Supporting information

Manuscript title:

A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells

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Supporting Figure and Figure Legends

Table S1

All assays employed in the screening cascade are listed with detailed information about their purpose and statistical significance. Additional details about these assays are provided as Supplementary Methods.

Assay	Assay Rationale	Z'	S:B
Nox1 HT29 Primary assay (HTS Maybridge Library)	The purpose of this assay is to measure the ability of compounds to inhibit Nox1 activity.	0.48 +/- 0.08	18.5 +/- 4.4
H2O2-based counterscreen	The purpose of this assay to eliminate H202 scavenger compounds that would behave as false positives in the primary screening assay	0.9	30
Nox1 HT29 confirmatory assay	The purpose of this assay is to confirm the ability of compounds to inhibit Nox1 activity.	0.52	19
Cytotoxicity assay (Cell Titer Glo)	The purpose of this assay to eliminate cytotoxic compounds that would behave as false positives in the primary screening assay	0.8	27
HEK293-Nox1 dose response confirmatory assay	The purpose of this assay is to determine the potency of compounds to inhibit Nox1 activity in the Nox -293 transfection format.	0.55	18.5
HEK293-Nox2, -Nox3 and -Nox4 selectivity assays	The purpose of this assay is to evaluate the ability of compounds to inhibit Nox-2, -3 or -4 activity in the Nox -293 transfection format	0.55 +/- 0.12	16.4 +/- 2.2
Xanthine Oxidase specificity assay	The purpose of this assay is to evaluate the ability of compounds to inhibit ROS production by another cellular sources such as xanthine oxidase	0.85	96

Table S2

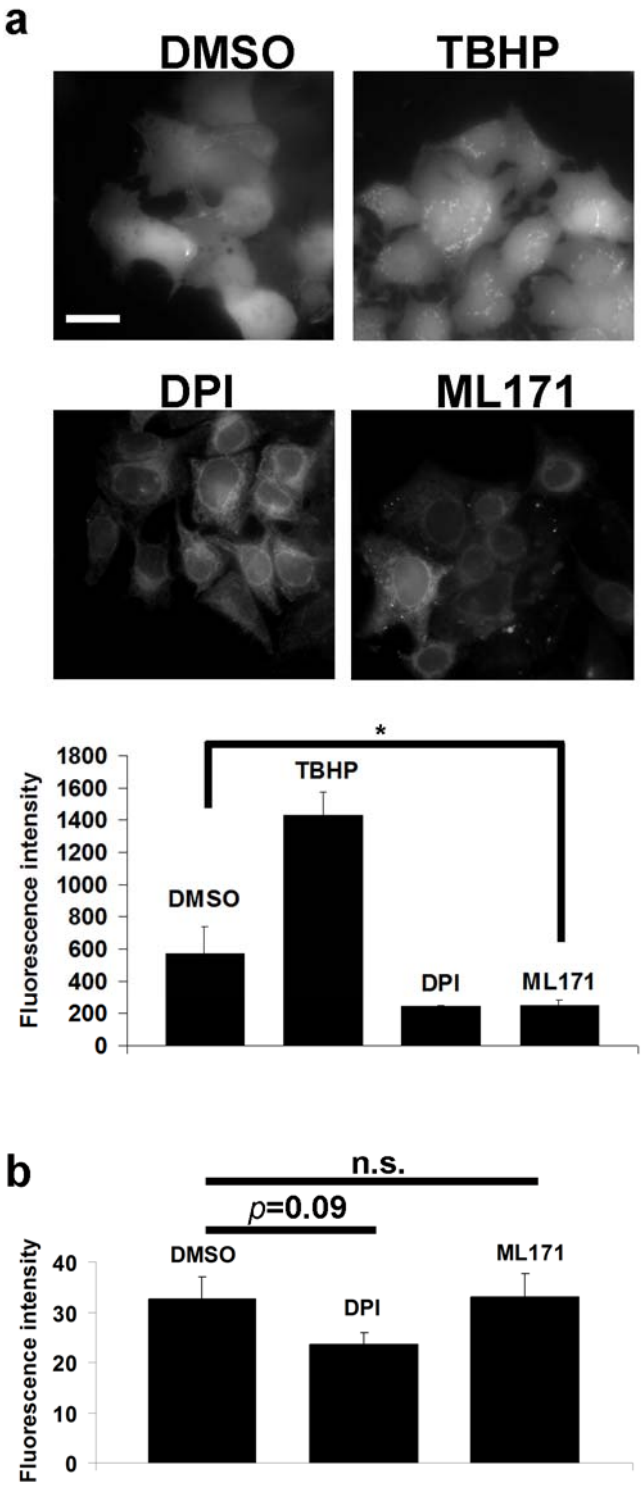
% of inhibition and Ki values for 2-APT towards a large battery of human or rodent G-protein coupled receptors (GPCRs), channels and transporters expressed in the CNS are reported. Ki values (nM) were generated only when the % of inhibition in the primary assay was > 60%

Receptor	% inhibition	Ki (nM)
5ht1a	34.4	
5ht1b	11.3	
5ht1d	16.1	
5ht1e	13.1	
5ht2a	33.2	
5ht2b	84.3	556
5ht2c	62.4	2964
5ht3	1.4	
5ht4		>10,000
5ht5a	45.6	
5ht6	30.8	
5ht7	-5.7	
A1		
A2		
A2B2	-4	
A2B4	4	
A3B2	4	
A3B4	0	
A4B2	1	
A4B2**	-2	
A4B4	7	
Alpha1A	36.3	
Alpha1B	38.4	
Alpha1D	23.8	
Alpha2A	50.7	6918.3
Alpha2B	58.8	2719
Alpha2C	98.2	5,757.0(AVE)
AMPA*		
B2		
Beta1	23.5	
Beta2	-0.9	
Beta3	3.5	
BZP Rat Brain Site	44.8	
Ca+Channel	-19.4	
CB1	5	
CB1 H		
CB2	3.8	
D1	23.6	
D2	22.6	
D3	59.2	9,056.00
D4	34.7	
D5	16.8(AVE)	
DAT	16	
DOR	-0.5	
EP1		
EP2		
EP3	0	
EP4	45.5	

Receptor	%inhibition	Ki (nM)
GabaA	25.0(AVE)	
GABAa a1	16.2	
GABAa a2		
GABAa a3		
GABAa a5		
GABAa a6		
GabaB		
H1	46.4	
H2	43	
H3	-2.4	
H4	10.1(AVE)	
Imidazoline 1		
KA-R*		
KOR	16.3	
M1	1.1	
M2	22.4	
M3	-5.8	>10,000
M4	19.3	>10,000
M5	2.3	>10,000
mGlur5		
mGluR5_RatBrain		>10,000
MOR	-18	
NA+Channel N/A		
NET	60.5	3943
NMDA PCP site	25	
NTS1		
NTS2		
Oxytocin	38.3(AVE)	>10,000
PBR	32.1	
PKCa		
PKCb		
PKCd		
PKCe		
PKCg		
SERT	16.4	10,000.0(AVE)
Sigma 1	50.1	>10,000
Sigma 2	9	
V1A	4	
V1B	8.9	
V2	-5.5	
VMAT1 N/A		
VMAT2 N/A		
Y2		

Figure S1

ML171 specifically blocks ROS generation. (a) ML171 blocks ROS generation measured by carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2-DCFDA) staining. In the upper panel, DLD1 cells were treated as indicated with DMSO, 10 μM ML171 or DPI and 5 μM *tert*-Butyl hydroperoxide (TBHP) used as positive control for the staining. After 1 hr, cells were washed with HBSS, stained with DCFDA for 30 min and visualized by epifluorescence microscopy (60X). Scale bars, 10 μm. One representative image is shown. In the lower panel, the quantification of the experiment shown in the upper panel is given: fluorescence intensity was obtained as the average of intensities calculated using Metamorph software from 10 random fields/condition. In the graph, error bars represent SD. *p <0.005. (b) ML171 does not significantly affect mitochondrial ROS generation. DLD1 cells were treated with DMSO control, 10 μM ML171 or 10 μM DPI. After 1 hr, cells were washed with HBSS, stained with MitoSOX Red mitochondrial superoxide indicator and visualized by epifluorescence microscopy (60X). Fluorescence intensity for each condition was obtained as the average of intensities calculated using Metamorph software from 10 random fields/condition. In the graph, error bars represent SEM. n.s. = not significant.


a
DMSO
TBHP
DPI
ML171
*
Fluorescence intensity
1800
1600
1400
1200
1000
800
600
400
200
0
DMSO
TBHP
DPI
ML171
b
n.s.
p=0.09
Fluorescence intensity
40
30
20
10
0
DMSO
DPI
ML171

Supporting Methods

Reagents

The following reagents were purchased from Sigma: H_2O_2 (95321), NAC (A7250), rotenone (R8875), xanthine oxidase (XO) (X4376), hypoxanthine (HX) (H9377).

Nox1 HT29 cell-based primary assay

HT29 cells are cultured in 150mm diameter plate (Falcon) and when 70-80% confluence is reached, cells are trypsinized, harvested in HBSS and counted. $4*10^4$ cells are dispensed into individual wells in 30µl final volume (384 well plates, white Corning 3704) by using a robotic liquid handler (FlexDrop, Perkin Elmer). Cells are treated for 60 min at 37^0C with 50 nL of DPI, DMSO and library compounds which are automatically dispensed into individual wells (Biomek FX, Beckman Coulter) from their respective assay plates. This will correspond to a final concentration of 10 µM DPI or library compounds, and 0.1% DMSO. 20 µl of a mixture containing 200 µM luminol plus 0.32 units of HRP (final concentration) is added. Luminescence is quantified using a 384-well plate luminometer (EnVision). The data output consisting of the emission intensities for each well is imported into a spread-sheet program (such as Excel) for further processing. As designed, compounds that inhibit Nox1 activity will reduce cellular ROS production, leading to reduced probe-ROS interactions and reduced well luminescence. Compounds were considered 'hits' and further processed when light emission was blocked > 75%

than DMSO wells (DMSO and DPI wells were set to 0% and 100% respectively). Compounds were tested in singlicate at a concentration of 10 μM.

H_2O_2-based counterscreen

This cell-free assay utilizes a luminol probe-based CL assay to measure the ability of primary hits to scavenge H_2O_2. The general antioxidant N-acetylcysteine (NAC) is used as positive control. H_2O_2 is freshly prepared by diluting stock solution 1/100,000 (0.088 mM final concentration) in HBSS. A 100 mg/ml NAC working solution in HBSS is made and used to prepare NAC assay plate. 30 μl of diluted H_2O_2 are dispensed in 384-well plate (White Corning) by using a robotic liquid handler (FlexDrop, Perkin Elmer). 50 nL of NAC/HBSS or library compounds are dispensed by Biomek FX into each individual wells. After 10 minutes incubation at room temperature, 20 μl of a mixture in HBSS of 1 mM luminol plus 8 units of HRP is added. Luminescence is quantified using a 384-well plate luminometer (EnVision). As designed, compounds that scavenge hydrogen peroxide will reduce well luminescence. Compounds were considered as false positive ROS scavengers when light emission was blocked > 15% than vehicle wells (vehicle and NAC wells were set to 0% and 100% respectively). Compounds were tested in singlicate at a concentration of 10 μM.

Nox1 HT29 cell-based confirmatory assay

Same as the Primary screen except that the compounds were tested in triplicate at 6.7 and 2.2 micromolar. DMSO solutions of the hit compounds were cherry picked from the Maybridge Library.

Cytotoxicity assay

In this assay, cells were incubated for 1 hour with test compound or rotenone used as a positive control. Cell viability was measured with the Cell Titer Glo reagent (Promega) that measures cellular ATP following the manufacturer's instructions. As designed, toxic compounds will reduce well luminescence. Compounds were considered as toxic when light emission was blocked > 25% than vehicle wells (vehicle and rotenone wells were set to 0% and 100% respectively).

HEK293-Nox1 dose response confirmatory assay

$5*10^5$ HEK293 cells are seeded in 6-well plates (Falcon) in media without antibiotics the day before transfection. Cells were transfected with expression vectors for Nox1, NoxA1, NoxO1 and constitutive active Rac1 (Rac1-Q61L) using Lipofectamine 2000 (InVitrogen) following manufacturer's instructions. Briefly, 2 µl Lipofectamine 2000/well were diluted in 100 µl Optimem and 2 µg total DNA/well were diluted in 100 µl Optimem. After 5 min of incubation at room temperature, Lipofectamine 2000 was added to diluted DNA. After 20 min of incubation at room temperature, Lipofectamine/DNA mixture was added to cells. After 16-20h tissue culture incubation, cells are trypsinized, resuspended in HBSS and counted. $5*10^4$ cells in 30 µl HBSS are

dispensed in 384-well plate (white Corning) by FlexDrop. 50 nL of DPI, DMSO or library compounds are dispensed by Biomek FX into each individual well. 20 µl of a mixture of 1 mM luminol plus 8 units of HRP in HBSS is added and luminescence quantified using a 384-well plate luminometer (EnVision). Chemiluminescence was measured for 30 minutes. Compounds were tested in triplicate.

HEK293-Nox2, -Nox3, -Nox4 dose-response selectivity assay

In these selectivity assays the cells are prepared in the same manner as the HEK293-Nox1 transfection assay except that the cells are transfected with the appropriate expression vectors for each Nox subtype. Nox2: Nox2, $p67^{phox}$, $p47^{phox}$ and Rac2-Q61L. Nox-3: Nox3, NoxO1, NoxA1 and Rac1-Q61L. Nox-4: Nox4 and $p22^{phox}$. The expression vectors for all Nox isoforms and their cytosolic regulators were previously described[1]. Nox activity was determined by chemiluminescence as for the HEK293-Nox1 assay. Compounds were tested in triplicate.

Xanthine oxidase (XO) dose-response specificity assay

This cell-free assay utilizes a luminol probe-based CL assay to measure the ability of compounds to block XO activity. 0.25U/ml XO and 2 mM HX solutions are prepared fresh at the moment. 25 µl of freshly-prepared XO solution is dispensed in 384-well plate (white Corning) by FlexDrop. 50 nL of DPI, DMSO or library compounds are dispensed by Biomek FX into each individual well. Upon 10 minutes of incubation at room temperature, 20 µl of a mixture in HBSS of 1 mM luminol plus 8 units of HRP is added

and luminescence is quantified using a 384-well plate luminometer (EnVision). Compounds were tested in triplicate.

Carboxy-H2-DCFDA staining

Carboxy-H2-DCFDA staining was performed using the Image iT-Live Green Reactive Oxygen Species Detection Kit (Molecular Probes, I36007) following the manufacturer's instruction. DLD1 cells were treated as indicated in figure legends for 1 hr, washed with HBSS and stained with carboxy-H2-DCFDA for 30 min. Cells were washed three times with warm HBSS and visualized by microscopy.

Detection of mitochondrial ROS generation

Detection of ROS generation in mitochondria was performed using MitoSOX Red Mitochondria superoxide indicator 'for live cell imaging' (Molecular Probes, M36008) following the manufacturer's instruction. DLD1 cells were treated as indicated in figure legends for 1 hr, washed with HBSS and stained with MitoSOX Red Mitochondria superoxide indicator for 10 min. Cells were washed three times with warm HBSS and visualized by microscopy.

NIMH Psychoactive Drug Screening Program (PDSP)

% of inhibition values and Ki determinations for CNS receptors and channels were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2008-00025-C (NIMH PDSP). The NIMH

PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA.

Statistical significance

Z-factor was calculated for each plate as described[2]

Supporting information References

1. **Gianni, D. *et al.* Novel p47(phox)-related organizers regulate localized NADPH oxidase 1 (Nox1) activity. *Science signaling* 2, ra54 (2009).**
2. **Zhang, J.H., Chung, T.D. & Oldenburg, K.R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* 4, 67-73 (1999).**