KAT8 compound inhibition inhibits PINK1/Parkin-dependent mitophagy and initiates mitochondrial delivery to lysosomes

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Abstract

It has recently been shown that *KAT8*, a genome-wide association study (GWAS) candidate risk gene for Parkinson's Disease, is involved in PINK1/Parkin-dependent mitophagy. The *KAT8* gene encodes a lysine acetyltransferase and represents the catalytically active subunit of the non-specific lethal (NSL) epigenetic remodeling complex. In the current study, we showed that contrary to KAT5 inhibition, dual inhibition of KAT5 and KAT8 via the MG149 compound inhibited the initial steps of the PINK1-dependant mitophagy process. More specifically, our study showed that MG149 inhibited PINK1-dependent mitophagy initiation by impairing PINK1-autophosphorylation and activation, thus preventing phosphorylation of Parkin and ubiquitin. While preventing PINK1-dependent mitophagy initiation, MG149 promoted the downstream recruitment of the autophagy receptor p62 and initiated mitochondrial delivery to the lysosomes independently of PINK1. Altogether, our study provided additional support for KAT8 inhibition as a regulator of mitophagy and autophagy processes.

Introduction

Like other neurodegenerative diseases, Parkinson's disease (PD) can either be inherited or stem from more variable sporadic origins. The Mendelian forms of PD have been of major importance for a better understanding of the pathophysiology of the disease. A few of the causative Mendelian PD mutations have been identified in genes playing a key role in the mitophagy process, namely *PINK1* and *Parkin*, respectively encoding a serine/threonine kinase (PTEN-induced kinase 1) and an E3 ubiquitin ligase (Deas, E., *et al*., 2011) (Park, J., *et al*, 2006) (Clark, I. E., *et al*., 2006). This identified dysfunctional mitophagy as an important underlying pathomechanism in familial PD.

Mitochondria are essential for a wide range of cellular processes such as ATP production through oxidative phosphorylation, calcium homeostasis, reactive oxygen species (ROS) production, release and consumption, and apoptosis (Lopez-Dom, *et al.*, 2021) (Brand, M. D., *et al.*, 2013). When heavily damaged, mitochondria can release toxic ROS throughout the cell, promoting oxidative stress and ultimately cell death (Guo, C. Y., *et al.*, 2013). As a result, mitochondrial quality control processes are essential, serving to identify, isolate, and remove

damaged mitochondria, thus limiting the accumulation of toxic mitochondrial components (Pickles *et al*., 2018). PINK1/Parkin-dependent mitophagy is one of several types of selective autophagy aimed towards the degradation of heavily toxic mitochondria (Wang, L., *et al*., 2020). In cells with a healthy mitochondrial network, PINK1 is maintained at low levels through constitutive and rapid degradation by the proteasome via the N-end rule pathway (Yamano, K., *et al*., R. J., 2013). Upon mitochondrial damage and associated depolarization of the mitochondrial membrane, PINK1 accumulates at the mitochondrial surface, where it becomes activated through dimerization and autophosphorylation (Rasool, S., *et al*., 2018). Activated PINK1 phosphorylates ubiquitin already present at the outer mitochondrial membrane (OMM) at serine 65 (pUb(Ser65)) (Kazlauskaite, A., *et al*., 2014) (Kane, L. A., *et al*., 2014) (Koyano, K., *et al*., 2014) (Shiba-Fukushima, K., *et al.*, 2014). Parkin is then recruited from the cytosol to those pUb(Ser65) sites and is subsequently phosphorylated by PINK1 at serine 65 (Kazlauskaite, A., *et al*., 2014) (Kondapalli, C., *et al*., 2012). PINK1-dependent phosphorylation of Parkin (pParkin) is required for the full activity of the E3 ubiquitin ligase (McWilliam, T. G., *et al*., 2018) (Wang, L., *et al*., 2020). pParkin mediates the ubiquitination (and subsequent clearance) of a wide range of OMM proteins, generating more ubiquitinated sites for PINK1 to phosphorylate, creating a phosphorylation / ubiquitination positive feedback loop at the OMM of damaged mitochondria. Autophagy adapters such as p62 can bind to pUb(Ser65) ubiquitin chains, facilitating autophagosome formation around the mitochondria and ultimate fusion to the lysosomes for degradation (Tanida, I., *et al*., 2008). Recent description of impairments in PINK1-dependent mitophagy associated with loss of function in idiopathic PD GWAS risk gene candidates *KANSL1* and *KAT8* has suggested impairments in PINK1-mitophagy in the more common idiopathic forms of the disease (Soutar, M., *et al*., 2022). KAT8, which serves as the catalytic subunit of the male-specific lethal (MSL) and the non-specific lethal (NSL) complex (Radzisheuskaya, A., *et al*., 2021), is a key player in the regulation of nuclear gene transcription (Sheikh, B. N., *et al.*, 2019). KAT8 was shown to regulate PINK1-Parkin dependent mitophagy by regulating *PINK1* gene transcription levels, levels of PINK1 protein and subsequent PINK1-dependant phosphorylation of Parkin and ubiquitin (Soutar, M., *et al*., 2022).

The acetylation process essentially works by removing a hydrogen atom from lysine's $NH3^+$ side chain and subsequently directly transferring an acetyl group to lysine from acetyl co-enzyme A (acetyl-CoA). This results in neutralization of lysine residues positive charge and in turn protein conformational changes which may amongst other things impact regulation of enzymatic activity (Farria, A., *et al*., 2015). By modulating the activity of lysine acetyltransferases (KATs) and lysine deacetylases, acetylation levels can be monitored, demonstrating the potential of protein acetylation as a "regulatory switch" for cellular metabolic processes (Farria, A., *et al*., 2015), (Xu, W., *et al*., 2014), (Christensen, D. G., *et al*., 2019). Playing on the inhibition or activation of different KATs and KDACs has been previously used in therapies (Farria, A., *et al*., 2015). This knowledge, combined with the newfound importance of KAT8 in PINK1-dependent mitophagy, prompted us to investigate the impact of small molecule KAT8 inhibitors on PINK1-dependant mitophagy. Of particular interest for this study is MG149, a dual KAT5 and KAT8 inhibitor whose inhibition is competitive towards the acetyl-CoA binding site in both KAT5 and KAT8 (Ghizzoni, M., *et al*., 2012). KAT5 is another lysine acetyltransferase involved in cellular processes including transcriptional regulations and DNA damage repair. Due to the unavailability of a KAT8 specific inhibitor, we used a specific

KAT5 inhibitor, NU9056, as a control for MG149's activity mediated through KAT5 (Wang J., *et al*., 2020).

Here we showed that KAT8 inhibition with MG149 decreased PINK1 kinase activity in PINK1-dependent mitophagy initiation while seemingly inducing a secondary PINK1-independent autophagy mechanism.

Results

MG149 inhibition of KAT8 reduces PINK1/Parkin-dependent mitophagy initiation.

The consequence of KAT8 inhibition on PINK1-dependant mitophagy initiation was first determined by assessing PINK1-dependent phosphorylation of ubiquitin at serine 65 (pUb(Ser65)) following mitochondrial depolarization. The effect of MG149 and NU9056 on pUb(Ser65) was tested by immunofluorescence in Flag-Parkin over-expressing SH-SY5Y neuroblastoma cells (POE SH-SY5Ys). POE SH-SY5Ys were pre-treated for 1, 3, 24, and 48hr with either 10µM NU9056 (targeting KAT5 at IC_{50} of 2µM – 100µM of NU9056 proved toxic for cells) (Li, L., *et al*., 2020) or 100µM MG149 (targeting KAT5 and KAT8 at IC_{50} values of 74µM and 47µM respectively) (Wang J., *et al*., 2020), prior to inducing mitophagy with a combination of 1µM oligomycin (an inhibitor of ATP synthase / mitochondrial complex V) and 1µM antimycin (an inhibitor of the mitochondrial complex III) (O/A) for 3hr. Mitochondrial depolarization effectively induced PINK1/Parkin-mediated mitophagy, as per deposition of pUb(Ser65) with treatment with O/A, when compared to DMSO (Figure 1A top panel, see quantification for control cells in Figure 1B). MG149 pre-treatment was shown to decrease O/A-induced pUb(Ser65) at 1hr and 3hr whereas NU9056 had no effect at any time point (Figure 1A,B). Notably, 1hr and 3hr MG149 pre-treatment led to a small, non-significant increase in pUb(Ser65) deposition in the absence of O/A treatment (Figure 1A bottom left panel, quantified in Figure 1B). These data suggest that KAT8 inhibition may induce a decrease in PINK1-depedent mitophagy initiation while inducing a subtle activation of mitophagy independent of O/A treatment. The effect of MG149 on pUb(Ser65) levels was confirmed by immunoblotting of POE SH-SY5Y cells pre-treated for 3hr with 100µM of MG149 (Figure 1C,D) prior to treatment with O/A for 3hr. The inhibitory effect of KAT8 compound inhibition on PINK1/Parkin-dependent mitophagy initiation was further validated using other markers, namely PINK1-dependent phosphorylation of Parkin at serine 65 (pParkin(Ser65)) and ubiquitination of mitofusin 2 (MFN2). The latter is a substrate for endogenous Parkin (Jin, S. M., *et al*., 2012) which undergoes ubiquitination and degradation in early stages of mitophagy and is therefore often used as a marker for mitophagy initiation (Wang, L., *et al*., 2020) (Figure 1C,D). Importantly, the KAT5 inhibitor NU9056 had no effect on any of these mitophagy markers, strongly suggesting that PINK1-dependent mitophagy initiation is modulated by KAT8, as opposed to KAT5, as shown previously (Soutar, M., *et al*., 2022).

MG149 inhibition of KAT8 does not prevent lysosomal mitochondrial clearance

Besides the best characterized PINK1/Parkin-dependent mitophagy process, PINK1/Parkin-independent pathways also play significant roles in mitochondrial clearance (reviewed in Cummins, N., *et al*., 2018 and Whiten, D. R., *et al*., 2021). In order to understand whether the impairments in PINK1-mitophagy initiation observed following MG149 treatment were associated with reductions in delivery of mitochondria to the lysosomes for clearance, and/or whether activation of compensatory pathways might have been initiated, we used the pH-

sensitive mitochondrial protein mitoKeima (mt-Keima), which fluorescence was shown to shift from green to red as mitochondria are delivered to the lysosomes (Katayama, H., *et al*., 2011), in POE-SH-SY5Y cells. Cells were simultaneously treated with either 10µM NU9056 or 100µM MG149, and with either DMSO or 1µM O/A, prior to live imaging for 9hr. MG149 did not alter the mitophagy index of O/A-treated cells, suggesting that despite the reduction in PINK1-dependent activation of mitophagy initiation, mitochondrial delivery to the lysosomes was not affected. These data highlight the potential activation of PINK1-independent secondary clearance mechanisms, which is supported by the fact that MG149 treatment led to a significant increase in mitochondrial delivery to the lysosome in the absence of O/A treatment (Figure 2A,B)

MG149 inhibition of KAT8 stimulates PINK1-independent mitochondrial clearance

During autophagy, adapters with LC3-interacting regions (LIR), such as p62, bind to ubiquitin chains, initiating autophagosome formation (Johansen, T., *et al*., 2020). In parallel, a cytosolic form of Light Chain 3 (LC3-I) forms LC3-phosphatidylethanolamine conjugate (LC3-II). LC3-II is recruited to autophagosome membranes and binds to the autophagy adaptors via the LIRs. Subsequently, autophagosome formation is initiated around the cargo, ultimately leading to fusion to the lysosomes for degradation (Tanida, I., *et al*., 2008). The LC3-II:LC3-I ratio that is widely used to monitor autophagy (Klionsky, D. J., *et al*., 2021) was significantly increased in the presence of MG149 with or without O/A treatment (Figure 3A,B), an increase that may be indicative of altered autophagic induction and subsequent lysosomal delivery. The effect of MG149 on LC3-II:LC3-I ratio was associated with an increase in p62 puncta recruitment to the mitochondria (Figure 3C,D). The recruitment of p62, an autophagy adaptor, at the OMM, indicates the initiation of an autophagy mechanism, and the increase in LC3-II:LC3-1 ratio points towards a potential increase in the autophagic activity in response to MG149.

In order to confirm whether the alterations in ubiquitin and Parkin phosphorylation following MG149 treatment were PINK1-dependent or not, PINK1 was knocked down (KD) in POE SH-SY5Y mtKeima cells treated with either DMSO or 1µM O/A, prior to live imaging for 9hr. In O/A treated cells, the mitochondrial delivery to the lysosomes was decreased upon PINK1 siRNA KD when compared to non-treated (NT) or scramble (SCR) siRNA control cells, regardless of MG149 treatment (Figure 3G,H). These data confirmed that MG149 decreasef mitophagy initiation in a PINK1-dependent manner in O/A-treated cells. In cells treated with MG149 alone (without O/A), the increase in mitophagy index remained unaffected by PINK1 siRNA KD (Figure 3E,F), further suggesting a PINK1-independent mechanism of mitophagy induction by MG149.

MG149 inhibition of KAT8 does not prevent O/A-induced mitochondrial membrane depolarization

We next attempted to explore possible mechanism(s) of action for MG149-dependent inhibition of PINK1/Parkin-dependent mitophagy initiation. First, we hypothesized that MG149 could inhibit O/A-induced mitochondrial membrane depolarization required for PINK1 stabilization at the OMM (Yamano, K., *et al*., 2016). In order to test this hypothesis, mitochondrial membrane potential ($\Delta\Psi m$) was measured in MG149-treated POE SH-SY5Y cells treated with DMSO or O/A, using live confocal fluorescence imaging and TMRM (tetramethyl rhodamine methyl ester) staining (Figure 4A,B). O/A treatment induced mitochondrial depolarization both in control cells and in MG149 treated cells. In fact, cells pre-treated with MG149 and then O/A showed more depolarized $\Delta\Psi m$ than cells treated with

O/A alone, suggesting that the inhibitory effect of MG149 on PINK1-mitophagy initiation was not due a lack of mitochondrial depolarization. Interestingly, MG149 caused a significant depolarization of the mitochondrial membrane potential without O/A treatment (Figure 4A, bottom left panel, quantified in Figure 4B). This effect was confirmed looking at OPA1 cleavage in immunoblotting as MG149 alone caused complete cleavage of L-OPA1 into S-OPA1, comparable to observations made following treatment with O/A (Figure 4C,D). The O/A-independent effect of MG149 on $\Delta\Psi m$ is likely to explain the small, non-significant increase in pUb(Ser65) deposition (Figure 1A,B) and subsequent mitochondrial recruitment of PINK1 (see Figure 5D below) following MG149 treatment without O/A.

KAT8 inhibition attenuates PINK1 autophosphorylation

We have shown previously that KAT8 siRNA KD POE SH-SY5Ys have reduced levels of *PINK1* mRNA (Soutar, M., *et al*, 2022). Thus, another possible mechanism for decreased mitophagy initiation in MG149-treated cells was a decrease of PINK1 transcription. In order to test this second hypothesis, we have measured the effect of MG149- and NU9056-mediated KAT8/KAT5 and KAT5 inhibition respectively, on *PINK1* transcription, using real-time quantitative polymerase chain reaction (RT-qPCR). Neither of the compound inhibitors significantly impacted *PINK1* mRNA levels (Figure 5A), suggesting that the impairments in PINK1-dependent pUb(Ser65) and p-Parkin(Ser65) following acute treatment with MG149 were unlikely to be associated with altered transcription of the *PINK1* gene. Next, we assessed whether PINK1 protein accumulation at the mitochondria was altered following treatment of POE SH-SY5Y cells with MG149 using immunoblotting of mitochondrial enriched fractions. In line with the mRNA data, PINK1 protein accumulation was not prevented following MG149 treatment (Figure 5B,C). In fact, MG149 treatment led to a significant increase of PINK1 accumulation at the mitochondria, independent of O/A, corroborating the subtle increase of pUb(Ser65) (Figure 1A) and mitochondrial depolarization (Figure 4) in cells treated with MG149. Finally, we hypothesized that KAT8 compound inhibition could decrease PINK1 activation. To test this hypothesis, phosphorylation of PINK1 at serine 228 (pPINK1(Ser228)), an autophosphorylation mark necessary for PINK1 activation, was assessed by immunoblotting in mitochondrial enriched fractions from SH-SY5Y cells overexpressing with HA-tagged PINK1 (PINK1-HA SH-SY5Y) (Okatsu, K., *et al*., 2013). In line with observations in SH-SY5Ys expressing endogenous levels of PINK1 (Figure 5B,C), MG149 alone evoked an increase in mitochondrial PINK1 recruitment in PINK1-HA SH-SY5Ys (Figure 6D). Despite this robust mitochondrial recruitment of PINK1 in MG149 treated cells, O/A-induced PINK1 phosphorylation at Ser228 was decreased when compared to control, and the mitochondrial PINK1 accumulating with MG149 alone did not appear substantially autophosphorylated either (Figure 6D,E). These data suggest that while MG149 treatment doesn't prevent neither mitochondrial depolarization nor PINK1 recruitment, it reduces PINK1's autophosphorylation, therefore decreasing its kinase activity and subsequent PINK1-mitophagy initiation.

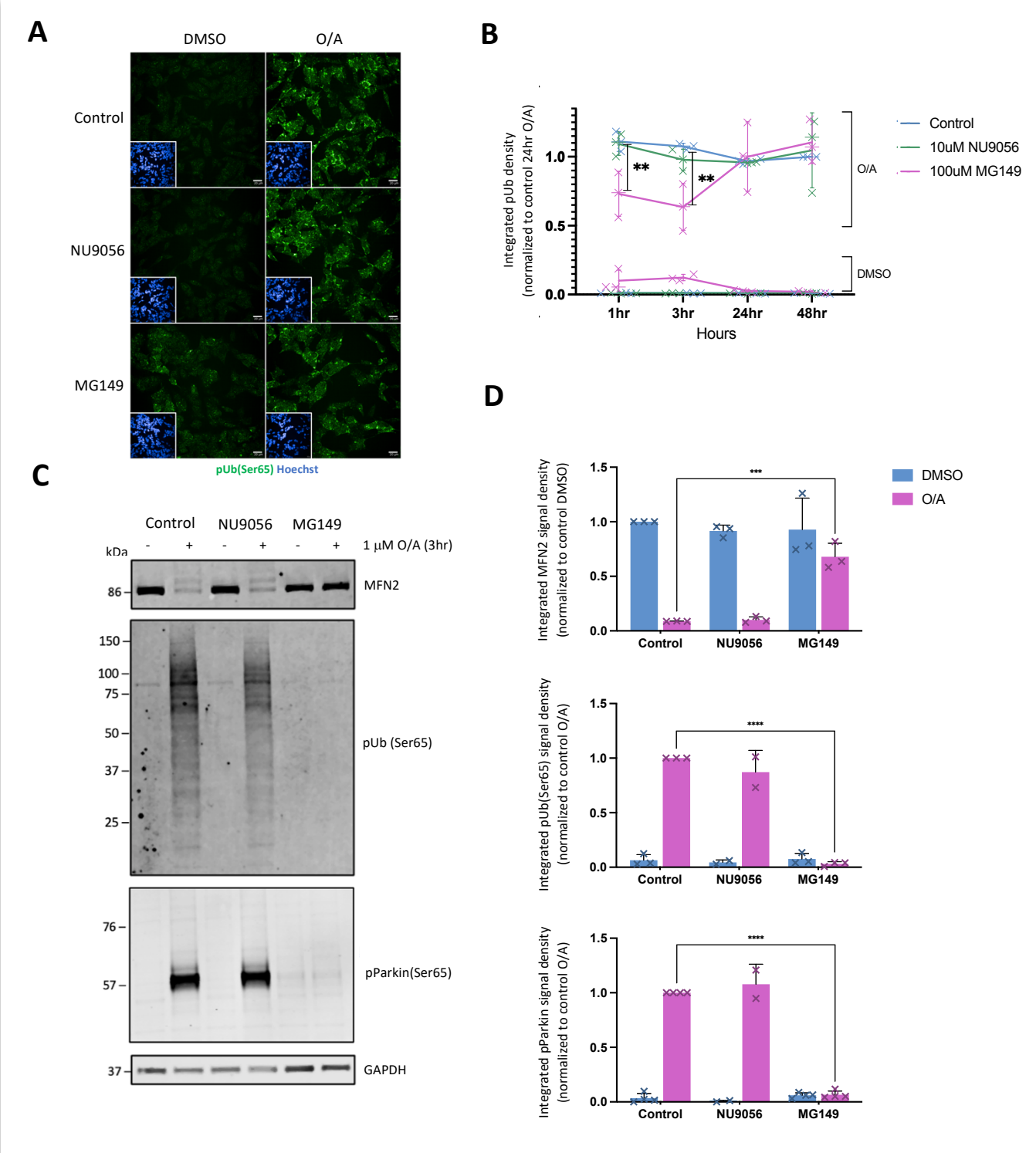


Figure 1

KAT8 inhibition reduces PINK1/Parkin-dependent mitophagy initiation.

A. Representative images of parkin-overexpression (POE) SH-SY5Y cells pre-treated with DMSO, 10µM NU9056 (KAT5 inhibitor) or 100µM MG149 (KAT5/KAT8 inhibitor) for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr to induce mitophagy. Cells were then immuno-stained with Hoechst (blue) and anti-pUb (Ser65) (green). Scale bar: 20µm.

B. Quantification of integrated pUb(Ser65) signal density normalized to control 24hr O/A in A. Data compared to control conditions (n=3, two-way ANOVA with Dunnett's correction).

C. Representative IB of POE SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr. Blots probed for pUb, GAPDH, pUb(Ser65), pParkin(Ser65).

D. Quantification of pUb, MFN2, pParkin signal density in C, compared to control conditions (n=3, two-way ANOVA with Dunnett's correction).

Data shown as mean +/- SD.

MG149 inhibits PINK1/Parkin-dependent mitophagy

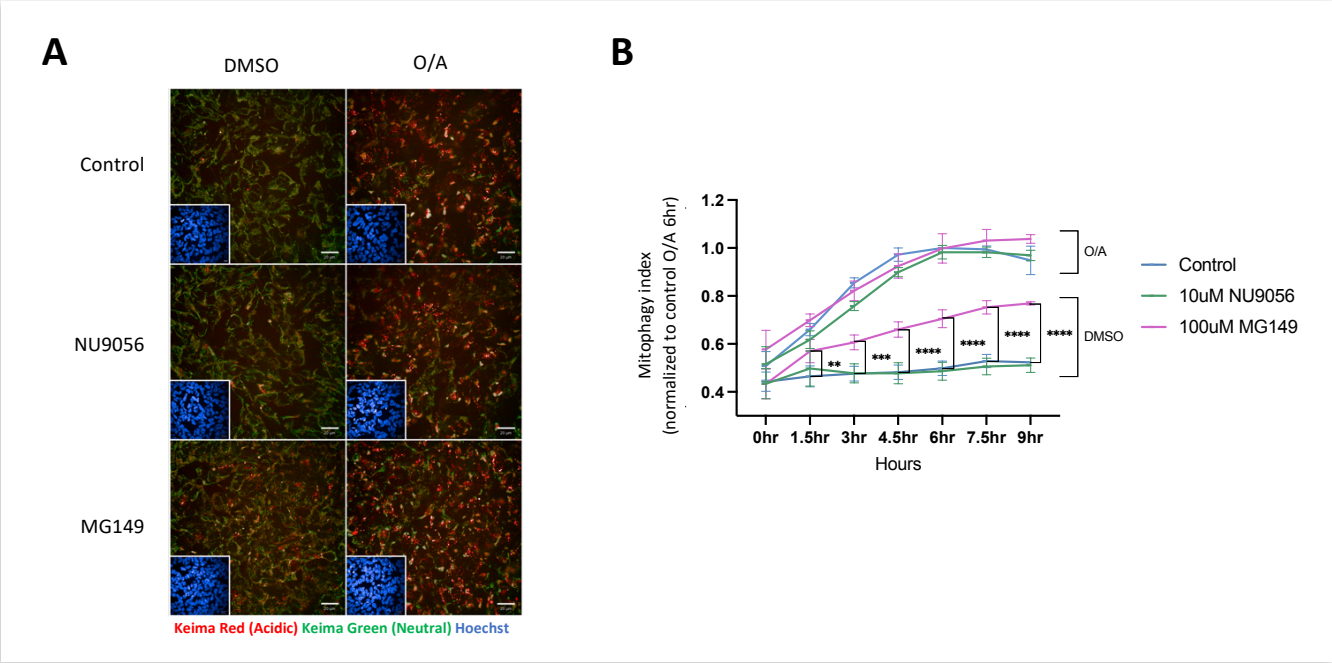


Figure 2

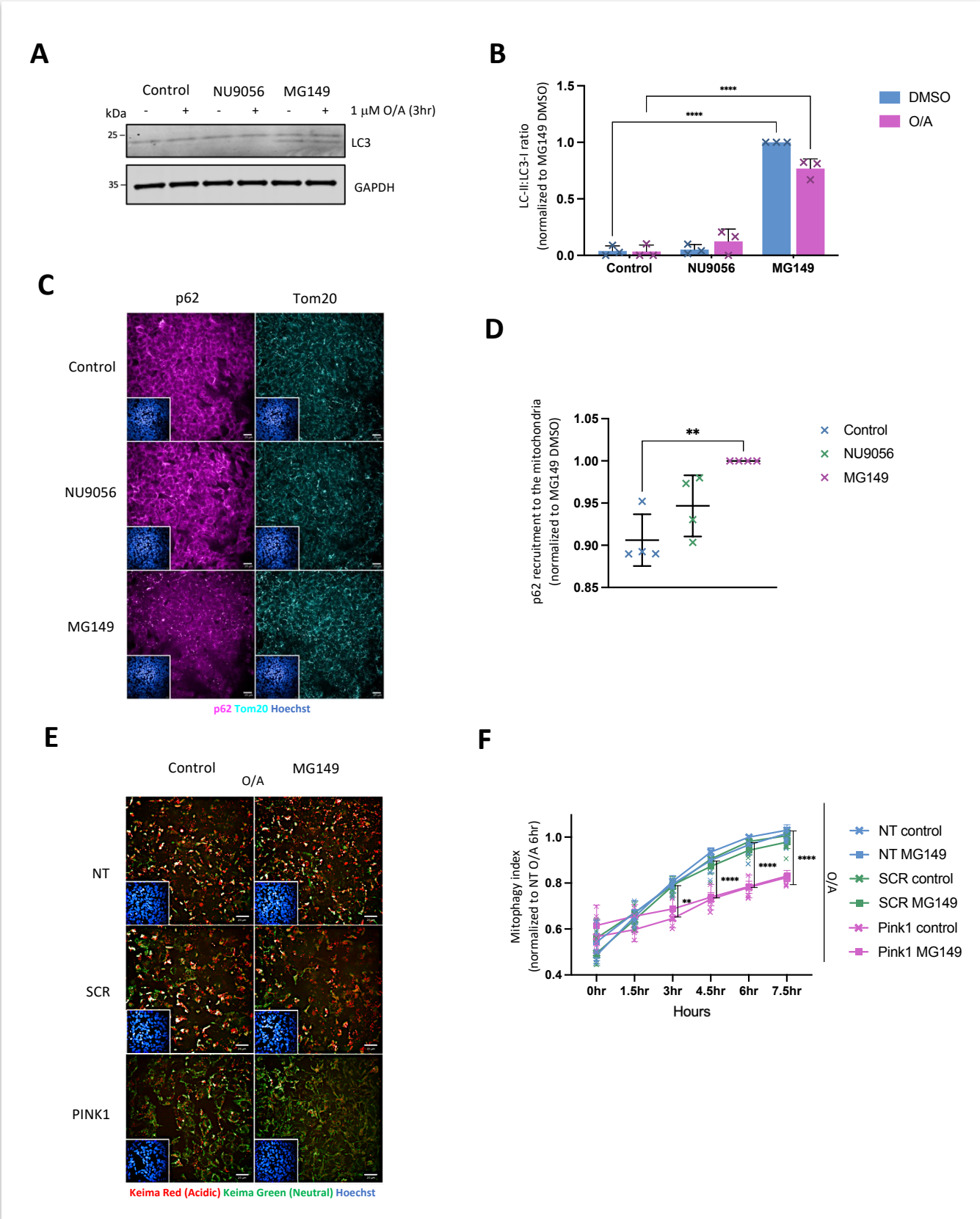
KAT8 inhibition initiates mitochondrial delivery to the lysosome

A. Representative images of mitoKeima cells pre-treated with DMSO, 10µM NU9056 or 100µM MG149 for 6hr and with DMSO or 1µM O/A for 3hr to induce mitophagy. The neutral Keima-green signal (green) is counterstained with the acidic lysosomal Keima-red signal (red). Scale bar: 20µm.

B. Quantification of the mitophagy index, calculated as the ratio of the area of lysosomal mt-Keima signal to total mt-Keima signal (sum of lysosomal and neutral mt-Keima signal), and normalized to control 6hr O/A, in A. Data compared to control conditions (n=4, two-way ANOVA with Dunnett's correction).

Data shown as mean +/- SD.

MG149 inhibits PINK1/Parkin-dependent mitophagy



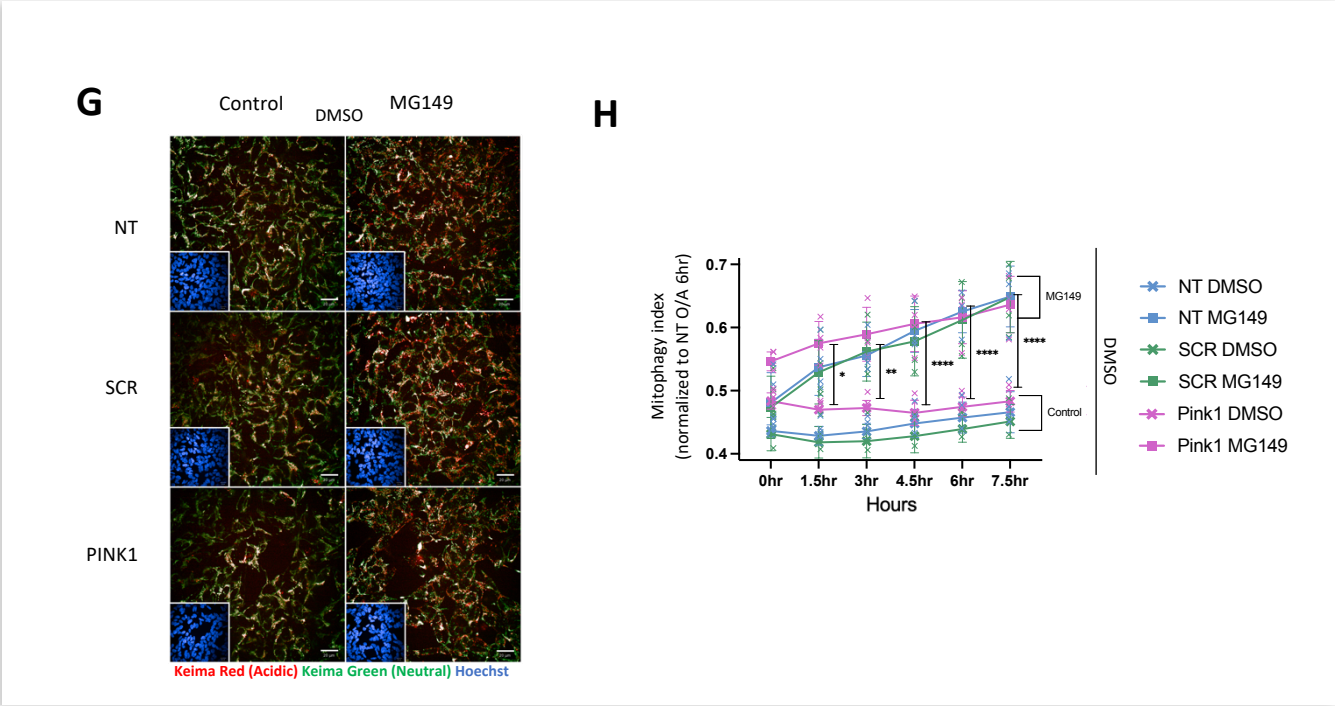


Figure 3

The mitochondrial clearance initiated in MG149 treated cells is happening independently of PINK1

A. Representative IB of POE SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr.

B. Quantification of LC3-II:LC3-I ratio signal intensity normalized to MG149 DMSO in A and compared to control conditions (n=3, two-way ANOVA with Dunnett's correction).

C. Representative images of POE-SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr. Cells were immuno-stained for Hoechst and p62.

D. Quantification of p62 recruitment to the mitochondria, calculated as the ratio of mitochondrial to cytoplasmic p62 intensity normalized to MG149 DMSO, in E. Data compared to control DMSO (n=3, one-way ANOVA with Dunnett's correction).

E. Representative images of mitoKeima in NT, SCR, and PINK1 siRNA KD POEs after pre-treated with DMSO, 10µM NU9056 or 100µM MG149 for 3hr and subsequently treated with 1µM O/A for 3hr to induce mitophagy. The neutral Keima-green signal (green) is counterstained with the acidic lysosomal Keima-red signal (red). Scale bar: 20µm.

F. Quantification of mitophagy index calculated as the ratio of the area of lysosomal mt-Keima signal to total mt-Keima signal (sum of lysosomal and neutral mt-Keima signal) in G. Data compared to NT (n=3, two-way ANOVA with Tukey correction).

G. Representative images of mitoKeima in NT, SCR, and PINK1 siRNA KD POEs after pre-treated with DMSO, 10µM NU9056 or 100µM MG149 for 3hr and subsequently treated with DMSO for 3hr. The neutral Keima-green signal (green) is counterstained with the acidic lysosomal Keima-red signal (red). Scale bar: 20µm.

H. Quantification of mitophagy index calculated as the ratio of the area of lysosomal mt-Keima signal to total mt-Keima signal (sum of lysosomal and neutral mt-Keima signal) in E. Data compared to NT (n=3, two-way ANOVA with Tukey correction).

Data shown as mean +/- SD.

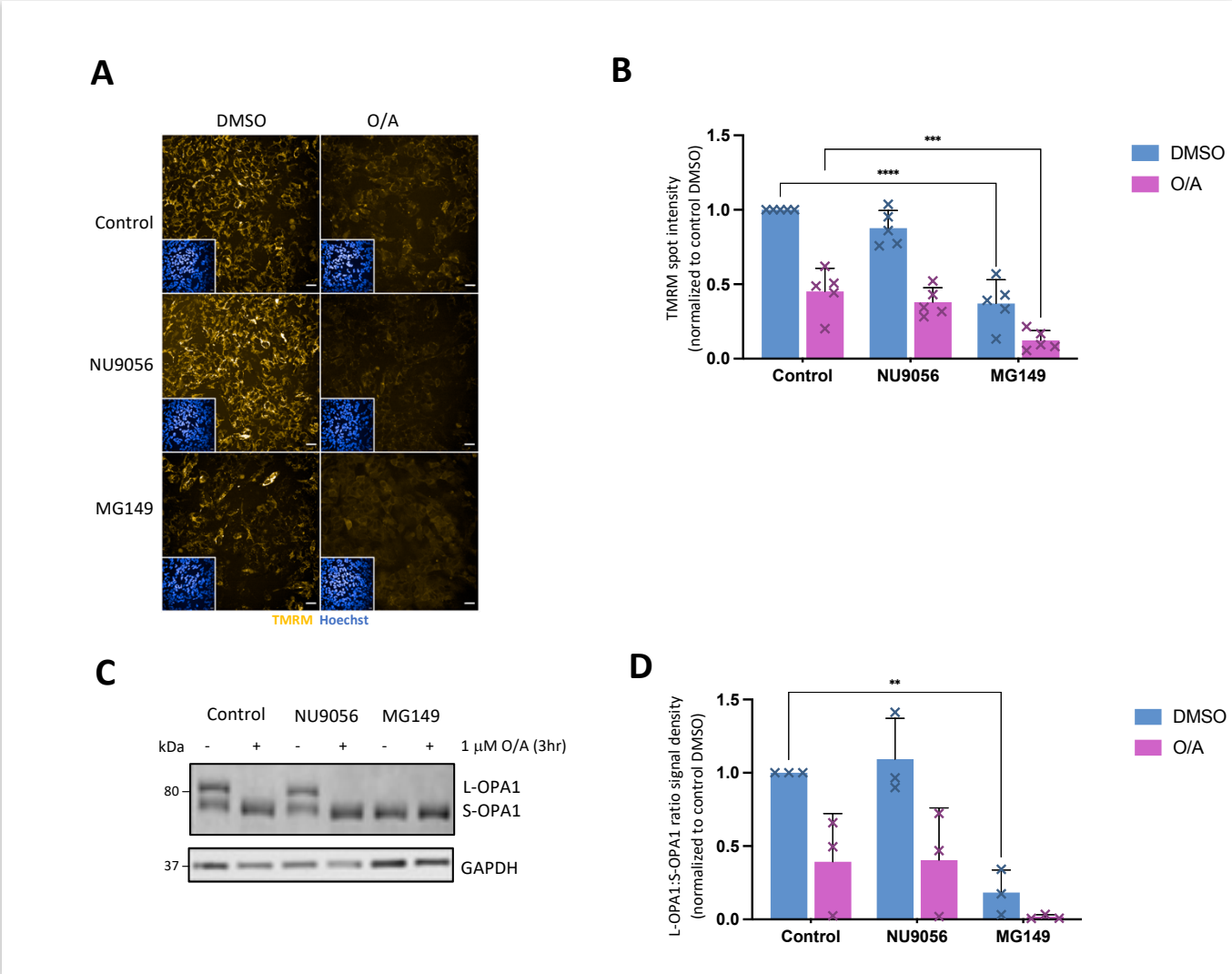


Figure 4

MG149 effectively reduces mitochondrial membrane potential

A. Representative images of TMRM after pre-treatment with DMSO, 10µM NU9056 or 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr to induce mitophagy. Scale bar: 20µm.

B. Quantification of TMRM spot intensity normalized to control DMSO and compared to control conditions, in A (n=5, two-way ANOVA).

D. Representative OPA1 IB of POE SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr.

D. Quantification of OPA1 calculated as the ratio of the signal intensity of L-OPA1 to S-OPA1 and normalized to control DMSO in C. Data compared to control conditions (n=3, two-way ANOVA with Dunnett's correction).

Data shown as mean +/- SD.

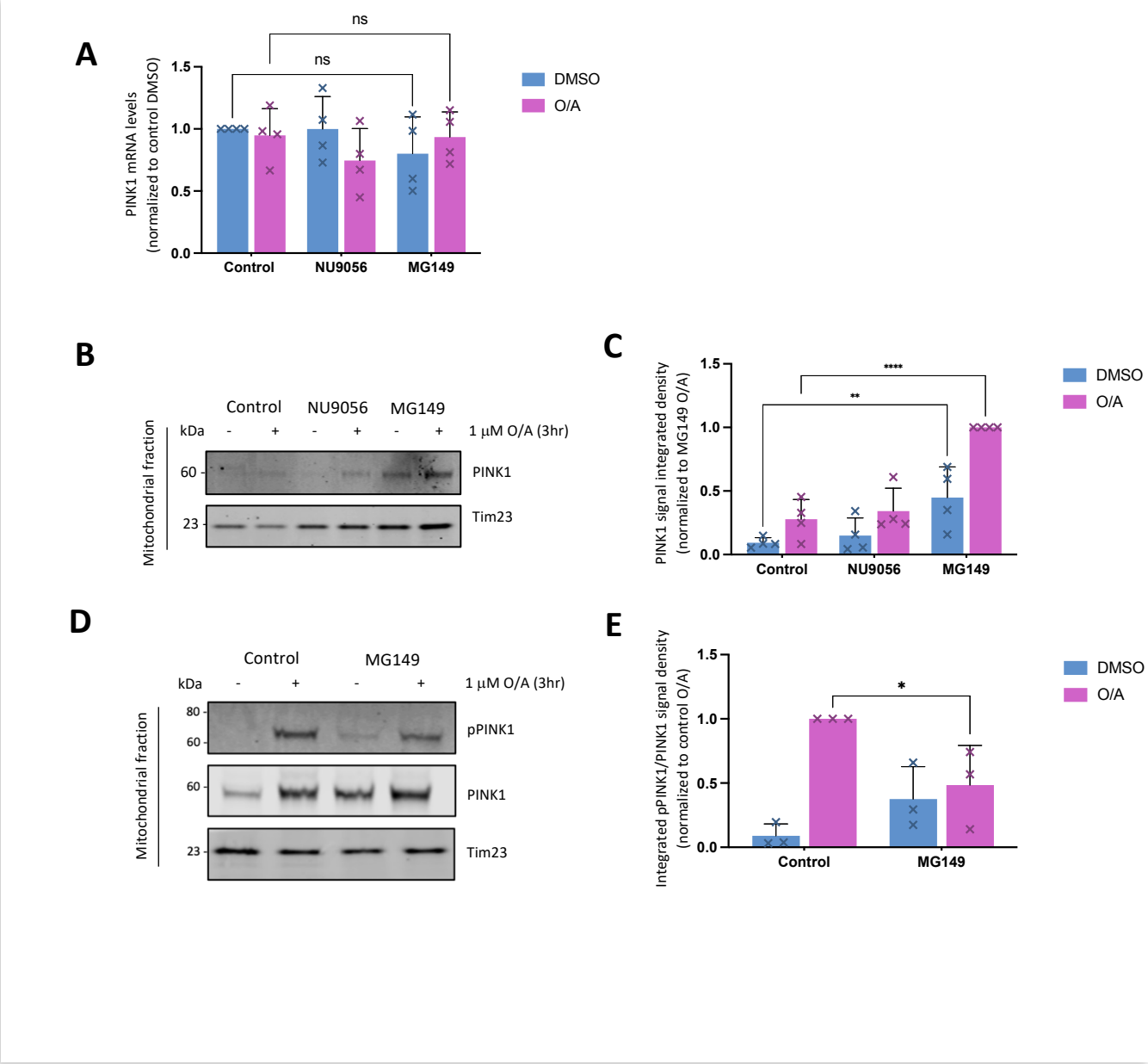


Figure 5

MG149 reduces PINK1's ability to phosphorylate, therefore decreasing PINK1-dependent mitophagy initiation

A. Quantification of PINK1 mRNA levels in POE SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 3hr and subsequently treated with DMSO (control) or 1µM O/A (to induce mitophagy). Data compared to controls (n=5, two-way ANOVA with Dunnett's correction).

B. Representative PINK1 IB of mitochondrial fractions from POE SH-SY5Y cells pre-treated with DMSO, 10µM NU9056, 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr.

C. Quantification of PINK1 signal intensity in B, compared to controls (n=3, two-way ANOVA with Dunnett's correction).

D. Representative pPINK1 and PINK1 IB of mitochondrial fractions from HA SH-SY5Y cells pre-treated with DMSO and 100µM MG149 for 3hr and subsequently treated with DMSO or 1µM O/A for 3hr.

E. Quantification of pPINK1 at the mitochondrial membrane, calculated as the ratio of pPINK1 to PINK1 signal intensity, and compared to controls, in D (n=3, two-way ANOVA with Sídák correction).

Data shown as mean +/- SD.

DISCUSSION

Dysregulation of the mitochondrial quality control machinery exacerbates toxicity throughout the cells, and has been linked to many pathologies, amongst them PD (Brand, M. D., *et al*., 2013). Impairments in mitophagy-associated genes have been identified in several human neurodegenerative diseases such as PD, frontotemporal dementia (FTD) and Alzheimer's disease (AD) (van der Zee, J., *et al*., 2014), (Lou. G., *et al*, 2019). KAT8 has been identified as both a PD-GWAS candidate risk gene and as being involved in the PINK1/Parkin-dependent mitophagy pathway (Soutar, M., *et al*., 2022). This study explored the impact of MG149, a dual KAT5 and KAT8 inhibitor, on PINK1/Parkin-dependent mitophagy. The absence of a KAT8-specific inhibitor led us to explore the role of MG149 alongside NU9056, a KAT5 specific inhibitor (Li, L., *et al.*, 2020).

MG149 significantly decreased all markers of PINK1-dependent mitophagy initiation following O/A treatment (as per pUb(ser65), pParkin(ser65) and MFN2 degradation) without affecting mitochondrial delivery to the lysosomes. MG149 can also increase mitochondrial delivery to the lysosomes independently of O/A, suggesting it could increase autophagy. In fact, a recent study has shown that MG149 can increase LC3-II to LC3-I ratio, LC3 delivery to the lysosome and subsequent autophagic degradation in HepG2 cells (Moon, B., *et al*, 2022). KAT8 KD has also been linked to increased autolysosome formation (Hale, C. M., *et al*., 2016), and decreased PINK1-dependent mitophagy (Soutar, M., *et al*., 2022). All together, these studies highlight the complex interplay between different autophagy pathways initiated following MG149 treatment.
We have previously shown that KAT8 siRNA KD decreases *PINK1* gene transcription and subsequent PINK1-mitophagy (Soutar, M., *et al*., 2022). Here we show that while KAT8 compound inhibition also prevents PINK1-mitophagy, MG149 effect is mediated by altering PINK1 protein kinase activity, rather than *PINK1* gene transcription. Induction of autophagy via starvation was previously associated with KAT8 degradation, subsequent reductions in H4K16 acetylation, and ultimate downregulation of autophagy genes (Füllgrabe, J., *et al*., 2013). The acute, rather than chronic, effect induced by MG149 may explain the lack of transcriptional impact observed. It is possible that prolonged KAT8 compound inhibition may be required for the acetylation mark to be removed and for the *PINK1* gene to be silenced. All together, our data suggest that KAT8 may play a role in PINK1 mitophagy both by mediating PINK1 gene expression (Soutar, M., *et al*., 2022) and PINK1 protein activity.

It was previously reported that impairment in one macroautophagic pathway may result in upregulation of other degradation pathways, essentially initiating a compensatory feedback loop (Chu, C. T., 2019) (Xilouri, M., *et al*., 2009). It is possible that by decreasing the initiation of the PINK1-mitophagy pathway, MG149 may increase receptor-mediated mitophagy or bulk autophagy through a cause-and-effect reaction, ultimately leading to mitochondrial clearance. Receptor-mediated mitophagy is a process whereby receptors localized at the OMM and containing LIRs interact with LC3 to mediate selective autophagy (Liu, L., *et al*., 2014). In fact, other OMM mitophagy receptors have been shown to play a role in PINK1/Parkin-independent mitophagy, such as AMBRA1, Bcl2L13, nix/BNIPL3, BNIP3, FKBP8 and FUNDC1 which all contain an LC3-interacting region (Liu, L., *et al*., 2014) (Whiten, D. R., *et al*., 2021). LC3 may bind directly to these receptors through their LIRs, subsequently inducing mitophagy independently of PINK1 and Parkin. The induction of such a compensatory

mechanism after loss of PINK1-dependent mitophagy has been recently studied in the context of PD (Park, J.-S., *et al*., 2017) (Koentjoro, B., *et al*., 2017). Increased levels of Nix were shown to restore cellular mitophagy and compensate for the loss of PINK1-dependent mitophagy in cell lines derived from PINK1/Parkin-deficient PD patients. In this study, we show that KAT8 compound inhibition can modulate both PINK1-dependent mitophagy initiation and PINK1-independent mitochondrial delivery to lysosomes. Our findings may open new avenues for the design of novel therapeutic approaches targeting KAT8 for neurodegenerative diseases such as PD, in which defective PINK1-mitophagy plays a central role.

KAT8 inhibition may be a promising intervention for treating diseases by modulating other pathways than mitophagy. Playing on the acetylation patterns of histones and proteins may be central in providing new therapies for a variety of diseases. KAT8 inhibition may provide neuroprotective effects for AD by increasing lysosome formation (Yuhong, L., *et al*., 2023). Interestingly, KAT8 was identified as one the risk candidate genes for AD (Bellenguez, C., *et al*., 2022). KAT8 inhibition may also have selective toxicity and antiproliferative activity against cancer cells (Zhu, H., *et al*., 2021), (Fiorentino, F., *et al*., 2023) and potentially normalize myocardial functions in the context of cardiovascular diseases and ischemia-reperfusion injury (Yu, L., *et al*., 2018). By looking at MG149 in the context of mitophagy, we have underlined a new area in which modulating KAT8 inhibition may be of use in the study of PD. The lack of specificity of MG149 as a KAT8 inhibitor leaves room for new efficient and potent selective KAT8 inhibitors, such as those described in the recently published study looking new KAT8 inhibitors in the context of cancer treatment (Fiorentino, F., *et al*., 2023). Hopefully, the development of optimized KAT8 inhibitors may help give a more concrete overview of the therapeutic potential of KAT8 inhibition.

Methods

Reagents

Oligomycin and antimycin A (inhibitor of mitochondrial complex V and III respectively) were purchased from Sigma-Aldrich (O4876 and A8674 respectively).

For immunoblotting, the following antibodies were used: rabbit anti phospho-ubiquitin (Ser65) (Cell Signaling, 62802, RRID:AB_2799632 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, RRID:AB_2716838 1:1000), mouse anti OPA1 (BD Biosciences, 612607, RRID:AB_399889 1:1000), rabbit anti-LC3B (Novus Biologicals, NB100-2220, RRID:AB_10003146 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 accessible as descried in Soutar, M., *et al*., 2018.

For immunofluorescence the following antibodies were used: mouse anti TOM20 (Santa Cruz, sc-17764, RRID:AB_628381 1:1000), rabbit anti phospho-ubiquitin (Ser65) (Cell Signaling, 62802, RRID:AB_2799632 1:1000), mouse anti p62 Lck Ligand (BD Biosciences, 610832, RRID:AB_398151 1:1000), AlexaFluor 488 goat anti rabbit (Invitrogen, A11008, 6 RRID:AB_143165, RRID:AB_ 1:2000), AlexaFluor 568 goat anti mouse (Invitrogen, A11004, 7 RRID:AB_2534072, 1:2000).

Cell culture

All cells were maintained in culture in a humified 5% CO_2 incubator at 37°C, in Dulbecco's modified Eagle's medium (DMEM, Gibco, 11995-065) with 4.5 g/L D-glucose and L-glutamine, supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gilbco, 10500-064). Cell medium was refreshed and changed every 3 days.

The human neuronal cell line used for this study was the SH-SY5Y cell line:

The Parkin over-expression (POE) SH-SY5Y cells were a gift from H. Ardley (Ardley H. *et al*., 2003), the mtKeima POE SH-SY5Y cells were a gift from C. Luft (Soutar M., *et al*., 2019), and the PINK1-HA overexpression SH-SY5Y cells were a gift from E. Deas (Deas E., *et al*., 2011).

Cells were split at 75% to 90% confluency using 2mL of Trypsin. The flasks were split in such a way that each of their confluency would be approximately the same at the time of manipulations.

Mitophagy Initiation

In order to initiate mitophagy, cells were treated with 1µM oligomycin/ 1µM antimycin for 3hr before collecting / fixing for downstream experiments. Dimethyl Sulfoxide (DMSO, Santa Cruz, sc-202581) was used as a control.

Small molecule modulators

MG149 targets KAT5 and KAT8 with IC_{50} values of 74µM and 47µM respectively (Li L., *et al*., 2020). NU9056 targets KAT5 with an IC_{50} value of 2µM. Both small molecules were reconstituted in DMSO (Santa Cruz, sc-202581) before treatments.

For immunofluorescence, both compounds were diluted in DMEM (Gibco, 11995-065) so that 25uL of each diluted compound was added per well of a 96-well plate (PhenoPlate 96-well microplate, PerkinElmer, 6055302) to make up a total concentration of 10µM for NU9056 and 100µM for MG149.

For immunoblotting, concentrations of 10µM for NU9056 and 100µM for MG149 were added to corresponding wells, choosing a sub-toxic concentration.

Equal volumes of DMSO (Santa Cruz, sc-202581) were used as no treatment controls.

Mitochondrial enrichment

POE SH-SY5Y cells were fractionated into cytoplasmic and mitochondria-enriched preparations to facilitate detection of mitochondrial localized proteins of interest. Cells were incubated overnight at −80 °C in mitochondrial homogenization buffer (250mM sucrose, 1mM EDTA, 10mM Tris (pH 7.4) supplemented with protease (cComplete™, EDTA-free Protease Inhibitor Cocktail, Roche, 05056489001) and phosphatase (PhosSTOP™, Roche, 04906837001) inhibitors) before being thawed on ice. The content of the wells were scraped into 1.5ml centrifuged tubes, triturated 20 times and frozen at −80 °C for 1hr. After being thawed on ice, cell debris / non-lysed cells were removed by centrifugation (1500g for 20 minutes at 4°C) and the supernatant centrifuged again to pellet the mitochondria (12500g for 20 minutes at 4°C). The resulting supernatant was kept, making up the cytoplasmic fraction, and the pellet washed twice with mitochondrial homogenization buffer to remove any cytoplasmic contamination, making up the mitochondrial fraction. Isolated mitochondria pellets were suspended in 1X LDS (NuPAGE™ LDS Sample Buffer (4X), Invitrogen, NP0008) supplemented with 10 mM dithiothreitol (DTT). LDS preps were heated at 70°C for 10 mins.

Cell lysis

Cells were lysed 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 (cComplete™, EDTA-free Protease Inhibitor Cocktail, Roche, 05056489001) and phosphatase (PhosSTOP™, Roche, 04906837001) inhibitors) on ice and incubated overnight at -80°C. Lysates were cleared by centrifugation (16000g for 20 minutes at 4°C) and protein quantified by BSA assay then LDS preps were made with 10mM DTT prior to heating at 70 °C for 10 mins.

Western blotting

LDS preparations were separated by SDS-PAGE and transferred to nitrocellulose membrane. Primary antibodies were diluted in 3% milk in PBS-Tween and incubated overnight at 4°C. The following day, membranes were washed and incubated at room temperature for 1hr in secondary antibodies diluted in PBS-Tween. Protein bands were detected using Odyssey CLx LI-COR.

Immunofluorescence

Cells were fixed with 4% Paraformaldehyde (PFA, Sigma-Aldrich) for 20 minutes at room temperature (RT), then blocked and permeabilized with a mix of 10% FBS, 0.25% Triton X-100 in PBS for 1 h. Cells were then immunostained with pUb(Ser65) and TOM20 primary antibodies or with p62 primary antibody diluted in 10% FBS/PBS for 2hr at RT. The wells were washed three times using PBS 1X before the secondary antibodies were added and incubated for 1hr: AlexaFluor 568 anti-mouse, 488 anti-rabbit secondary antibodies, and Hoechst 33342 (Thermo Scientific) were all at a 1:2000 dilution in 10% FBS/PBS. The wells were washed three times using PBS 1X, and the plates were imaged using Opera Phenix (PerkinElmer). Images were subsequently analysed using Columbus 2.8 analysis system (PerkinElmer) to measure either the integrated intensity of pUb(Ser65) within the whole cell, or the mitochondrial to cytoplasmic p62 intensity ratio.

Measurement of mitochondrial membrane potential using TMRM

POE-SHSY5Y cells were seeded in a 96-well plate (PhenoPlate 96-well microplate, PerkinElmer, 6055302) before being incubated live in 25 nM of Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM, Sigma Aldrich, T5428) diluted in cell media for 40 mins at 37 °C and 4% CO_2.

Cells were imaged 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.

The images were then analysed using the Columbus 2.8 analysis system (PerkinElmer) to measure the TMRM spot intensity within the whole cell.

Measurement of mitochondrial delivery to lysosomes using mitoKeima reporter

mt-Keima expressing POE SHSY5Y cells were seeded in a 96-well plate (PhenoPlate 96-well microplate, PerkinElmer, 6055302) and treated according to desired conditions (MG149, NU9056, O/A as described above).

Before imaging, cell media was replaced with phenol-free DMEM (Gibco, 31053-028) supplemented with 10% FBS (Gilbco, 10500-064) containing the treatments previously administered, and adding Hoechst 33342 (1:10000) to stain the nuclei.

Cells were immediately imaged on the Opera Phenix (PerkinElmer) at 37 °C with 5% CO_2, acquiring multiple single-plane fields of view, using the 63X water objective.
Images were then analysed using the Columbus 2.8 analysis system (PerkinElmer) to measure the mitophagy index, which is the ratio between the total area of lysosomal mitochondria (561nm) to the total area of mt-Keima (sum of cytoplasmic (488nm) and lysosomal (561nm) mt-Keima area) per well.

Statistical Analysis
Intensity measurements from imaging experiments were normalized as stated for each experiment and presented as a fraction. N numbers are shown in figure legends and refer to the number of independent, replicate experiments. A minimum of 3 biological replicates were used for each experiment. GraphPad Prism 6 (La Jolla, California, USA, RRID:SCR_002798) was used for statistical analyses and graph production. Data were subjected to two-way ANOVA, unless otherwise stated. Data was expressed as the mean ± standard deviation (SD) from replicate experiments and statistical significance was set at p-value of <0.05.

The above methods are detailed in protocols.io: https://www.protocols.io/view/the-impact-of-mg149-a-kat8-inhibitor-on-mitophagy-cuw7wxhn

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