Supplementary Information for

Screening the ReFrame drug repurposing library to discover new drugs for treating sickle cell disease

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(August 22, 2022)

Description of video of cells sickling. Images were collected every 15 min for 16 hrs, so the 26 second video has been sped up by about a factor of ~2,000. The contrast increase early in the video is due to the change in the absorption spectrum of hemoglobin from oxy (peak at 415 nm) to deoxy (peak at 430 nm, the center of the band pass filter).

Table S1: List of compounds that inhibit sickling at 10 μM[b]

name	IC50 (μM)[a]	name	IC50 (μM)[a]
5,7-Dihydroxyflavone [c]	12 ± 2	Exifone	7.0 ± 1.9
Triclabendazole	13 ± 2	Tolfenamic Acid	11 ± 1
Merbromin [c,d,e]	NA[f]	CGP-35949 [g]	4.4 ± 1.6
Ticlatone [g]	9.5 ± 0.2	Propenidazole	9.7 ± 2.2
Diphenadione [c]	17 ± 5	Onconoxib	4.6 ± 1.6
Bonaphthone [d,e,g]	8.7 ± 1.7	Tesimide	12 ± 2
Pimobendan [g]	11 ± 2.7	BMS-817378 (disputed),BMS-794833 [c,h]	11
Bedaquiline	10 ± 2.5	PH-CBR-2016-001-HVAC-03620-0	4.9 ± 2.5
WBI-1001	6.3 ± 2.2	Alexidine [c,d,e]	NA[f]
Tibenzonium	10 ± 2	TCD-717 [c]	NA[f]
Entrectinib	10 ± 1	K-20160 E	6.3 ± 3.2
MI-773 [d]	4.0 ± 1.9	Wortmannin	10 ± 1.4
Fursultiamine	4.3 ± 2.7	Itanoxone [c]	14 ± 3
ABX-464	8.1 ± 0.7	MK-8141 [c,d,e,g]	9.6 ± 7.12
Exifone	10 ± 2	KF-4939	4.7 ± 2.1
Sepantronium bromide [c]	8.7 ± 5.6	SLV-330 [c]	8.5 ± 3.2
Phanquinone [c]	4.9 ± 2.5	Eseroline	7.6 ± 2.4
Talarozole	14 ± 0.6	Sanguinarium Chloride [c,d,e,g]	7.8 ± 3.2
Voxelotor [d,i]	3.5 ± 1.7	Ethopropazine hydrochloride	9.9 ± 3.1
DRF-3188 [c,d]	7.2 ± 0.8	Niflumic acid [c,h]	4.6
MS-322	6.9 ± 3.5	Cdk1 Inhibitor IV, RO-3306 [c]	NA[f]
Dyclonine HCl	4.0 ± 0.6	Ponalrestat [c,h]	14

Adibendan	12 ± 2.3	NITD609	9.9 ± 3.5
E-3030 [c]	13 ± 0.8	SB 225002	6.5 ± 2.7
Pamicogrel	5.6 ± 2.1	Tribromsalan [c]	8.2 ± 3.5
1045U85 [c]	11 ± 2.9	VE-822	10 ± 1.6
KP-496 [c]	8.8 ± 4.4	COH-29	5.7 ± 2.0
PD 150606	4.6 ± 2.8	Phenothiazine	6.1 ± 2.1

[a]concentration that produces 50% inhibition
[b]lowest concentration of statistically significant inhibition
[c]LIC is anti-sickling for cells from 2 of 3 donors
[d]compound causes lysis at high concentration, anti-sickling at lower concentration
[e]incubated for 8 hours
[f]IC50s could not be determined for these 4 compounds because of an insufficient number of data points higher than 0 % inhibition
[g]IC50s could only be determined for 2 of the 3 donor samples for these compounds because of an insufficient number of data points higher than 0 % inhibition
[h]IC50s could only be determined for 1 of the donor samples for these compounds because of an insufficient number of data points higher than 0 % inhibition

Additional details of screen

Organization of well plates. Corning 384 well plates were shipped to Calibr at Scripps Institute in La Jolla, CA and returned to NIH after spotting 100 picomoles of all 12,657 compounds of the ReFrame library. 10 μL of the sickle trait red cell suspension was added to give a compound concentration of 10 μM. Compounds that reduced sickling were then studied in a second set of experiments that measured the percent inhibition as a function of the compound concentration (i.e., dose response measurements). For these experiments, 9 wells were spotted with 9 concentrations of a compound from 10 femtomoles to 100 picomoles spaced at approximately factors of 3 intervals to produce final concentrations from ~1 nM to 10 μM. Triplicate plates for both the initial screen and dose-response measurements were sent by Calibr in order to assess variation in the inhibitory response from 3 different donors. Wells in rows 2-15 and columns 4-21 of a 384 (16 rows, 24 columns) well plate were spotted with compounds. Cells in rows 2-15 and columns 3 and 22 containing no test compound served as negative controls. Cells in a 170 milli-osmolar PBS buffer in rows 2-15 and columns 2 and 23 containing no test compound served as positive controls. Because of imperfect homogeneity in the control of the gas atmosphere above the well plate in the humidified chamber, the rate of deoxygenation is not exactly the same for every well of the plate, with the rate decreasing with increasing row number. Consequently, only the cell suspensions for the negative and positive controls in the same row and the 2 rows adjacent to the row with the test compound were

considered (or 1 adjacent row for rows 2 and 15). Also, image analysis of the cells in rows 1 and 16 and edge columns 1 and 24 were not considered because of edge effects.

Selection of compounds for dose-response measurements. In the analysis of the first step of the screen at 10 μM, the average of the sickling curves for all 28 negative control wells on the plate were used to determine whether a compound is anti-sickling. Singular value decomposition (SVD) was used to make this determination. Using the $\left(\langle \mathrm{V1_{nc}} \rangle - \mathrm{V1_{cpd}}\right)/\sigma_{\mathrm{nc}} > 3$ criterion from the SVD analysis for statistically significant inhibition, 335 compounds were further studied in dose-response experiments for each one at 9 compound concentrations between ~1 nM and 10 μM, spaced by approximately factors of 3. Of the 335, 62 compounds lysed cells at 10 μM, which were pursued because swelling red cells is a viable therapeutic strategy, so it was important to study these compounds at concentrations less than the 10 μM lysis concentration. A later analysis using only control wells in the same row as the drug wells and the two adjacent rows, dose response measurements for these 335 compounds showed that 192 were false positives. The reason for this is that the sickling curve is sensitive to the rate of deoxygenation, which was later discovered to decrease in the Lionheart humidified chamber as the row number increases, presumably because of imperfect homogeneity in the percent oxygen as a function of time in the atmosphere above the well plate. Adding a cover to the well plate with an 11 by 7 array of 1.7 mm holes spaced 9.0 mm apart improved the homogeneity but did not make it perfectly homogenous. The sickling curves for 12,657 compounds were therefore completely reanalyzed with the more limited number of wells as negative controls, which also uncovered 92 false negatives, i.e., compounds with $\left(\langle \mathrm{V1_{nc}} \rangle - \mathrm{V1_{cpd}}\right)/\sigma_{\mathrm{nc}} > 3.$

The Lionheart microscope was not designed to be optimal for our assay. In addition to the lack of homogeneity in the time dependence of the % oxygen in the humidified chamber of the Lionheart instrument, a troublesome defect was the laser auto-focusing cube, which was not stable in many experiments. We suspect that a lens mount in the cube moves when the nitrogen flow is high, as it is at the start of the experiment, but then returns to its original position when the nitrogen flow is slowed considerably upon obtaining the final desired percent oxygen. Consequently, the first 5-10 images from each well can become badly out of focus before the focus returns to its original setting at the start of the nitrogen flow. For this reason, the dose response measurements had to be repeated on 70 of the compounds with de-focused images.

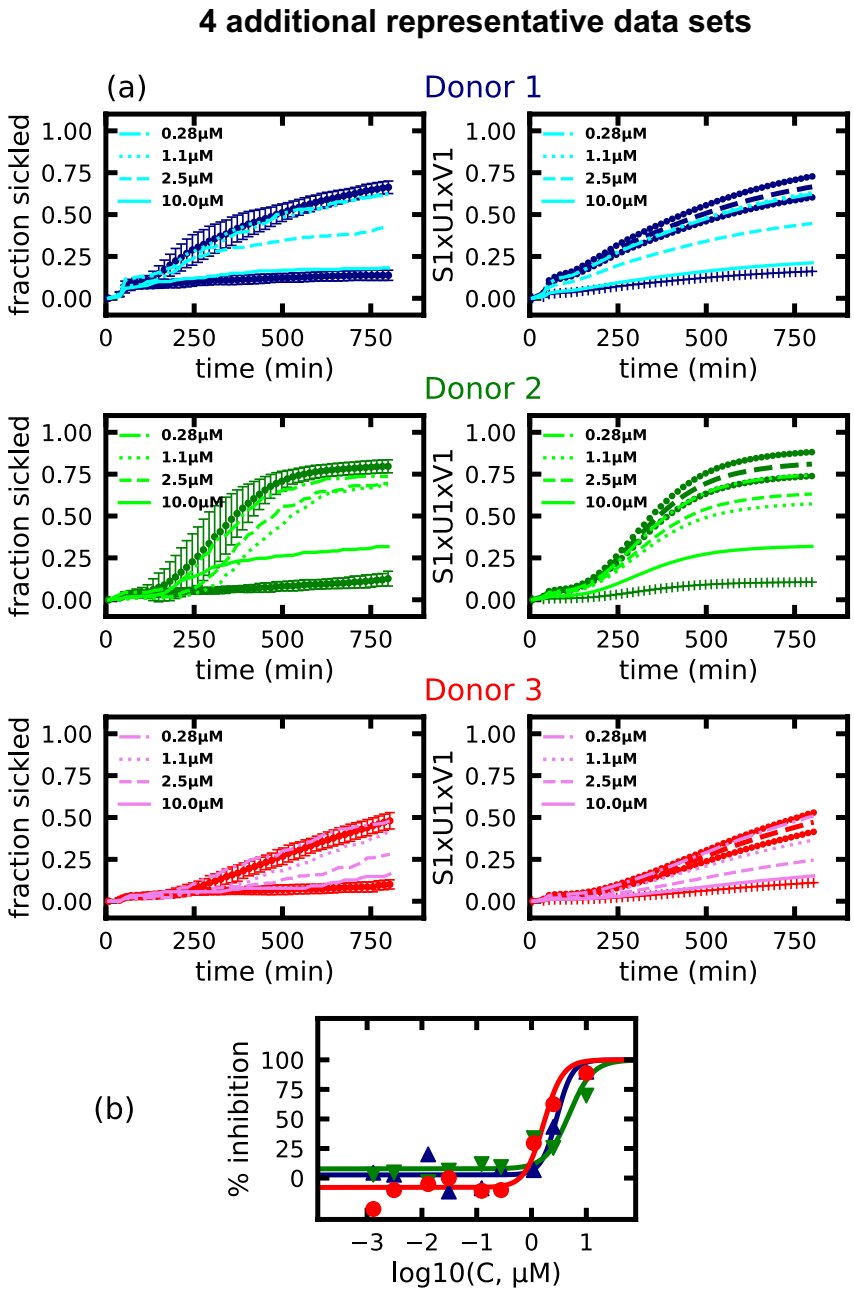


Figure S1. Representative dose response data for ReFrame compound TS-80 (a) Time course of the fraction sickled following the start of deoxygenation with nitrogen to induce HbS polymerization and sickling. Sickling curves are shown at four different concentrations of TS-80. Panels on the left are the measured sickling curves, while panels on the right show the curves constructed using only the first component of the SVD.The light blue, light green, and light pink curves (solid, large dash, small dash, and large/small dash) in both left and right panels are the sickling curves for the single wells containing the test compound. The three colors represent samples from three different sickle trait donors with hemoglobin compositions as percentage of total hemoglobin: Donor 1 (blue): 59.8 %HbA, 35.5 %HbS, 3.8 %HbA2, <1.0 %HbF, MCHC 30.0 g/dL; Donor 2 (green): 59.8 %HbA, 36.4 %HbS, 3.6 %HbA2, <1.0 %HbF, MCHC 28.5 g/dL; Donor 3 (red): 58.4 %HbA, 38.0 %HbS, 3.3 %HbA2, <1.0 %HbF, MCHC 26.9 g/dL. In the left panels, the upper darker blue, green, and red points are the average for 12 negative controls (no compound) and standard deviations from the average, while the lower darker blue, green, and

red points are the average for 6 positive controls (osmotically swollen cells) and standard deviations from the average. In the right panels, the darker blue, green, and red dashed curves are the average sickling curves (S1xU1xV1) of the negative controls with standard deviations indicated by the dotted curves of the same color. The lower points (+) are the positive control averages. (b) Points are measured percent inhibition defined by equation (1) for cells from 3 different donors, while the continuous curves are the fits to the data using equation (2) (see **Methods** section for equations).

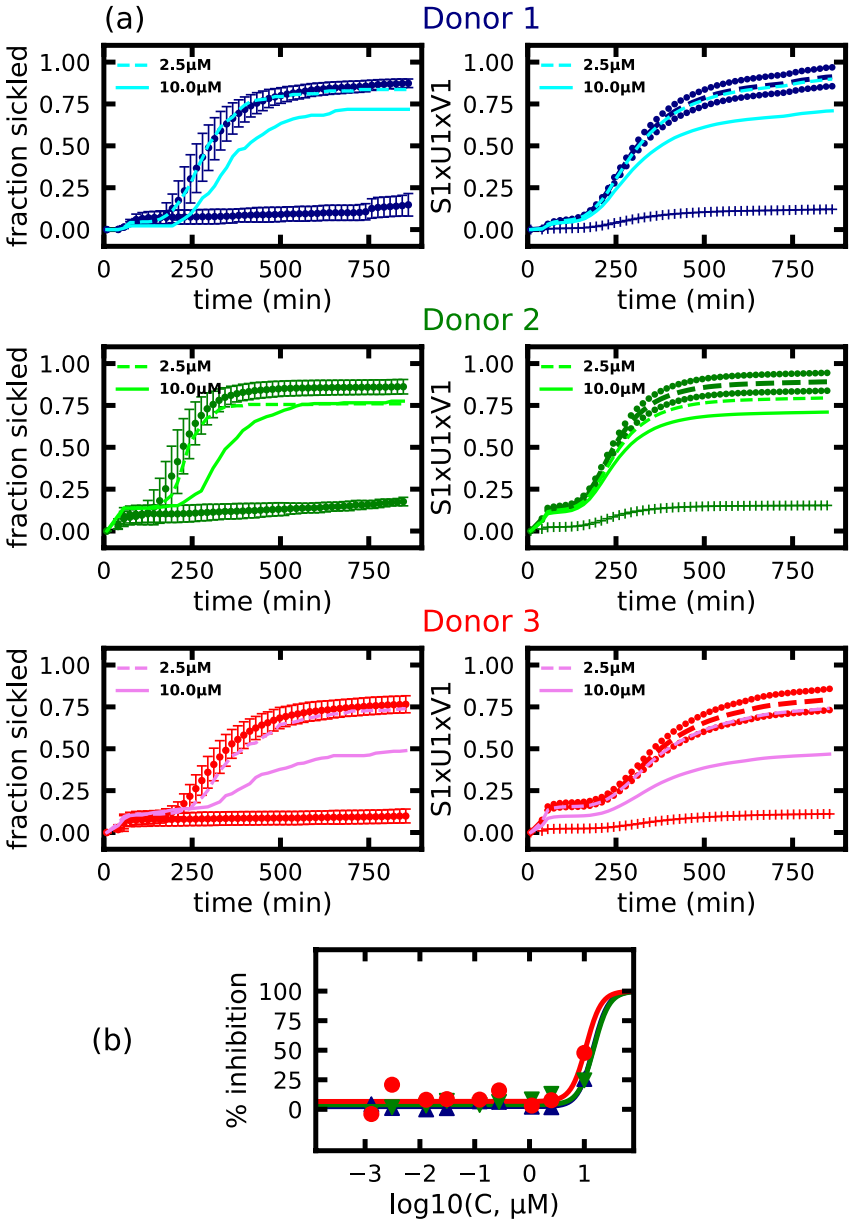


Figure S2. Representative dose response data for ReFrame compound triclabendazole The hemoglobin compositions are: blue donor: 56.1 %HbA, 40 %HbS, 3.7 %HbA2, <1.0 %HbF, MCHC 29.5 g/dL; green donor: 62.7 %HbA, 33.4 %HbS, 3.5 %HbA2, <1.0 %HbF, MCHC 27.6 g/dL; red donor: 59.8 %HbA, 36.4 %HbS, 3.6 %HbA2, <1.0 %HbF, MCHC 28.5 g/dL. Remainder of legend is same as for Figure S1.

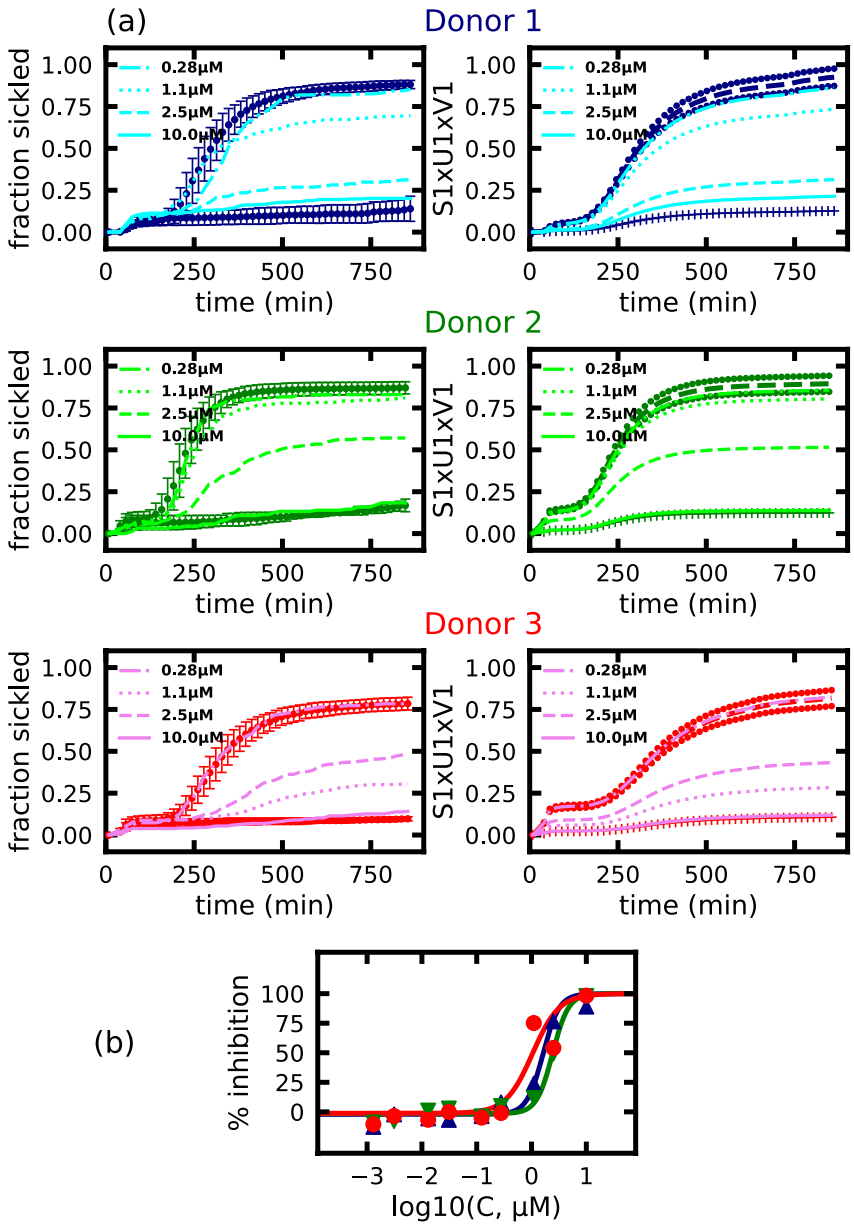


Figure S3. Representative dose response data for ReFrame compound cyclovalone. The hemoglobin compositions are: blue donor: 59.8 %HbA, 35.5 %HbS, 3.8 %HbA2, <1.0 %HbF, MCHC 30.0 g/dL; green donor: 59.8 %HbA, 36.4 %HbS, 3.6 %HbA2, <1.0 %HbF, MCHC 28.5 g/dL; red donor: 58.4 %HbA, 38.0 %HbS, 3.3 %HbA2, <1.0 %HbF, MCHC 26.9 g/dL. Remainder of legend is same as for Figure S1.

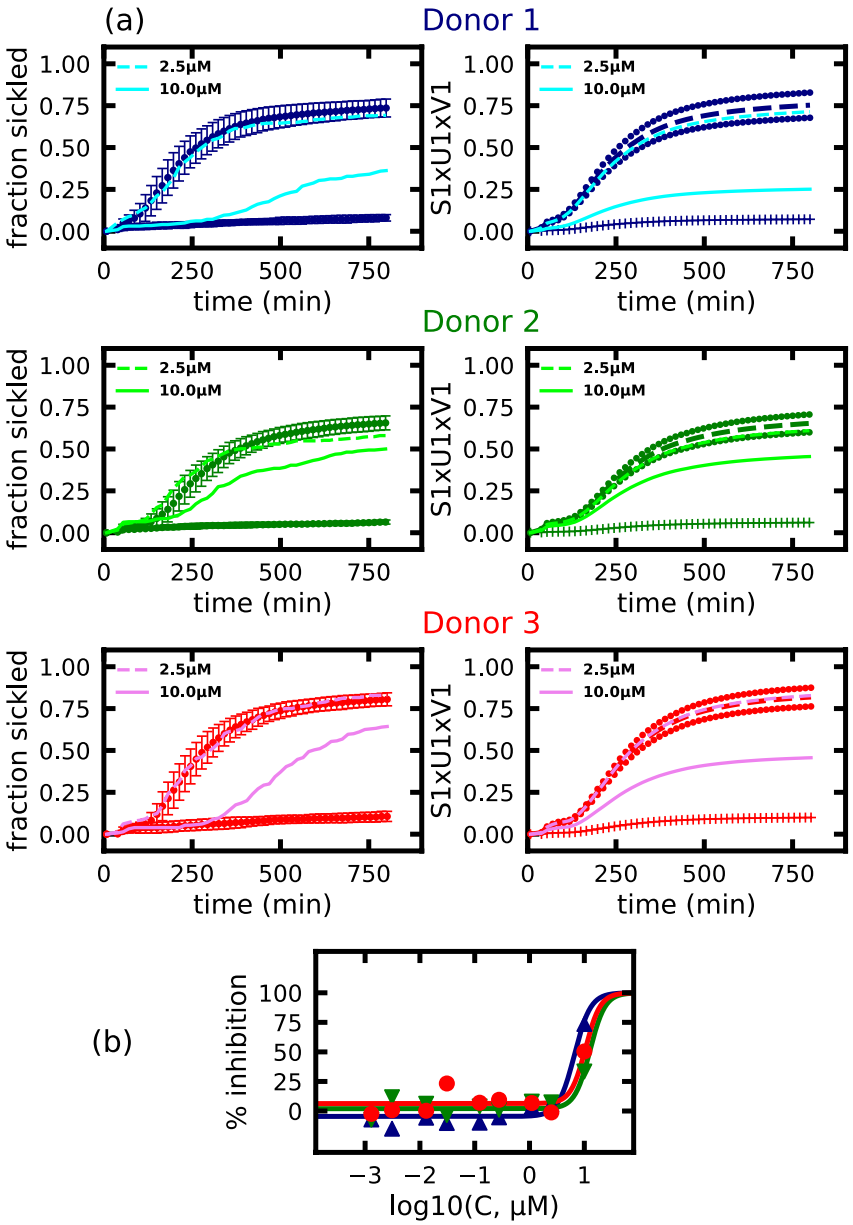


Figure S4. Representative dose response data for ReFrame compound ethopropazine hydrochloride. The hemoglobin compositions are: blue donor: 59.8 %HbA, 35.5 %HbS, 3.8 %HbA2, <1.0 %HbF, MCHC 30.0 g/dL; green donor: 59.7 %HbA, 37.8 %HbS, 3.8 %HbA2, <1.0 %HbF, MCHC 26.9 g/dL; red donor: 58.1 %HbA, 37.6 %HbS, 3.6 %HbA2, <1.0 %HbF, MCHC 27.8 g/dL. Remainder of legend is same as for Figure S1.

The compounds listed in Table S2 showed lysis at 10 µM but no anti-sickling at a lower concentration. They should be further investigated after 24 hrs of pre-incubation before the start of deoxygenation.

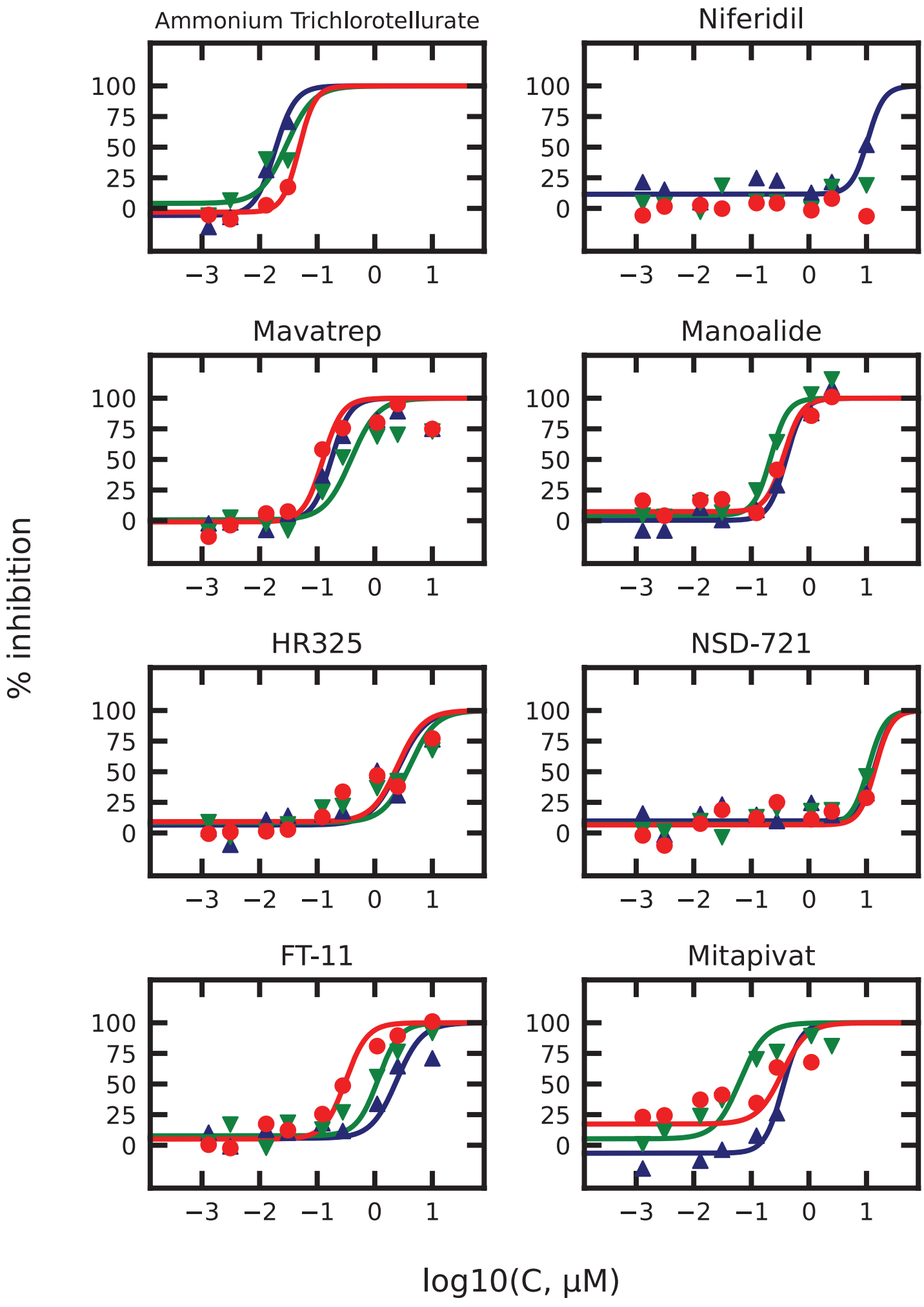
Table S2. List of compounds that lyse cells at 10 μM but do not inhibit sickling at lower concentrations

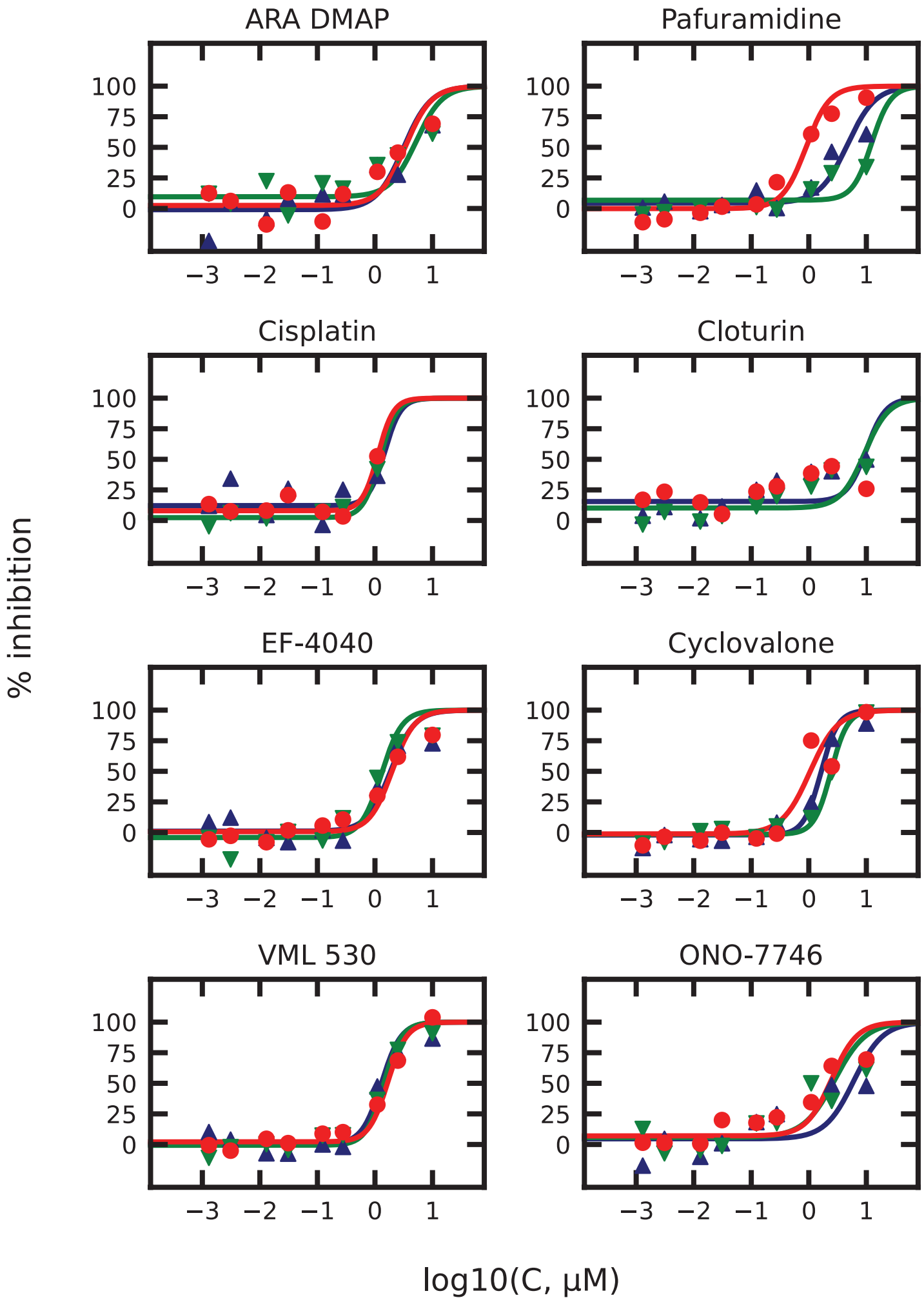
Alectinib Hydrochloride	Provecta [a]	R-213129	Fendosal
Anidulafungin	AGN-194310	AGN 203818	PLD-147
Tannic Acid	OT 4003	Xanthohumol	Purvalanol A
ST-1859	OT 4003 [a]	Cethexonium Bromide	Mercuric Chloride
Ebselen	Mercufenol Chloride [a]	Mecetronium Ethylsulfate	Elacestrant (dihydrochloride)

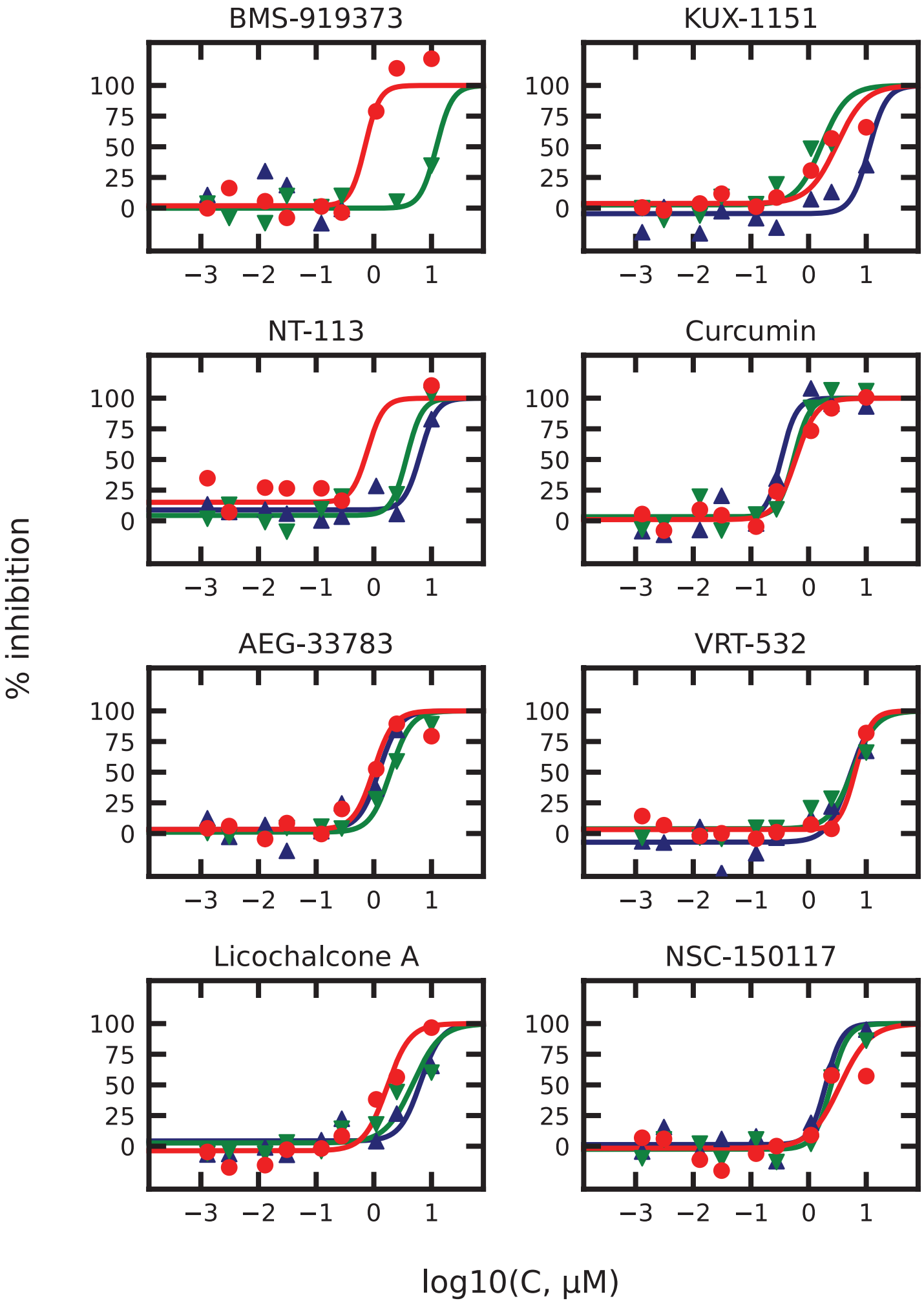
[a]Incubated for 8 hours

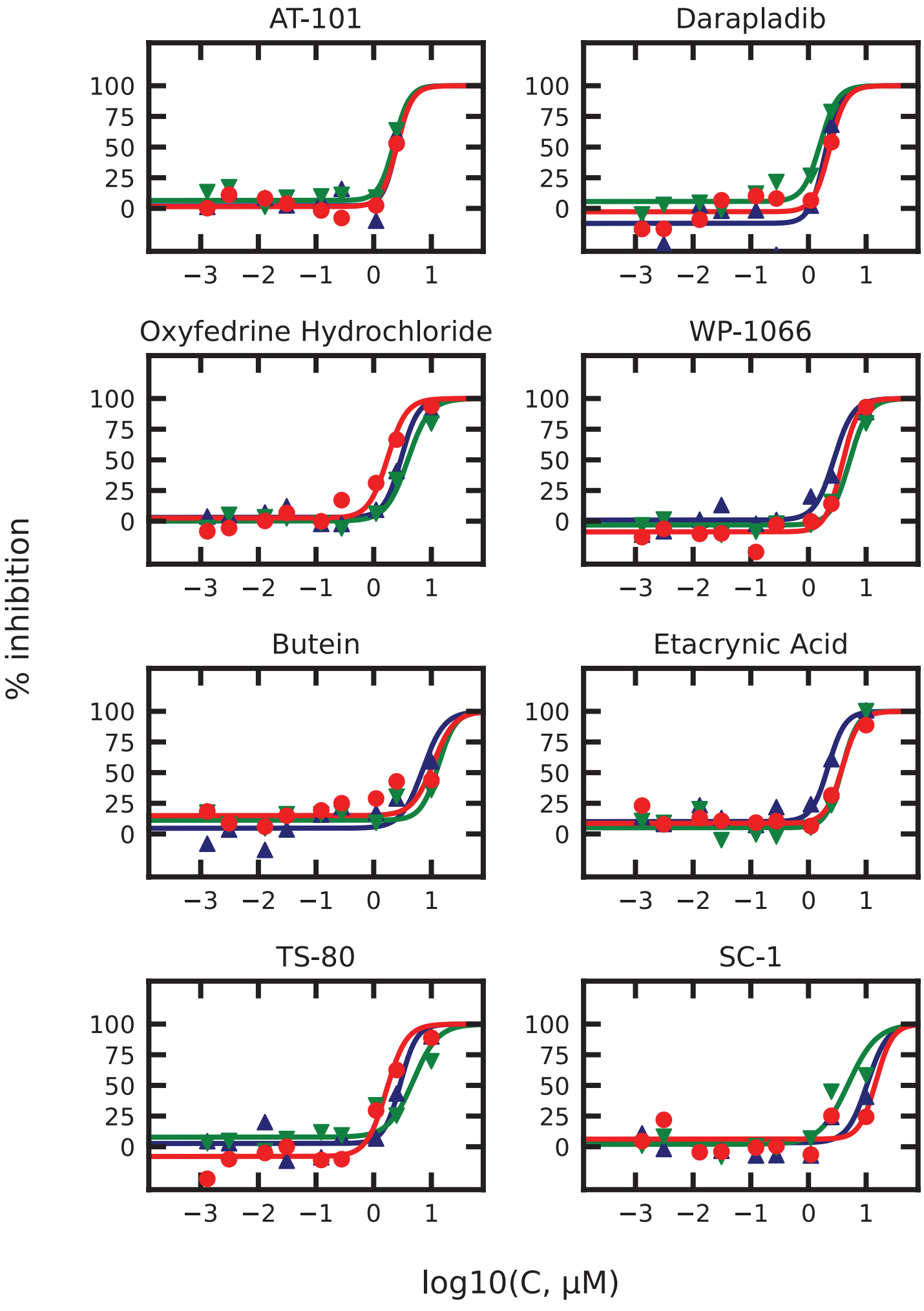
The following pages are the dose response plots for 99 of the 106 anti-sickling compounds

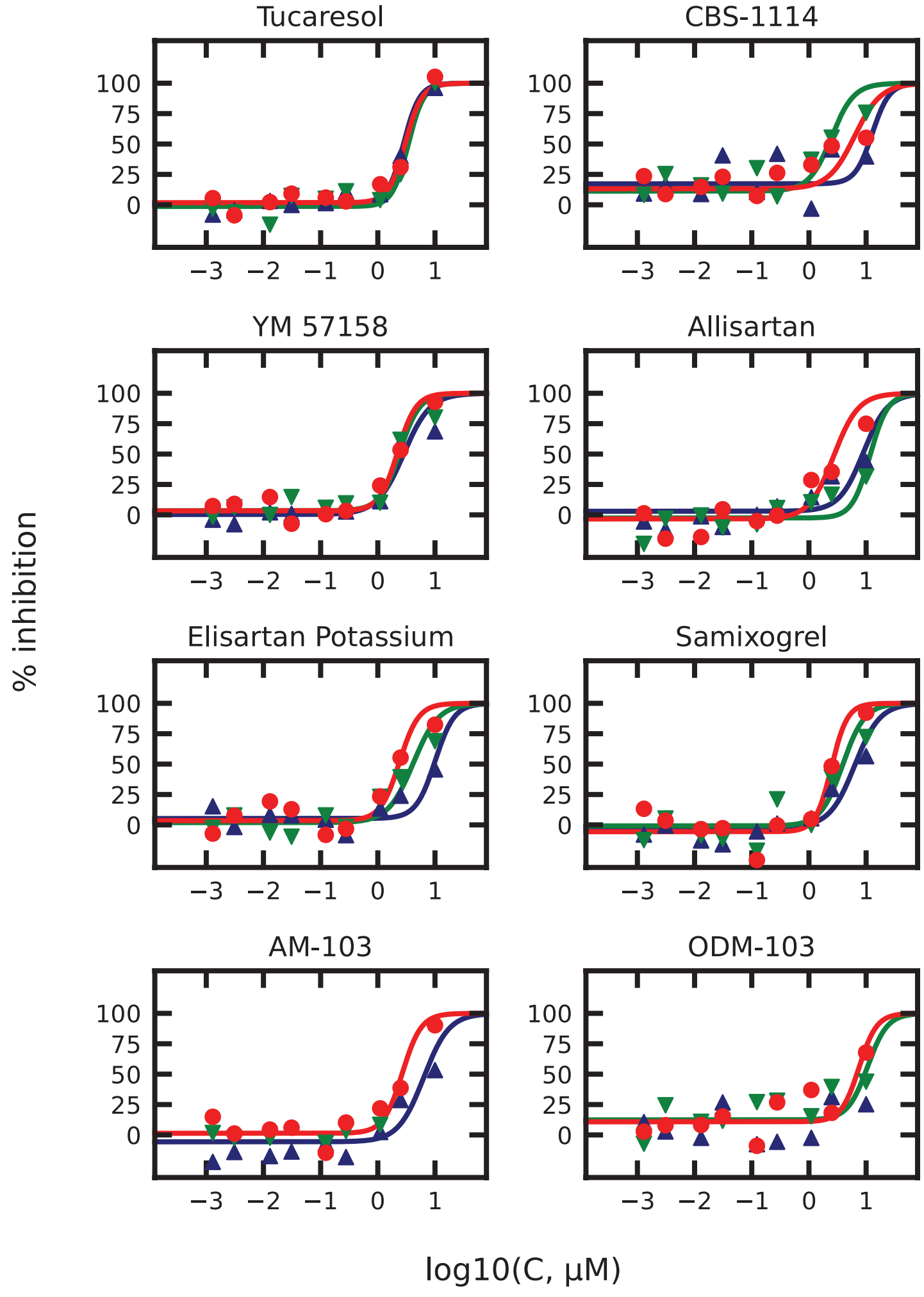
(see footnote d in Table 1 of main text and in Table S1)

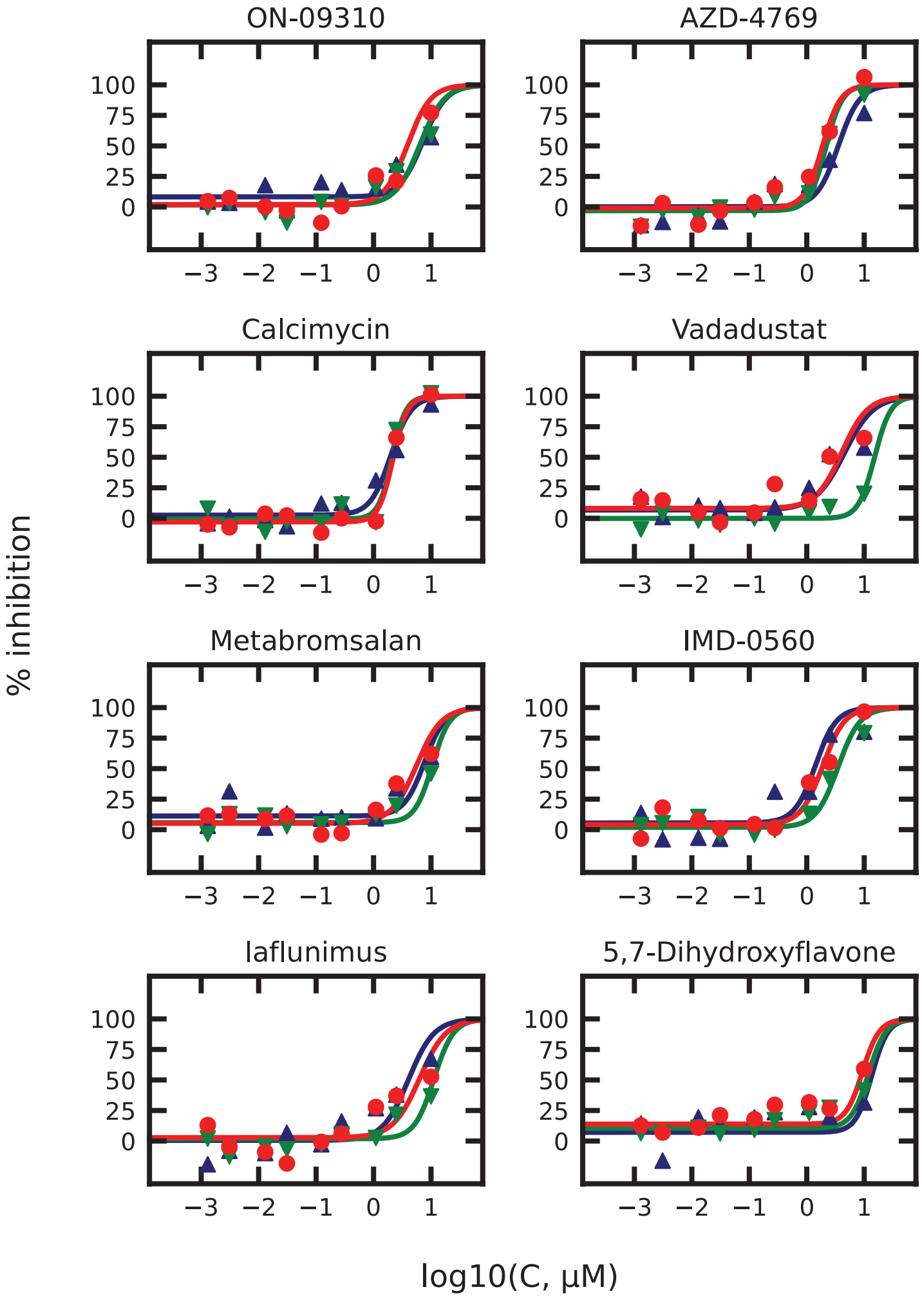

Ammonium Trichlorotellurate
Niferidil
Mavatrep
Manoalide
HR325
NSD-721
FT-11
Mitapivat
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1

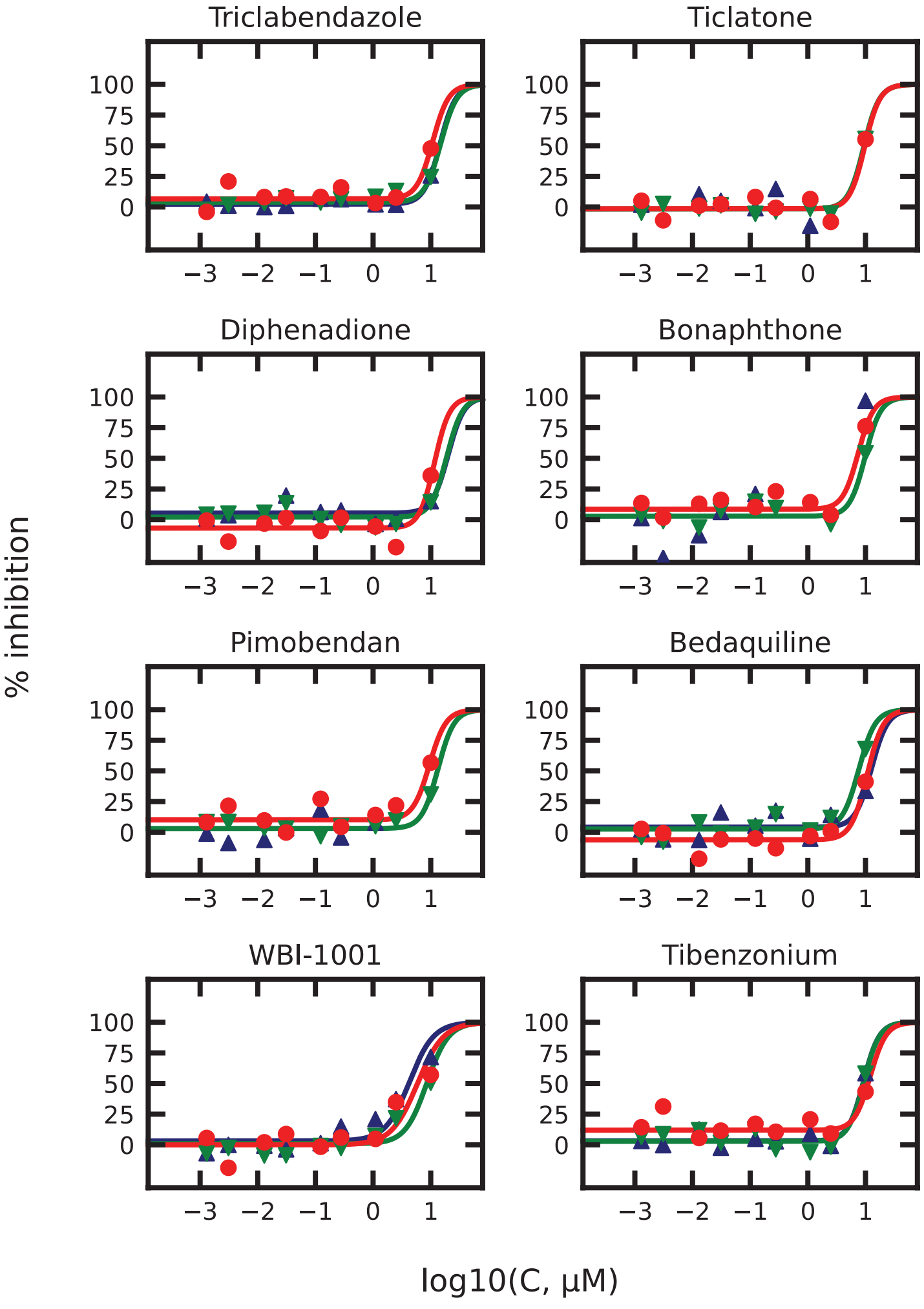

ARA DMAP
Pafuramidine
Cisplatin
Cloturin
EF-4040
Cyclovalone
VML 530
ONO-7746
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
1

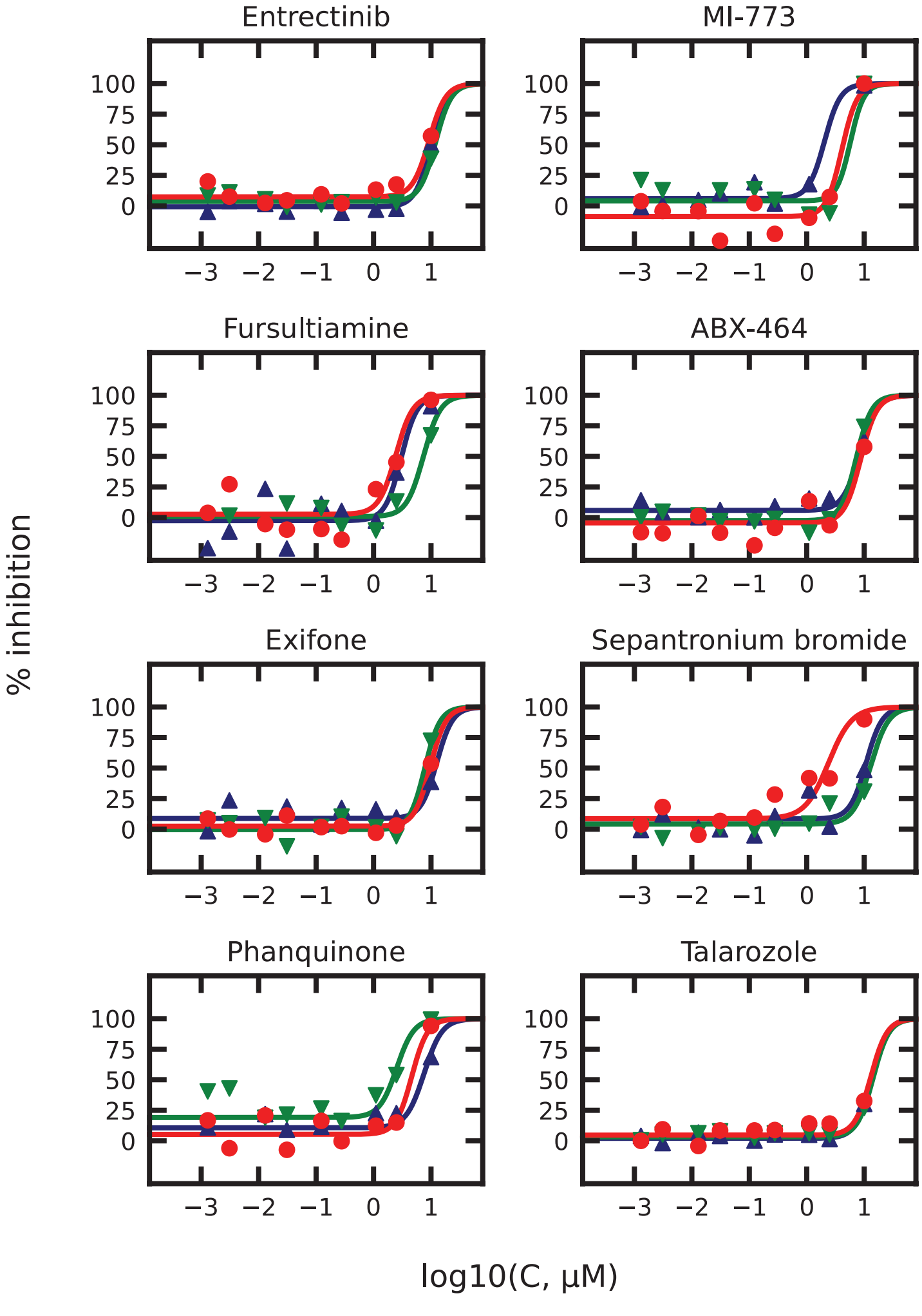

BMS-919373
KUX-1151
NT-113
Curcumin
AEG-33783
VRT-532
Licochalcone A
NSC-150117
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1

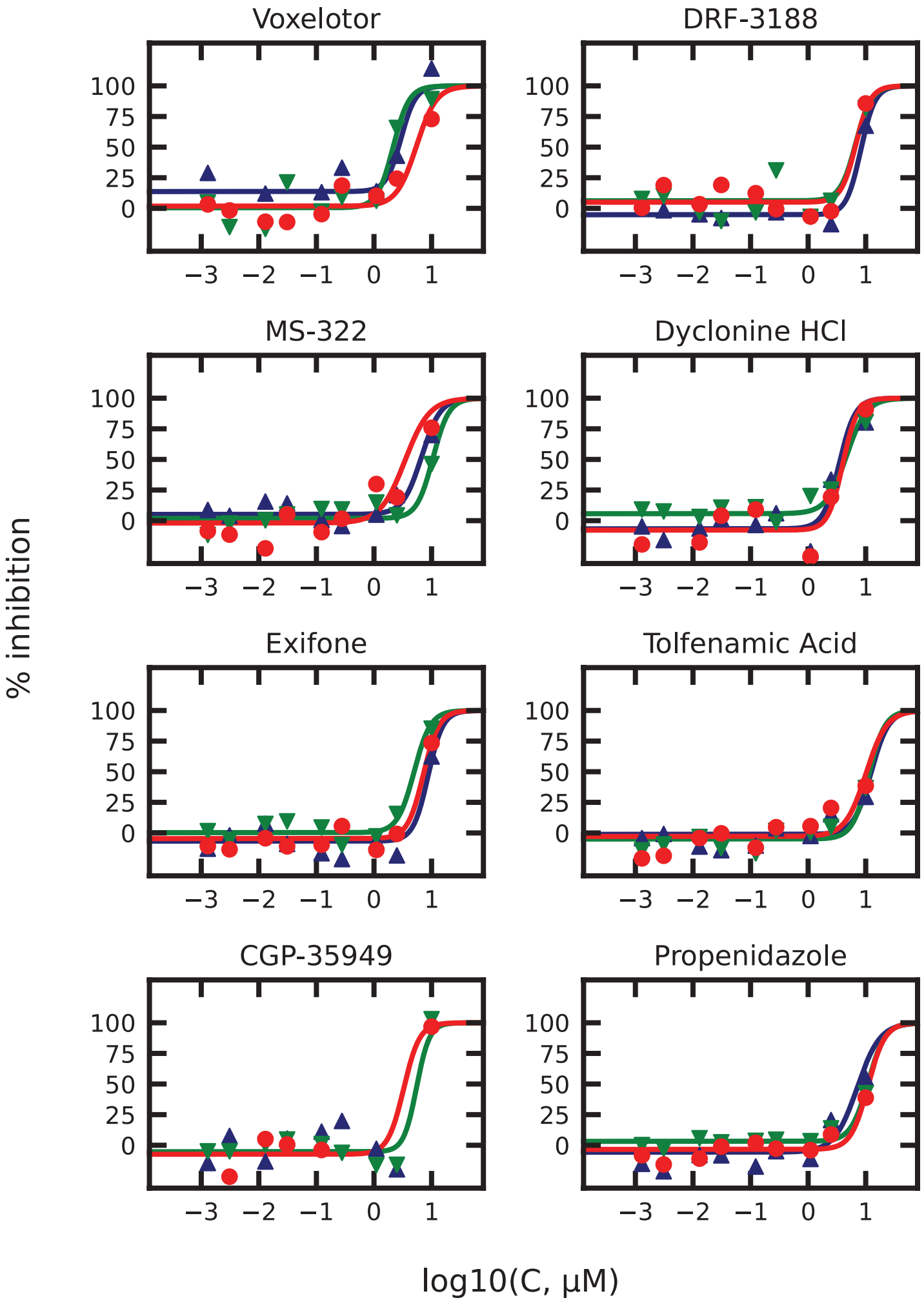

AT-101
Darapladib
Oxyfedrine Hydrochloride
WP-1066
Butein
Etacrynic Acid
TS-80
SC-1
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1

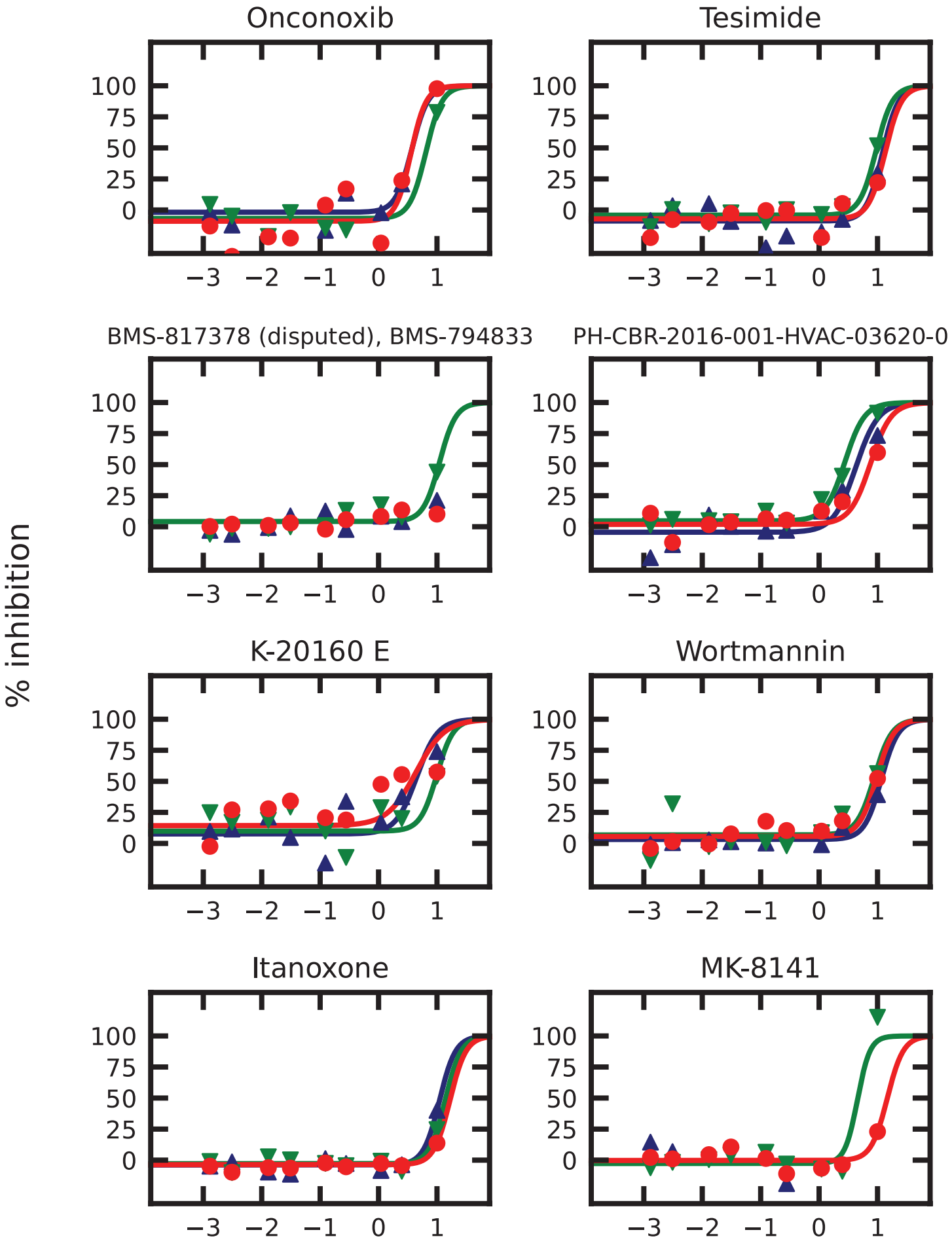

Tucaresol
CBS-1114
YM 57158
Allisartan
Elisartan Potassium
Samixogrel
AM-103
ODM-103
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1

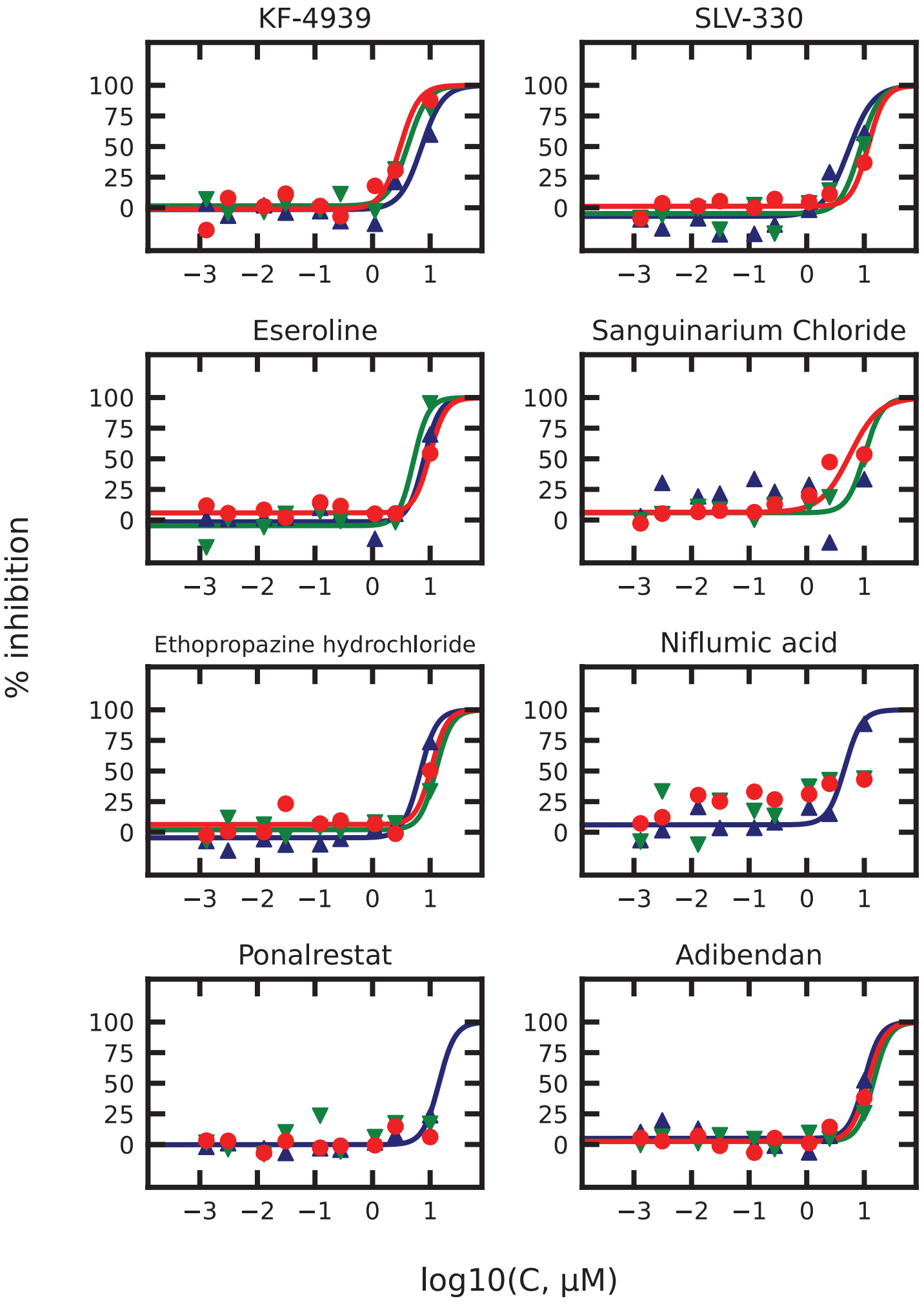

ON-09310
AZD-4769
Calcimycin
Vadadustat
Metabromsalan
IMD-0560
laflunimus
5,7-Dihydroxyflavone
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
1

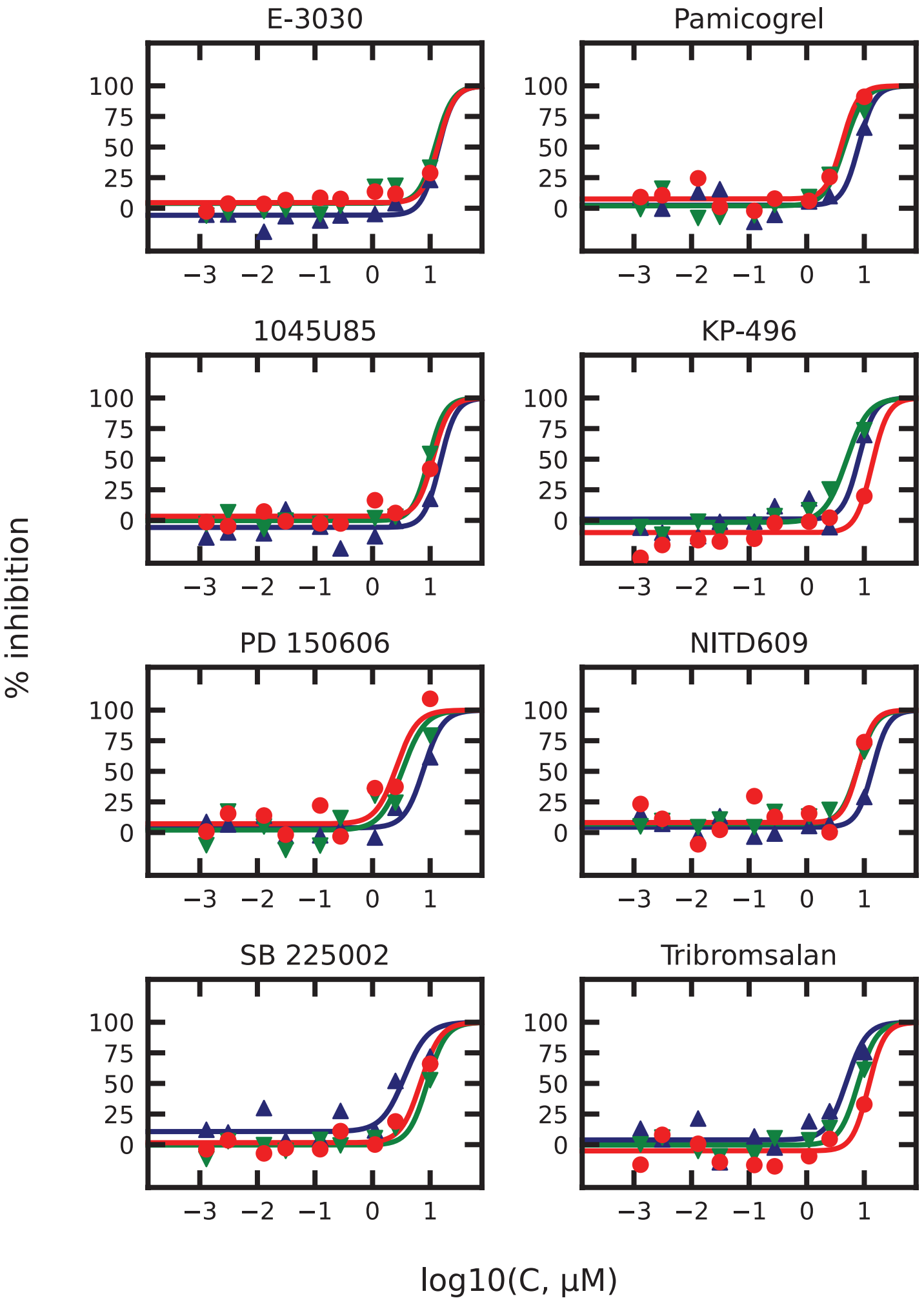

Triclabendazole
Ticlatone
Diphenadione
Bonaphthone
Pimobendan
Bedaquiline
WBI-1001
Tibenzonium
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1


Entrectinib
MI-773
Fursultiamine
ABX-464
Exifone
Sepantronium bromide
Phanquinone
Talarozole
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
1


Voxelotor
DRF-3188
MS-322
Dyclonine HCl
Exifone
Tolfenamic Acid
CGP-35949
Propenidazole
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
1


Onconoxib
Tesimide
BMS-817378 (disputed), BMS-794833
PH-CBR-2016-001-HVAC-03620-0
K-20160 E
Wortmannin
Itanoxone
MK-8141
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
1


KF-4939
SLV-330
Eseroline
Sanguinarium Chloride
Ethopropazine hydrochloride
Niflumic acid
Ponalrestat
Adibendan
% inhibition
log10(C, μM)
100
75
50
25
0
−3
−2
−1
0
1


E-3030
Pamicogrel
1045U85
KP-496
PD 150606
NITD609
SB 225002
Tribromsalan
% inhibition
log10(C, µM)
100
75
50
25
0
−3
−2
−1
0
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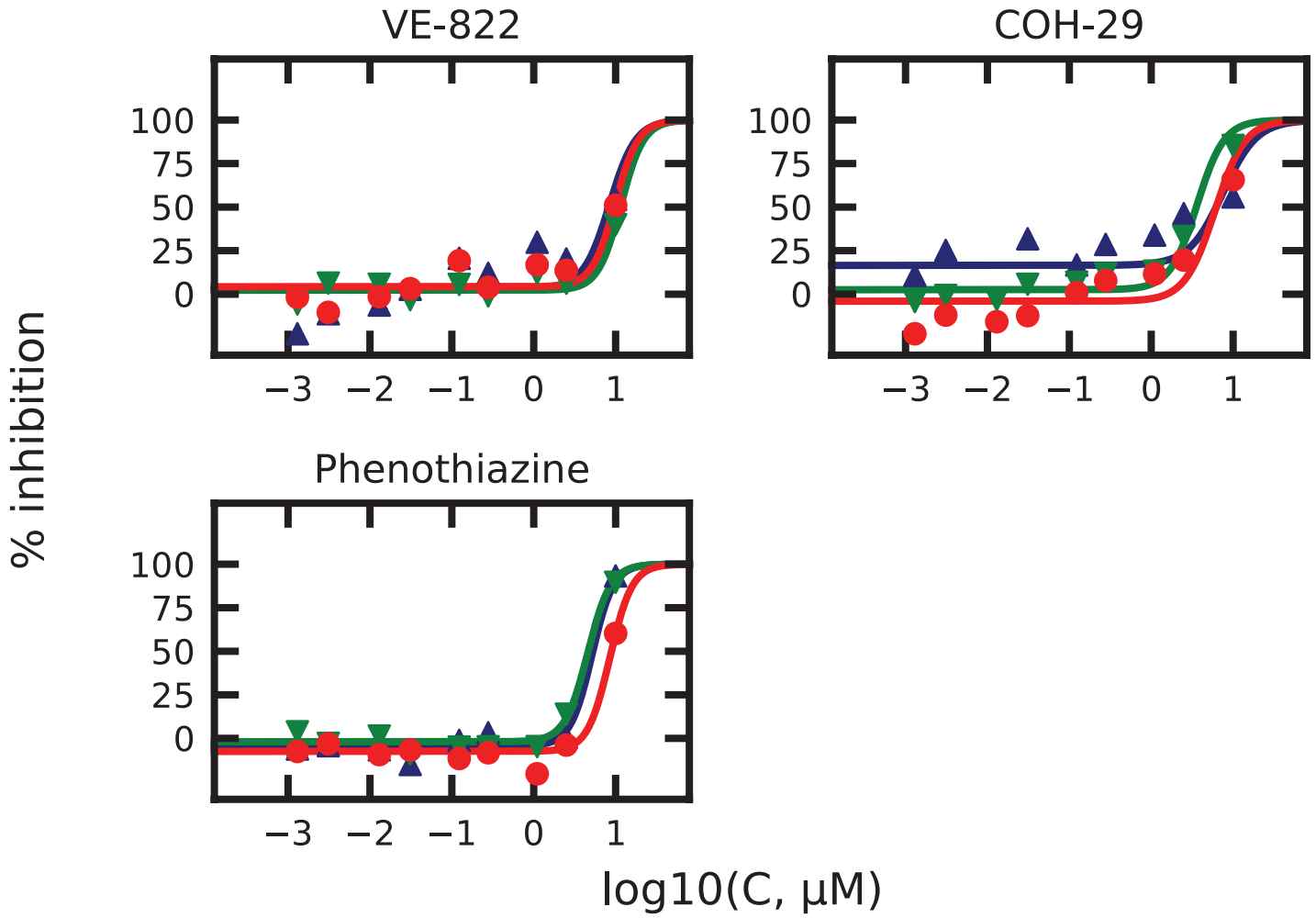
VE-822
COH-29
Phenothiazine
% inhibition
100
75
50
25
0
−3
−2
−1
0
1
log10(C, μM)