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🌐 KAT8 compound inhibition inhibits the initial steps of PINK1-dependant mitophagy V.1
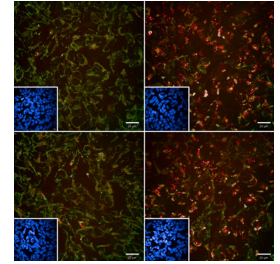
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We use this protocol and it's working

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Abstract

It has recently been shown that KAT8, a genome-wide association study candidate risk gene for Parkinson's Disease, is involved in PINK1/Parkin-dependant mitophagy. The KAT8 gene encodes a lysine acetyltransferase and represents the catalytically active subunit of the non-specific lethal epigenetic remodelling complex. In the current study, we show that contrary to KAT5 inhibition, dual inhibition of KAT5 and KAT8 via the MG149 compound inhibits the initial steps of the PINK1-dependant mitophagy process. More specifically, our study shows that following mitochondrial depolarisation induced by mitochondrial toxins, MG149 treatment inhibits PINK1-dependant mitophagy initiation by impairing PINK1 activation, and subsequent phosphorylation of Parkin and ubiquitin. While this inhibitory effect of MG149 on PINK1-activation is potent, MG149 treatment in the absence of mitochondrial toxins is sufficient to depolarise the mitochondrial membrane, recruit PINK1 and promote partial downstream recruitment of the autophagy receptor p62, leading to an increase in mitochondrial delivery to the lysosomes. Altogether, our study provides additional support for KAT8 as a regulator of mitophagy and autophagy processes.

Materials

Mitochondrial toxins

Oligomycin (inhibitor of mitochondrial complex V) was purchased from Cayman Chemicals (11341) and from Sigma-Aldrich (O4876)

Antimycin A (inhibitor of mitochondrial complex III) was purchased from Sigma-Aldrich (A8674)

Antibodies used for immunoblotting

rabbit anti-phospho-ubiquitin (Ser65) (Cell Signaling, 37642, 1:1000)

mouse anti-TIM23 (BD Biosciences, 611223, RRID:AB_398755, 1:1000)

mouse anti-TOM20 (Santa Cruz, sc-11415, RRID:AB_2207533,1:1000)

mouse anti-GAPDH (Abcam, ab110305, RRID:AB_10861081, 1:1000)

rabbit anti-pParkin(Ser65) (Abcam/Michael J.Fox Foundation, MJF17, 1:1000)

rabbit anti-MFN2 (Cell Signaling, 9482, 1:1000)

mouse anti-OPA1 (BD Biosciences, 612607, 1:1000)

rabbit anti-LC3B (Novus Biologicals, NB100-2220, 1:1000)

IRDye 680LT donkey anti mouse (LI-COR Biosciences, 925-68022, RRID:AB_2814906, 1:20000)

IRDye 800CW donkey anti rabbit (LI-COR Biosciences, 925-32213, RRID:AB_2715510, 1:20000)

rabbit monoclonal anti-PINK1 antibody generated as described in the following manuscript: Sci Rep. 2015. DOI: https://doi.org/10.1038/s41598-018-26949-6

Antibodies used for immunofluorescence

mouse anti TOM20 (Santa Cruz, sc-17764, RRID:AB_628381 1:1000)

rabbit anti phospho-ubiquitin (Ser65) (Cell Signaling, 37642, 1:1000)

mouse anti-p62 (BD Biosciences, 610832, 1:1000)

AlexaFluor 488 goat anti rabbit (Invitrogen, A11008, 6 RRID:AB_143165,1:2000)

AlexaFluor 568 goat anti mouse (Invitrogen, A11004, 7 RRID:AB_2534072, 1:2000)

Cell Culture

1   Maintain cells in culture in a humified 5% CO_2 incubator at ⚡ 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco, 11995-065) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco). Change cell medium every 3 days.

> **Note**
>
> Parkin over-expressing (POE) SHSY5Y cells are a gift from H. Ardley (Ardley, H., *et al.*, 2003).
> DOI: https://doi.org/10.1091/mbc.e03-02-0078
>
> Mt-Keima POE SHSY5Y cells were a gift of C. Luft (Soutar, M., *et al.*, 2019).
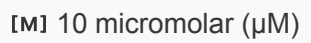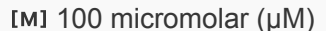> DOI: https://doi.org/10.1080/15548627.2019.1603549
>
> PINK1-HA overexpressing SHSY5Y cells were a gift from E. Deas (Deas, E.., *et al.*, 2011).
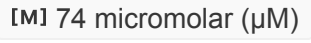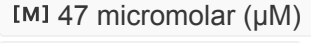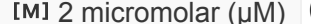> DOI: https://doi.org/10.1093/hmg/ddq526

2   Split cells at 75% to 90% confluency using 🧪 2 mL of Trypsin.
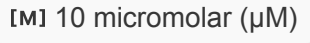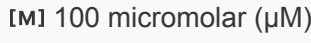
Cell Treatment (Small Molecule Modulator and Mitophagy Induction)

3   Dilute each compound in DMSO to reach concentrations determined according to their speculated toxicity and IC_{50}, [M] 10 micromolar (µM) for NU9056 and [M] 100 micromolar (µM) for MG149.

> **Note**
>
> MG149 targets KAT5 and KAT8 with IC_{50} values of [M] 74 micromolar (µM) and [M] 47 micromolar (µM) respectively; NU9056 targets KAT5 with an IC_{50} value of [M] 2 micromolar (µM) (Li, L., *et al.*, 2020).
> DOI: https://doi.org/10.1172/JCI131145

4   Treat cells with DMSO, [M] 10 micromolar (µM) NU9056, or [M] 100 micromolar (µM) MG149 for ⏱ 06:00:00 before collecting / fixing for downstream experiments.   6h

5   Treat cells with either DMSO or [M] 10 micromolar (μM) oligomycin/ [M] 10 micromolar (μM) antimycin for ⏱ 03:00:00 to induce mitophagy before collecting / fixing for downstream experiments.

            3h

> **Note**
>
> Note that both small molecule modulator and O/A are often added to the same wells / cells to look at the impact of MG149 on mitophagy initiation.

Mitochondrial Enrichment   `2h 10m`

6   Incubate cells ⏱ Overnight at 🌡 -80 °C in mitochondrial homogenization buffer (250mM sucrose, 1mM EDTA, 10mM Tris (pH 7.4) supplemented with protease and phosphatase inhibitors).

7   Thaw, scrape into 1.5mL centrifuged tubes, and triturate 20x, keeping samples 🌡 On ice .

8   Freeze tubes at 🌡 -80 °C for ⏱ 01:00:00 , then thaw 🌡 On ice .

        1h

9   Remove cell debris / non-lysed cells by centrifugation ⊕ 1500 rcf, 4°C, 00:20:00 .

        20m

10   Centrifuge the supernatant ⊕ 12500 x g, 4°C, 00:20:00 to collect the mitochondrial enriched pellet (aka the mitochondrial fraction) and save the supernatant (aka the cytoplasmic fraction).

        20m

11   Centrifuge the cytoplasmic fraction ⊕ 12500 x g, 4°C, 00:20:00 to remove membrane contamination and wash the mitochondrial fraction twice in 🧪 500 μL of fractionation buffer (⊕ 12500 x g, 4°C, 00:10:00 x 2) to remove cytoplasmic contaminants.

        30m

12   Determine the relative ratios of sample differences for the mitochondrial enriched fraction using the protein concentration of the cytoplasmic fraction (BSA assay). Re-suspend the mitochondrial enriched pellet accordingly using 1x NuPAGE® LDS sample buffer supplemented with 10 mM dithiothreitol (DTT).

13 Sonicate the mitochondrial fraction (10 microns power amplitude for 2 x 10 seconds, "flick" in between) and heat to 🌡 70 °C for ⏱ 00:10:00 . `10m`

Cell Lysis `1h 30m`

14 Lyse cells in whole-cell lysis buffer (50mM Tris (pH 7.4), 0.1mM EGTA, 1mM EDTA, 0.27M Sucrose, 1% triton-x-100, supplemented with protease and phosphatase inhibitors) 🌡 On ice and incubated ⏱ Overnight at 🌡 -80 °C .

15 Centrifuge the lysates ⚫ 1600 x g, 4°C, 00:20:00 and protein quantify the supernatant by BSA assay. `20m`

16 Make LDS preps with 10 mM dithiothreitol (DTT), and heat to 🌡 70 °C for ⏱ 00:10:00 . `10m`

Western Blotting `1h 30m`

17 Separate the LDS preps (either after cell lysis or mitochondrial enrichment) by SDS-PAGE and transfer them to nitrocellulose membrane.

18 Incubate the membrane ⏱ Overnight at 🌡 4 °C with primary antibodies diluted in 3% milk in PBS-Tween.

19 Wash and incubate the membrane for ⏱ 01:00:00 at 🌡 Room temperature in secondary antibodies diluted in PBS-Tween. `1h`

20 Detect the protein bands using Odyssey CLx LI-COR.

Immunofluorescence `4h 20m`

21 Fix cells with 4% Paraformaldehyde (PFA, Sigma-Aldrich) for ⏱ 00:20:00 at 🌡 Room temperature . `20m`

22 Remove PFA solution, block and permeabilize the cells with a mix of 10% FBS, 0.25% Triton X-100 in PBS for ⏱ 01:00:00 at 🌡 Room temperature . `1h`

23 Immunostain the cells with pUb(Ser65) and TOM20 primary antibodies diluted in 10% FBS/PBS for ⏱ 02:00:00 at 🌡 Room temperature . `2h`

> **Note**
>
> rabbit anti-pUb(Ser65) (Cell Signaling, 37642) and mouse anti-TOM20 (Santa Cruz, sc-17764) primary antibodies are both added at a 1:1000 dilution in 10% FBS/PBS.

24 Wash wells three times using PBS 1X and incubate with secondary antibodies for ⏱ 01:00:00 at 🌡 Room temperature , before washing again three times using PBS 1X.

1h

> **Note**
>
> AlexaFluor 568 anti-mouse, 488 anti-rabbit secondary antibodies, and Hoechst 33342 (Thermo Scientific) are all added at a 1:2000 dilution in 10% FBS/PBS.

25 Image cells on the Opera Phenix (Perkin Elmer), acquiring confocal z-stacks for multiple fields of view across 3 individual wekks per experimental condition, using the 40X water objective.

> **Note**
>
> Analyse images using Columbus 2.8 analysis system (Perkin Elmer) to measure the integrated intensity of pUb(Ser65) within the whole cell.
>
> For visualisation purposes, select brightness and contrast settings on control and apply the same settings to all other conditions.

Measuring Mitochondrial Membrane Potential using TMRM

40m

26 Seed POE-SHSY4Y cells in a 96-well CellCarrier Ultra plate.

27 Incubate live cells in [M] 25 nanomolar (nM) Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) (Sigma Aldrich) diluted in cell media for ⏱ 00:40:00 at 🌡 37 °C and 4% CO_2.

40m

28 Image cells on the Opera Phenix (PerkinElmer) at 🌡 37 °C and 4% CO_2, acquiring confocal z-stacks for multiple fields of view across 3 individual wells per experimental condition using the 40X water objective.

> **Note**
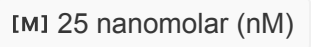>
> Analyse images using the Columbus 2.8 analysis system (Perkin Elmer) to measure the TMRM signal intensity of maximum intensity projections.

Measuring Mitochondrial Delivery to Lysosomes using mitoKeima Reporter `40m`

29 Seed mt-Keima expressing POE SHSY5Y cells in a 96-well CellCarrier Ultra plate and treat them according to desired conditions (MG149, NU9056, O/A as described in ⭤ go to step #3).

30 Before imaging, replace cell media with phenol-free DMEM + 10% FBS containing the treatments previously administered ⭤ go to step #29 , and adding Hoechst 33342 (1:10000) to stain the nuclei.

31 Image cells immediately on the Opera Phenix (PerkinElmer) at 🌡 37 °C with 5% CO_2, acquiring multiple single-plane fields of view, using the 63X water objective.

> **Note**
>
> Analyse images in using the Columbus 2.8 analysis system (Perkin Elmer) to measure the mitophagy index.
> To analyse:
> - Identify cells using the Hoechst 33342 channel, before measuring the area of the cytoplasmic and lysosomal mitoKeima
> - Calculate the mitophagy index = the ratio between the total area of lysosomal mitochondria and the total area of mitoKeima (cytoplasmic + lysosomal areas) per well.

RT-qPCR

32 Extract total RNA from cells using the Monarch Total RNA Miniprep Kit (New England Bioscience) with inclusion of the optional on-column DNAse treatment.

33 Reverse transcribe RNA with SuperScript™ IV reverse transcriptase and random hexamers (Invitrogen).

34 Dilute the cDNA product then subject to quantitative real-time PCR (qPCR) using Power SYBR™ Green Master Mix (Applied Biosystems) and gene specific primer pairs on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems).

	A	B	C	D
		Sequence 5'-3'		
	Gene Target	**Forward**	**Reverse**	**Product Size / bp**
	RPL18A	CCCACAACATGTACCGGGAA	TCTTGGAGTCGTGGAACTGC	180
	PINK1	GTGGAACATCTCGGCAGGTT	CCTCTCTTGGATTTTCTGTAAGTGAC	129

List of primer pairs used for RT-qPCR of target genes

35 Calculate relative mRNA expression levels using the $2^{-\Delta\Delta Ct}$ method and *RPL18A* as the housekeeping gene.

Statistical Analysis

36 Use a minimum of 3 biological replicates for each experiments (N numbers shown in legends refer to the number of replicate experiments).
- For each imaging experiments, calculate the mean values of every condition from a minimum of 3 technical replicates.
- Normalise intensity and integrated density measurements to control conditions.

37 Subject data to a two-way ANOVA with Dunnett's post-hoc analysis for multiple comparisons unless stated otherwise.
- Express data as the mean ± standard deviation (SD) from replicate experiments.
- Set statistical significance at a p-value of <0.05.

> **Note**
>
> Use GraphPad Prism 6 (La Jolla, California, USA) for statistical analyses and graph production.
> (Present data with error bars showing mean ± standard deviation (SD) from replicate experiments).

Measuring p62 recruitment to the OMM and mitochondrial delivery to lysosomes using mitoSRAI

3h

38 Seed mitoSRAI POE SHSY5Y cells in a 96-well CellCarrier Ultra plate and treat them according to desired conditions (MG149, NU9056, O/A as described in ⮌ go to step #3).

39 Fix cells as described in ⮌ go to step #21 to 22

40 Immunostain the cells with p62 primary antibodies diluted in 10% FBS/PBS for 🕐 02:00:00 at 🌡 Room temperature .

2h

> **Note**
>
> mouse anti-SQSTM1/p62 (Abcam, ab56416) primary antibody added at a 1:1000 dilution in 10% FBS/PBS.

41 Wash wells three times using PBS 1X and incubate with secondary antibodies for 🕐 01:00:00 at 🌡 Room temperature , before washing again three times using PBS 1X.

1h

> **Note**
>
> AlexaFluor 568 anti-mouse secondary antibodies, and draq5 (Thermo Scientific) are all added at a 1:2000 dilution in 10% FBS/PBS.

42 Image cells on the Opera Phenix (Perkin Elmer), acquiring confocal z-stacks for multiple fields of view across 3 individual wells per experimental condition, using the 63X water objective.

> **Note**
>
> Analyse images using Columbus 2.8 analysis system (Perkin Elmer) to measure the SRAI mitophagy index
> - corresponds to the ratio between the area of lysosomal mitochondria (corresponding to the total YPet negative TOLLES area) and the total mitochondrial area (corresponding to the total TOLLES area) per well.
>
> Analyse images using Columbus 2.8 analysis system (Perkin Elmer) to measure p62 recruitment to teh mitochondria using TOLLEs as a mitochondrial marker
> - calculated as the ratio of p62 signal intensity inside vs. outside TOLLES
>
> For visualisation purposes, select brightness and contrast settings on control and apply the same settings to all other conditions.

43

Protocol references

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