SUPPORTING INFORMATION

Selective modulation of autophagy, innate immunity and adaptive immunity by small molecules

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Supplementary Methods

Annotated compound library

The screened compounds include the Prestwick Chemical library of marketed drugs (Prestwick Chemical, 2 mg/mL stock concentration); Spectrum Collection of known bioactives (MicroSource Discovery Systems, 10 mM stock); Institute of Chemistry and Cell Biology Bioactives collection (Enzo Life Sciences, variable concentrations); and Biomol-NT (Neurotransmitter) collection of neurotransmitter drugs and bioactives (Enzo Life Sciences, 10 mM stock). For the screen, 153 nL was transferred into 200 µL volume in 96-well plates; for compounds at 10 mM stock concentration, this corresponds to a screening concentration of ~7.7 µM. Follow-up assays used re-ordered compounds (Sigma, St. Louis, MO) and fresh compound stocks.

LC3: *Salmonella* co-localization assay

HeLa cells, stably expressing eGFP-LC3 were seeded overnight in 96-well glass bottom plates at 10,000 cells per well in IMDM plus 10% FBS (Hyclone) and 2 mM L-glutamine (GlutaMax, Life Technologies). On the following day, the medium was removed and replaced with complete

IMDM containing compound in DMSO, and the cells were incubated at 37^{0}C. After three hours, infections with *Salmonella enterica* sbsp *enterica* serotype Typhimurium strain SL1344 (*S. Typhimurium*) carrying DsRed2 expression plasmid (Clontech) were performed as previously described.*(1, 2)* After 20 minute infection period, the cells were washed once in medium containing 50 μg/ml gentamycin sulfate, then incubated in fresh IMDM containing gentamycin and compound for 40 minutes. The medium was aspirated, and the cells were fixed in 2% paraformaldehyde in 1% phosphate buffered saline (PBS) overnight. The following day, the cells were stained with 10 ug/ml Hoechst H33342 (Invitrogen). Images were obtained on a Molecular Devices ImageXpress Micro at 20x magnification with the total number of cells, total number of bacteria per cell, and the number of GFP-LC3 positive bacteria assessed in 6 fields of view per well and analyzed by Cell Profiler software.*(3)* The numbers of GFP-LC3 positive bacteria were then calculated as a percentage of total bacteria. The average number of nuclei in each of the 6 fields of view was calculated to determine compound toxicity in each experimental well.

Evaluation of Oxidative Stress/ROS production

Murine bone marrow-derived macrophages (BMDM) were generated as described above. After 5 days in culture with M-CSF, BMDM were harvested and replated at 70x10^3 cells per well in 96 well imaging plates. Cells were primed with IFN-γ (100ng/ml) overnight and then stimulated with LPS (3ng/ml) and MDP (10ug/ml) in the presence of the indicated compounds for 6 hours. Compounds were administered at a dose of 5 μM with the exception of rotenone (1 μM) and caspase inhibitors (20 μM). Following stimulation, cells were washed twice in PBS and labeled for 30 minutes at 37^{o}C with CM-H_2DCFDA (Invitrogen) at a concentration of 5 μM in phenol red-free DMEM. After thorough washing in PBS, cells were lightly fixed in paraformaldehyde (2%) for 3 minutes and stained with Dapi (1 μg/ml) for 5 minutes. Fluorescence intensity of H_2DCFDA was measured using ImageXpress high content imaging system (Molecular Devices), and

analysis was performed with the cell scoring module within MetaXpress software (Molecular Devices). Data represent the average intensity of H_2DCFDA fluorescence per Dapi-positive cell. Two fields per well were imaged, and each field contained an average of 650 cells. Compound treatments were performed in quadruplicate.

ATP stimulation for IL-1β release

Day 5 BMDMs were primed for 4h with 500ng/ml LPS followed by a 1h treatment with 2.5mM ATP. (Small molecule inducers of autophagy were present during both LPS and ATP phases). IL-1β was then measured from cell supernatants by ELISA as described in Methods. Error bars represent the standard deviation from quadruplicate samples.

Flow cytometry and data analysis

Cells were stained with blue LIVE/DEAD stain (Invitrogen, USA) to discriminate live cells, fixed with Foxp3 Permeabilization Buffer (eBioscience, USA), stained with FITC anti-CD4 (clone RM4-5, Biolegend, USA) and PE anti-Foxp3 (clone FJK-16S, eBioscience) and acquired on a FACSVerse (BD Biosciences, USA). Absolute cell numbers were enumerated by adding 5000 10mm beads (Spherotech, USA) to each well. Analysis was performed using Flowjo (Treestar, USA). Each set of experiments contained ≥ 16 DMSO controls and ≥ 16 positive controls. Compound effects on the differentiated percentage of a given subset ($\%T_{H(compd)}$) were expressed in units of the standard deviation observed under DMSO treatment (SD DMSO), and normalized to the percentage of the subset differentiated under positive control conditions ($\%T_{H(pos)}$): $[(\%T_{H(compd)} - \%T_{H(DMSO)})/\text{SD DMSO}] \div [(\%T_{H(pos)} - \%T_{H(DMSO)})/\text{SD DMSO}]$ Doses that caused significant toxicity were excluded from analysis.

Heatmap generation

Heatmap was generated for IL-1β inhibition, *Salmonella* autophagy and killing using the Hierarchical Clustering Module of Genepattern (genepattern.broadinstitute.org), in which rows were clustered using Euclidean distance with row normalization; other settings used default values.

Atg16L1 knockdown in Hela Cells

For Atg16L1 knockdown, HeLa cells were transfected in 96 well plates with 5pmol of either non targeting control siRNA or ATG16L1 siRNA (Life Technologies) as previously described *(2)*. 48h post-transfection cells were either harvested for RNA isolation and qRT-PCR to evaluate siRNA knockdown efficiency or infected with bioluminescent Salmonella to quantitate Salmonella survival over time as described in Methods.

Supplementary Tables

Table S1. Initial top-scoring compound sets from CSEA. The leading edge describes the fraction of compounds in a compound set that CSEA identified as contributing to set enrichment. For instance, in the digitalis glycosides set evaluated for GFP+ punctae, 7 out of the 9 set members occurred between ranked list positions 3547 and 3713 (strong negative enrichment). For the digitalis glycosides evaluated in the autolysosome distribution, 7 out of the 9 set members occurred between ranked list positions 1 and 195 (strong positive enrichment). Compounds that did not show enhanced autophagic flux (by LC3-II Western blot) are listed at the bottom of the table.

Supplementary Data file S1: Small molecule membership for compound sets identified by CSEA.

Supplementary Figures

Fig. S1. Normalized enrichment scores (NES) of top-scoring compound sets by CSEA. NES for GFP+ punctae are plotted along the x-axis, and NES for mCherry+ GFP- (autolysosome) punctae are plotted along the y-axis. These sets were chosen based on CSEA analysis of the initial screen. If a compound set showed enrichment for only GFP+ but not mCherry+ GFP- autolysosomes, then its NES was plotted as zero along the autolysosome axis (and vice versa).

Fig. S2. Western blot analysis for LC3-II. Compounds were tested at 10 μM and 1 μM, and plus or minus the lysosomal inhibitors E64D/pepstatin A. GAPDH loading controls are shown. Each compound is shown with corresponding control lanes from that experiment: DMSO, torin (positive control for enhanced autophagic flux, i.e., increased LC3-II vs. DMSO, and further increase in LC3-II with E64D/pepstatin), and bafilomycin (lysosome inhibitor that prevents autophagosome maturation, i.e., increased LC3-II vs. DMSO but no further increase in LC3-II with E64D/pepstatin).

Fig. S3. Sytox Green assay performed in parallel with IL-1β assay, demonstrating minimal compound toxicity between 1.25 - 5 μM, and modest decrease in viability for certain compounds at 10 μM. Results are shown as fluorescence intensity/cell with error bars indicating S.D. of quadruplicate wells.

Fig. S4. ROS production in BMDM stimulated with LPS and MDP. BMDMs were primed with IFN-γ (100ng/ml) overnight and then stimulated with LPS (3 ng/ml) and MDP (10 μg/ml) in the presence of the indicated compounds at 5 μM for 6 hours. Cells were then stained with CM-

H_2DCFDA (Invitrogen) at a concentration of 5 μM and fluorescence intensity was measured. Values plotted are the mean and standard deviation of four replicates.

Fig S5. Effects of small-molecule enhancers of autophagy on IL-1β production from BMDMs using LPS/ATP stimulation. Compounds were all tested at 5 μM. IL-1β production was stimulated using 4h LPS priming followed by 1h ATP treatment. P-values are shown for the comparison of each compound with control cells stimulated with LPS/ATP in the presence of DMSO ("stim"). "No stim" = cells primed with LPS but not treated with ATP.

Fig. S6. Co-localization of LC3 punctae with DsRed Salmonella, and cell viability at multiple compound doses. A. Fraction co-localization of LC3:*Salmonella* for each compound at doses of 1.25, 2.5, 5 and 10 μM. B. Quantitation of DAPI-labeled cell nuclei in the identical wells as panel A, at multiple compound doses.

Fig. S7. Absence of direct bactericidal effects of compounds in Salmonella bioluminescence assay (in absence of cells). Gentamicin is a positive control for a bactericidal compound. Compound treatment is associated with preserved viability as assessed by bacterial bioluminescence.

Fig. S8 Differing activity of digitalis cardiac glycosides in *Salmonella* viability assay, as assessed by a bioluminescence assay for bacterial viability.

Fig. S9. Representative FACS plots for T cell differentiation assays showing compound effects. The observed percent of the indicated subpopulation is indicated on each plot, for T_{reg} (panel A), T_H17 (panel B), and T_H1 (panel C) subsets.

Fig. S10. *Salmonella* viability in the presence of Atg16L1 knockdown in HeLa cells. A. siRNA against Atg16L1 results in ~80% knockdown by quantitative PCR. B. Only digoxin and strophantine cause a statistically significant decrease in *Salmonella* viability in Atg16L1 knockdown HeLa cells. P values are for the comparison with DMSO treated knockdown cells.

Figure S11. Chemical structures of compounds shown in Figure 7a.

REFERENCES

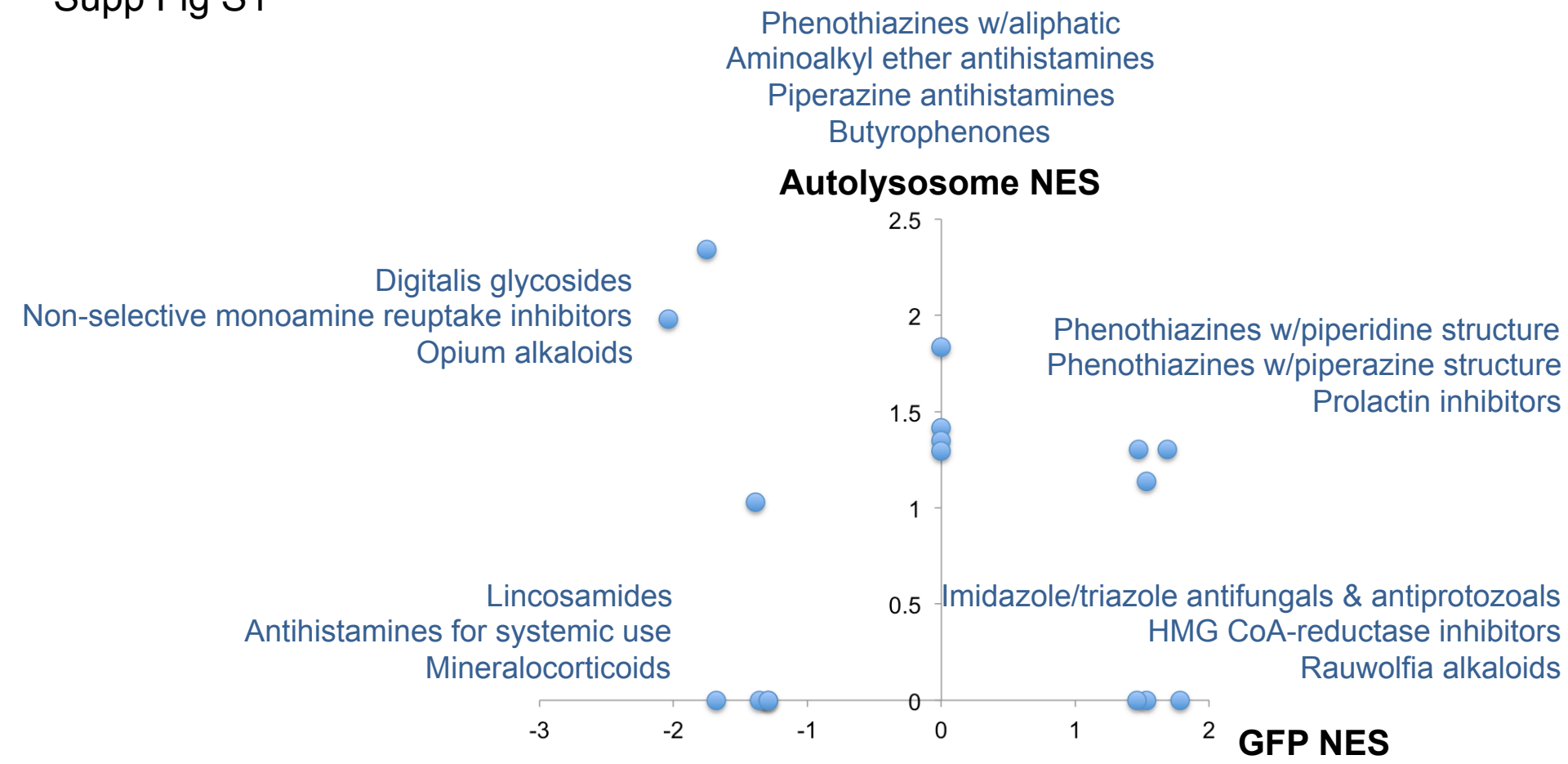
1. Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M. M., Datta, L. W., Shugart, Y. Y., Griffiths, A. M., Targan, S. R., Ippoliti, A. F., Bernard, E. J., Mei, L., Nicolae, D. L., Regueiro, M., Schumm, L. P., Steinhart, A. H., Rotter, J. I., Duerr, R. H., Cho, J. H., Daly, M. J., and Brant, S. R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis, *Nat Genet 39*, 596-604.
2. Kuballa, P., Huett, A., Rioux, J. D., Daly, M. J., and Xavier, R. J. (2008) Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant, *PLoS One 3*, e3391.
3. Carpenter, A. E., Jones, T. R., Lamprecht, M. R., Clarke, C., Kang, I. H., Friman, O., Guertin, D. A., Chang, J. H., Lindquist, R. A., Moffat, J., Golland, P., and Sabatini, D. M. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes, *Genome Biol 7*, R100.

SUPPLEMENTAL TABLE S1

Set	Enrichment	Leading Edge (fraction of set above or below a rank list position) (Total 3713 compds)	p-value	Representative compound
Digitalis glycosides	GFP down	7/9 @ 3547	< 0.001	Digoxin
Digitalis glycosides	Autolysosome up	7/9 @ 195	0.004	Digoxin
Mineralocorticoids	GFP down	4/4 @ 3295	0.003	Fludrocortisone
Opium alkaloids	GFP down	2/4 @ 3560	0.089	Noscapine
Piperazine phenothiazines	GFP up	7/10 @ 510	0.049	Prochlorperazine
Piperidine phenothiazines	GFP up	4/5 @ 314	0.064	Thioridazine
Piperidine phenothiazines	Autolysosome up	5/5 @ 1457	0.163	Thioridazine
Aliphatic phenothiazines	Autolysosome up	4/4 @ 178	< 0.001	Chlorpromazine
Rauwolfia alkaloids	GFP up	2/2 @ 29	0.002	Reserpine
Prolactin inhibitors	GFP up	2/5 @ 44	0.005	Metergoline
Prolactin inhibitors	Autolysosome up	4/5 @ 390	0.175	Metergoline
Systemic antihistamines	GFP down	9/34 @ 3387	0.061	Clemastine
Piperazine antihistamines	Autolysosome up	2/4 @ 633	0.153	Cetirizine
Butyrophenones	Autolysosome up	3/4 @ 736	0.232	bromperidol
Compounds that did not induce autophagic flux by LC3-II Western blot				
Non-selective monoamine uptake inhibitor antidepressants	GFP down	6/14 @ 3360	0.005	Protriptyline
Non-selective monoamine Uptake inhibitor antidepressants	Autolysosome up	9/14 @ 372	0.004	Protriptyline

Set	Enrichment	Leading Edge (fraction of set above or below a rank list position) (Total 3713 compds)	p-value	Representative compound
Lincosamides	GFP down	2/2 @ 3067	0.138	Clindamycin
Imidazole/Triazole antifungals	GFP up	4/17 @ 55	0.010	Sertaconazole
HMG CoA reductase inhibitors	GFP up	2/5 @ 151	0.029	simvastatin
Aminoalkylether antihistamines	Autolysosome up	3/7 @ 542	0.123	diphenhydramine

Supp Fig S1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Aminoalkyl ether antihistamines
Piperazine antihistamines
Butyrophenones
Autolysosome NES
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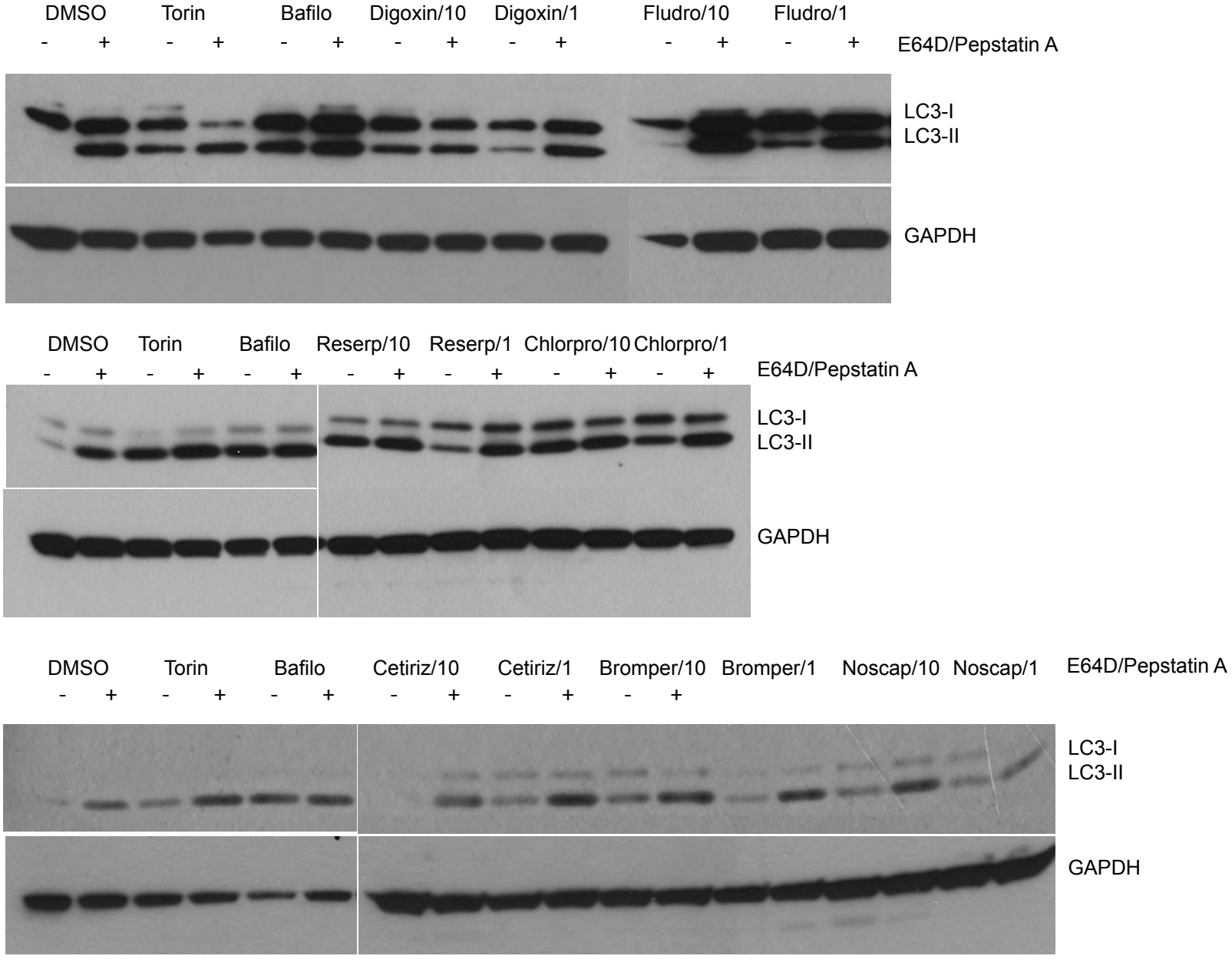
2
1.5
1
0.5
0
-3
-2
-1
0
1
2
GFP NES
Digitalis glycosides
Non-selective monoamine reuptake inhibitors
Opium alkaloids
Phenothiazines w/piperidine structure
Phenothiazines w/piperazine structure
Prolactin inhibitors
Lincosamides
Antihistamines for systemic use
Mineralocorticoids
Imidazole/triazole antifungals & antiprotozoals
HMG CoA-reductase inhibitors
Rauwolfia alkaloids

Supp Fig S2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Torin
Bafilo
Digoxin/10
Digoxin/1
Fludro/10
Fludro/1
- + - + - + - + - + - + - + E64D/Pepstatin A
LC3-I
LC3-II
GAPDH
DMSO
Torin
Bafilo
Reserp/10
Reserp/1
Chlorpro/10
Chlorpro/1
- + - + - + - + - + - + - + E64D/Pepstatin A
LC3-I
LC3-II
GAPDH
DMSO
Torin
Bafilo
Cetiriz/10
Cetiriz/1
Bromper/10
Bromper/1
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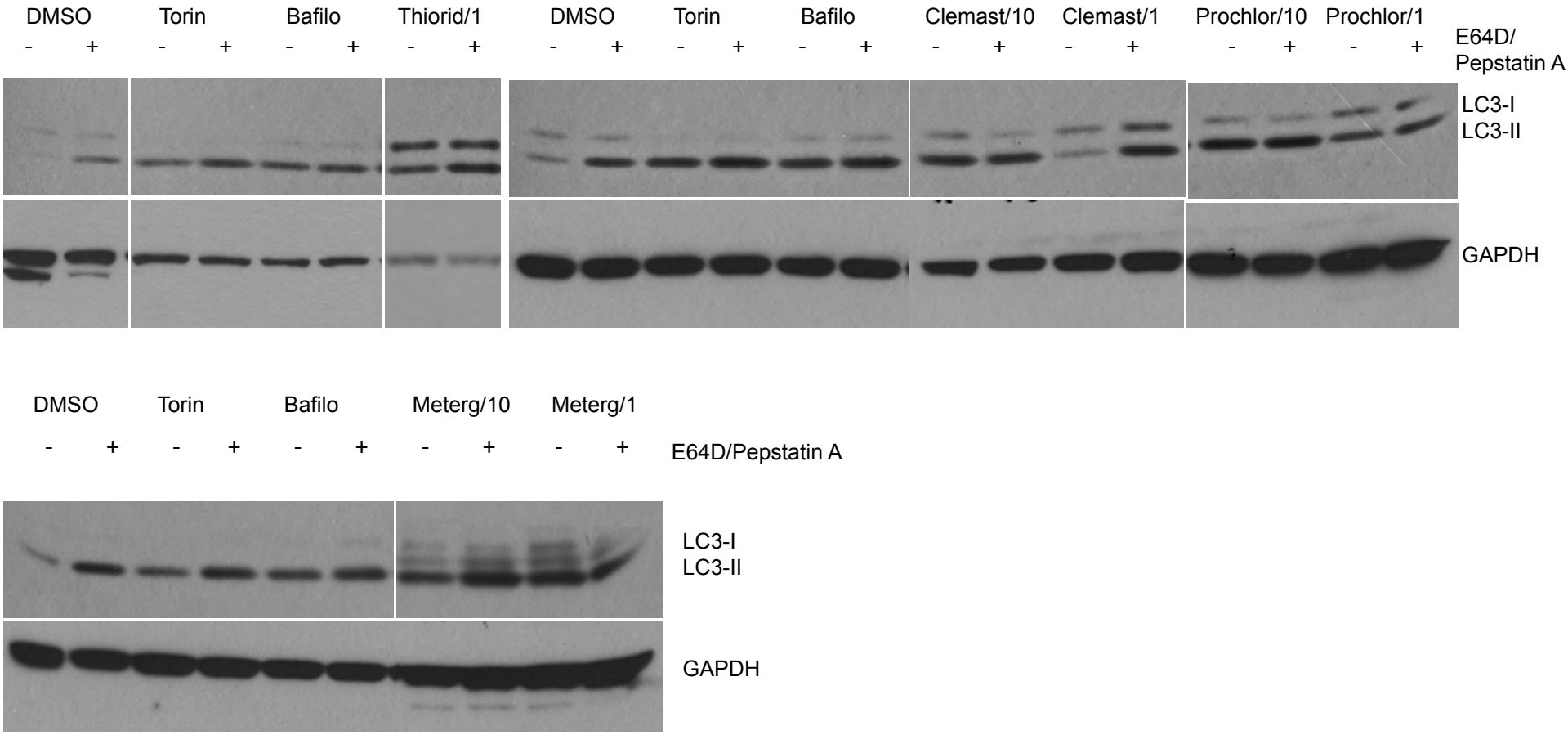
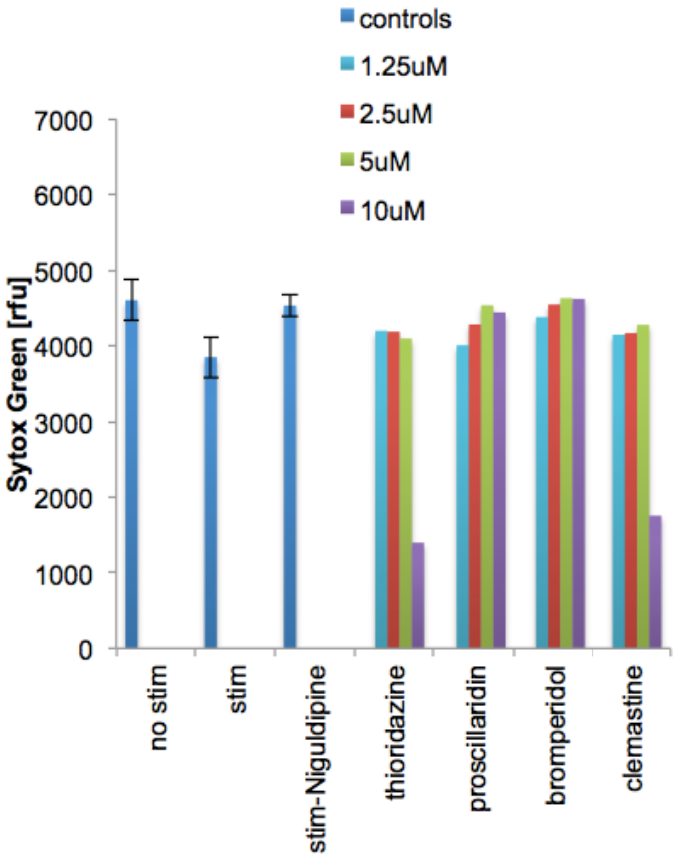
LC3-I
LC3-II
GAPDH

Supp Fig S2 (cont.)



Supp Fig S3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2.5uM
5uM
10uM
Sytox Green [rfu]
7000
6000
5000
4000
3000
2000
1000
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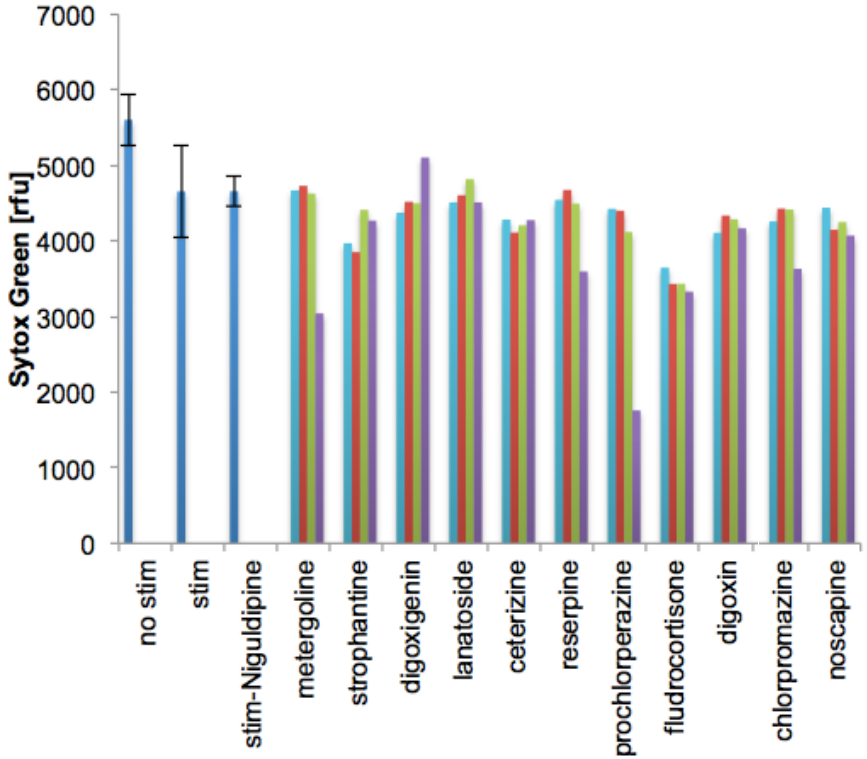
stim
stim-Niguldipine
thioridazine
proscillaridin
bromperidol
clemastine


Sytox Green [rfu]
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6000
5000
4000
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stim
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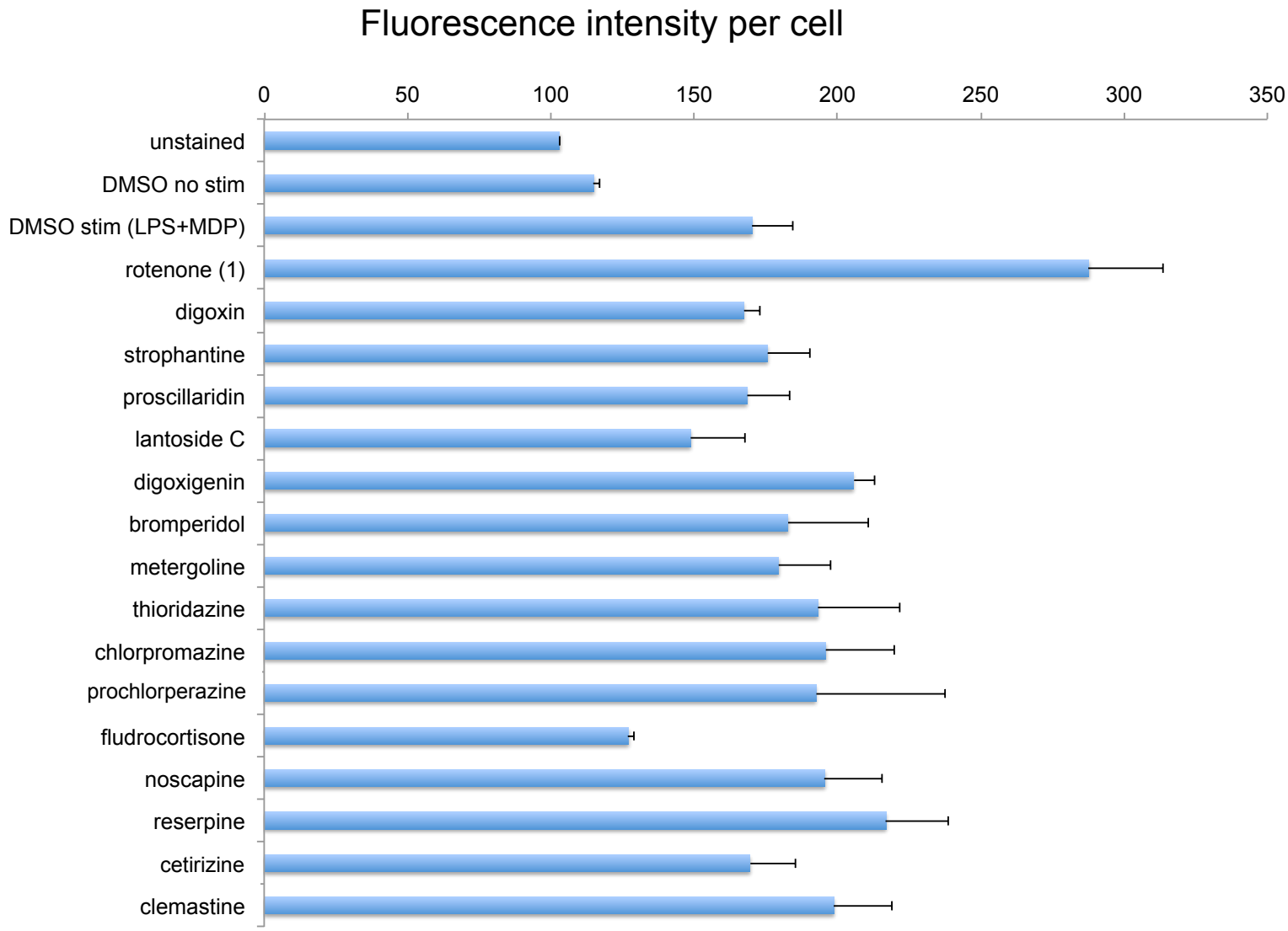
fludrocortisone
digoxin
chlorpromazine
noscapine

Supp. Fig. S4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50
100
150
200
250
300
350
unstained
DMSO no stim
DMSO stim (LPS+MDP)
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digoxin
strophantine
proscillaridin
lantoside C
digoxigenin
bromperidol
metergoline
thioridazine
chlorpromazine
prochlorperazine
fludrocortisone
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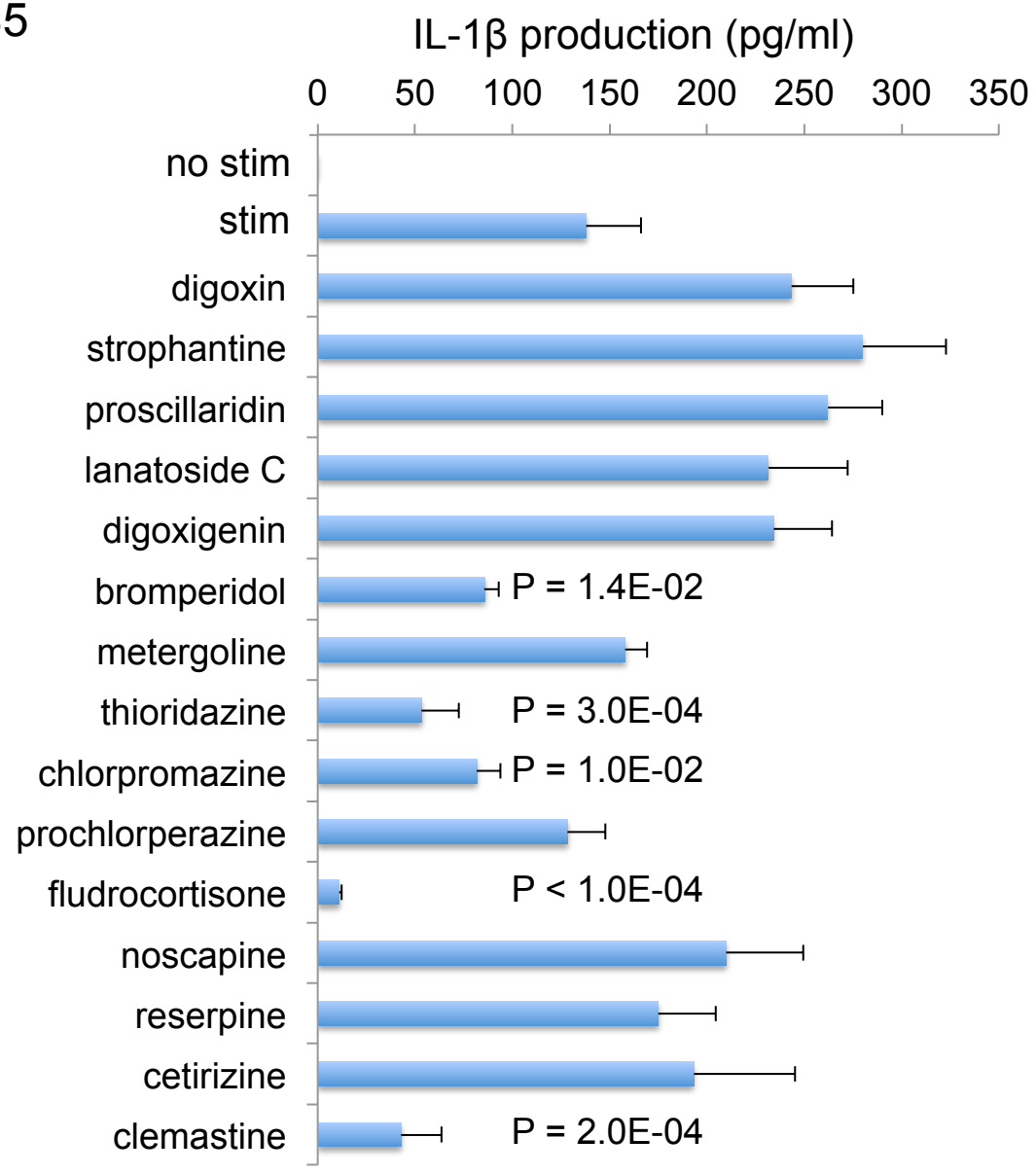
reserpine
cetirizine
clemastine

Supp Fig S5


IL-1β production (pg/ml)
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50
100
150
200
250
300
350
no stim
stim
digoxin
strophantine
proscillaridin
lanatoside C
digoxigenin
bromperidol
P = 1.4E-02
metergoline
thioridazine
P = 3.0E-04
chlorpromazine
P = 1.0E-02
prochlorperazine
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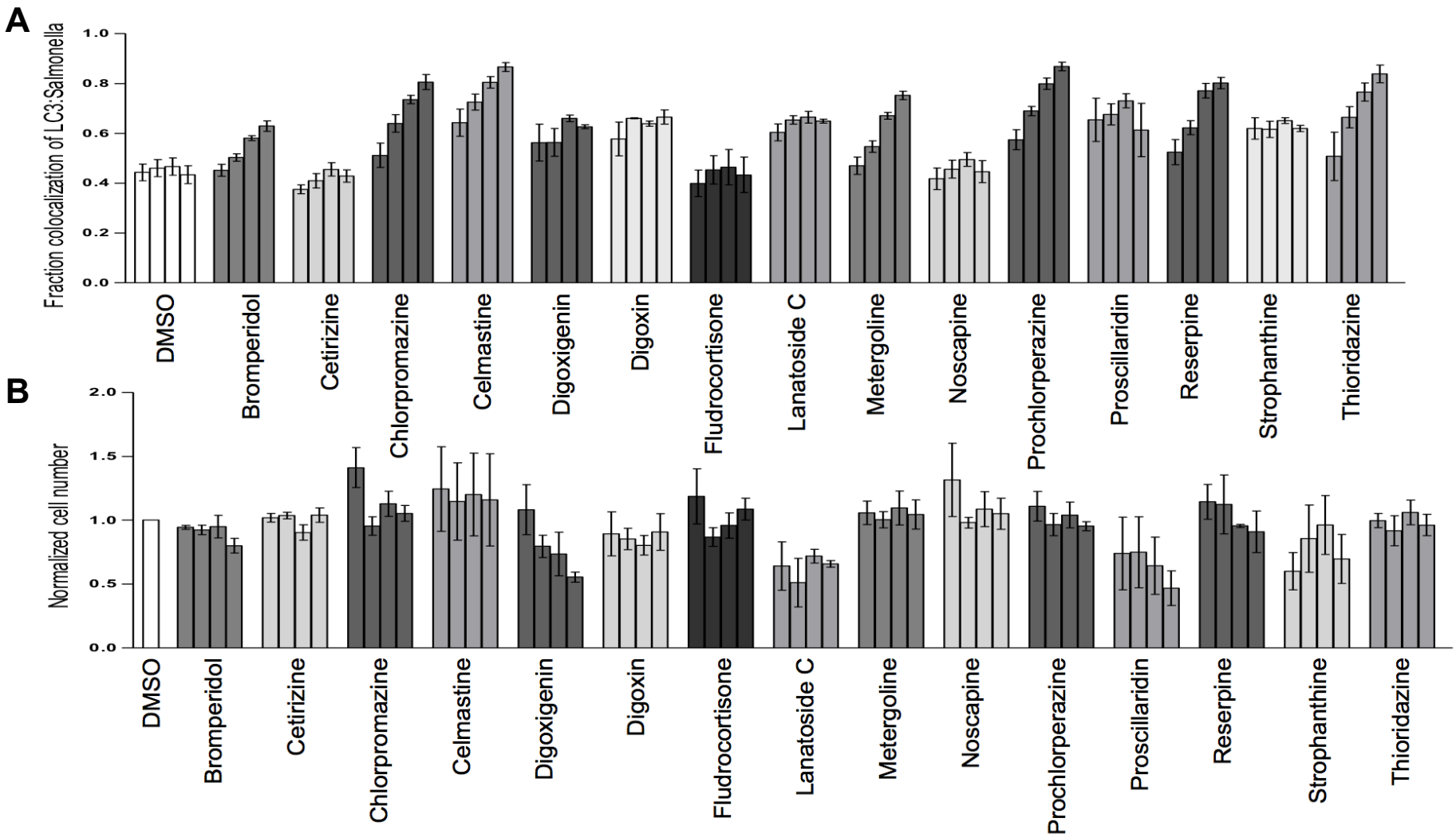
reserpine
cetirizine
clemastine
P = 2.0E-04

Supp Fig S6


A
Fraction colocalization of LC3:Salmonella
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0.6
0.4
0.2
0.0
DMSO
Bromperidol
Cetirizine
Chlorpromazine
Celmastine
Digoxigenin
Digoxin
Fludrocortisone
Lanatoside C
Metergoline
Noscapine
Prochlorperazine
Proscillaridin
Reserpine
Strophanthine
Thioridazine
B
Normalized cell number
2.0
1.5
1.0
0.5
0.0
DMSO
Bromperidol
Cetirizine
Chlorpromazine
Celmastine
Digoxigenin
Digoxin
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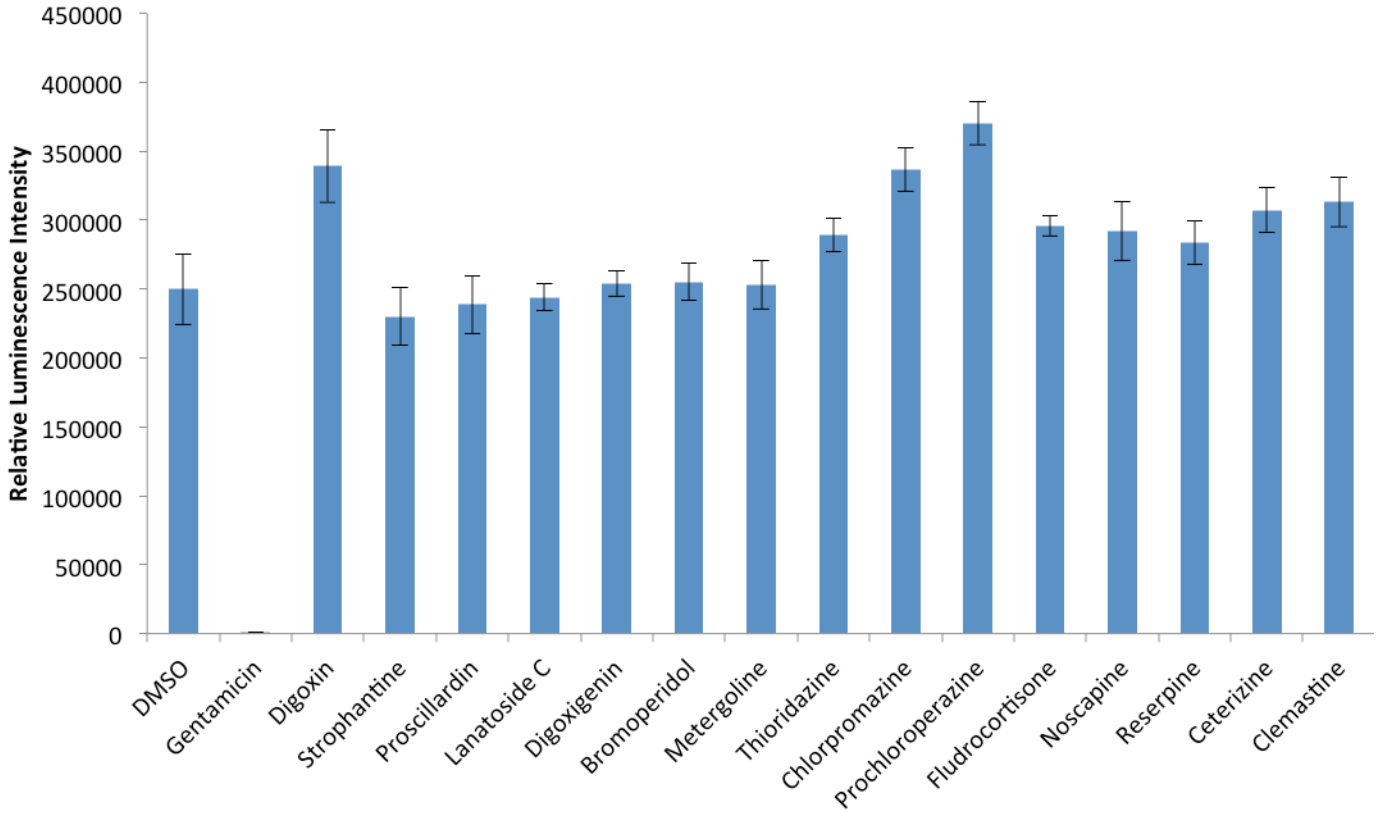
Strophanthine
Thioridazine

Supp Fig S7


Relative Luminescence Intensity
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
DMSO
Gentamicin
Digoxin
Strophantine
Proscillardin
Lanatoside C
Digoxigenin
Bromoperidol
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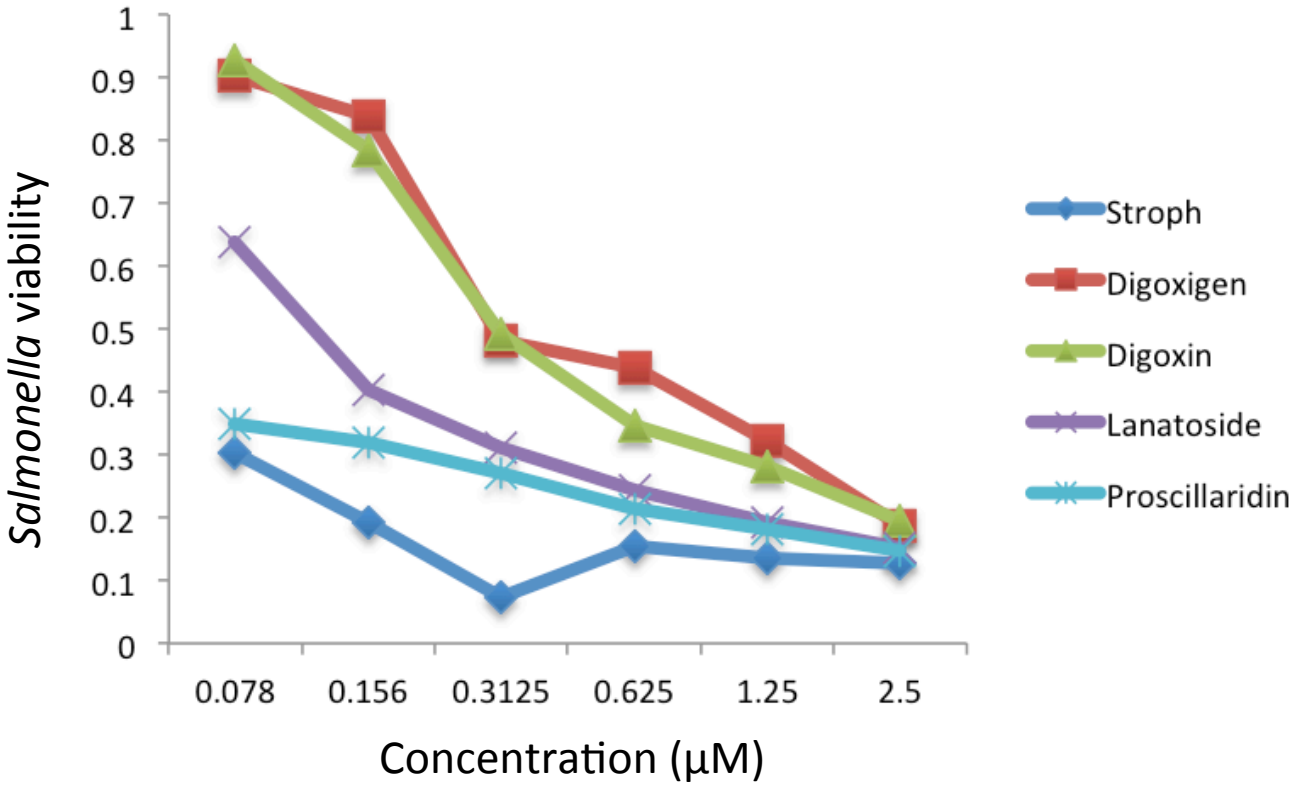
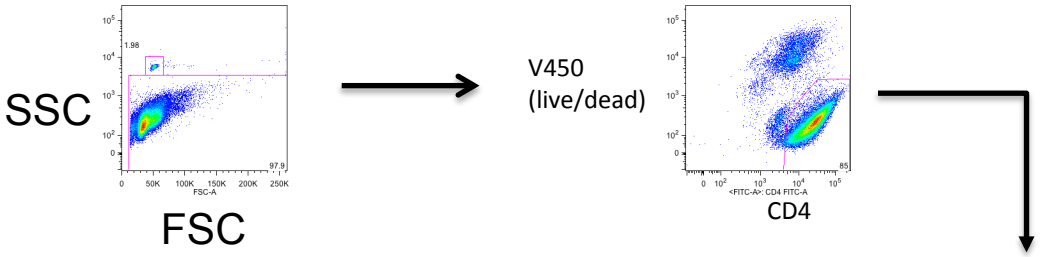
Reserpine
Ceterizine
Clemastine

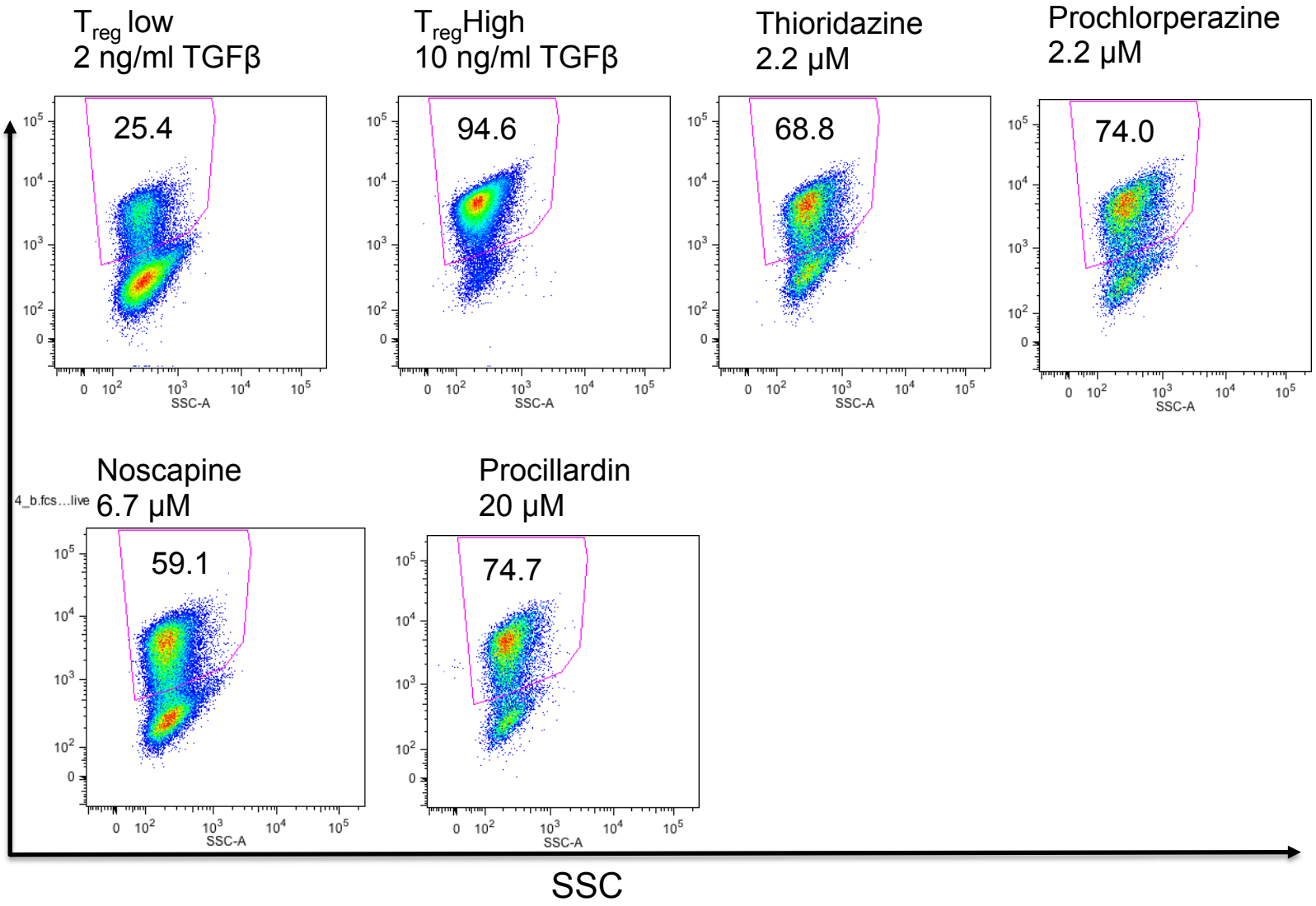
Supp Fig S8


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Salmonella viability
0.078
0.156
0.3125
0.625
1.25
2.5
Concentration (µM)
Stroph
Digoxigen
Digoxin
Lanatoside
Proscillaridin

Supp Fig S9a

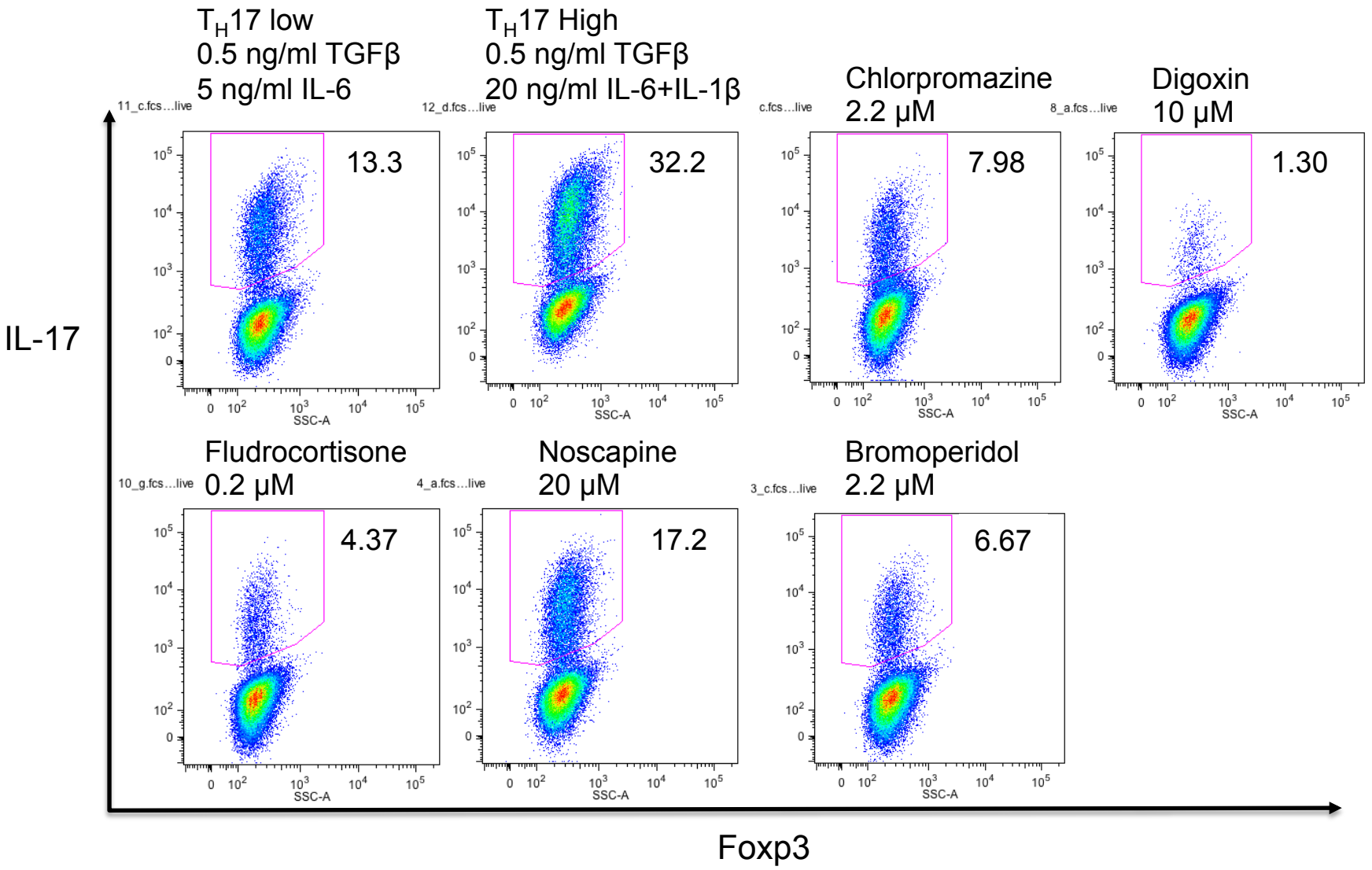
a T_{reg}





Supp Fig S9b

b



Supp Fig S9c

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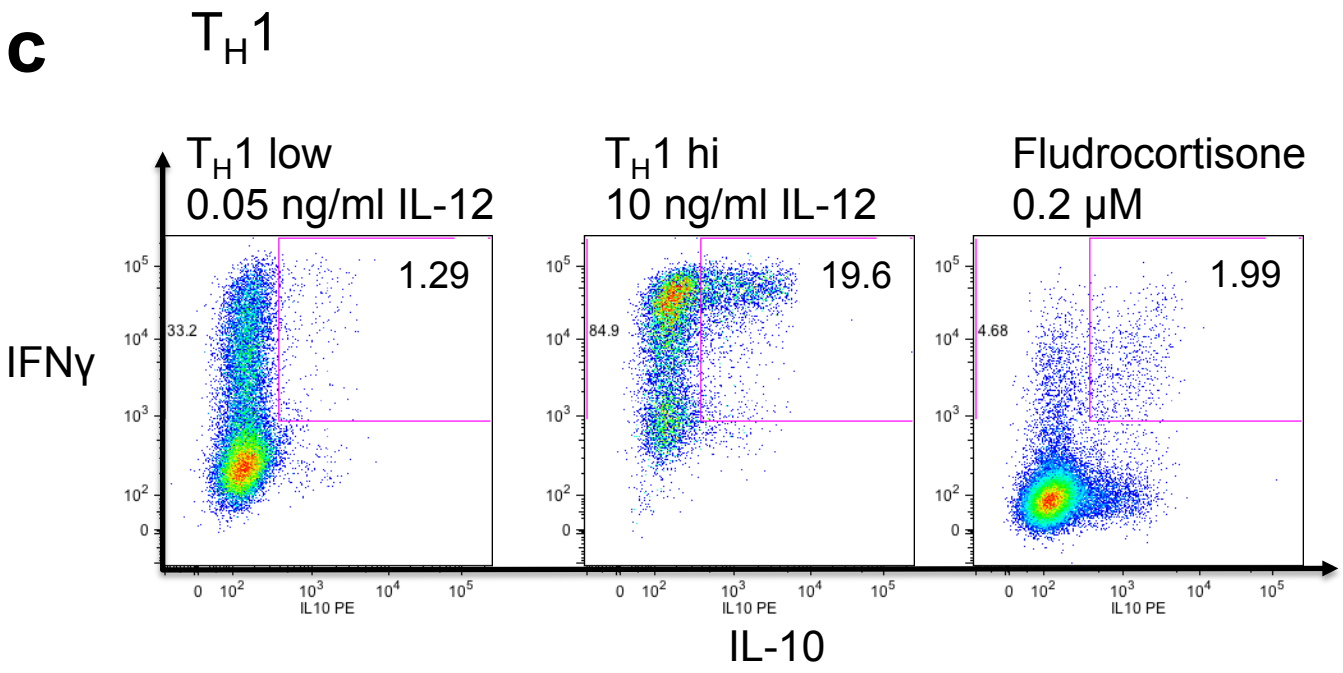
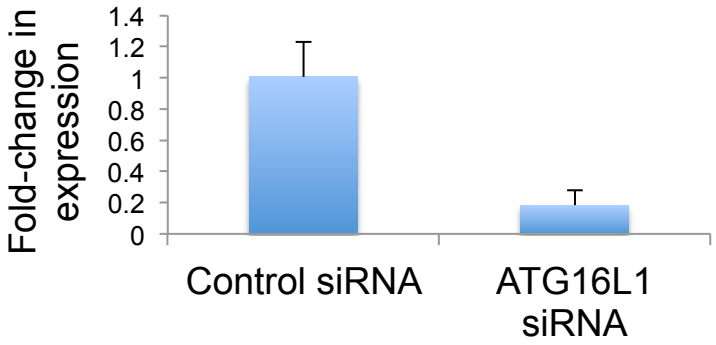
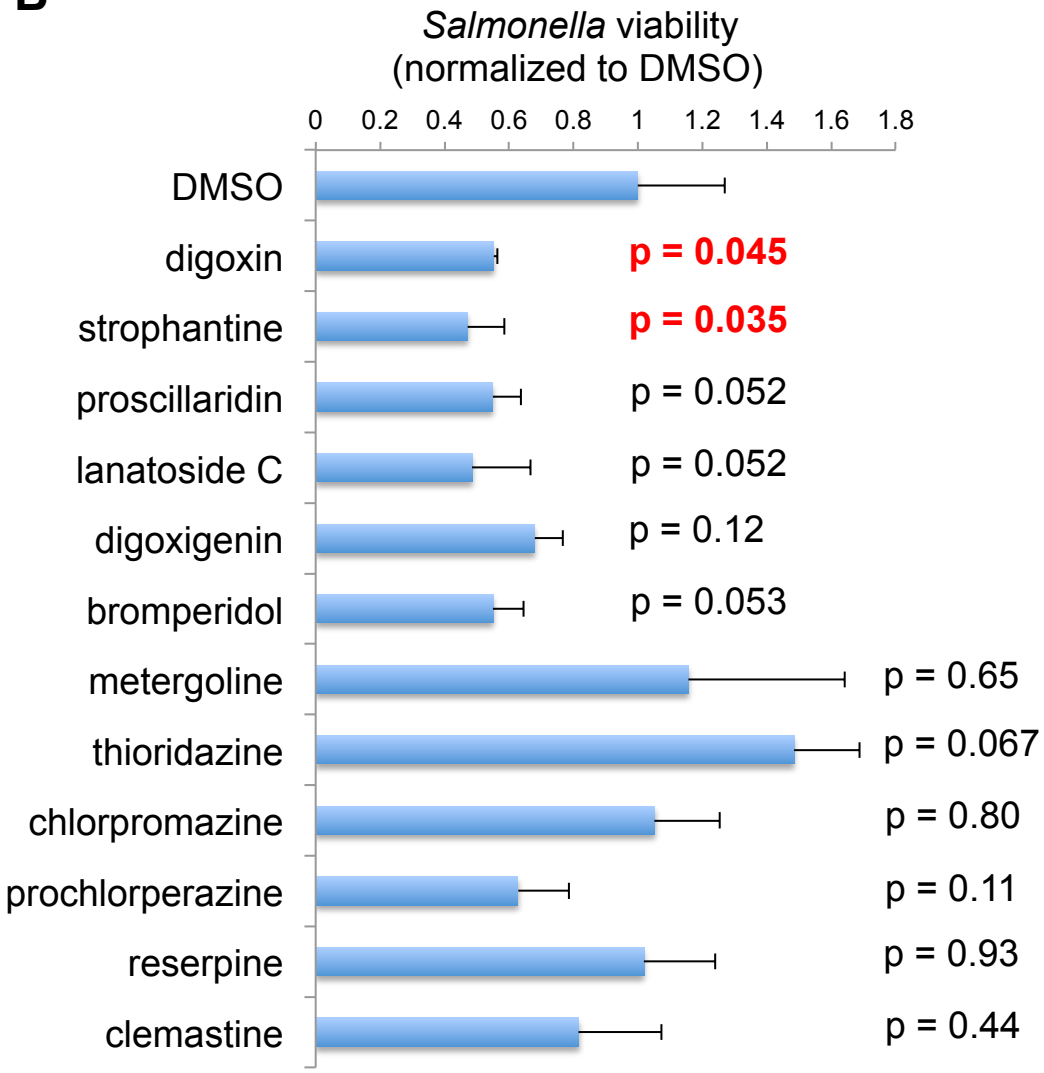


Supp Fig S10

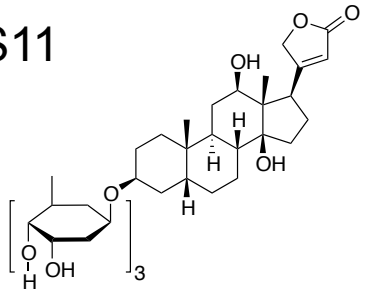
A



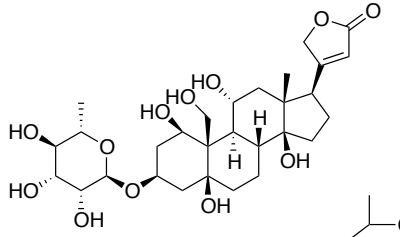
B



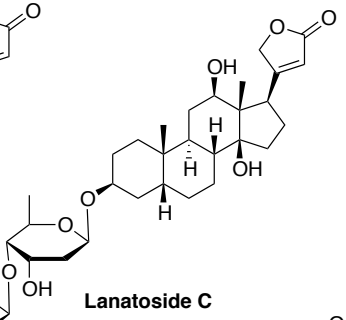
Supp. Fig. S11



Digoxin



Strophanthine



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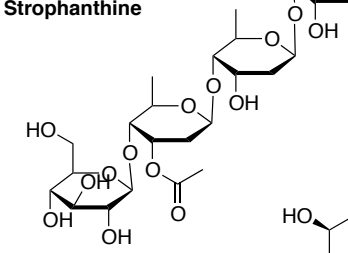
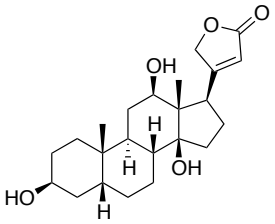
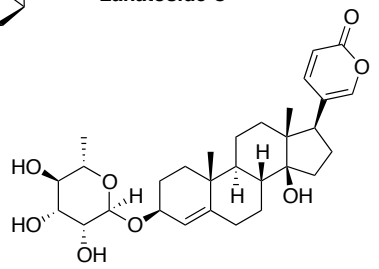
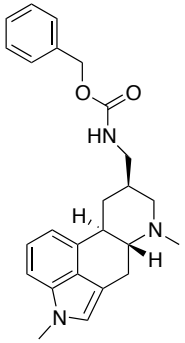




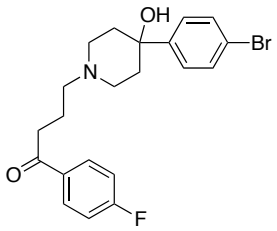
Digoxigenin



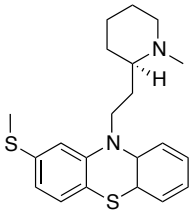
Proscillaridin



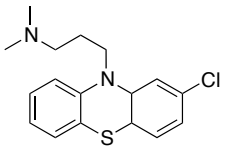
Metergoline



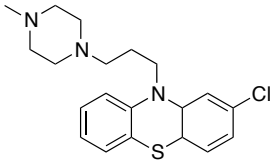
Bromperidol



Thioridazine



Chlorpromazine



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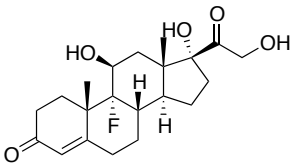
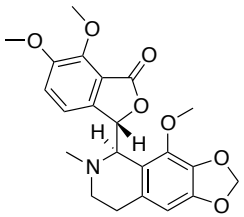
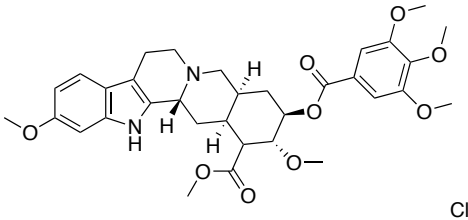


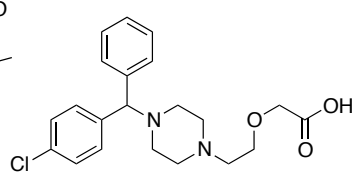
Fludrocortisone



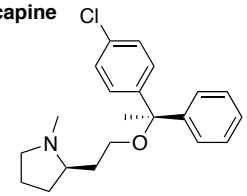
Noscapine



Reserpine



Cetirizine



Clemastine