

4 June 13

**Supplementary Material for:**

**A high-throughput functional screen identifies small molecule regulators of heat- and mechano-sensitive  $K_{2P}$  channels**

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**Inventory of Supplementary Material:**

Supplementary Figures S1-S6

Supplementary Tables S1-S2

Supplementary Methods

Supplementary References

## Figure S1

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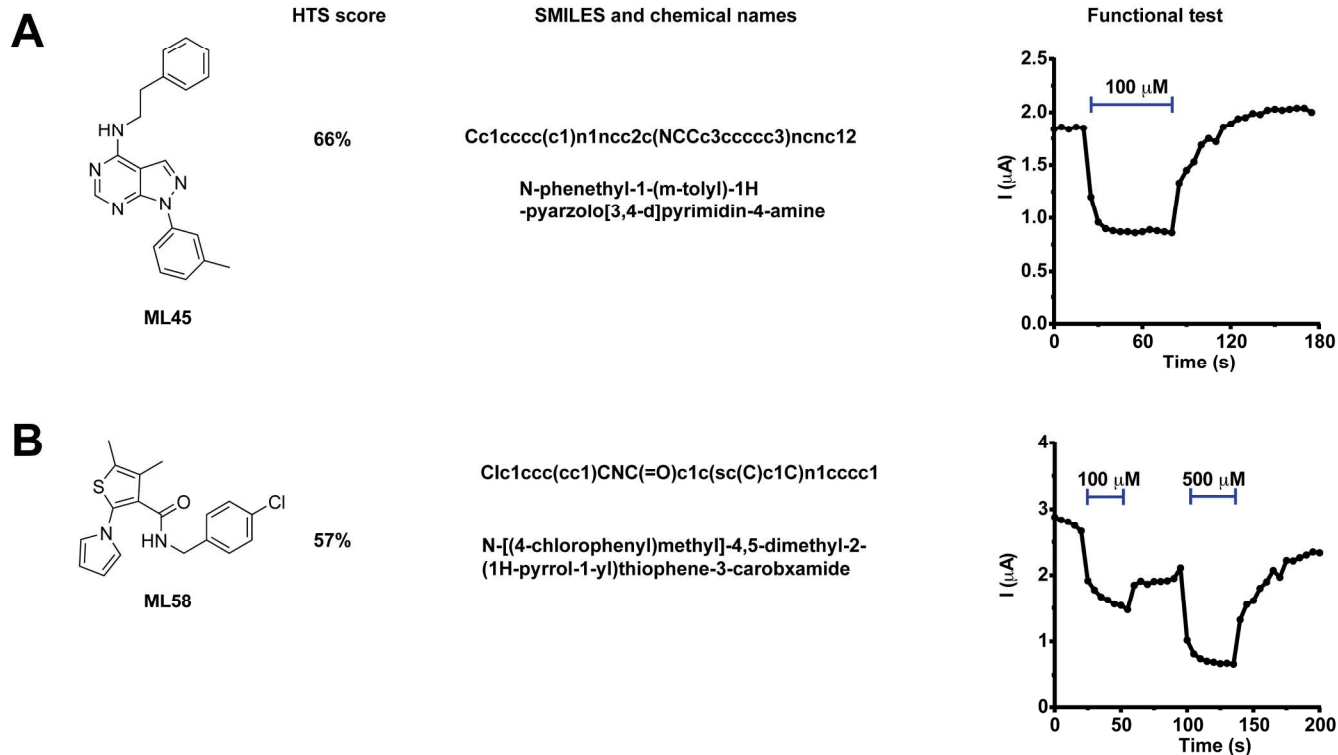
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
A	0	5	20	21	1	4	-7	16	-13	97	-6	-1	-8	3	-18	71	-12	11	15	31	17	-13	102
B	10	2	27	-7	-111	-8	-1	17	4	25	17	16	47	-3	23	10	29	-2	-5	14	24	-32	101
C	-5	3	-22	-1	-8	-2	-11	24	-10	-5	-8	7	6	-9	-7	3	-14	-6	24	22	-30	57	102
D	7	-1	-2	-1	11	17	6	-1	-1	-6	89	-9	-10	-9	1	-7	-8	-3	10	15	-43	84	101
E	-10	-8	-17	29	-27	-25	-23	-13	-12	40	5	-9	-19	-120	-13	18	-40	-12	2	14	3	-30	97
F	-5	-3	-6	4	-3	-34	-28	-16	17	22	-5	-21	-6	-13	11	-13	-12	-26	-9	-13	28	-11	98
G	-2	3	-4	-13	-13	-1389	10	10	15	20	20	18	11	93	-2	14	5	16	-14	1	-13	26	98
H	17	4	50	-4	35	-9	6	-2	5	-19	27	17	98	-6	14	12	4	-12	24	-15	22	-34	101
I	1	-2	-6	-8	10	22	-6	-11	-8	19	-7	-8	31	53	-9	-9	12	26	-11	0	22	6	101
J	9	-5	18	-6	2	-2	-3	-12	17	-11	11	-11	-2	8	-6	-5	-6	-12	-14	-9	12	-32	100
K	-4	-7	-16	-15	-9	3	-24	-8	-10	-14	2	-16	-15	-7	15	48	-12	-25	-16	24	19	29	99
L	-2	-2	84	-6	-106	-15	13	-17	8	-3	-16	-12	7	-22	-6	-21	38	-23	-10	-6	5	-42	101
M	-5	5	23	1	-3	3	-1	12	18	2	-2	-4	0	23	-10	-4	-2	3	2	12	21	-33	101
N	14	0	3	0	96	-5	17	1	42	2	-2	-8	-523	-4	36	-13	32	-4	6	29	15	-13	100
O	-9	4	-15	-4	-3	3	-5	2	4	6	-15	-9	3	14	-26	-6	1	53	-12	13	18	-21	98
P	-6	-8	-5	-9	14	-6	-9	-16	-2	-3	55	7	10	-15	-11	-8	-12	1	-1	-1	2	17	99

0% inhibition control (1% DMSO), columns 1–2 numbers: % growth inhibition  
 Library Compounds, columns 3–22  
 100% inhibition control (0.1% SDS), column 23

	0% Inhibition control	100% inhibition control	Z'
Alamar Blue mean fluorescence, AU	60.6	14.4	0.76
s.d.	3.05	0.712	

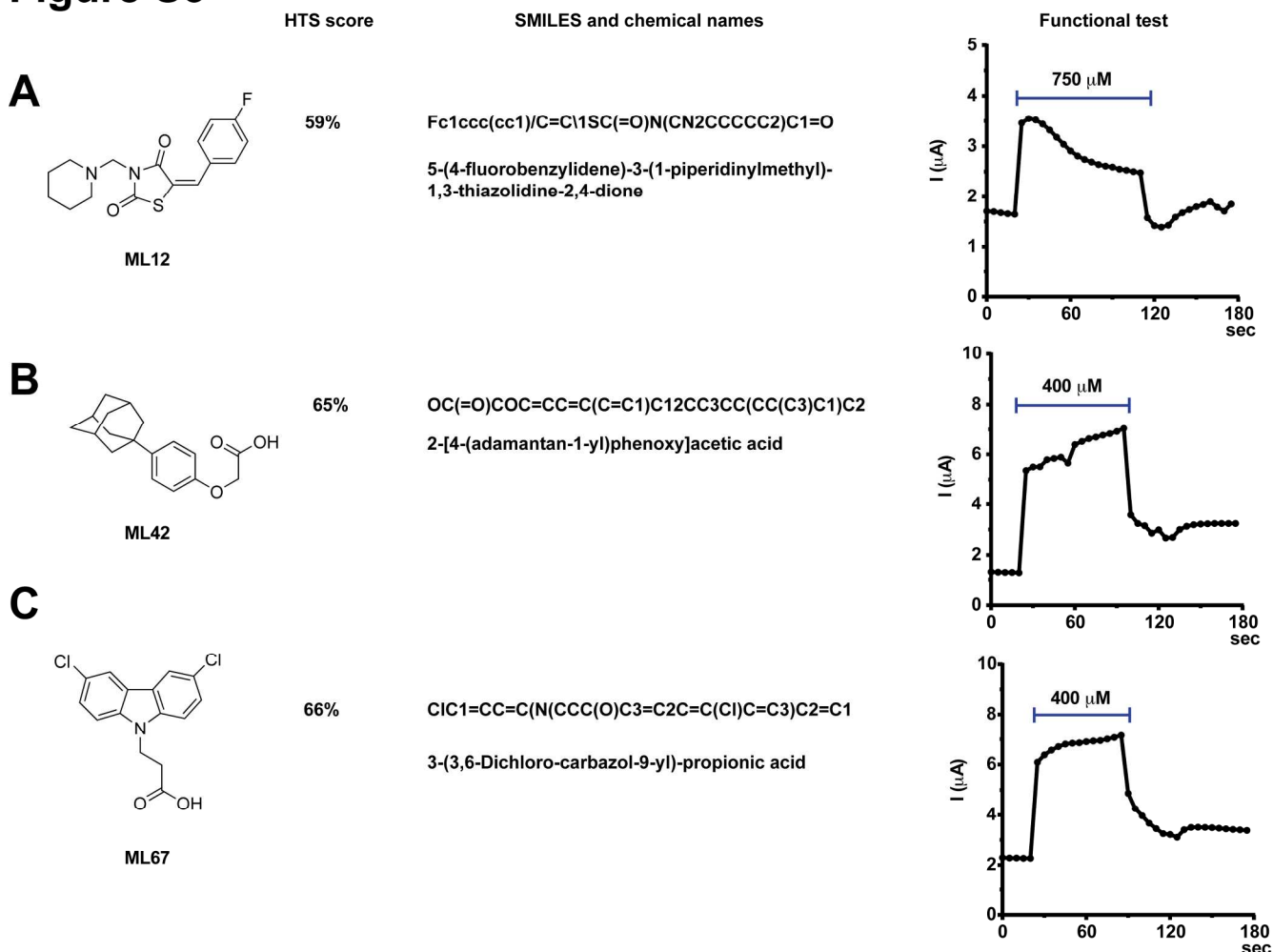
**Supplementary Figure S1** *Upper panel*, A 384-well plate showing calculated growth of SGY1528 yeast expressing K<sub>2p2.1</sub> (TREK-1) after 48 hour incubation in 2 mM KCl media in the presence of library compounds (10 μM, 1% DMSO) and controls. Growth was assessed by measuring fluorescence (560 nm excitation / 590 nm emission) after the addition of the vital dye Alamar Blue at the end of incubation. The numbers show percent of growth inhibition caused by the compounds relative to 1% DMSO or 0.1% SDS (0 and 100% inhibition controls, respectively). *Lower panel*, Summary table showing assay performance statistics.

## Figure S2

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**Supplementary Figure S2** Exemplar  $K_{2p2.1}$  (TREK-1) inhibitors **A**, ML45 and **B**, ML58 identified in the high-throughput yeast screen. Panels show from left to right: chemical structure; HTS score, percent of growth inhibition for the indicated compound in the high-throughput screen; SMILES and chemical names; and activity. Activity plots show the effect of the compounds at the indicated concentrations against  $K_{2p2.1}$  (TREK-1) measured by two-electrode voltage clamp in *Xenopus* oocytes in 90 mM  $[K^+]_o$ , pH 7.4. Currents were elicited by a ramp from  $-100$  to  $50$  mV from a  $0$  mV holding potential. Values shown were taken at  $+40$  mV.

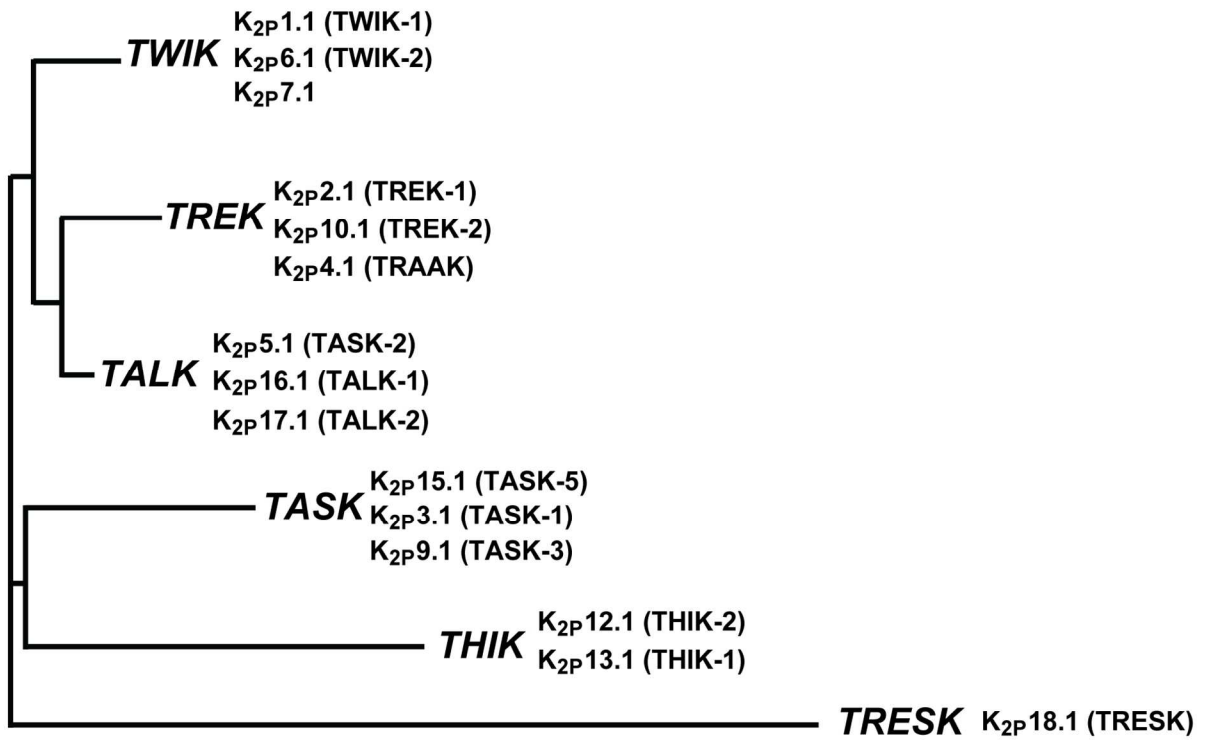
## Figure S3



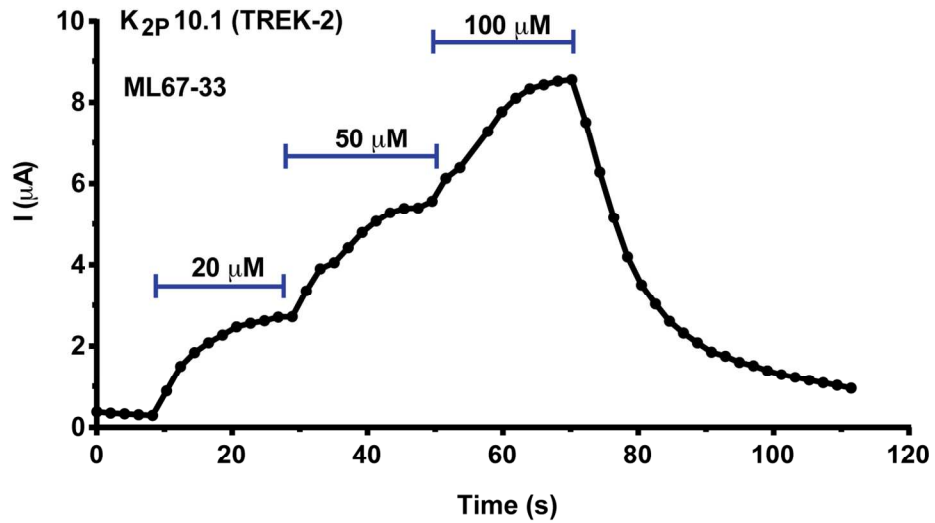
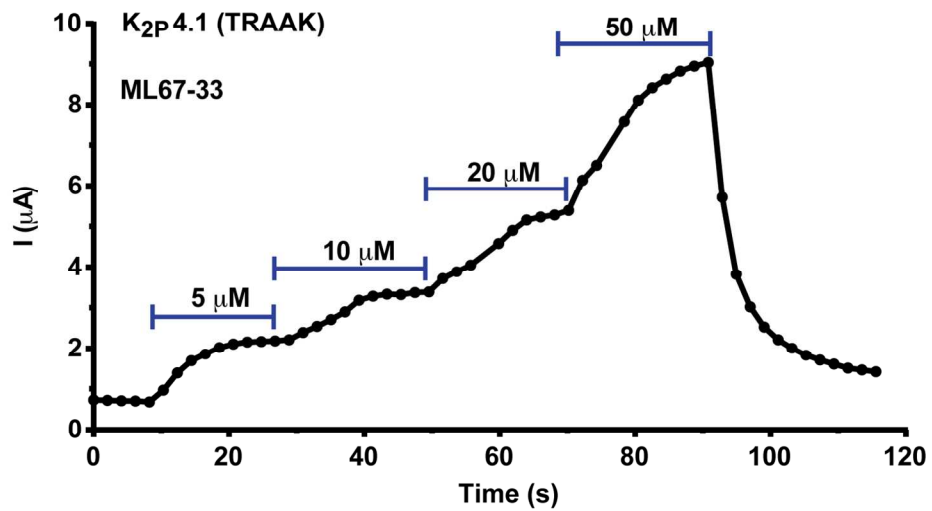
**Supplementary Figure S3** Exemplar  $K_{2p2.1}$  (TREK-1) activators **A**, ML12, **B**, ML42, and **C**, ML67 identified in the high-throughput yeast screen. Panels show from left to right: chemical structure; HTS score, percent of growth inhibition for the indicated compound in the high-throughput screen; SMILES and chemical names; and activity. Activity plots show the effect of the compounds at the indicated concentrations against  $K_{2p2.1}$  (TREK-1) measured by two-electrode voltage clamp in *Xenopus* oocytes in 90 mM  $[K^+]_o$ , pH 7.4. Currents were elicited by a ramp from -100 to 50 mV from a 0 mV holding potential. Values shown were taken at +40 mV.

# Figure S4

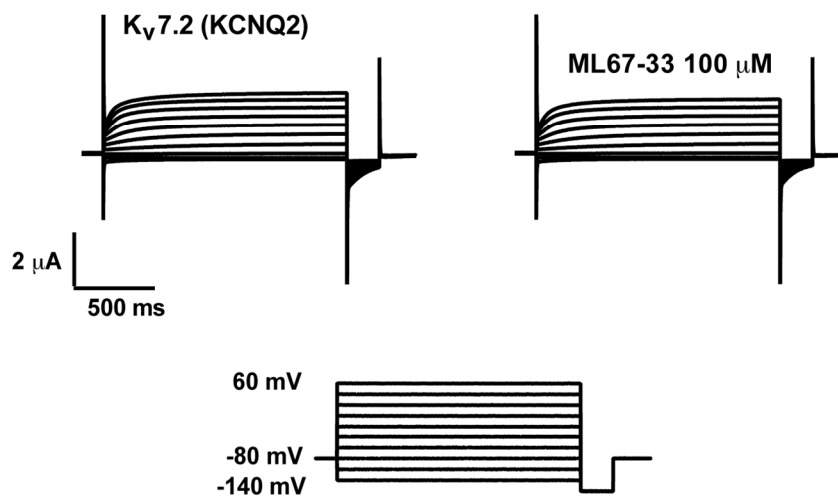
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Supplementary Figure S4 Dendrogram of K<sub>2</sub>P channels based on Enyedi and Czirjak<sup>1</sup> and Lesage and Barhanin<sup>2</sup>.

**Figure S5****Bagriantsev *et al.*****A****B**

**Supplementary Figure S5** Exemplar responses (at 0 mV) of **A**,  $\text{K}_{2\text{P}}10.1$  (TREK-2) and **B**,  $\text{K}_{2\text{P}}4.1$  (TRAAK) to the indicated concentration of ML67-33 measured by two-electrode voltage clamp in *Xenopus* oocytes in 2 mM  $[\text{K}^+]_o$  pH 7.4. Currents were elicited by a voltage ramp from -150 to 50 mV, from a holding potential of -80 mV.

**Figure S6****Bagriantsev *et al.***

**Supplementary Figure S6** Exemplar I-V curves showing the effect of 100  $\mu$ M ML67-33 on Kv7.2 (KCNQ2) measured by two-electrode voltage clamp in *Xenopus* oocytes in 2 mM  $[K^+]_o$ , pH 7.4. Currents were elicited by a step protocol from -120 to 60 mV, in 20 mV increments, from a holding potential of -80 mV.

**Supplementary Table S1** Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	cell-based (yeast strain SGY1528)
	Target	mouse two-pore potassium channel K <sub>2p</sub> 2.1 (TREK-1)
	Primary measurement	fluorometric detection of K <sub>2p</sub> 2.1-expressing yeast viability in the presence of library compounds in potassium-limiting conditions
	Key reagents	'vital dye' Resazurin (Alamar Blue, <i>Invitrogen</i> )
	Assay protocol	see <i>Methods</i> section
	Additional comments	none
Library	Library size	106,281 small molecule compounds
	Library composition	104,121 – Diversity Collection ( <i>ChemBridge, ChemDiv, SPECS</i> ) 2,160– Bioactive Collection ( <i>Microsource Spectrum</i> )
	Source	Small Molecule Discovery Center, University of California, San Francisco
	Additional comments	none
Screen	Format	384-square-well
	Concentration(s) tested	10 μM, 1% DMSO
	Plate controls	1% DMSO (0% growth inhibition control) 0.1% SDS, 1% DMSO (100% growth inhibition control)
	Reagent/ compound dispensing system	FXp Liquid Handler ( <i>Beckman</i> ) WellMate Bulk Dispenser ( <i>Matrix</i> )
	Detection instrument and software	Analyst HT Plate Reader ( <i>Molecular Devices</i> )
	Assay validation/QC	Z' = 0.76
	Correction factors	none
	Normalization	see <i>Plate Controls</i>
	Average Z' for screen (± s.d.)	Z' = 0.52 ± 0.2 (number of plates = 334)
	Average Z for screen (± s.d.)	Z = 0.26 ± 0.41 (number of plates = 334)
Additional comments	Hit criteria: growth inhibition in the range 44–92%. 320 compounds were chosen for post-HTS analysis.	
Post-HTS analysis	Hit criteria	K <sub>2p</sub> 2.1-specific growth inhibition. Reference: Trk1p-expressing yeast. Hit criteria: 2-fold difference in the apparent IC <sub>50</sub> required to inhibit the growth of yeast expressing K <sub>2p</sub> 2.1 (TREK-1) versus those expressing Trk1p
	Hit rate	0.077% (81 compounds)
	Additional assay(s)	Direct electrophysiological measurements of the effect of the hit compounds on K <sub>2p</sub> 2.1 activity in <i>Xenopus</i> oocytes.
	Confirmation of hit purity and structure	resupplied as dry powders, QC by LC/MS > 90% purity
Additional comments	none	

Z' is the assay performance based on 0% inhibition controls and 100% inhibition controls. Z'-factor is a characteristic factor for the quality of the assay itself, without interventions of test compounds<sup>3</sup>.

Z measures the spread of test compounds away from the 100% inhibition controls<sup>3</sup>. Z-factor is often < Z'-factor. A positive Z-factor suggests that the bulk population of test compounds is reasonably different from the 100% inhibition control, hence, inhibitory hits can be identified from the assay.



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**Supplementary Table S2** clogP and clogD values for K<sub>2p</sub>2.1 (TREK-1) modulators

<b>Compound</b>	<b>clogP</b>	<b>clogD (pH 7.4)</b>
<b>Inhibitors</b>		
ML45	4.12	4.08
ML58	4.44	4.44
<b>Activators and derivatives</b>		
ML12	3.20	3.11
ML42	3.64	0.33
ML67	4.24	1.26
ML67-2	3.03	0.55
ML67-13	4.57	1.14
ML67-15	4.53	1.63
ML67-17	4.66	1.71
ML67-18	3.81	2.26
ML67-29	4.66	1.71
ML67-33	4.84	3.29
ML67-137	5.17	3.62

clogP and clogD values were calculated using the ‘weighted’ method in MarvinSketch v. 5.12.4 (ChemAxon, Ltd.)

‘P’ is defined as the octanol/water partition coefficient of the neutral species:

$$P = \frac{[X]_{\text{octanol}}}{[X]_{\text{water}}}$$

‘D’ is defined as the octanol/water distribution coefficient taking into account ionization:

$$D = \frac{[XH]_{\text{octanol}} + [X^-]_{\text{octanol}}}{[XH]_{\text{water}} + [X^-]_{\text{water}}}$$

## Supplementary methods

### *Yeast, media and compounds*

*Saccharomyces cerevisiae* strain SGY1528 (W303, MAT $\alpha$ , *ade2-1*, *can1-100*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *ura3-1*, *trk1::HIS3*, *trk2::TRP1*) was transformed using the lithium-chloride method and cultivated at 30°C using standard techniques<sup>4</sup> in synthetic liquid media without uracil (Ura, for plasmid selection) and methionine (Met, to drive K<sub>2p2.1</sub> (TREK-1) expression from the *MET25* promoter of the pYES2-MET25 vector). Synthetic media, estimated to contain ~5  $\mu$ M potassium<sup>5</sup> and designated as ‘0 mM KCl’, was supplemented with up to 50 mM KCl. YPAD, non-selective medium: 10 g/L yeast extract, 20 g/L dextrose, 20 g/L peptone, 24 mg/L adenine hemisulfate, 100 mM KCl. Plasmid-selective –Ura/–Met synthetic medium: 1.5 g/L –Ura/–Met dropout powder, 6.7 g/L yeast nitrogen base (without amino acids), 20 g/L dextrose, 100 mM KCl, pH 6.5 (adjusted with 1M Tris base). –Ura/–Met test medium: 2.1 g arginine (free base), 1.5 g/L dropout powder –Ura/–Met, 10 g/L dextrose, 1X trace minerals and vitamins (see 1000X stock recipes below), 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 0–50 mM KCl, pH 6.0 with adjusted with phosphoric acid. –Ura/–Met dropout powder: 6.0 g glutamic acid, 2.5 g adenine hemisulfate, 1.2 g arginine, 6.0 g aspartic acid, 1.8 g lysine, 3.0 g phenylalanine, 22.5 g serine, 12.0 g threonine, 2.4 g tryptophane, 1.8 g tyrosine, 9.0 g valine, 1.2 g histidine, 3.4 g leucine. 1000x Vitamin 1000X stock solution: 2 mg/L biotin, 400 mg/L *D*-panthothenic acid, 400 mg/L pyridoxine, 400 mg/L thiamin, 2 g/L inositol. Trace mineral 1000X stock solution: 500 mg/L boric acid, 40 mg/L CuSO<sub>4</sub>, 100 mg/L KI, 500 mg/L FeCl<sub>3</sub>, 400 mg/L MnSO<sub>4</sub>, 900 mg/L molybdcic acid, 400 mg/L ZnSO<sub>4</sub>, 10 ml concentrated HCl.

### *High-throughput screening*

SGY1528 was transformed with K<sub>2p2.1</sub> (TREK-1) or Trk1p plasmid and grown on the plasmid-selective –Ura/–Met synthetic medium. For each plasmid, a single colony was grown in –Ura/–Met synthetic medium with 100 mM KCl to saturation, diluted with the same media to optical density at 600 nm (OD<sub>600</sub>) = 0.3, and grown until OD<sub>600</sub> reached 0.8. Cells were pelleted, washed with water, and resuspended in –Ura/–Met test medium supplemented with 2 mM KCl to OD<sub>600</sub> 0.3. Using an automated dispenser, the cultures were aliquoted into 384-square-well plates (30  $\mu$ l per well) containing, per well: 3  $\mu$ l 10% DMSO (0% growth inhibition control), or 3  $\mu$ l 1% SDS (100% growth inhibition control), or 3  $\mu$ l of a 100  $\mu$ M library compound in 10% DMSO. Following 24 hour incubation 30°C at constant shaking, 5  $\mu$ l of the vital dye resazurin (Alamar Blue, Invitrogen) was dispensed into each well, and the plates were incubated for 3 hours in the same conditions to allow the

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dye penetrate into the cells. Resazurin signals were quantified using an automated plate reader at 560 nm excitation/590 nm emission settings.

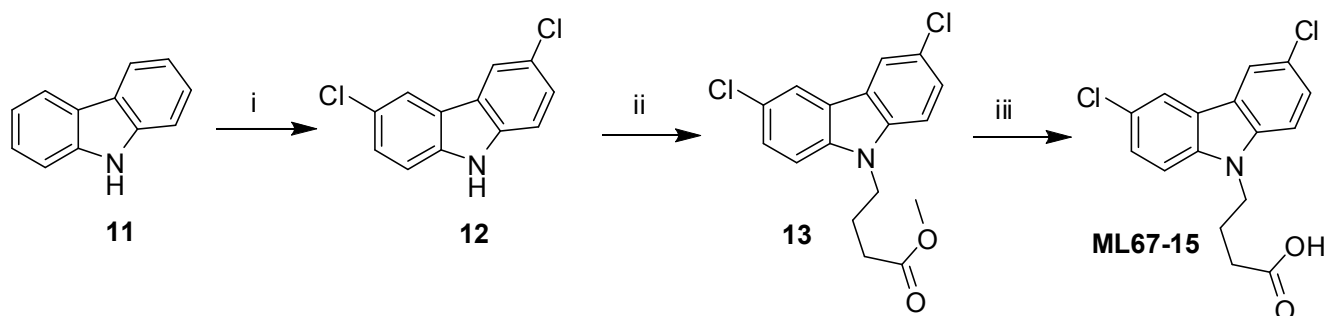
Compounds having screening scores of 44% - 92% inhibition were chosen for further evaluation as this range encompassed the range of  $1.25\sigma$ - $3\sigma$  above the mean inhibition of the test population (10% inhibition). Pilot screens showed that many of the compounds scoring 100% inhibition included many known antifungals. Hence, the cutoff of 92% was chosen to try to avoid enrichment of generally toxic compounds among the initial leads.

### ***Electrophysiology***

Two-electrode voltage clamp recordings were performed from defolliculated stage V-VI *Xenopus* oocytes 24-48 hours after injection with 0.015–6.0 ng cRNA, using microelectrodes (0.3–3.0 M $\Omega$ ) filled with 3M KCl. Data were acquired using a GeneClamp 500B (MDS Analytical Technologies) amplifier and pClamp software (Molecular Devices), and digitized at 1 kHz using Digidata 1332A (MDS Analytical Technologies). Recording solutions (mM): 2K (96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>), 90K (90 KCl, 8 NaCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>), were contained 5 mM HEPES pH 7.4. K<sub>2P</sub> currents were elicited by a 1 second ramp from –150 to +50 mV from a –80 mV holding potential of (2K), or –100 to +50 mV from a 0 mV holding potential (90K). KCNQ2 currents were elicited by a step protocol from –120 to 60 mV, in 20 mV increments, from a –80 mV holding potential. HEK—293T patch-clamp recordings were performed using microelectrodes (2-3 M $\Omega$ ) filled with 1M KCl. Data were acquired using the Axopatch 200B amplifier and pClamp software, and digitized at 1 kHz using Digidata 1332A. Recording solutions (mM): intracellular (145 KCl, 5 EGTA, 3 MgCl<sub>2</sub>), extracellular (150 NaCl, 5 KCl, 3 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>) were buffered with 5 mM HEPES pH 7.2 and 7.4, respectively. Times of half-maximal activation ( $t_{1/2act}$ ) and return to baseline ( $t_{1/2wash}$ ) correspond to the amount of time required to reach half-maximal channel activity upon compound application or removal, respectively. Currents were elicited by a 1 second ramp from –150 to +50 mV from a -80 mV holding potential. Data were fit with a modified Hill equation:  $I=I_{min}+(I_{max}-I_{min})/(1+10^{((LogEC_{50}-Log[C])*H)})$ ;  $I_{max}$  and  $I_{min}$  are maximal and minimal current values, respectively,  $EC_{50}$  is a half-maximal effective concentration, and H is the Hill coefficient.

## Compound synthesis

## Synthesis of ML67-15



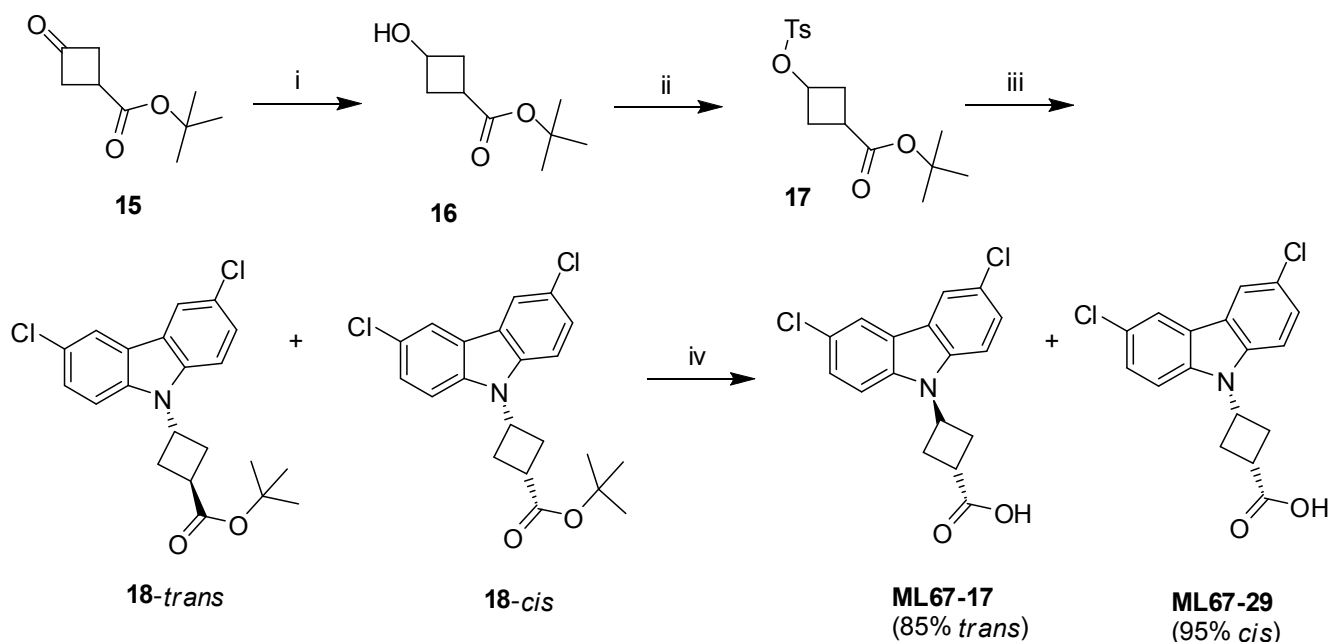
i)  $\text{SO}_2\text{Cl}_2$ ,  $\text{CH}_2\text{Cl}_2$ ; ii) methyl 4-bromobutanoate, NaH, DMF; iii) LiOH, MeOH, THF.

**3,6-dichlorocarbazole (12).** A round bottom flask was charged with 9H-carbazole (**11**, 20.0 g, 119.6 mmol) and dichloromethane (200 mL) and the mixture was stirred at 0 °C. Sulfuryl chloride (9.69 mL, 119.6 mmol) was slowly added at that temperature. The dark reaction mixture was stirred at 0 °C for 2h and then diluted with  $\text{CH}_2\text{Cl}_2$  and aq.  $\text{NaHCO}_3$ . The organic layer was separated and washed with aq.  $\text{NaHSO}_3$ , brine, and dried ( $\text{Na}_2\text{SO}_4$ ). The solution was then filtered and concentrated to afford the crude product as a thick oil. This was recrystallized from hexanes/ethyl acetate to afford 3,6-dichlorocarbazole (**12**) as a white solid (15.2 g, 54%).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  11.56 (s, 1H), 8.27 (d,  $J=1.8$  Hz, 2H), 7.50 (d,  $J=8.7$  Hz, 2H), 7.39 (d,  $J=8.7$  Hz, 2H).

**Methyl 4-(3,6-dichloro-9H-carbazol-9-yl)butanoate (13).** A round bottom flask was charged with 3,6-dichlorocarbazole (**12**, 175 mg, 0.74 mmole), sodium hydride (27 mg, 1.11 mmol) and DMF (5 mL) under nitrogen. The reaction mixture was stirred at 60° C for 30 minutes and methyl-4-bromobutyrate (83  $\mu\text{L}$ , 0.74 mmol) was added and the reaction stirred at 60° C over night. The reaction was cooled to room temperature and diluted with ethyl acetate and washed with water and brine. The organic solvents were removed under reduced pressure and the residue purified by flash chromatography over silica gel (0-30% ethyl acetate/hexanes) to afford methyl 4-(3,6-dichloro-9H-carbazol-9-yl)butanoate (**13**) as white solid (0.13g, 52%).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  7.97 (d,  $J=1.8$  Hz, 2 H), 7.43 (d,  $J=2.1$  Hz, 1H), 7.41 (d,  $J=2.1$  Hz, 1H), 7.35 (s, 1H), 7.31 (s, 1H), 4.34 (t,  $J=7.2$  Hz, 2H), 3.67 (s, 3H), 2.33 (t,  $J=7.2$  Hz, 2H), 2.20-2.14 (m, 2H). LCMS  $m/z$  337.2 (MH+).

**4-(3,6-Dichloro-9H-carbazol-9-yl)butanoic acid (ML67-15).** A round bottom flask was charged with methyl 4-(3,6-dichloro-9H-carbazol-9-yl)butanoate (**13**, 130 mg, 0.39 mmol), methanol (3 mL), THF (3 mL) and 1M lithium hydroxide (1.16 mL, 1.16 mmol) and the reaction stirred for 2 hours. The organic solvents were removed under reduced pressure and the aqueous residue was acidified with 3N HCl. The precipitate formed was collected by filtration and dried. The white solid obtained was purified by flash chromatography over silica gel (0-5% MeOH/ $\text{CH}_2\text{Cl}_2$ ) to afford 4-(3,6-dichloro-9H-carbazol-9-yl)butanoic acid (ML67-15) as white solid (97 mg, 77%).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  8.32 (d,  $J=2.1$  Hz, 2 H), 7.67 (s, 1H), 7.64 (s, 1H), 7.50 (d,  $J=2.1$  Hz, 1H), 7.47 (d,  $J=2.1$  Hz, 1H), 4.39 (t,  $J=7.5$  Hz, 2H), 2.25 (t,  $J=7.2$  Hz, 2H), 1.96-1.91 (m, 2H); LCMS  $m/z$  321.9 (M-1).

## Synthesis of ML67-17 and ML67-29



i) NaBH<sub>4</sub>, THF, MeOH; ii) TsCl, pyridine, CH<sub>2</sub>Cl<sub>2</sub>; iii) **12**, NaH, DMF; iv) LiOH, MeOH, THF.

**tert-butyl 3-(tosyloxy)cyclobutanecarboxylate (17).** A mixture of tert-butyl 3-oxocyclobutanecarboxylate (**15**, 1.50 g, 8.8 mmol) in THF:MeOH (3:1, 16 mL) was added dropwise to a stirring slurry of sodium borohydride (0.167 g, 4.4 mmol) in THF (8 mL) in round bottom flask cooled in an ice bath. The mixture was stirred at 0-5 °C for two hours. Water was added dropwise (10 mL) followed by aq. Na<sub>2</sub>CO<sub>3</sub>, and the mixture was extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate. After filtration, the organic layer was concentrated to give the crude tert-butyl 3-hydroxycyclobutanecarboxylate (**16**) as a white semi-solid (2.3 g, 100%), which was used in the next step without purification. p-Toluenesulphonyl chloride (4.201 g, 0.022 moles) was added to a stirring solution of crude tert-butyl 3-hydroxycyclobutanecarboxylate (**16**, 2.30 g, 8.8 mmol) in dry pyridine (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (20 mL) at 0 °C. The mixture was allowed to warm to room temperature and stirred under nitrogen overnight. The solvent was then removed under reduced pressure and the residue was partitioned between ethyl ether (100 mL) and 0.5 N aq. HCl (20 mL). The organic layer was separated and washed with saturated NaHCO<sub>3</sub> and brine, and dried (Na<sub>2</sub>SO<sub>4</sub>). After filtration, the solvent was removed under reduced pressure and the residue purified by silica gel flash chromatography (0-50% EtOAc-hexane) to afford tert-butyl 3-(tosyloxy)cyclobutanecarboxylate (**17**) as a colorless oil that slowly solidified at room temperature (2.6 g, 90% yield over 2 steps). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 7.79 (d, 2H, J = 8.4 Hz), 7.35 (d, 2H, J = 8.1 Hz), 4.72 (m, 1H), 2.60-2.30 (m, 8H), 1.44 (s, 9H).

**cis and trans-tert-butyl 3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylate (18).** To a stirred solution of 3,6-dichlorocarbazole (**12**, 694 mg, 2.94 mmol) in dry DMF (15 mL) under nitrogen was added 60% sodium hydride in mineral oil (127 mg, 3.19 mmol). The reaction mixture was stirred at room temperature for 20 min and then at 60 °C for 30 minutes, and then cooled to rt. Solid tert-butyl 3-(tosyloxy)cyclobutanecarboxylate (**17**, 800 mg, 2.45 mmol) was added and the reaction mixture was stirred at 60 °C overnight. The reaction mixture was then cooled to room temperature and quenched with water and extracted with ethyl acetate. The organic layer was separated and washed with brine,

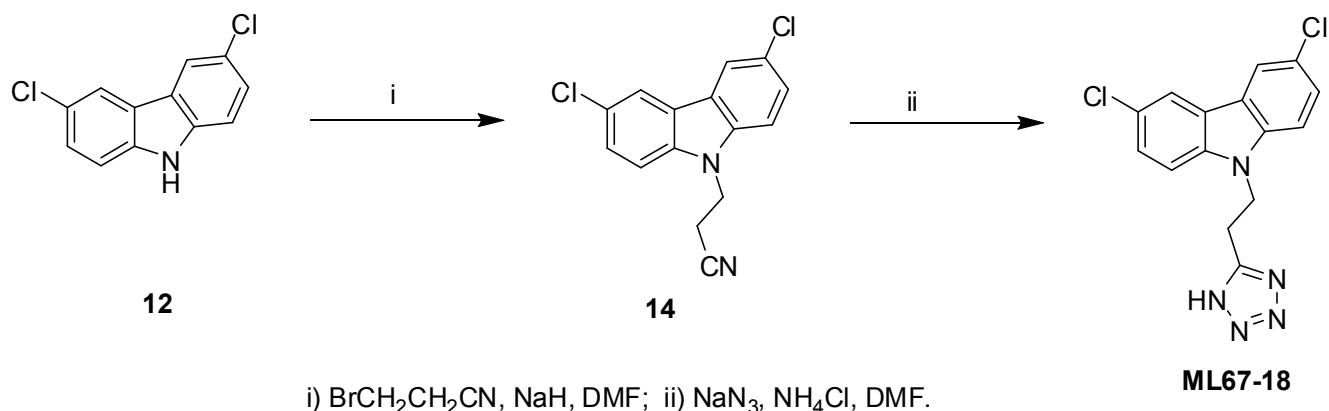
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dried ( $\text{Na}_2\text{SO}_4$ ), filtered, and concentrated. The crude residue was purified over silica gel (0-20% ethyl acetate/hexane) to afford **cis-18** as a light yellow syrup which solidified on standing (220 mg), and then **trans-18** as colorless syrup which solidified on standing (580 mg). **cis-18**:  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.99 (d, 2H,  $J = 1.8$  Hz), 7.47 (AB, 2H,  $J = 8.7$  Hz), 7.41 (AB d, 2H,  $J = 8.7, 1.8$  Hz), 5.45 (m, 1H), 3.35-3.15 (m, 3H), 3.00-2.80 (m, 2H), 1.57 (s, 9H). **trans-18**:  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.99 (d, 2H,  $J = 2.1$  Hz), 7.66 (d, 2H,  $J = 9.0$  Hz), 7.43 (dd, 2H,  $J = 8.7, 2.1$  Hz), 5.08 (m, 1H), 3.40-3.20 (m, 2H), 3.02 (5 peaks, 1H,  $J = 8.7$  Hz), 2.90-2.75 (m, 2H), 1.56 (s, 9H).

**cis-3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylic acid (ML67-29)**. A mixture of *cis*-tert-butyl 3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylate (**cis-18**, 90 mg, 0.23 mmol) and lithium hydroxide monohydrate (47 mg, 1.15 mmol) in THF-MeOH (1:1, 10 mL) was stirred at room temperature overnight. The reaction mixture was then concentrated and the residue treated with water, adjusted to pH  $\sim 3$  with 2N aq. HCl, and extracted with ethyl acetate. The organic layer was washed with brine, dried ( $\text{Na}_2\text{SO}_4$ ), filtered, and concentrated. The crude residue was recrystallized from EtOAc/hexane to afford *cis*-3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylic acid as a white solid that was further purified by preparative HPLC (C18 column, 40-80% ACN-water with 0.1%  $\text{HCO}_2\text{H}$ ) to afford the title compound (55 mg, 69% yield; 95% *cis*).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  12.45 (br s, 1H), 8.33 (d, 2H,  $J = 2.1$  Hz), 7.76 (d, 2H,  $J = 8.7$  Hz), 7.45 (dd, 2H,  $J = 8.7, 2.1$  Hz), 5.47 (m, 1H), 3.40-3.00 (m, 3H), 2.81 (m, 2H). LCMS  $m/z$  332.0 (M-1).

**trans-3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylic acid (ML67-17)**. A mixture of *trans*-tert-butyl 3-(3,6-dichloro-9H-carbazol-9-yl)cyclobutanecarboxylate (**trans-18**, 320 mg, 0.82 mmol) and lithium hydroxide monohydrate (336 mg, 8.2 mmol) in THF-MeOH (1:1, 10 mL) was stirred at room temperature overnight and then concentrated. The residue was treated with water and adjusted to pH  $\sim 3$  with 2N aq. HCl. The solids that precipitated out of this solution were collected by filtration, washed with water, and dried in air to afford the title compound (275 mg, >95% yield; 85% *trans*).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  8.33 (d, 2H,  $J = 2.1$  Hz), 7.87 (d, 2H,  $J = 9.0$  Hz), 7.47 (dd, 2H,  $J = 9.0, 2.1$  Hz), 5.32 (m, 1H), 3.15-2.90 (m, 3H), 2.85-2.65 (m, 2H); LCMS  $m/z$  331.8 (M-1).

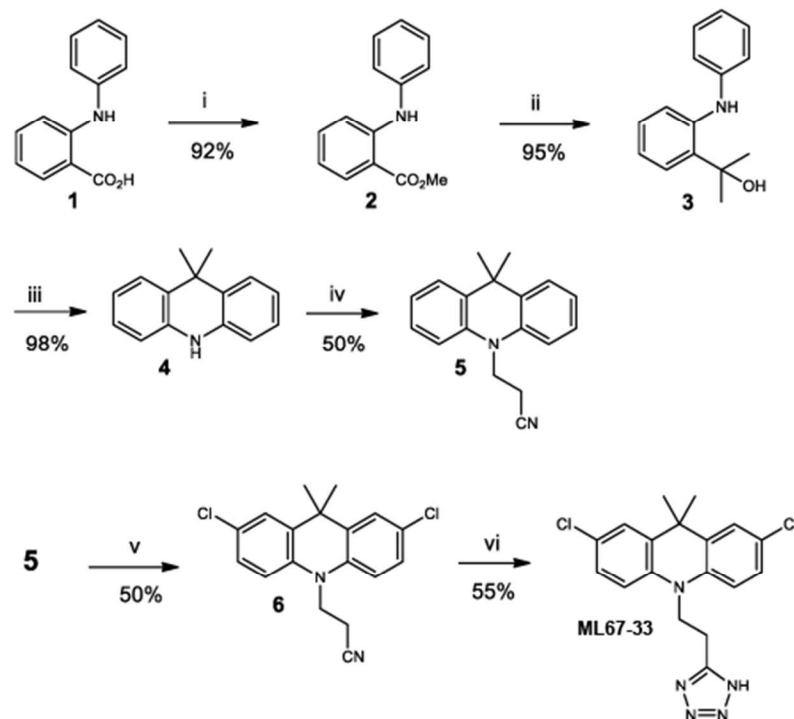
## Synthesis of ML67-18



**3-(3,6-dichloro-9H-carbazol-9-yl)propanenitrile (14).** A round bottom flask was charged with 3,6-dichlorocarbazole (**12**, 175 mg, 0.74 mmol), sodium hydride (27 mg, 1.11 mmol) and DMF (5 mL) under nitrogen. The reaction mixture was stirred at 60° C for 30 minutes and 3-bromopropanenitrile (62  $\mu\text{L}$ , 0.74 mmol) was added and the reaction mixture stirred at 60° C overnight. The reaction mixture was then cooled to room temperature, diluted with ethyl acetate, and washed with water and brine. The organic solvents were removed under reduced pressure and the residue purified by flash chromatography over silica gel (0-30% ethyl acetate-hexanes) to give 3-(3,6-dichloro-9H-carbazol-9-yl)propanenitrile (**14**) as white solid (140 mg, 65%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.00 (dd,  $J=2.1$  & 0.6 MHz, 2H), 7.49 (d,  $J=2.1$  Hz, 1H), 7.46 (d,  $J=1.8$  Hz, 1H), 7.35 (s, 1H), 7.33 (s, 1H), 4.63 (t,  $J=6.9$  Hz, 2H), 2.86 (t,  $J=6.9$  Hz, 2H).

**9-(2-(1H-tetrazol-5-yl)ethyl)-3,6-dichloro-9H-carbazole (ML67-18).** A mixture of 3-(3,6-dichloro-9H-carbazol-9-yl)propanenitrile (**14**, 140 mg, 0.48 mmol), sodium azide (94 mg, 1.45 mmol) and ammonium chloride (104 mg, 1.94 mmol) in DMF (5 mL) was stirred at 120 °C for 6h, after which time LCMS analysis indicated complete reaction. The reaction mixture was diluted with EtOAc (50 mL), washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. The crude residue was purified by flash chromatography over silica gel (0-10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) to afford 9-(2-(1H-tetrazol-5-yl)ethyl)-3,6-dichloro-9H-carbazole (**ML67-18**) as a beige solid (165 mg, 93%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.23 (d, 1H,  $J = 1.5$  Hz), 8.13 (d, 1H,  $J = 1.8$  Hz), 7.25 (t, 2H,  $J = 8.1$  Hz), 7.05 (t, 1H,  $J = 7.5$  Hz), 6.83 (d, 2H,  $J = 7.8$  Hz), 3.35 (s, 3H), 3.13 (t, 4H,  $J = 7.2$  Hz), 1.31-1.20 (m, 4H), 1.13-0.99 (m, 4H), 0.80 (t, 6H,  $J = 7.2$  Hz); LCMS  $m/z$  444.2 (MH+).

## Synthesis of ML67-33



i)  $\text{Me}_2\text{SO}_4$ ,  $\text{K}_2\text{CO}_3$ , acetone; ii) 3M MeLi, DEM; iii) 85%  $\text{H}_3\text{PO}_4$ ; iv) acrylonitrile, triton-B; v)  $\text{SO}_2\text{Cl}_2$ , DCM; vi)  $\text{NaN}_3$ ,  $\text{NH}_4\text{Cl}$ , DMF.

**Methyl 2-(phenylamino) benzoate (2)**<sup>6</sup>. Commercially available *N*-phenyl anthranilic acid (**1**) (2.0 g, 10 mmol) in acetone (30 mL) was refluxed with dimethyl sulphate (2.0 gr, 1.55 mL, 15 mmol) and potassium carbonate (1.38 g, 10 mmol) for 2 hrs. The progress of the reaction was monitored by TLC and when reaction was judged complete, the reaction mixture was allowed to cool to room temperature and poured into crushed ice. The aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 30mL), dried over anhydrous  $\text{MgSO}_4$ , filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with gradient elution (10 to 40% EtOAc-hexane) to afford the title compound as light yellow oil (2.1 g, 92% yield).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  9.29 (s, 1H), 7.88 (d, 1H,  $J = 7.8$  Hz), 7.45-7.30 (m, 3H), 7.28-7.18 (m, 3H), 7.07 (t, 1H,  $J = 7.5$  Hz), 6.80 (t, 1H,  $J = 7.5$  Hz), 3.85 (s, 3H). LCMS (ESI)  $m/z$  228 (MH<sup>+</sup>).

**2-(2-(phenylamino)phenyl)propan-2-ol (3)**. To a stirred solution of methyl 2-(phenylamino)benzoate (**2**, 1.0 g, 4.40 mmol) in dry THF (10 mL) at  $-78$  °C was added a 3.0M solution of methyllithium in diethoxymethane (4.40 ml, 13.2 mmol) over a period of 30 min. The mixture was stirred at  $-78$  °C for 30 min and then returned to room temperature and stirred for an additional 1 hour. The reaction mixture was then poured into crushed ice and extracted with EtOAc. The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with gradient elution (10-30% EtOAc-hexane) to afford the title compound as thick yellow oil (0.95 g, 95% yield).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  8.46 (s, 1H), 7.30-7.10 (m, 5H), 6.97 (d, 2H,  $J = 7.8$  Hz), 6.88-6.76 (m, 2H), 5.76 (s, 1H), 1.52 (s, 6H). LCMS (ESI)  $m/z$  228 (MH<sup>+</sup>).

**9,9-dimethyl-10H-acridine (4)**. A mixture of 2-(2-(phenylamino)phenyl)propan-2-ol (**3**, 1.0 g, 4.5 moles) in 85.0% phosphoric acid (15 ml) was stirred at  $35$  °C for 2h until judged complete by TLC. The reaction mixture was then poured onto crushed ice and the precipitate was filtered, washed with



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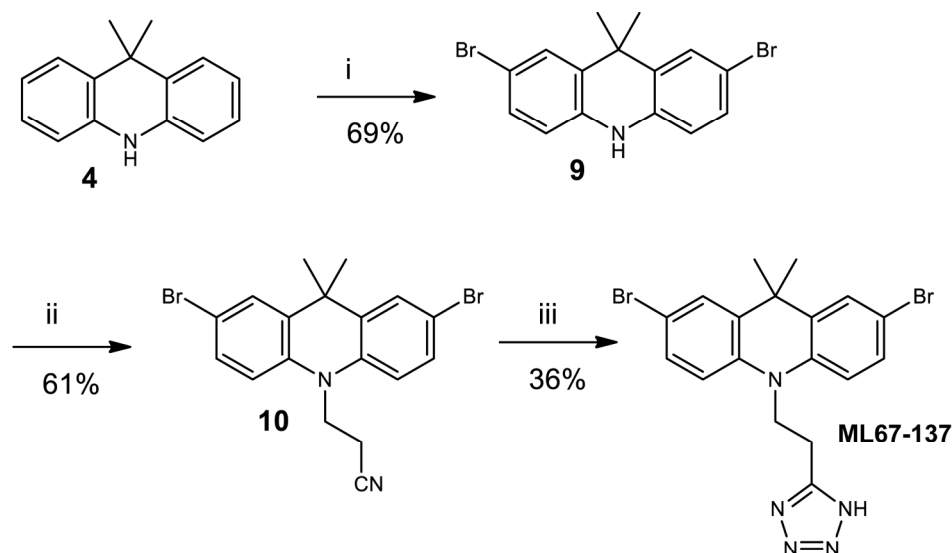
water, and dried to afford the title compound (0.90 g, 98% yield) as a white powder.  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.39 (d, 2H,  $J = 8.1$  Hz), 7.11 (t, 2H,  $J = 7.2$  Hz), 6.92 (t, 2H,  $J = 7.2$  Hz), 6.71 (d, 2H,  $J = 7.8$  Hz), 6.15 (br s, 1H), 1.61 (s, 6H); LCMS (ESI)  $m/z$  210 (MH+).

**3-(9,9-dimethylacridin-10-yl)propanenitrile (5).** To a stirred solution of 9,9-dimethyl-10H-acridine (4, 0.30 g, 1.0 mmol) in acrylonitrile (8 mL) was added benzyltrimethylammonium hydroxide solution (Triton-B, 50  $\mu\text{L}$ ) dropwise at room temperature. A vigorous exothermic reaction occurs, after which the reaction mixture was stirred for another hour and then dissolved in EtOAc (50 mL) and filtered through a pad of silica gel, washing with more EtOAc. The filtrate was evaporated and the crude product purified by silica gel column chromatography with gradient elution (0-20% EtOAc-hexane) to afford the title compound as off white crystals (0.27 g, 73% yield).  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$  1.60 (s, 6 H) 2.84 - 3.02 (m, 2 H) 4.32 - 4.48 (m, 2 H) 6.95 (d,  $J=8.29$  Hz, 2 H) 7.03 - 7.17 (m, 2 H) 7.24 - 7.39 (m, 2 H) 7.50 (dd,  $J=7.72, 1.51$  Hz, 2 H). LCMS (ESI)  $m/z$  263 (MH+).

**3-(2,7-dichloro-9,9-dimethylacridin-10-yl)propanenitrile (6).** To a stirred solution of 3-(9,9-dimethylacridin-10-yl)propanenitrile (5, 2.0 g, 7.62 mmol) in  $\text{CH}_2\text{Cl}_2$  (30 mL) at  $0^\circ\text{C}$  was added a solution of sulfonyl chloride (1.13 g, 0.68 mL, 8.4 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 mL) dropwise. The reaction mixture was stirred until starting material was consumed as judged by TLC, and then an additional 1.1 equivalents of sulfonyl chloride (1.13 g, 0.68 mL, 8.4 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 mL) was added dropwise. The dark solution was stirred until the monochloro adduct had been consumed, as judged by TLC. Aqueous  $\text{NaHCO}_3$  was added to the reaction mixture carefully until the solution was pH  $\sim 8$ , and the organic layer was then separated, dried over anhydrous  $\text{MgSO}_4$ , filtered, and evaporated under reduced pressure. The crude residue was adsorbed to silica gel and purified by silica gel column chromatography with gradient elution (0 to 40% EtOAc-hexane) to afford the title compound as a light brown oil (1.5 g, 60% yield).  $^1\text{H NMR}$  (300 MHz,  $\text{DMSO}-d_6$ )  $\delta$  ppm 1.51 (s, 6 H) 2.97 (t,  $J=6.50$  Hz, 2 H) 4.35 (t,  $J=6.69$  Hz, 2 H) 7.20 (d,  $J=8.85$  Hz, 2 H) 7.29 (dd,  $J=8.76, 2.35$  Hz, 2 H) 7.45 (d,  $J=2.26$  Hz, 2 H). LCMS (ESI)  $m/z$  332 (MH+).

**9,9-dimethyl-10-[2-(1H-1,2,3,4-tetrazol-5-yl)ethyl]acridine (ML67-33).** A sealed tube was charged with 3-(9,9-dimethylacridin-10-yl)propanenitrile (5, 0.1 g, 0.46 mmol), 1.2 equivalents of sodium azide (30 mg, 0.46 mmole), ammonium chloride (25 mg, 0.46 mmol) and DMF (5 mL). The tube was sealed under nitrogen and stirred at  $120^\circ\text{C}$  overnight. After cooling, the reaction was diluted with water (20 mL) and adjusted to pH  $\sim 5$  with aqueous 1N HCl. The aqueous solution was then extracted with EtOAc and the organic layer was washed with brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with gradient elution (0 to 5% MeOH- $\text{CH}_2\text{Cl}_2$ ) to afford the title compound as an off white solid (40 mg, 35% yield).  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm 1.48 (s, 6 H) 3.49 - 3.60 (m, 2 H) 4.42 - 4.54 (m, 2 H) 6.93 - 7.03 (m, 2 H) 7.07 (d,  $J=8.10$  Hz, 2 H) 7.15 - 7.25 (m, 2 H) 7.42 (dd,  $J=7.72, 1.51$  Hz, 2 H). LCMS (ESI)  $m/z$  306 (MH+).

## Synthesis of ML67-137



i) Trimethylphenylammonium tribromide (PTT), THF; ii) acrylonitrile, Triton-B; iii) NaN<sub>3</sub>, NH<sub>4</sub>Cl, DMF.

**2,7-dibromo-9,9-dimethyl-10H-acridine (9).** To a stirred solution of 9,9-dimethyl-10H-acridine (**4**, 0.50 g, 2.4 mmol) in dry THF (10 mL), was added trimethylphenylammonium tribromide (PTT) (1.8 g, 4.8 mmol) in one portion. The reaction mixture was stirred overnight at room temperature. After the reaction was judged complete (TLC), the reaction mixture was poured in water (30 mL) and extracted with EtOAc (2 x 30 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography with gradient elution (0 to 30% EtOAc-hexane) to afford the title compound as a light brown oil (0.6 g, 69% yield). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 1.47 (s, 6 H) 6.74 (d, *J*=8.48 Hz, 2 H) 7.22 (dd, *J*=8.48, 2.26 Hz, 2 H) 7.46 (d, *J*=2.07 Hz, 2 H) 9.18 (s, 1 H). LCMS (ESI) *m/z* 368 (MH<sup>+</sup>).

**3-(2,7-dibromo-9,9-dimethylacridin-10-yl)propanenitrile (10).** To a stirred solution of 2,7-dibromo-9,9-dimethyl-10H-acridine (**9**, 0.20 g, 0.54 mmol) in acrylonitrile (5 mL) was added benzyltrimethylammonium hydroxide solution (50 μL) at room temperature. After the initial vigorous exothermic reaction subsided, the reaction mixture was stirred at room temperature for 1 hour. The thick reaction mixture was then diluted with EtOAc (50 mL) and the mixture filtered through a pad of silica gel and washed with more EtOAc. The filtrate was evaporated and the crude product purified by silica gel column chromatography with gradient elution (0-20% EtOAc-hexane) to afford the title compound as a light brown foam (139 mg, 61% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 1.55 (s, 6 H) 2.82 - 2.94 (m, 2 H) 4.33 (t, *J*=7.35 Hz, 2 H) 6.80 (d, *J*=8.67 Hz, 2 H) 7.38 (dd, *J*=8.67, 2.26 Hz, 2 H) 7.54 (d, *J*=2.26 Hz, 2 H). LCMS (ESI) *m/z* 421 (MH<sup>+</sup>).

**2,7-dibromo-9,9-dimethyl-10-[2-(1H-1,2,3,4-tetrazol-5-yl)ethyl]acridine (ML67-137).** A sealed tube was charged with 3-(2,7-dibromo-9,9-dimethylacridin-10-yl)propanenitrile (**10**, 0.1 g, 0.24 mmol), sodium azide (31 mg, 0.48 mmol), ammonium chloride (26 mg, 0.48 mmol) and DMF (5 mL). The tube was sealed under nitrogen and stirred at 120 °C overnight. After cooling, the reaction mixture was diluted with water (20 mL) and adjusted to pH~5 with 1N aq. HCl, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. The residue was purified by silica gel column chromatography with gradient elution (0 to 5% MeOH-CH<sub>2</sub>Cl<sub>2</sub>) to afford the title compound as a light brown foam (40 mg, 36% yield). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 1.34

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(s, 6 H) 3.32 (t,  $J=7.16$  Hz, 2 H) 4.40 (t,  $J=7.25$  Hz, 2 H) 7.15 (d,  $J=8.85$  Hz, 2 H) 7.39 (dd,  $J=8.67$ , 1.88 Hz, 2 H) 7.50 (d,  $J=2.07$  Hz, 2 H). LCMS (ESI)  $m/z$  464 (MH<sup>+</sup>).

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