SUPPLEMENTARY INFORMATION

Suppressors of superoxide production from mitochondrial complex III

Adam L. Orr[1,3], Leonardo Vargas[2], Carolina N. Turk[2], Janine E. Baaten[2], Jason T. Matzen[2], Victoria J. Dardov[2], Stephen J. Attle[2], Jing Li[2], Douglas C. Quackenbush[2], Renata L. S. Goncalves[1], Irina V. Perevoshchikova[1], H. Michael Petrassi[2], Shelly L. Meeusen[2], Edward K. Ainscow[2], Martin D. Brand[1,*]

[1]Buck Institute for Research on Aging, Novato, California 94945, USA

[2]Genomics Institute of the Novartis Research Foundation, San Diego, California 92121, USA

[3]Current address: Gladstone Institutes, San Francisco, California 94158, USA

*Corresponding author. E-mail address: mbrand@buckinstitute.org (Martin D. Brand)

Nature Chemical Biology: doi:10.1038/nchembio.1910

SUPPLEMENTARY RESULTS

Supplementary Table 1. Summary of screening strategy to identify S3QELs

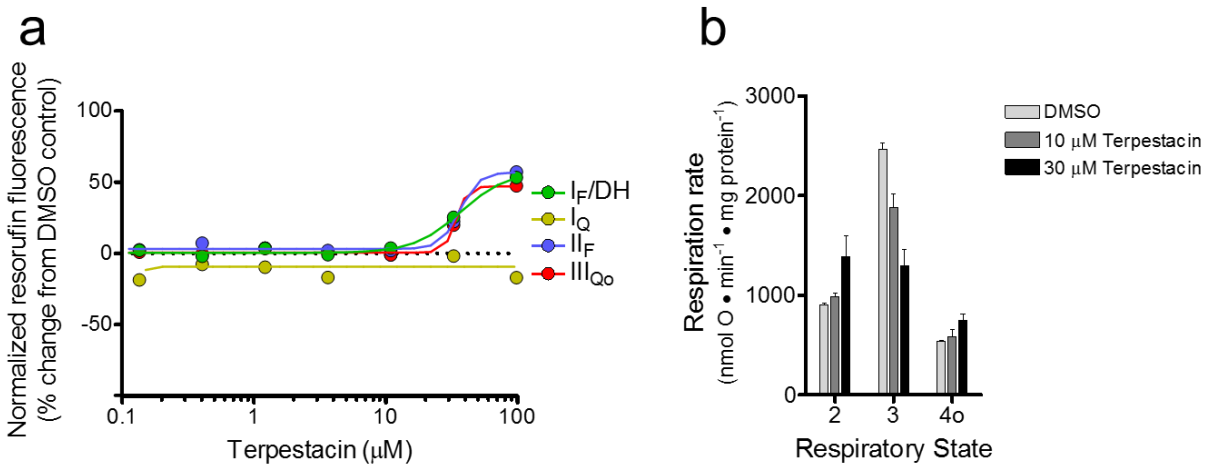
Step	Assay	Description	Concentrations tested (μM)	Criteria for selectivity	Remaining compounds
1	Primary Screen	Endpoint H_2O_2 production from sites III_{Qo}, I_Q, II_F against 635,000 compounds	10	>40% inhibition of III_{Qo}; <45% inhibition of II_F, <50% inhibition of I_Q	6,674
2	Confirmation screen	Endpoint H_2O_2 production from sites III_{Qo}, I_Q, II_F. Triplicate determinations	10	>35% inhibition of $IIIQ_o$; <30% inhibition of I_Q, II_F	1097
3	$\Delta\psi_m$	Endpoint $\Delta\psi_m$ assay	10	<±30% effect	1094
4	GalacTox	Endpoint cell viability after 72 h exposure	10	<20% decrease in cell viability	995
5	Dose-response rescreen	Determine IC_{50} against III_{Qo} superoxide production	Up to 10	Progressive inhibition of III_{Qo}; (IC_{50} <3.2 μM)	103
6	Expanded rescreen	Determine selectivity in six H_2O_2 and two $\Delta\psi_m$ endpoint assays	Up to 32 (10x IC_{50})	>30% suppression of III_{Qo}; <30% inhibition in any other assay	71
7	Respiration	State 2, 3, 4o respiration	Up to 32	<30% inhibition of state 3; <30% increase in state 4o	63
8	Dose-response with fresh compounds	Confirm selectivity in six H_2O_2 and two $\Delta\psi_m$ endpoint assays	Up to 32	Progressive, near complete suppression of III_{Qo}; <20% effect in any other assay	20

Nature Chemical Biology: doi:10.1038/nchembio.1910

Supplementary Table 2. Small molecule screening data

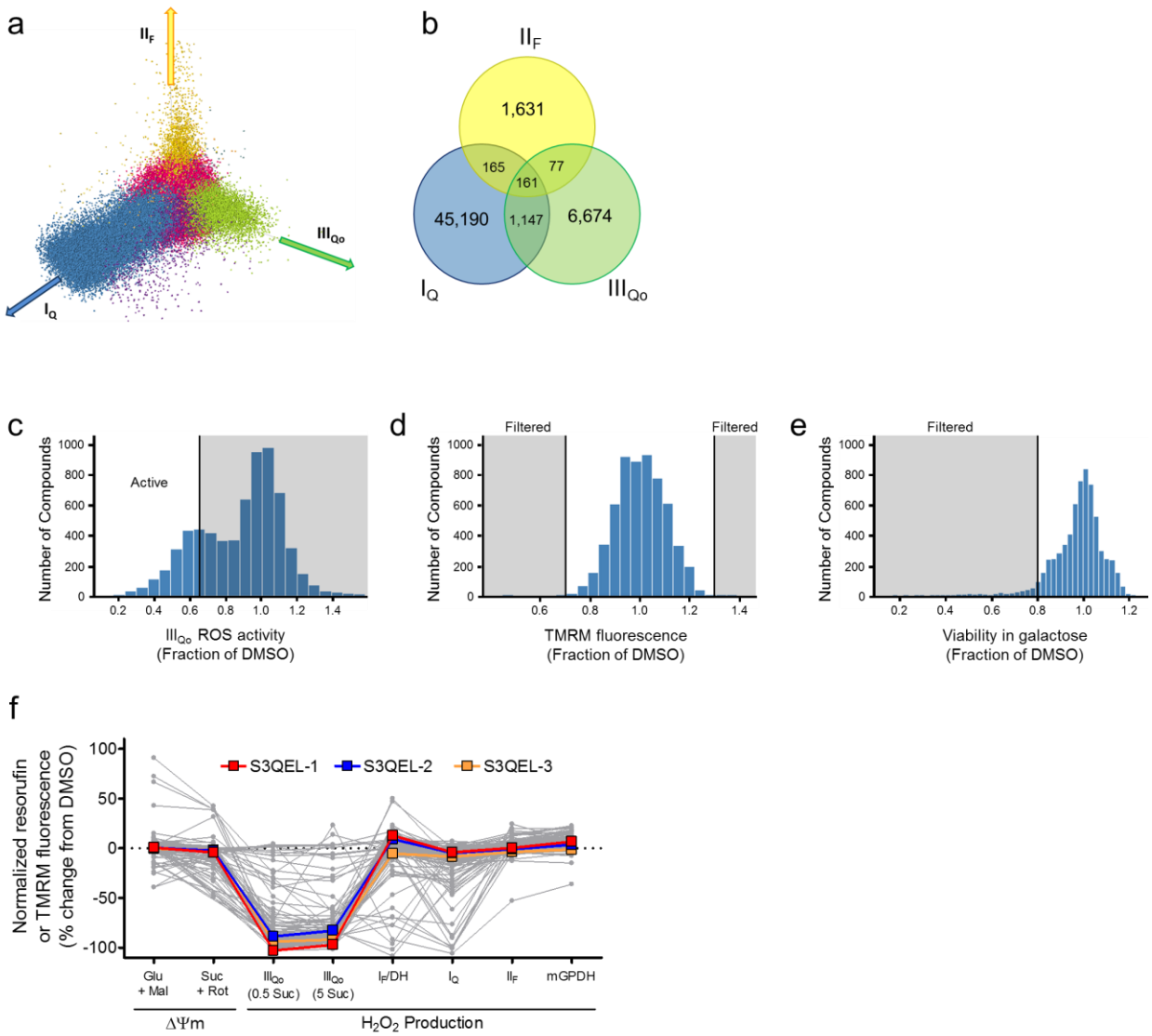
Category	Parameter	Description
Assay	Type of assay	In vitro biochemical assay using intact isolated mitochondria and an Amplex UltraRed-based H_2O_2 detection system.
	Target	The outer ubiquinone binding site of mitochondrial complex III (site of III_{Qo} cytochrome *bc*$_1$ complex; EC 1.10.2.2).
	Primary measurement	Endpoint measurement of superoxide/H_2O_2 produced from site III_{Qo} induced by succinate in the presence of antimycin A and rotenone.
	Key reagents	Amplex UltraRed (Life Technologies), Superoxide dismutase (Sigma), Horseradish peroxidase (Sigma)
	Assay protocol	Described in the "Online Methods" section under the heading "Ultra high-throughput primary screen: sites III_{Qo}, I_Q and II_F".
	Additional comments	Parallel screens of superoxide/H_2O_2 produced from the ubiquinone binding site of complex I (site I_Q of NADH:ubiquinone oxidoreductase; EC 1.6.5.30) and the flavin binding site of complex II (site II_F of succinate dehydrogenase; EC 1.3.5.1) were used to identify hits selective only for site III_{Qo} superoxide/H_2O_2 production. See Ref 23 for details of all assays in 96-well format: Orr, A.L. et al. Free Rad. Biol. Med. 65, 1047-1059 (2013).
Library	Library size	635,000
	Library composition	The GNF Academic Screening Collection consists of a collection of compounds that were selected after applying proprietary algorithms designed to select for optimal compound properties and eliminate undesirable functional groups.
	Source	Composed from multiple sources.
	Additional comments	
Screen	Format	1536 well plates.
	Concentration(s) tested	10 µM, 1% v/v DMSO.
	Plate controls	DMSO vehicle as negative control.
	Reagent/ compound dispensing system	GNF Systems Washer Dispenser for reagents; GNF Systems Pintool for compounds.
	Detection instrument and software	BMG Labtech PHERAstar Plus microplate reader (λ_{ex} = 540 nm, λ_{em} = 590 nm); Data was analyzed and hit selection performed using GNF proprietary software.
	Assay validation/QC	Median Z' score = 0.43, Median assay window = 4.9.
	Correction factors	Signal normalized to median signal across plate.
	Normalization	Endpoint measurements normalized as percent of DMSO controls.
	Additional comments	
Post-HTS analysis	Hit criteria	Hits in the III_{Qo} assay were first selected based on >40% inhibition. Compounds that also had <50% effect in the I_Q and <45% inhibition in the II_F assay were chosen as selective suppressors of superoxide production by site III_{Qo} and tested further.
	Hit rate	1.05% (6674/635,000)
	Additional assay(s)	Additional counterscreens and secondary screens outlined in the "Online Methods" section and summarized in Supplementary Table 1
	Confirmation of hit purity and structure	LC-MS from powder supply at reconfirmation
	Additional comments	

Nature Chemical Biology: doi:10.1038/nchembio.1910



Supplementary Figure 1. Terpestacin is not a selective suppressor of site III_{Qo} superoxide production. (a) Dose-response curves for terpestacin tested in four H_2O_2 endpoint assays using the Amplex UltraRed detection system (mean of two biological replicates). (**b**) Effect of 10 and 30 µM terpestacin on respiration rates of rat skeletal muscle mitochondria driven by succinate in the presence of rotenone (respiratory state 2) followed by the sequential additions of ADP (phosphorylating state 3) and oligomycin (non-phosphorylating state 4o) (mean ± SE, n = 8 technical replicates).

Nature Chemical Biology: doi:10.1038/nchembio.1910

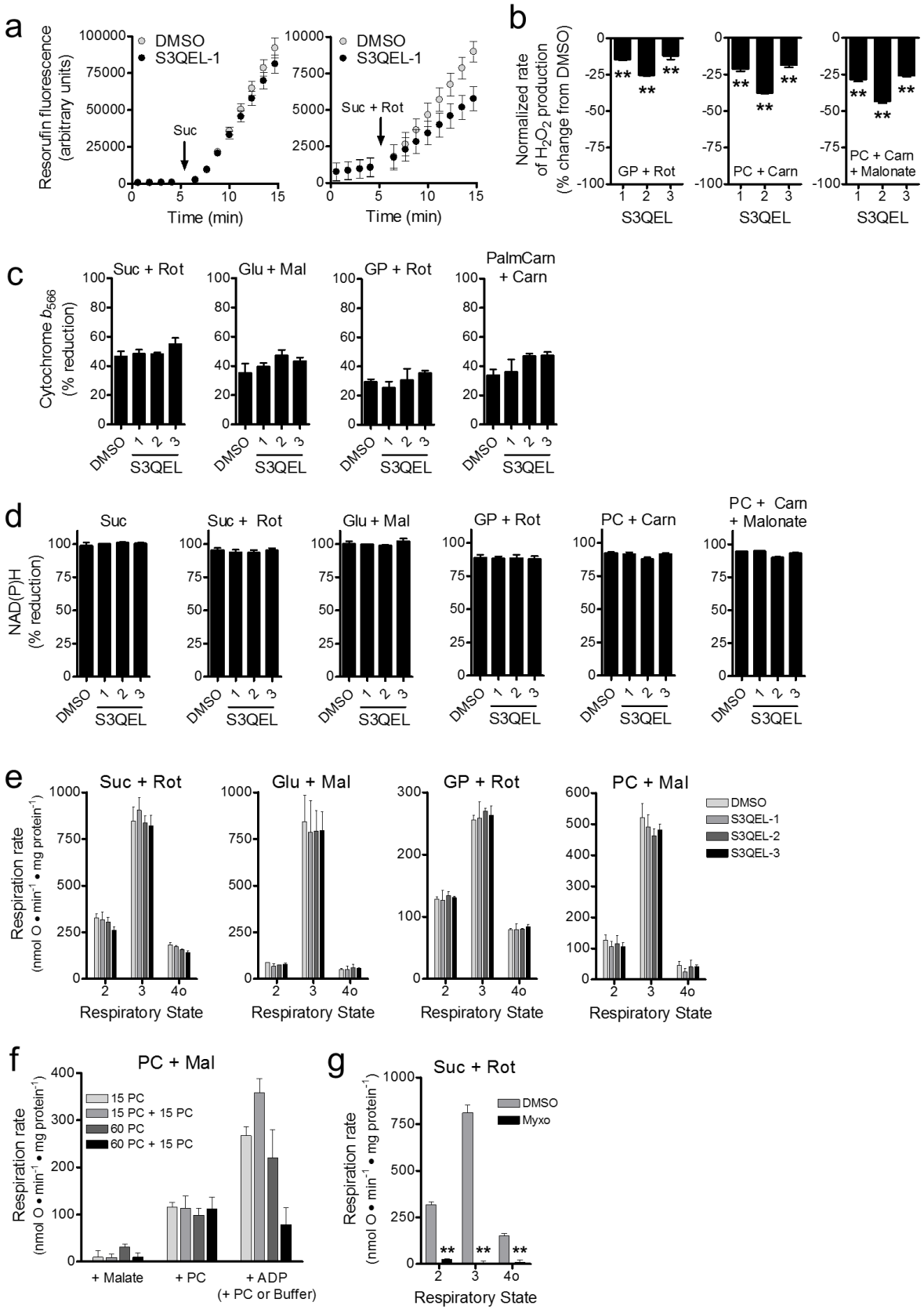


Supplementary Figure 2. Chemical screening using isolated mitochondria identifies inhibitors of site III_{Qo} superoxide production. (a) 3D-scatterplot of the effects of 635,000 small molecules at 10 μM on H_2O_2 production from three sites, measured using the Amplex UltraRed assay, in which horseradish peroxidase uses H_2O_2 to oxidize non-fluorescent Amplex UltraRed to a fluorescent resorufin product. Compounds that selectively inhibited production at sites III_{Qo}, I_Q or II_F are shaded in green, blue or yellow, respectively. Specific inhibition at each site is represented as displacement away from the origin on the relevant axis. **(b)** Subgroup classification of >55,000 inhibitors identified in the primary screen. **(c - e)** Activity profiles for primary hits retested against (c) site III_{Qo} superoxide production and (d) $\Delta\psi_m$ in isolated mitochondria and (e) cell viability in HEK293 cells cultured with galactose as the primary carbohydrate. Data are median values for triplicate repeats, normalized to the intra-plate median signal. Compounds with activity of <0.65 in the III_{Qo} assay (>35% inhibition) were classed as being active. Compounds that changed $\Delta\psi_m$ ± 0.3 or decreased cell viability by > 0.2 were removed from further testing. These cutoffs were approximately 3 standard deviations from the group mean. **(f)** Selectivity profiles for 103 compounds retested against two $\Delta\psi_m$ and six H_2O_2

Nature Chemical Biology: doi:10.1038/nchembio.1910

endpoint Amplex UltraRed screening assays at 10x IC_{50} against III_{Qo} H_2O_2 production.

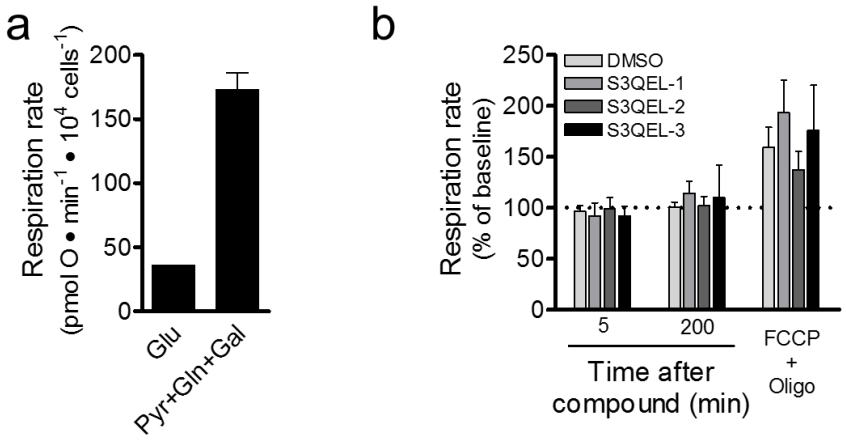
Nature Chemical Biology: doi:10.1038/nchembio.1910



Nature Chemical Biology: doi:10.1038/nchembio.1910

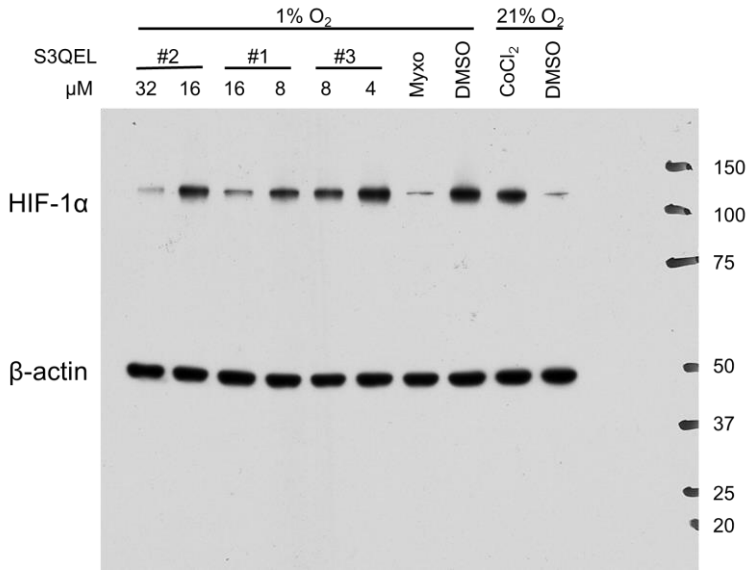
Supplementary Figure 3. S3QELs suppress superoxide production by site III_{Q_o} in isolated mitochondria without altering energy metabolism. (a) Kinetic traces of H_2O_2 production (Amplex UltraRed assay) in the presence of 8 µM S3QEL-1 in mitochondria respiring on succinate alone, when overall H_2O_2 production is predominantly from site I_Q, or with rotenone added to eliminate site I_Q (mean ± SE, n = 3 biological replicates). **(b - d)** Effects of S3QELs 1-3 on (b) the steady-state rate of H_2O_2 production measured using the Amplex UltraRed assay, (c) reduction state of cytochrome b_{566}, and (d) reduction state of the matrix NAD(P)H pool, with the substrate/inhibitor combinations indicated (normalized mean ± SE, n = 3 biological replicates). **(e)** Effect of S3QELs on respiration rates driven by succinate plus rotenone, glutamate plus malate, glycerol phosphate plus rotenone, or palmitoylcarnitine plus malate (respiratory state 2) followed by the sequential additions of ADP (phosphorylating state 3) and oligomycin (non-phosphorylating state 4o) (mean ± SE, n = 3 biological replicates). **(f)** Effect of adding palmitoylcarnitine to mitochondria respiring on malate. Sequential additions of 15 µM palmitoylcarnitine yielded the highest state 3 rates (mean ± SE for 4 technical replicates). **(g)** Effect of myxothiazol on respiratory states 2, 3, and 4o driven by succinate and rotenone (mean ± SE, n = 4 biological replicates). Concentrations of S3QELs 1-3 were 8, 16, and 4 µM in (a – e). **p < 0.01; one-way ANOVA with Dunnett's posttest (S3QELs) or Student's *t*-test with Welch's correction (myxothiazol). The values for S3QELs were not significantly different from the value for DMSO in (c – e). GP, glycerol phosphate; Rot, rotenone; PC, palmitoylcarnitine; Carn, carnitine; Glu, glutamate; Mal, malate; Suc, succinate; Myxo, myxothiazol.

Nature Chemical Biology: doi:10.1038/nchembio.1910



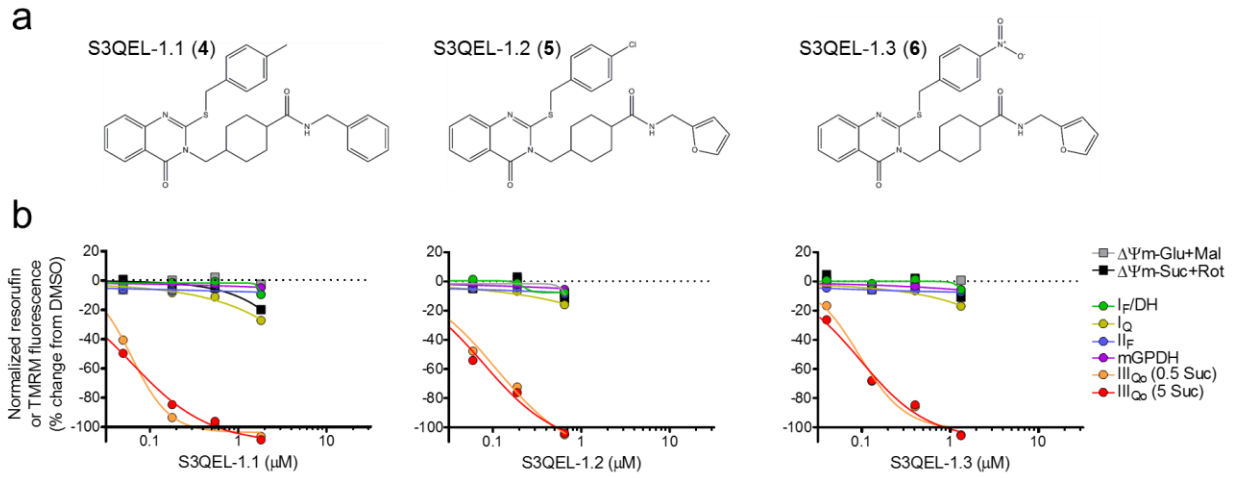
Supplementary Figure 4. S3QELs do not alter cellular respiration. (a) HEK-293 cells cultured in medium containing pyruvate, glutamine and galactose had higher basal respiration than when cultured in glucose-based media (mean of two biological replicates for Glu and mean ± SE, n = 3 biological replicates for Pyr+Gln+Gal). **(b)** Respiration of HEK-293 cells was not significantly changed after acute addition of S3QELs 1-3 at 20x their mitochondrial IC_{50} (mean ± SE, n = 3 biological replicates). Glu, glucose; Pyr, pyruvate, Gln, glutamine; Gal, galactose; Oligo, oligomycin.

Nature Chemical Biology: doi:10.1038/nchembio.1910



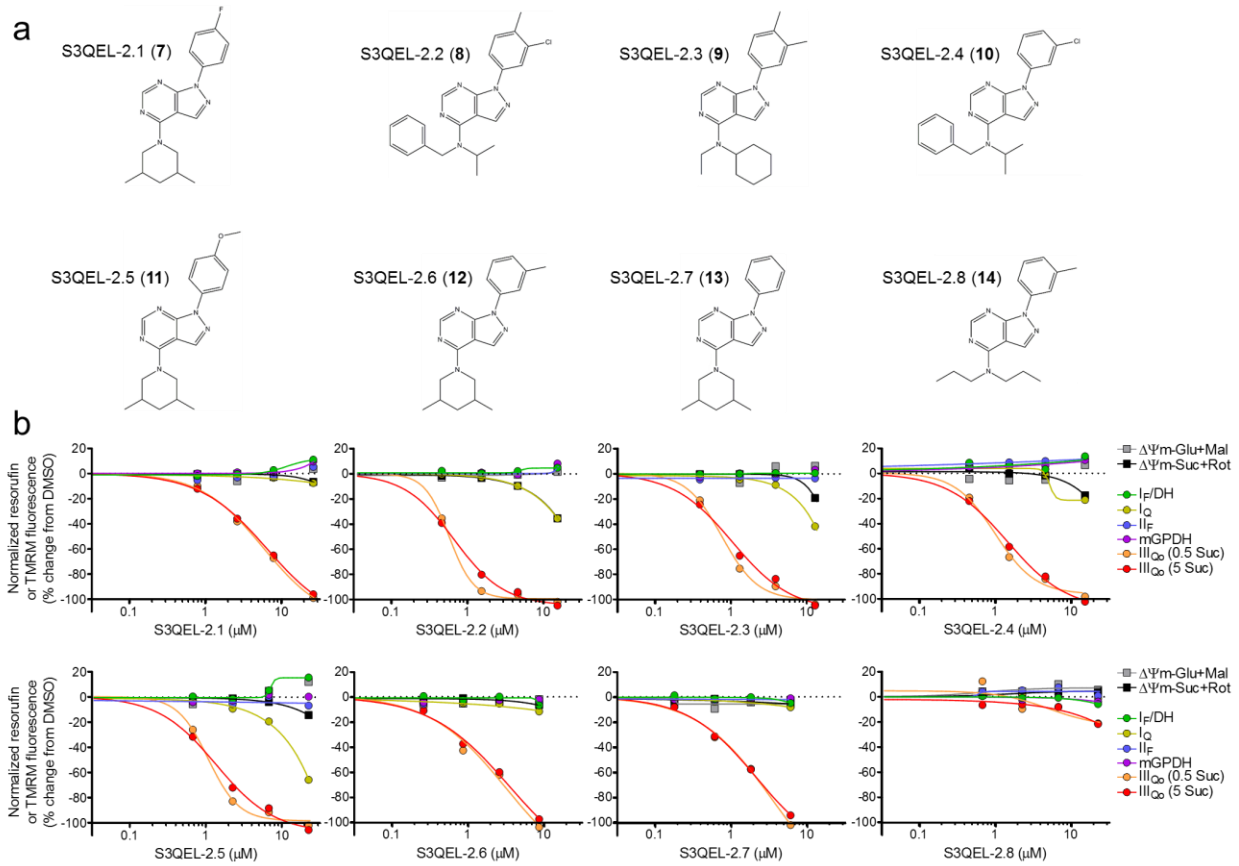
Supplementary Figure 5. S3QELs modulate the cellular hypoxic response. Representative blot of HIF-1α and β-actin levels in HEK-293 cells following 3.5 h normoxia, $CoCl_2$-induced pseudohypoxia, or hypoxia. Cells were treated with DMSO, S3QELs 1-3 at 10 or 20x their mitochondrial IC_{50} or 2 μM myxothiazol during the hypoxic challenge. This blot is representative of three biological replicates. Myxo, myxothiazol.

Nature Chemical Biology: doi:10.1038/nchembio.1910



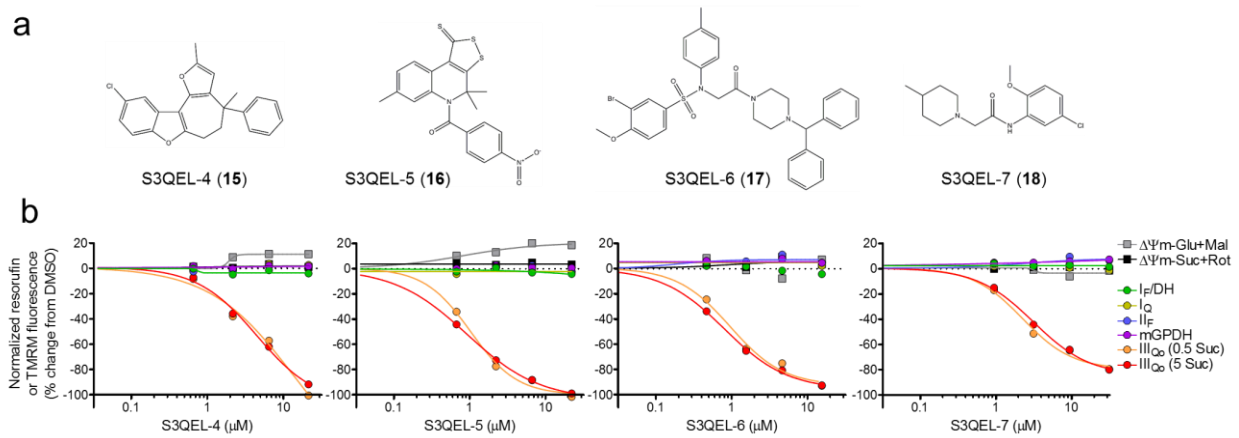
Supplementary Figure 6. Structure-activity relationships for the sulfanyloxoquinazoline structural group that includes S3QEL-1. (a) Structures of S3QELs 1.1-1.3. **(b)** Corresponding dose-response curves for S3QELs 1.1-1.3 tested in two $\Delta\psi_m$ and six H_2O_2 endpoint assays (n = 1). Glu, glutamate; Mal, malate; Suc, succinate; Rot, rotenone.

Nature Chemical Biology: doi:10.1038/nchembio.1910



Supplementary Figure 7. Structure-activity relationships for the pyrazolopyrimidine structural group that includes S3QEL-2. (a) Structures of S3QELs 2.1-2.8. **(b)** Corresponding dose-response curves for S3QELs 2.1-2.8 tested in two $\Delta\psi_m$ and six H_2O_2 endpoint assays (n = 1). Glu, glutamate; Mal, malate; Suc, succinate; Rot, rotenone.

Nature Chemical Biology: doi:10.1038/nchembio.1910



Supplementary Figure 8. S3QELs are structurally diverse. (a) Structures of S3QELs 4-7. **(b)** Corresponding dose-response curves for S3QELs 4-7 tested in two $\Delta\psi_m$ and six H_2O_2 endpoint assays (n = 1). Glu, glutamate; Mal, malate; Suc, succinate; Rot, rotenone.

Nature Chemical Biology: doi:10.1038/nchembio.1910

Supplementary Note

Compound Characterization

S3QELs were obtained from Chemdiv unless specified otherwise: S3QEL-1 (catalog ID K284-4710), S3QEL-1.1 (K284-4711), S3QEL-1.2 (K284-4767), S3QEL-1.3 (K284-4794), S3QEL-2 (K405-3102), S3QEL-2.1 (Life Chemicals, F1886-0120), S3QEL-2.2 (Life Chemicals, F1886-0426), S3QEL-2.3 (K405-3096), S3QEL-2.4 (K405-3741), S3QEL-2.5 (K402-1025), S3QEL-2.6 (K402-0937), S3QEL-2.7 (K402-0893), S3QEL-2.8 (K402-0508), S3QEL-3 (Maybridge, JFD03367), S3QEL-4 (3377-0061), S3QEL-5 (3389-0595), S3QEL-6 (3786-1206), S3QEL-7 (8010-6022). Purity of powder stocks was confirmed by HPLC MS and HPLC purity at 254 nm was greater than 95% and confirmed as M+H or M+Na.

Nature Chemical Biology: doi:10.1038/nchembio.1910