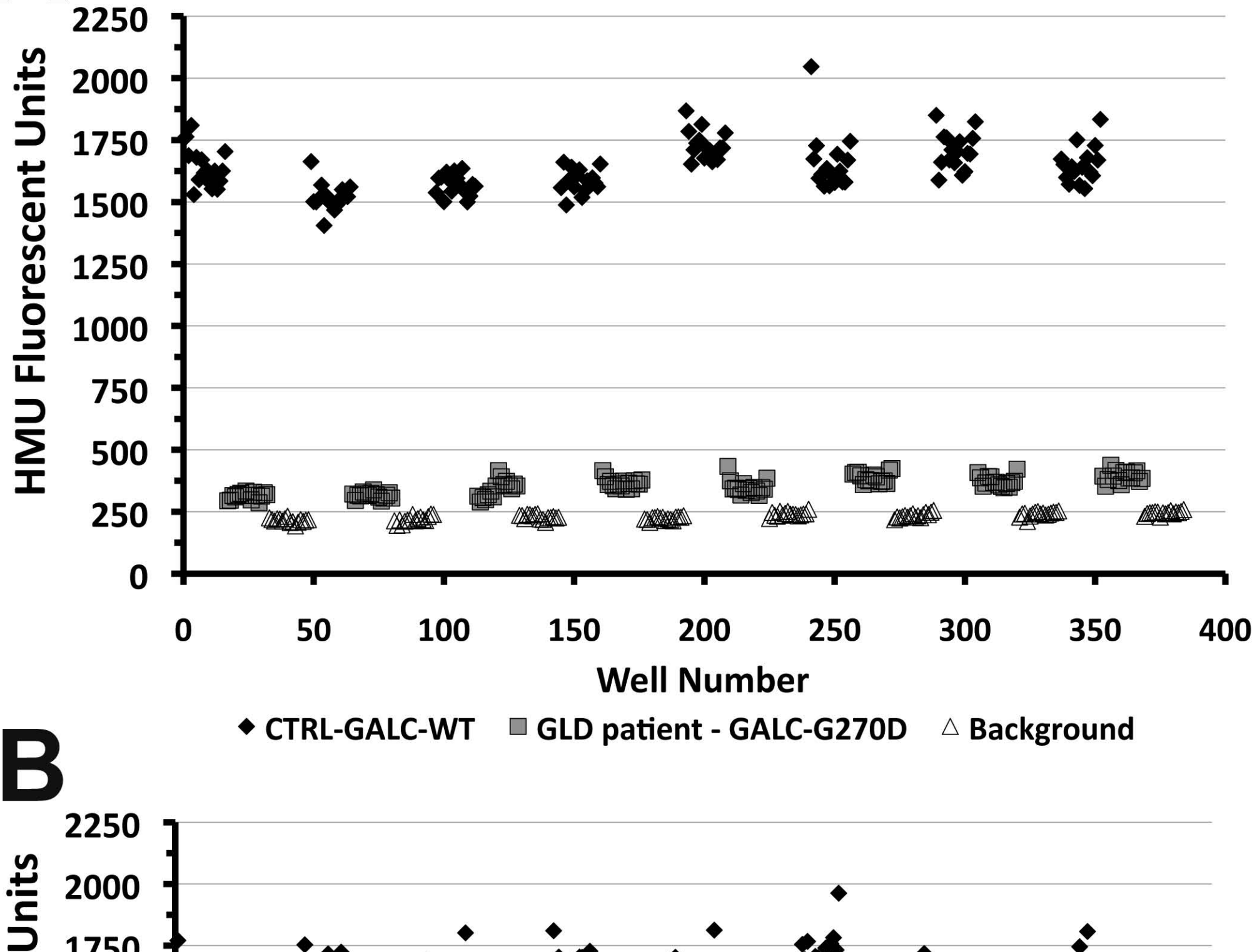
bitartrate **(**SM-8; **C)** and 4-Chloro-DL-phenylalanine methyl ester HCl **(**SM-9; **D)** showed CRCs of class 2.2. Edrophonium HCl (SM-10; **E**) and bromopheniramine maleate (SM-11; **F**) showed CRCs class 2.4. Pyrostigmine HCl (SM-15; **G**), R(-)-2,10,11-Trihydroxy-N-propyl-noraporphine hydrobromide hydrate (SM-16; **H**), Nialamide (SM-17; **I**), (+/-)-cis-Piperidine-2 (SM-18; **J**) and Alloxazine (SM-19; **K**) showed CRCs class 3.

Figure S4. Dose-response GALC assays in primary fibroblasts from GLD patients and control fibroblasts treated with small molecules with active concentration-response curves in the HTS for GALC. Two GLD patient primary fibroblast lines, GALC-G270D and GALC-G553R and control (GALC-WT) were treated with small molecules (SM) with concentration-response curves (CRCs) of classes 2 and 3 at concentrations ranging from 1.9 to 150 μM. Guanabenz acetate (SM-5**; A**)**,** 4-Cyclohexylmethoxy-2,6-diamino-5-nitrosopyrimidine (SM-6; **B**), 3-bromo-7-nitroindazole (SM-7; **C**), isoproterenol bitartrate (SM-8; **D**), 4-Chloro-DL-phenylalanine methyl ester HCl (SM-9; **E**), bumetamide (SM-13; **F**), pyrostigmine HCl (SM-15; **G**) and nialamide (SM-17; **H**) are also shown. In each primary fibroblast cell line, the average level of protein concentration per well each 96-well plate was used to standardize the HMU fluorescence signals.

Figure S5. Cell viability assays in cultured fibroblasts treated with different small molecule that showed active CRCs in the cell-based HTS for GALC. Cell viability results in SV40T GLD (GALC-G270D) fibroblasts (**A**), used in the primary screening, were treated with 46 μM (light grey columns) and 240 μM (dark grey columns) of small molecule (SM) for 48 hs. The primary GLD patient fibroblasts (GALC-G270D; **B**) and the primary fibroblast control (GALC-WT) (**C**) were also tested at the same conditions. The small molecules SM-2, SM-3, SM-5, SM-6, SM-8, SM-12 and SM-14 showed to some level of cytotoxic at higher concentrations in the three different cells.

Figure S6. Compound inherent fluorescence in presence or absence of HMU substrate. Some of the small molecules from the LOPAC showed to emit fluorescence at the assay conditions for GALC. However, after performing the GALC assay with HMU substrate, the compound fluorescence was quenched. In 384-well plate, primary GLD patient cells (GALC-G270D) were treated with one of the candidate small molecule from the primary screen (SM-12) and fluorescence reading was performed before **(A)** and after dispensing the HMUGal substrate (**B**). Absolute HMU signals were shown in histograms in panels **A** and **B**. Quenching of the SM-12 inherent fluorescence signals were observed in several concentrations of the compound.

A


HMU Fluorescent Units
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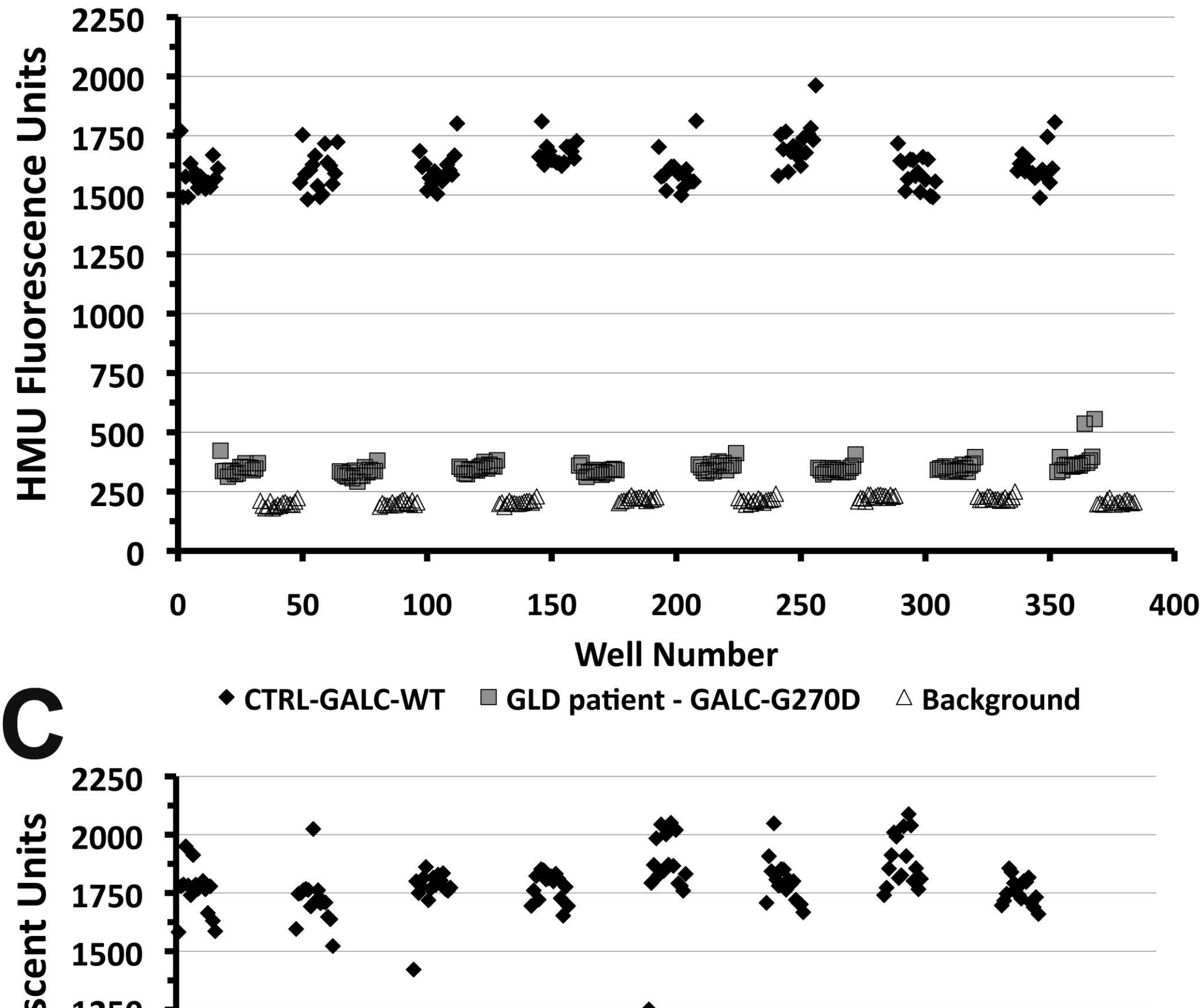
2000
1750
1500
1250
1000
750
500
250
0
0
50
100
150
200
250
300
350
400
Well Number
CTRL-GALC-WT
GLD patient - GALC-G270D
Background

B


HMU Fluorescence Units
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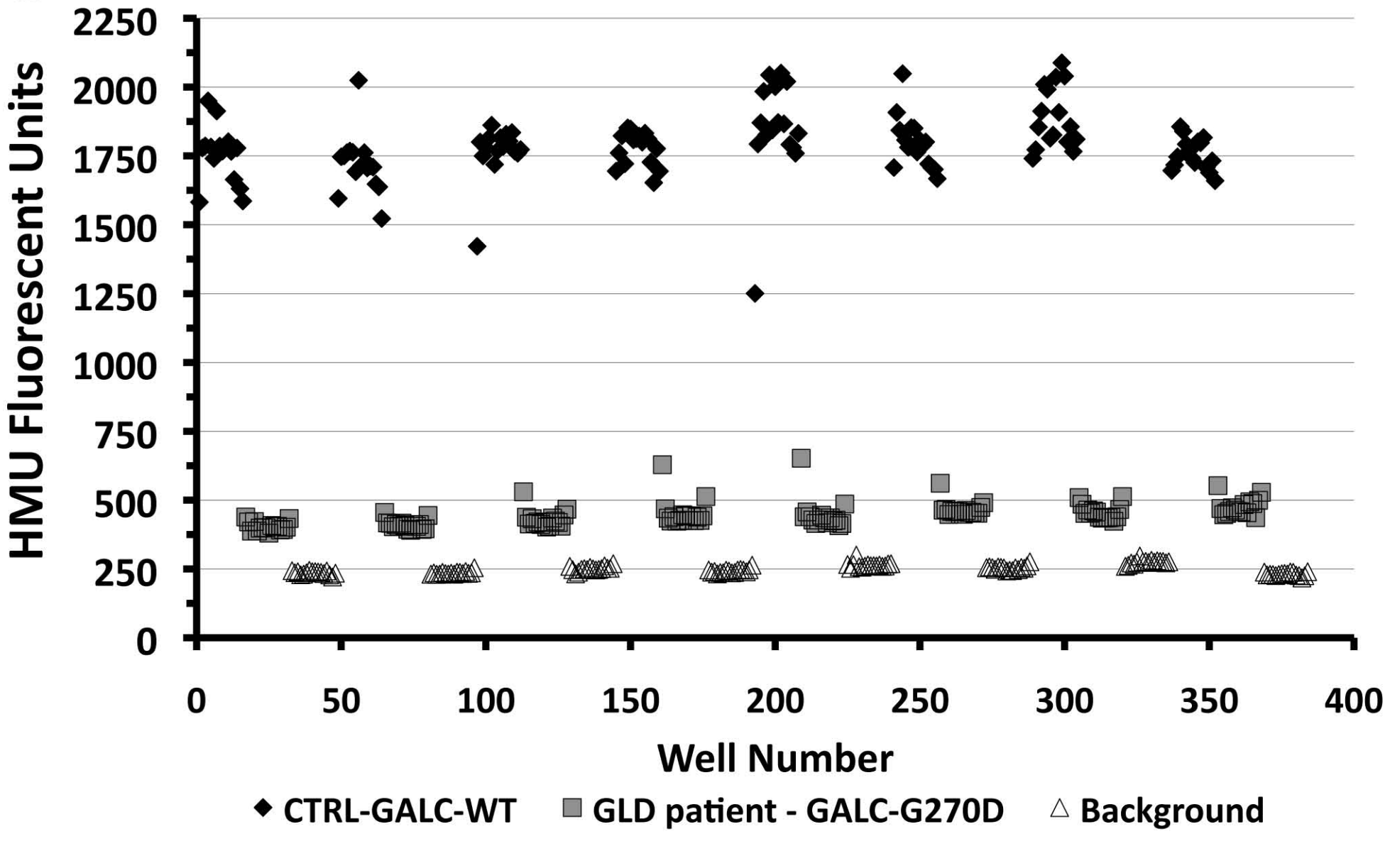
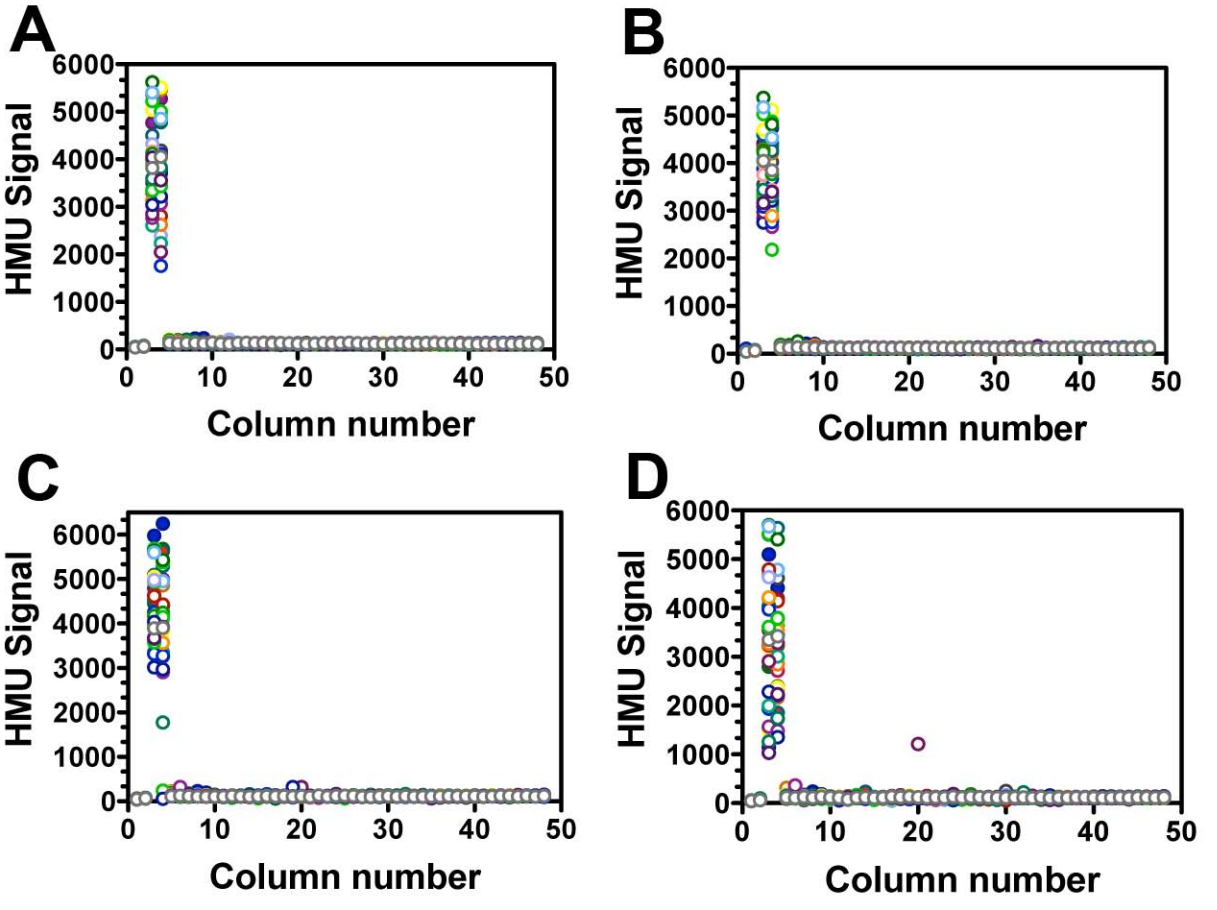
2000
1750
1500
1250
1000
750
500
250
0
0
50
100
150
200
250
300
350
400
Well Number
CTRL-GALC-WT
GLD patient - GALC-G270D
Background

C


HMU Fluorescent Units
2250
2000
1750
1500
1250
1000
750
500
250
0
0
50
100
150
200
250
300
350
400
Well Number
CTRL-GALC-WT
GLD patient - GALC-G270D
Background


A
B
C
D
HMU Signal
Column number
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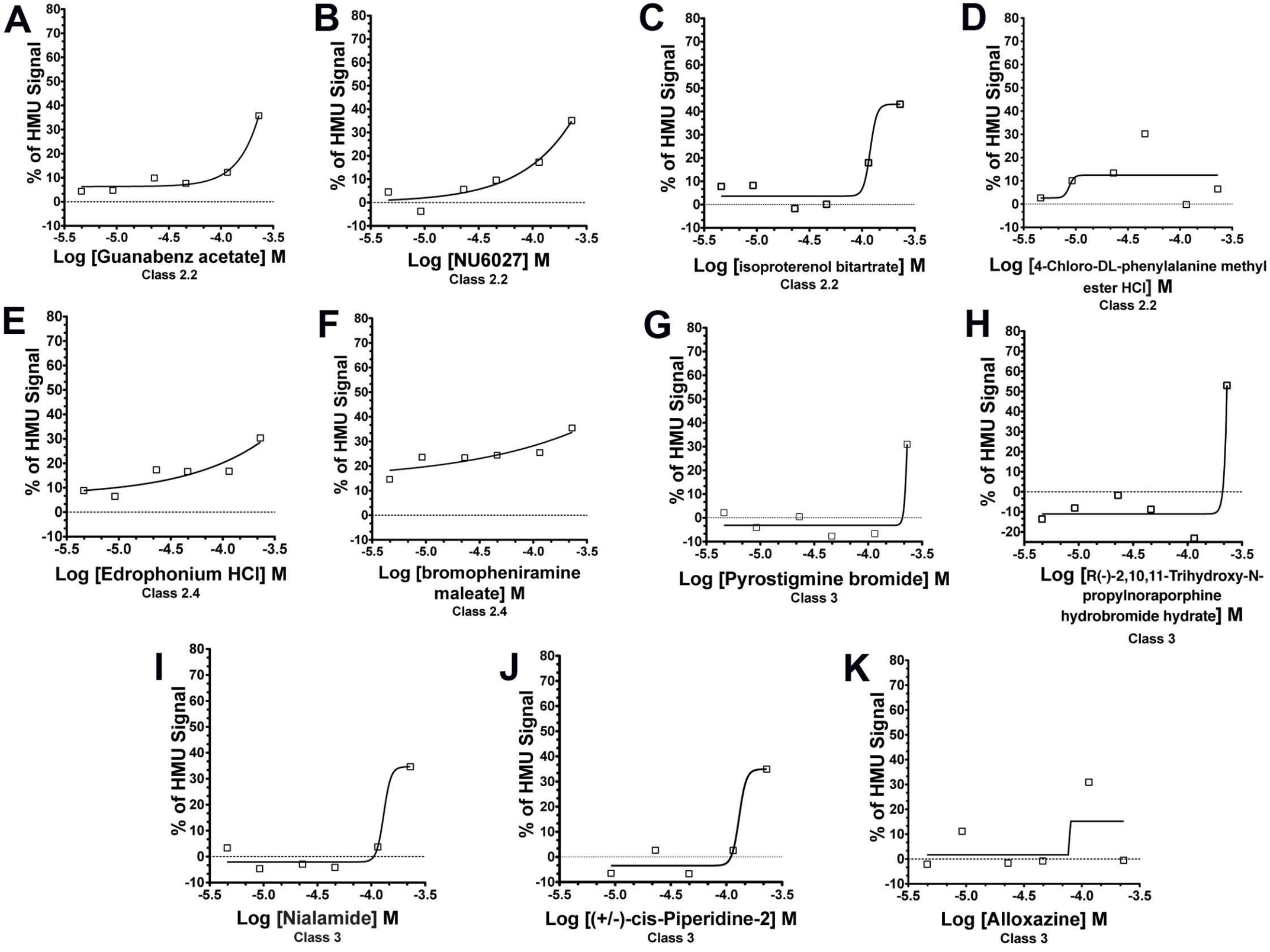
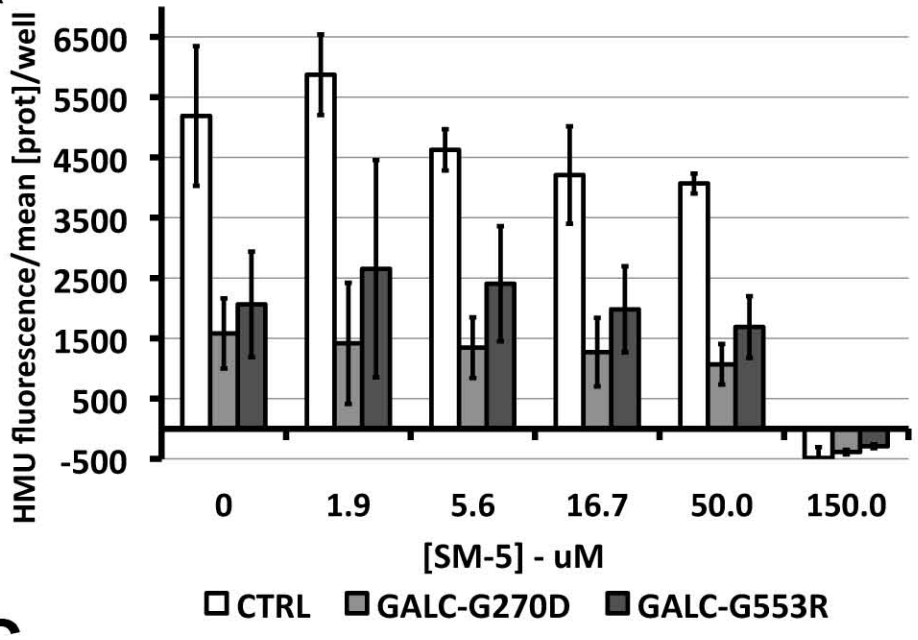
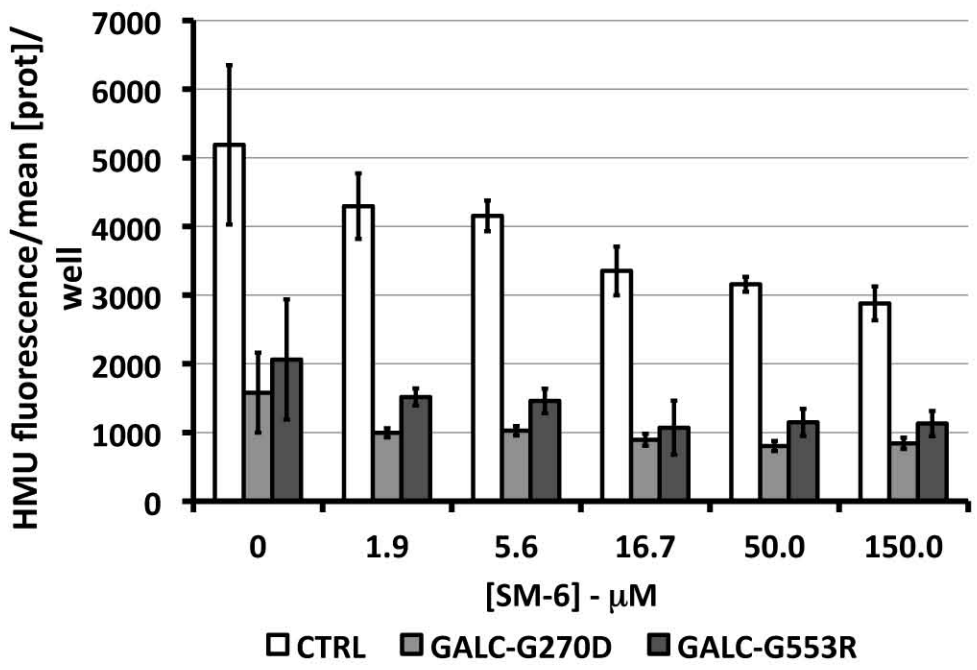
2000
3000
4000
5000
6000
0
10
20
30
40
50


A
% of HMU Signal
Log [Guanabenz acetate] M
Class 2.2
B
% of HMU Signal
Log [NU6027] M
Class 2.2
C
% of HMU Signal
Log [isoproterenol bitartrate] M
Class 2.2
D
% of HMU Signal
Log [4-Chloro-DL-phenylalanine methyl ester HCl] M
Class 2.2
E
% of HMU Signal
Log [Edrophonium HCl] M
Class 2.4
F
% of HMU Signal
Log [bromopheniramine maleate] M
Class 2.4
G
% of HMU Signal
Log [Pyrostigmine bromide] M
Class 3
H
% of HMU Signal
Log [R(-)-2,10,11-Trihydroxy-N-propylnoraporphine hydrobromide hydrate] M
Class 3
I
% of HMU Signal
Log [Nialamide] M
Class 3
J
% of HMU Signal
Log [(+/-)-cis-Piperidine-2] M
Class 3
K
% of HMU Signal
Log [Alloxazine] M
Class 3

A


HMU fluorescence/mean [prot]/well
6500
5500
4500
3500
2500
1500
500
-500
0
1.9
5.6
16.7
50.0
150.0
[SM-5] - uM
CTRL
GALC-G270D
GALC-G553R

B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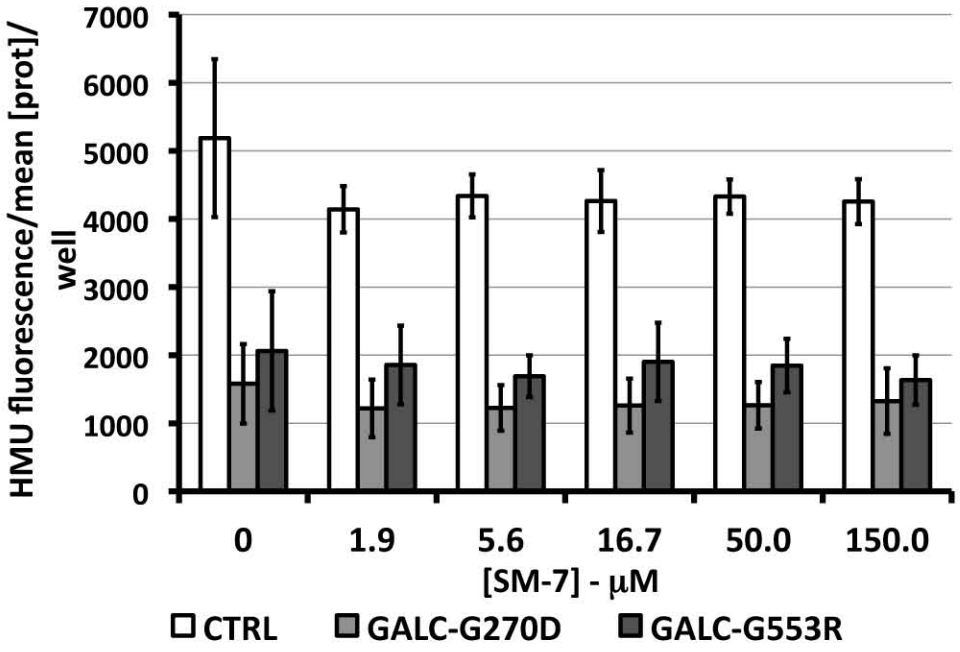
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-6] - µM
CTRL
GALC-G270D
GALC-G553R

C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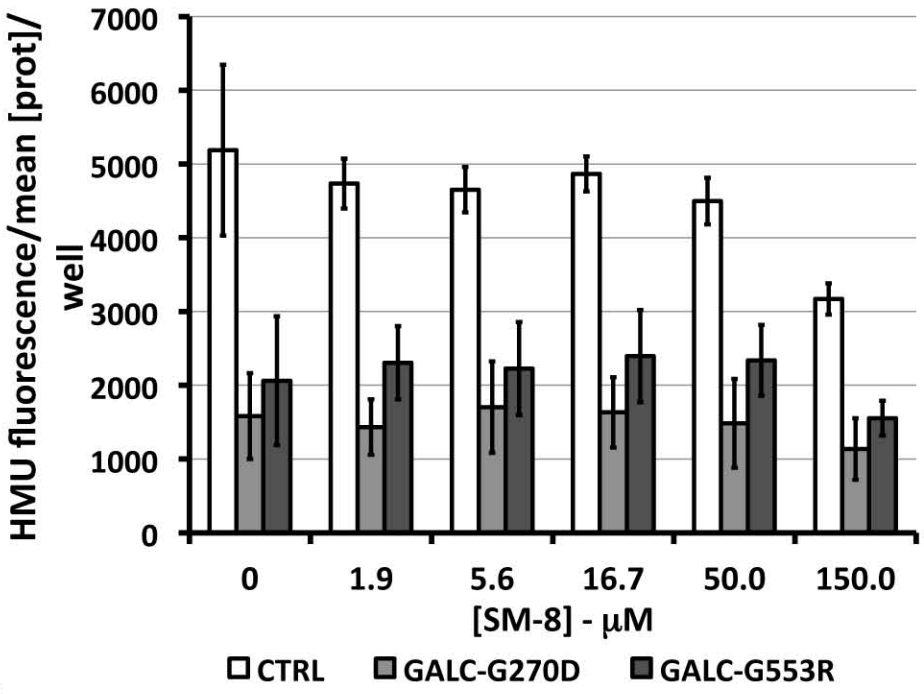
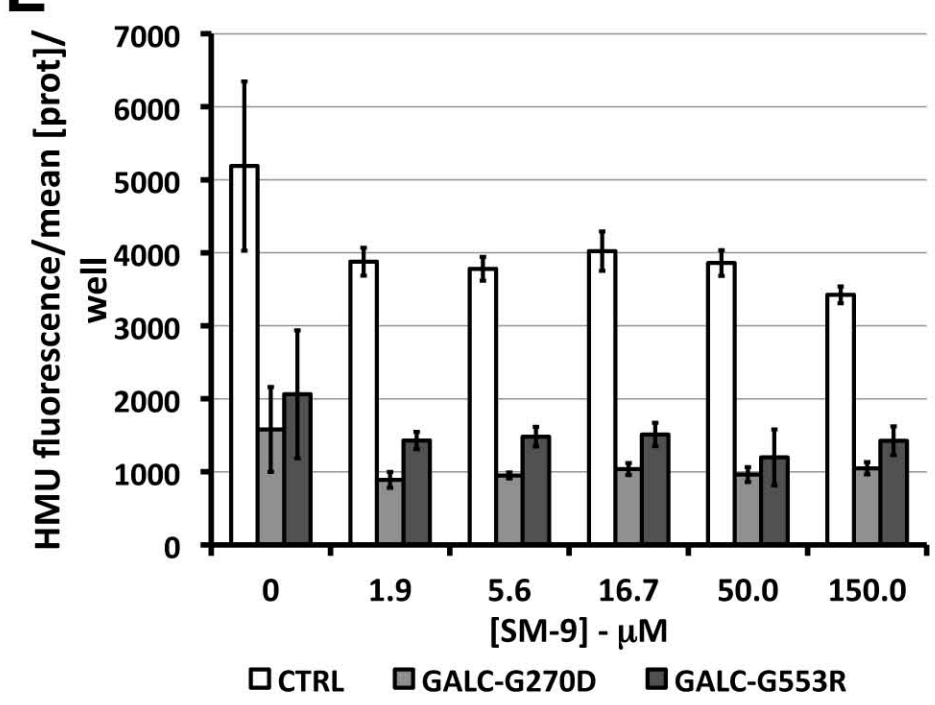
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-7] - µM
CTRL
GALC-G270D
GALC-G553R

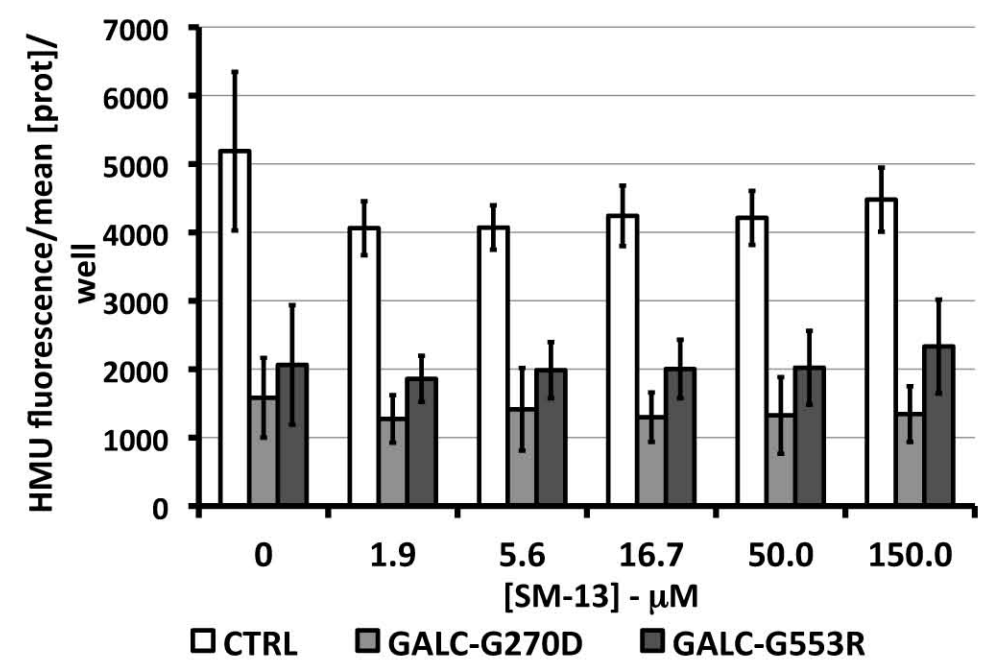
D


HMU fluorescence/mean [prot]/ well
7000
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-8] - µM
CTRL
GALC-G270D
GALC-G553R

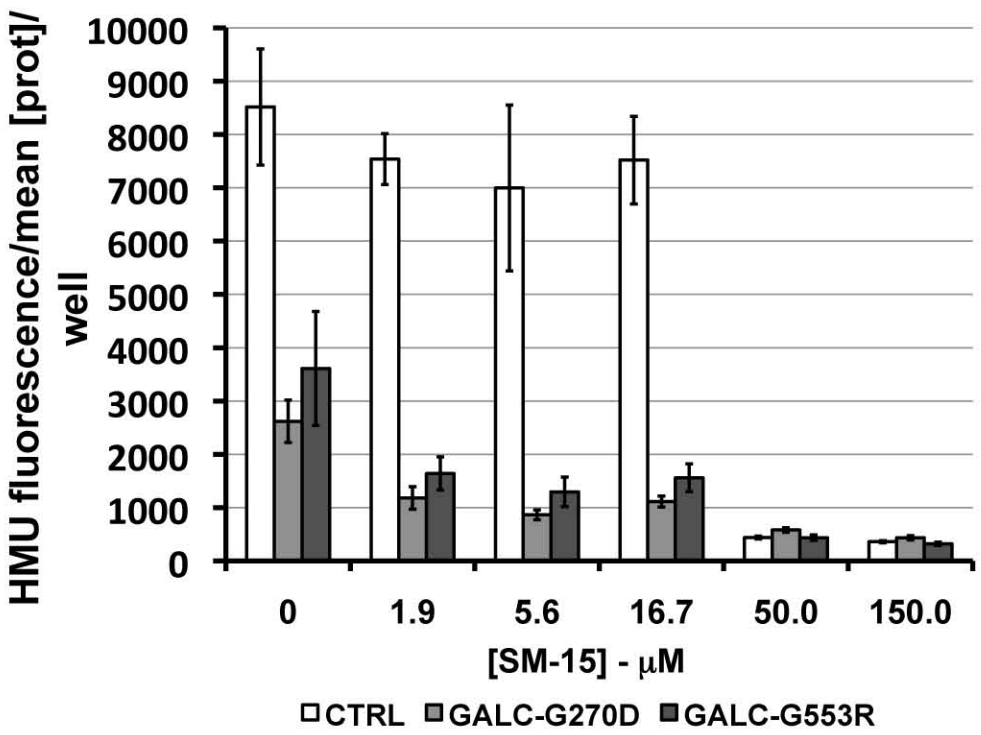
E


HMU fluorescence/mean [prot]/ well
7000
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-9] - µM
CTRL
GALC-G270D
GALC-G553R

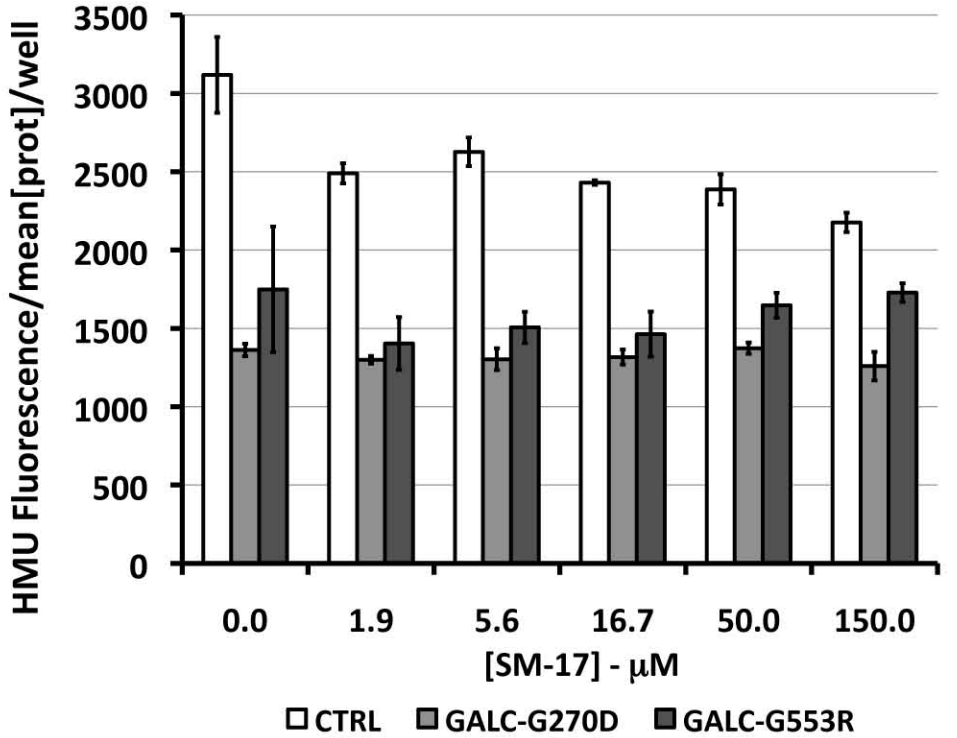
F

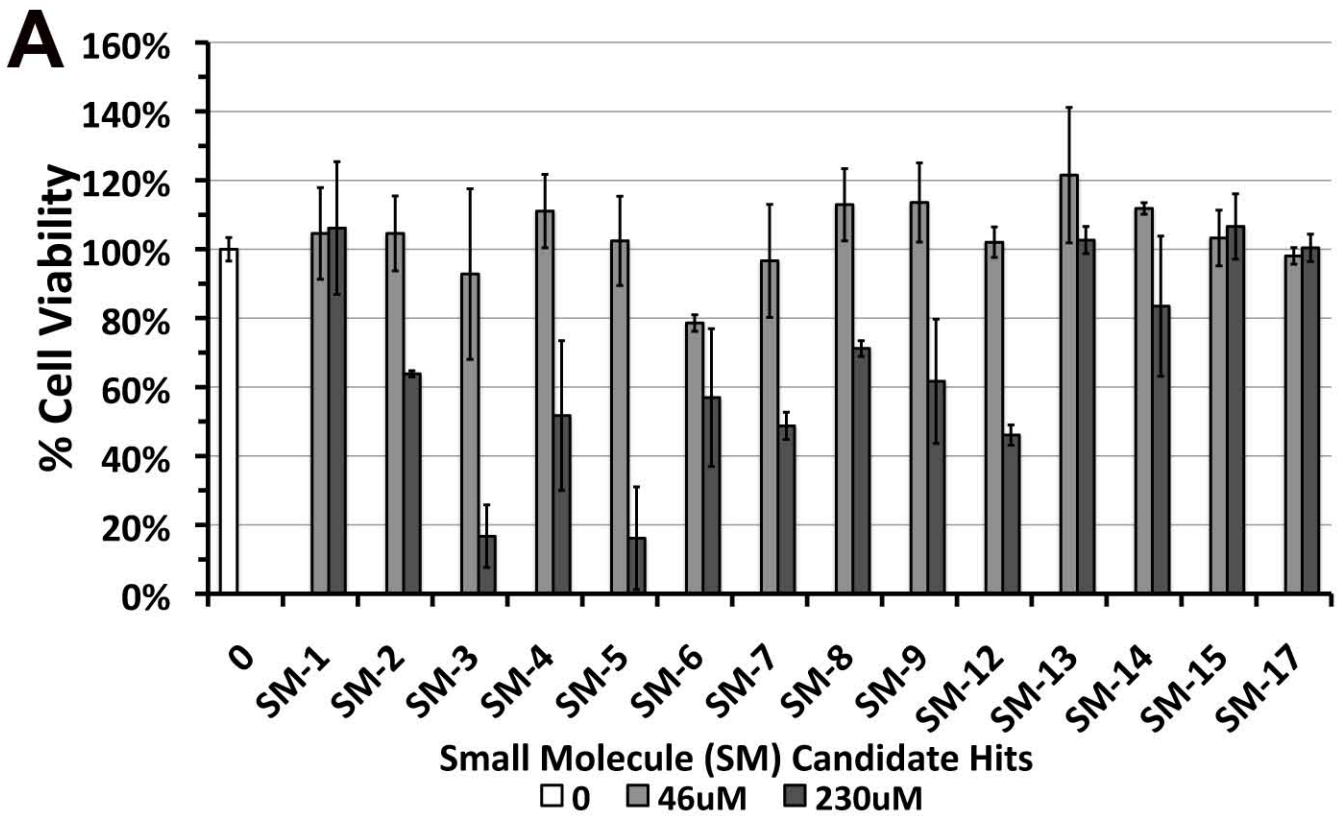

HMU fluorescence/mean [prot]/ well
7000
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-13] - µM
CTRL
GALC-G270D
GALC-G553R

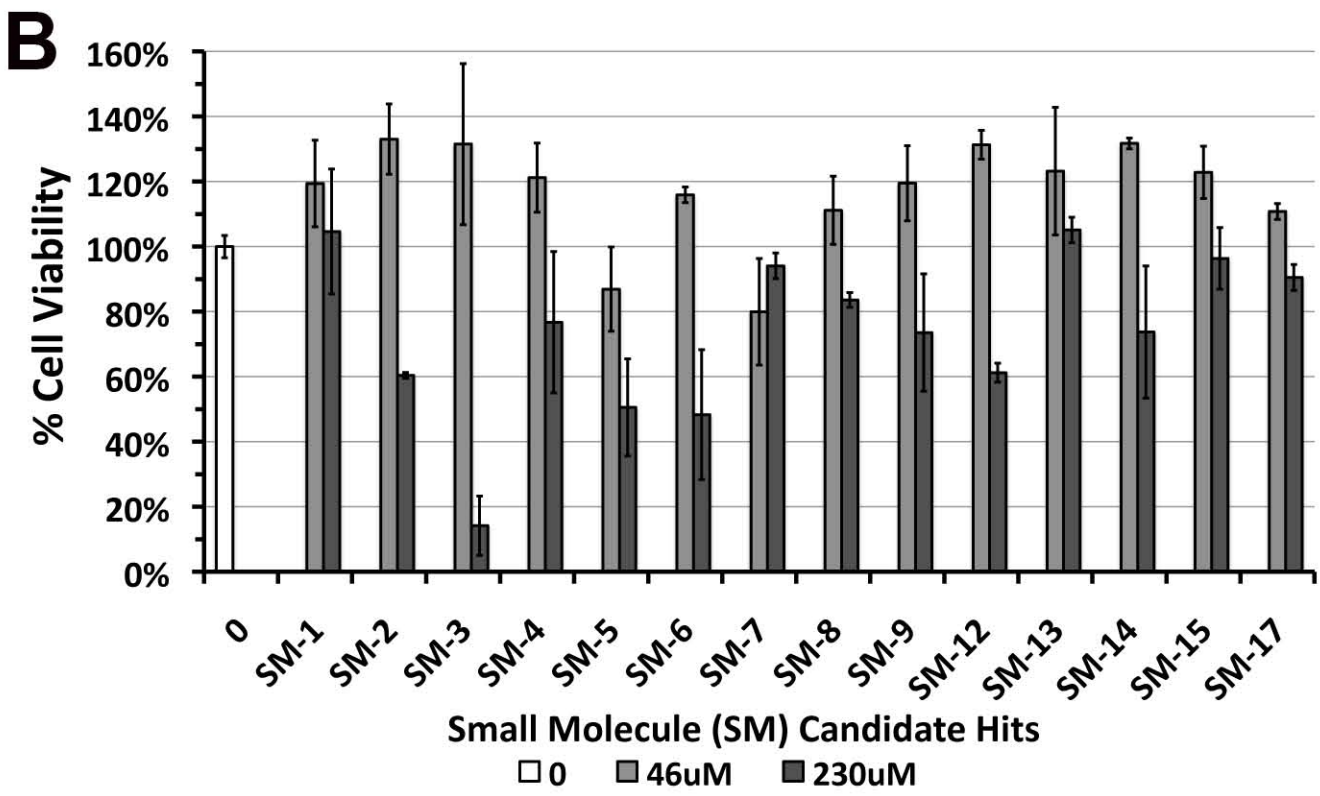
G


HMU fluorescence/mean [prot]/ well
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1.9
5.6
16.7
50.0
150.0
[SM-15] - µM
CTRL
GALC-G270D
GALC-G553R

H


HMU Fluorescence/mean[prot]/well
3500
3000
2500
2000
1500
1000
500
0
0.0
1.9
5.6
16.7
50.0
150.0
[SM-17] - µM
CTRL
GALC-G270D
GALC-G553R


A
160%
140%
120%
100%
80%
60%
40%
20%
0%
% Cell Viability
0
SM-1
SM-2
SM-3
SM-4
SM-5
SM-6
SM-7
SM-8
SM-9
SM-12
SM-13
SM-14
SM-15
SM-17
Small Molecule (SM) Candidate Hits
0
46uM
230uM


B
160%
140%
120%
100%
80%
60%
40%
20%
0%
% Cell Viability
0
SM-1
SM-2
SM-3
SM-4
SM-5
SM-6
SM-7
SM-8
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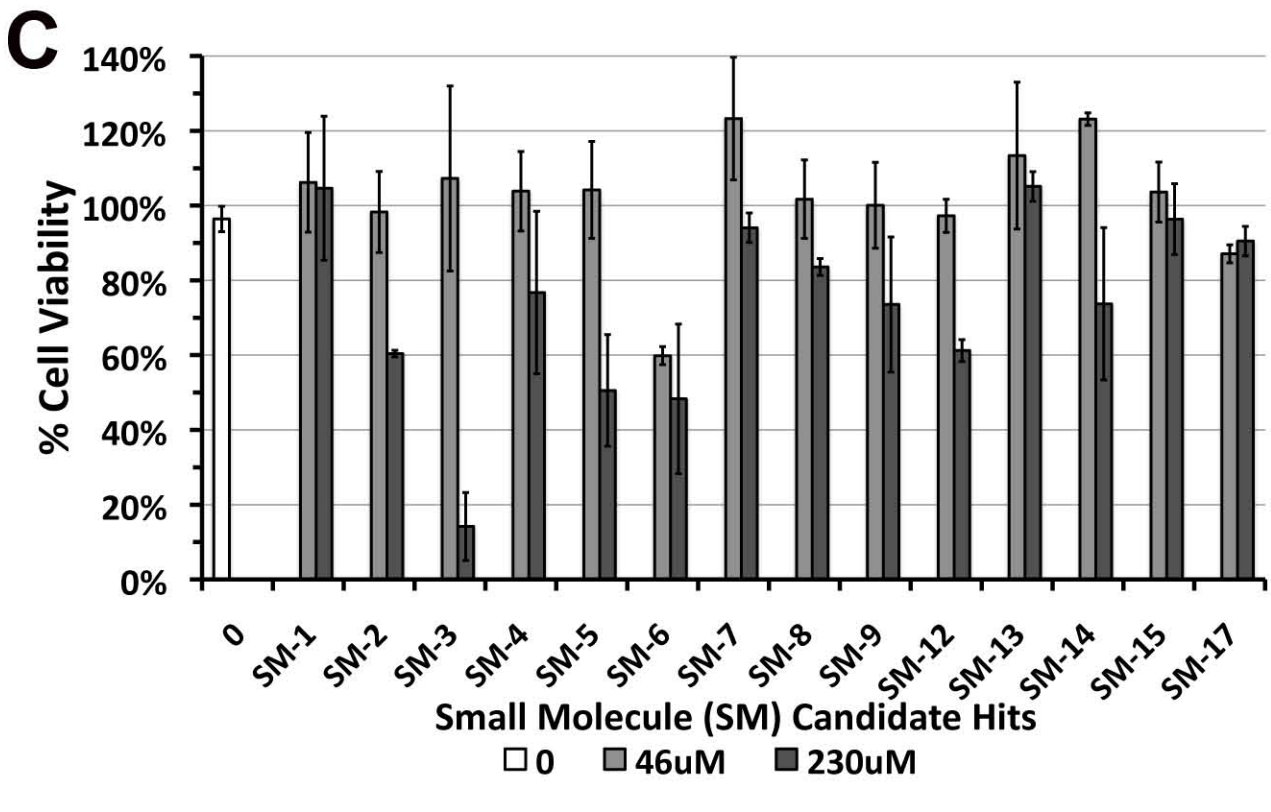
SM-12
SM-13
SM-14
SM-15
SM-17
Small Molecule (SM) Candidate Hits
0
46uM
230uM


C
140%
120%
100%
80%
60%
40%
20%
0%
% Cell Viability
0
SM-1
SM-2
SM-3
SM-4
SM-5
SM-6
SM-7
SM-8
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SM-13
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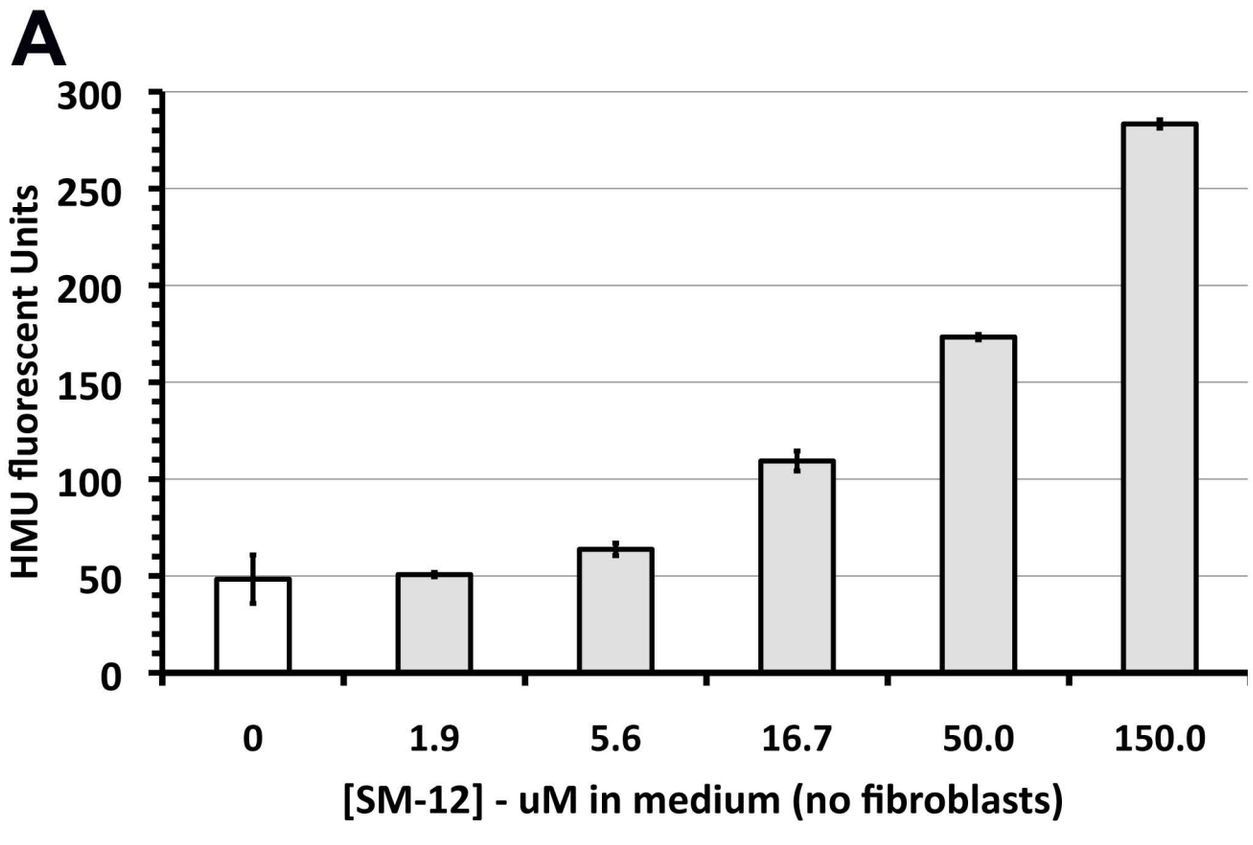
SM-17
Small Molecule (SM) Candidate Hits
0
46uM
230uM


A
300
250
200
150
100
50
0
HMU fluorescent Units
0
1.9
5.6
16.7
50.0
150.0
[SM-12] - uM in medium (no fibroblasts)

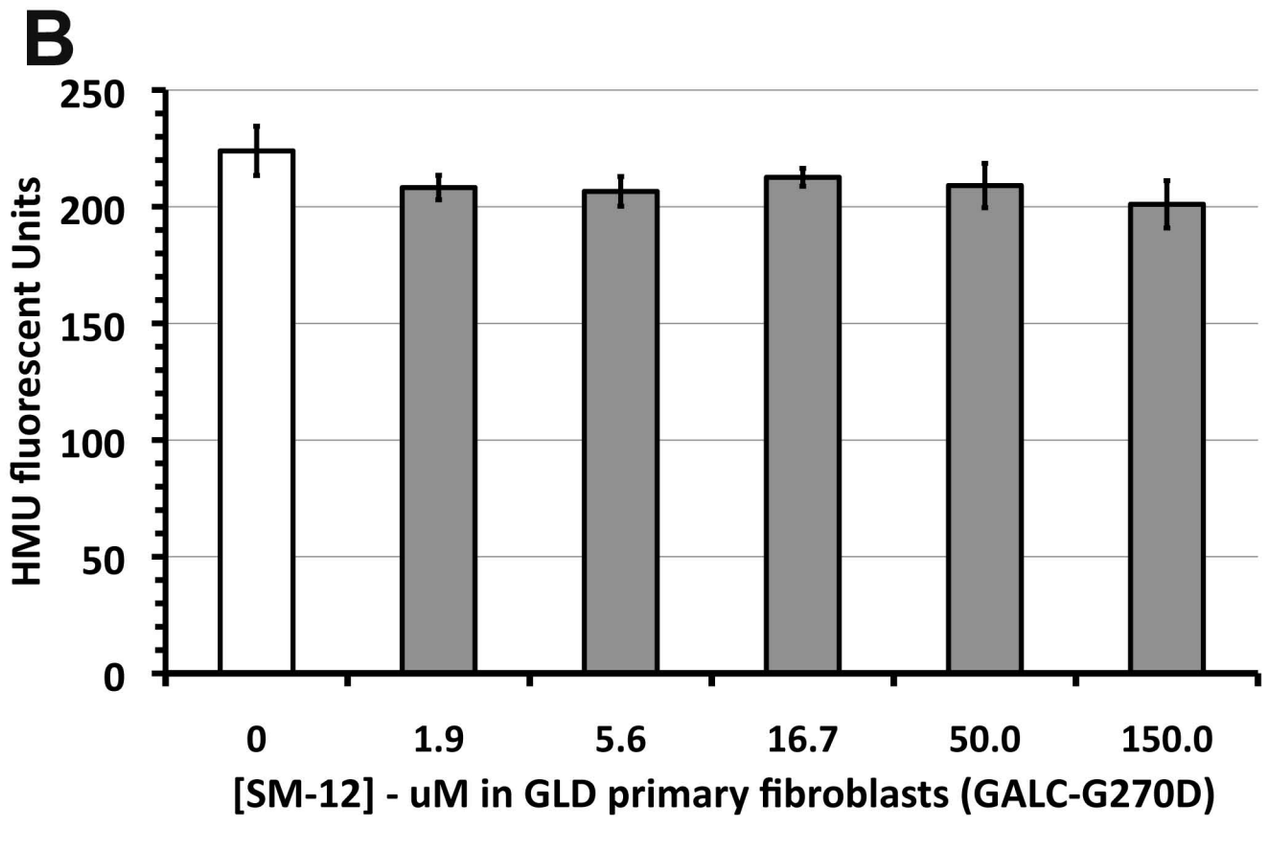

B
250
200
150
100
50
0
HMU fluorescent Units
0
1.9
5.6
16.7
50.0
150.0
[SM-12] - uM in GLD primary fibroblasts (GALC-G270D)

Table S1. Classification of Concentration-Response Curves (CRCs).

Curve Class	Asymptotes	r^2	Efficacy	Description
1.1	**2**	$\geq$ 0.9	>80%	Complete curve; good fit; high efficacy
1.2			Min - 80%	Complete curve; good fit; partial efficacy
1.3		$\leq$ 0.9	>80%	Complete curve; poor fit; high efficacy
1.4			Min - 80%	Complete curve; poor fit; partial efficacy
2.1	**1**	$\geq$ 0.9	>80%	Partial curve; good fit; high efficacy
2.2			Min - 80%	Partial curve; good fit; partial efficacy
2.3		$\leq$ 0.9	>80%	Partial curve; poor fit; high efficacy
2.4			Min - 80%	Partial curve; poor fit; partial efficacy
3	**1**		> Min.	Single point of activity
4	**None**		< Min	Inactive
5	**None**		$\leq$ Min	Inconclusive

* Original classification in Inglese J *et al.* 2006 [22]. Min, minimal.