Heliyon 8 (2022) e11179

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Identification of active natural products that induce lysosomal biogenesis by lysosome-based screening and biological evaluation

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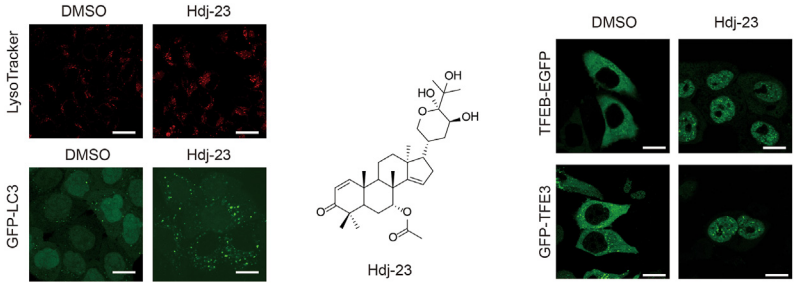
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Lysosomal biogenesis
Natural products
TFEB and TFE3
Lysosome-based screening
Autophagy

ABSTRACT

Lysosomal biogenesis is an essential adaptive process by which lysosomes exert their function in maintaining cellular homeostasis. Defects in lysosomal enzymes and functions lead to lysosome-related diseases, including lysosomal storage diseases and neurodegenerative disorders. Thus, activation of the autophagy-lysosomal pathway, especially induction of lysosomal biogenesis, might be an effective strategy for the treatment of lysosome-related diseases. In this study, we established a lysosome-based screening system to identify active compounds from natural products that could promote lysosomal biogenesis. The subcellular localizations of master transcriptional regulators of lysosomal genes, TFEB, TFE3 and ZKSCAN3 were examined to reveal the potential mechanisms. More than 200 compounds were screened, and we found that Hdj-23, a triterpene isolated

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https://doi.org/10.1016/j.heliyon.2022.e11179
Received 15 April 2022; Received in revised form 30 August 2022; Accepted 17 October 2022

from *Walsura cochinchinensis*, induced lysosomal biogenesis via activation of TFEB/TFE3. In summary, this study introduced a lysosome-based live cell screening strategy to identify bioactive compounds that promote lysosomal biogenesis, which would provide potential candidate enhancers of lysosomal biogenesis and novel insight for treating lysosome-related diseases.

1. Introduction

In the past, most drugs have been discovered by identifying the active ingredient from traditional medicines or by serendipitous discovery. As innovations in scientific discoveries and technological advances continue to increase, current new drug discovery strategies include phenotypic and target-based approaches [1]. Target-based drug discovery is more biased toward focus and investment, as it exhibits its strengths over phenotypic drug screening, such as shortened drug discovery and development time frames, which are more specific, as they are predominantly hypothesis-driven [2, 3]. Phenotypic screening in the discovery of innovative small molecule drugs is the most successful approach for first-in-class medicine. Phenotypic screening holds promise for unbiased identification of novel molecular mechanisms of action (MMOAs), therefore shedding light on the discovery of new therapeutic principles and molecular pathways of currently untreatable diseases [4, 5].

Lysosomes are single-membrane organelles that serve as the major degradative and signaling sites within the cell [6, 7, 8]. More than 60 soluble lysosomal hydrolases and accessory proteins and over 120 lysosomal membrane proteins and cargo receptors have been found [9]. The characterization of lysosomal proteins helps expand the understanding of lysosomes and their role in maintaining cellular homeostasis [10]. In addition to its canonical role as a degradative center, lysosomes play an antioxidant protective role by removing damaged proteins or organelles induced by oxidative damage [11]. Defects in genes encoding lysosomal proteins lead to lysosomal storage disorders (LSDs), while lysosomal dysfunction plays important roles in neurodegenerative diseases, autoimmune disorders and cancer [12]. Given that the understanding of lysosomal dysfunctions in diseases, more therapeutic options are available targeting lysosomes. For example, lysosomal inhibition would be the goal in the treatment of autoimmune diseases. Activation of autophagy and induction of lysosomal biogenesis have therapeutic potential in treating neurodegenerative diseases [13, 14].

In recent years, the study of master transcription factors, such as TFEB and TFE3, in the autophagy-lysosomal pathway has received much attention [15]. In response to starvation, TFEB and TFE3 actively transcribed a series of genes involved in autophagy and lysosome biogenesis. However, recent studies have revealed novel roles for these transcription factors in response to a variety of stress signals other than starvation [16]. Our recent study showed that in response to a small molecule compound (Hep-14), TFEB-dependent lysosome biogenesis is activated in a protein kinase C (PKC)-dependent and mTOR-independent mechanism, providing novel mechanistic insight into the application of PKC activators in the treatment of lysosome-related diseases [17].

In our previous work, limonoids isolated from *Walsura yunnanensis* were proven to induce G2/M cell cycle arrest and apoptosis in cancer cells [18]. In this study, we took advantage of a cell-based phenotypic screening system to search for natural-derived small molecule compounds that promote lysosomal biogenesis, an essential step for lysosomal adaptation to stress. Cell-based screening of increased lysosomal biogenesis by LysoTracker Red staining was utilized, and the subcellular locations of transcription factors such as TFEB, TFE3, and ZKSCAN3 were investigated. We identified a group of natural products that efficiently induced lysosomal biogenesis. More than 200 compounds were screened, and Hdj-23 was identified. Hdj-23 induced lysosomal biogenesis in a TFEB/TFE3-dependent manner. These findings suggested that natural products might be promising candidates for dissecting the signals underlying lysosomal biogenesis and for developing novel therapeutic strategies for lysosome related diseases.

2. Experimental

2.1. Cells, reagents, and materials

The compounds were collected from Professor Hao's laboratory. All the compounds were dissolved in DMSO to make a 20 mM storage solution. The HeLa cell line was kindly provided by Qinghua Kong (Kunming Institute of Botany, Chinese Academy of Sciences). pCMV-TFEB-EGFP, pCMV-GFP-TFE3, and pCMV-GFP-ZKSCAN3 plasmids were constructed. The cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone), 10,000 units/mL penicillin and 10 mg/mL streptomycin. LysoTracker Red DND 99 was purchased from Thermo Scientific.

2.2. Plant material

The leaves of *Walsura cochinchinensis* were collected from Yulin, Guangxi Province, P. R. China, in July 2019. The samples were identified by Prof. Hua Peng of Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (HXJ20190706) was deposited at State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

Air-dried leaves of *W. cochinchinensis* (4.6 kg) were extracted with 95% CH_3OH (10 L × 3) at room temperature and then concentrated under reduced pressure to give a crude extract (326.1 g). The extract was suspended in water and successively partitioned with petroleum ether (PE) and EtOAc. The EtOAc-soluble fraction (123.5 g) was subjected to silica gel column chromatography (CC) (200–300 mesh, PE/acetone, 200:1–0:1) to produce ten major fractions (1–10). Fr.5 (42.9 g) was subjected to reverse-phase separation (CH_3OH–H_2O, 2:8 to 9:1) and afforded 7 fractions (5A-5G). Fr.5C was chromatographed by Sephadex LH-20 CC and eluted with MeOH to give Fr.5C2 (338.1 mg), which was further purified by semi-preparative HPLC (70% CH_3CN in water) to yield Hdj-26 (4.2 mg) and Hdj-38 (9.3 mg). Fr.6 (19.7 g) was applied to an MCI gel column (CH_3OH–H_2O from 2/8 to 8/2) to yield 7 major fractions (6A-4G). Fr.6C (3.2 g) was applied to a silica gel column (300–400 mesh; PE/acetone, 100:1–5:1) to yield Fr.6C2 (20.7 mg), which was further purified by semipreparative HPLC (68% CH_3CN in water) to yield Hdj-35 (7.0 mg). Fr.6F (4.2 g) was applied to a silica gel column eluted with petroleum ether–acetone (from 100:1 to 2:1) to give Hdj-23 (116.8 mg) and subfraction 6F1 (180.0 mg). Subfraction 6F1 was further purified by Sephadex LH-20 CC (CH_3OH) and semipreparative HPLC (72% CH_3CN in water) to yield Hdj-27 (27.6 mg).

2.4. Screening for compounds that induce lysosomal biogenesis

HeLa cells with 70% cell density in 96-well plates were treated with individual compounds at 20 and 40 μM in triplicate. Three hours later, the cells were grown in fresh medium containing LysoTracker Red DND-99 (0.2 μM) for 30 min. Then, the medium was changed again to LysoTracker-free medium, and images were taken with ArrayScan Infinity (Cellomics, ArrayScan VTI HCS).

2.5. Confocal microscopy

HeLa cells transiently expressing TFEB-GFP, GFP-TFE3, GFP-ZKSCAN3, or RFP-GFP-LC3 were treated with the indicated compounds, and images were collected by confocal microscopy [19]. For live-cell imaging, cells grown on glass-bottom dishes (In Vitro Scientific) were observed directly. All samples were examined with Leica Confocal Microscope TCS SP8 X (Leica Microsystems).

2.6. Quantitative real-time PCR with reverse transcription (qRT–PCR)

RNA was isolated from HeLa cells using TRIzol Reagent (Invitrogen) as previously described [20]. A reverse-transcription kit (Promega) was used to reverse transcribe RNA (1 μg) in a 20 μL reaction mixture. Quantification of gene expression was performed using a real-time PCR system (7900HT Fast; Applied Biosystems) in triplicate. Amplification of the sequence of interest was normalized to the reference endogenous gene actin.

2.7. Western blots

Standard western blot assays were used to analyze the levels of protein [21]. The following antibodies were used in this study: anti-actin (Proteintech, 66009-1-Ig), anti-LC3 (Abcam, ab48394), anti-s6k (Proteintech, 14485-1-AP), anti-p-s6k (Cell Signaling Technology, #9205), anti-TFEB (Proteintech, 13372-1-AP), and anti-p-TFEB (Cell Signaling Technology, #37681).

2.8. Statistics and reproducibility

Data analyses were carried out using GraphPad Prism 7, and Student's *t*-test was employed for statistical analyses with a level of significance of $p < 0.05$.

3. Results and discussion

3.1. A lysosome-based screening for lysosomal biogenesis from natural products

Lysosomes play important roles in maintaining cellular homeostasis in response to environmental cues. Previously, it has been shown that lysosomes can function as signaling organelles sensing nutrient availability and activating lysosome-nucleus signaling pathways, such as TFEB, to mediate the starvation response and regulate energy metabolism [7]. However, the mechanisms underlying stress-induced lysosomal biogenesis are not fully understood. Here, we aimed to screen a natural small-molecule compound library in the search for compounds that would induce lysosomal biogenesis and further elucidate the mechanism underlying lysosomal biogenesis. The changes in lysosome number upon treatment with the natural compounds were examined using LysoTracker Red DND 99. LysoTracker Red is a red fluorescent dye with a hydrophobic weak base that selectively accumulates in acidic spherical organelles, predominantly in lysosomes [22]. The pH of lysosomes is acidic (approximately pH 5), which is required for the hydrolytic enzymes within the lysosome to exert their functions best. The LysoTracker probes are permeant to cell membranes and can irreversibly remain in lysosomes even with weakly basic cell-permeant compounds. The probe can effectively label live cells at nanomolar concentrations. We treated HeLa cells with more than 200 compounds of plant origin and monitored the changes in the intensity and number of fluorescent dots accumulated in the cytoplasm. We identified Hdj-23, which can effectively induce lysosomal biogenesis. The structures of Hdj-23 and its analogs are shown in Figure 1.

3.2. Subcellular localization of master transcription factors to characterize compounds inducing lysosomal biogenesis

Previous studies revealed that global transcriptional regulation by TFEB, TFE3 and ZKSCAN3 plays an important role in regulating

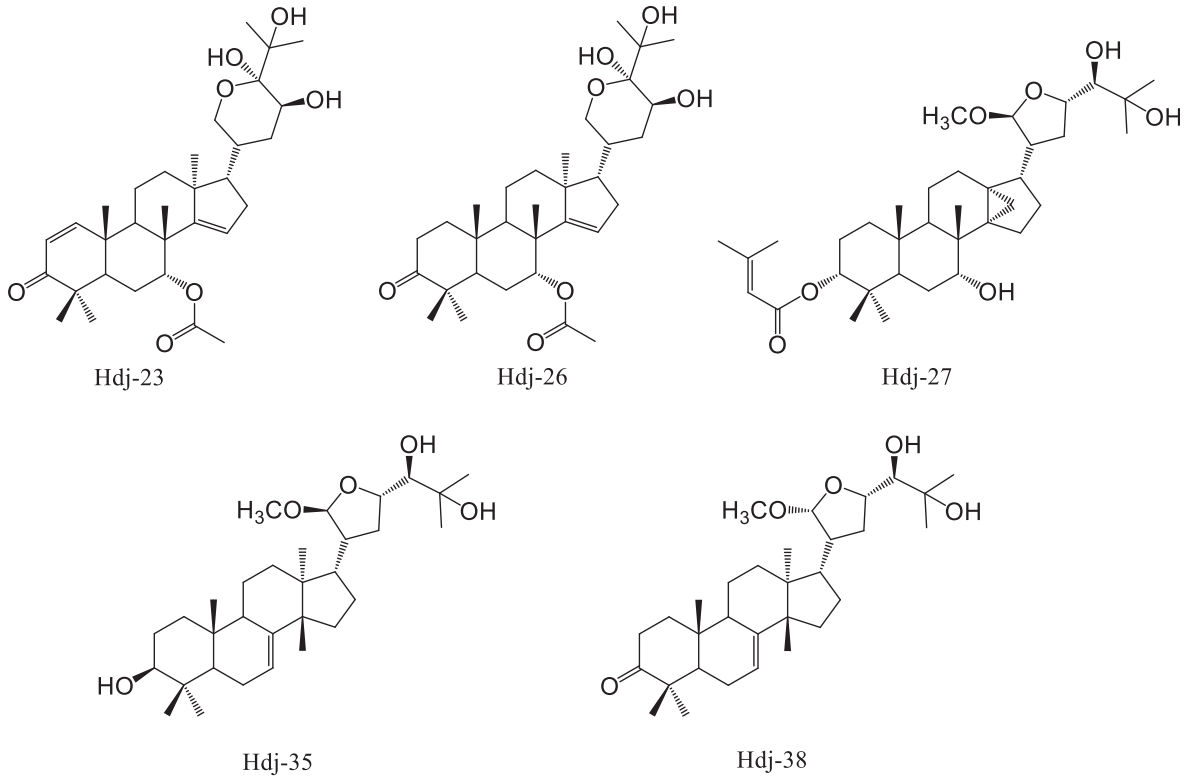

Figure 1. The structures of Hdj-23 analogs.

lysosomal biogenesis. TFEB and TFE3 are members of the MITF subfamily of transcription factors that bind E-box sequences (5′-CANNTG-3′) [15]. TFEB specifically recognizes the coordinated lysosomal expression and regulation (CLEAR) network (5′-GTCACGTGAC-3′) to positively regulate lysosomal gene transcription as well as other cellular degradative pathways, including autophagy [23]. Under physiological conditions, the majority of TFEB and TFE3 are located in the cytoplasm. However, under specific conditions, such as nutrient deprivation, TFEB and TFE3 rapidly translocate from the cytoplasm to the nucleus. Phosphorylated TFEB and TFE3 are mainly located in the cytoplasm, whereas dephosphorylated TFEB and TFE3 reside in the nucleus. The phosphorylation status of TFEB has been reported to be controlled by mTORC1, ERK2, PKCβ, PKCδ and GSK3β [24, 25, 26]. In contrast, ZKSCAN3 is a master transcriptional repressor of lysosomal and autophagy genes [27]. Phosphorylation of ZKSCAN3 at a putative nuclear export signal (NES) also leads to the cytoplasmic localization of ZKSCAN3, therefore alleviating transcriptional repression of its target genes.

Here, we transiently transfected HeLa cells with pCMV-TFEB-GFP, GFP-TFE3 or GFP-ZKSCAN3 to monitor the subcellular location of these master transcription factors in response to natural compound

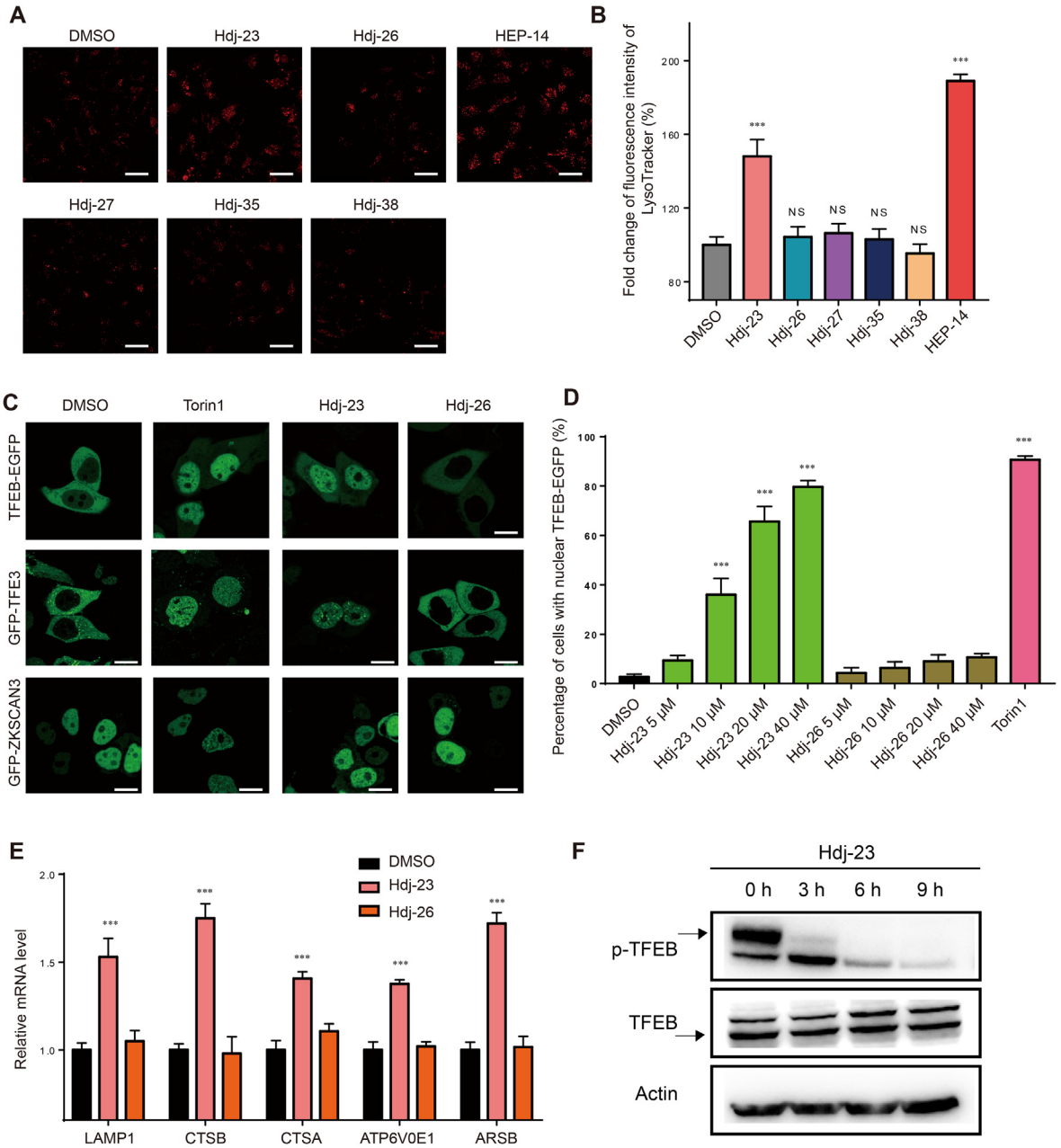

Figure 2. Hdj-23 promoted lysosomal biogenesis via activation of TFEB and TFE3. (A&B) Hdj-23 induced lysosomal biogenesis as measured by LysoTracker staining. (A) Representative images of lysosomes stained with LysoTracker. Cells were treated with the indicated compounds at 40 μM for 3 h. DMSO was added as a negative control and HEP-14 (20 μM, 3 h) was used as a positive control. (B) Quantification of the fold change in the fluorescence intensity of LysoTracker. (C) Hdj-23 promoted nuclear translocation of TFEB and TFE3, while it had no effect on the localization of ZKSCAN3. (D) Quantification of the percentage of cells with nuclear TFEB. Hdj-23 promoted the nuclear translocation of TFEB in a dose-dependent manner. DMSO was used as the negative control and Torin1 (1 μM, 3 h) was used as the positive control. (E) Hdj-23 induced the transcription of lysosomal genes. The bar graph shows the relative mRNA level of each gene, and β-actin served as the internal control. (F) Cells were treated with Hdj-23 at 20 μM. p-TFEB and TFEB were detected by western blot assay. Data are presented as the mean ± SD, and all the data were collected from 3 independent experiments. ***$p < 0.001$.

treatment. This would help provide clues to categorize the different compounds in their mechanisms of action. We found two types of subcellular localization of these transcription factors induced by our candidate compounds. HEP-14, which we used as a positive control, induced lysosomal biogenesis through TFEB activation and ZKSCAN3 inactivation. Hdj-23 could induce the nuclear translocation of both TFEB and TFE3, whereas ZKSCAN3 remained in the nucleus. The analogs of Hdj-23 did not have the same activity as Hdj-23.

3.3. Compound Hdj-23 induces lysosomal biogenesis through the TFEB/TFE3 axis

We tested a group of triterpenes that were isolated from *W. cochinchinensis* with similar structures to Hdj-23. As shown in Figure 2A and B, only Hdj-23 induced significant lysosomal biogenesis. We further evaluated the subcellular location of TFEB, TFE3 and ZKSCAN3. Similar to Torin1, a mTOR inhibitor, Hdj-23 induced the nuclear translocation of TFEB and TFE3 (Figure 2C). In addition, compound Hdj-23 induced TFEB nuclear translocation in a dose dependent manner (Figure 2D). The transcriptional levels of TFEB target lysosome-related genes, including lysosomal membrane protein (LAMP1), lysosomal hydrolytic enzymes (CTSB and CTSA), lysosomal ATPase (ATP6V0E1) and lysosomal sulfatase (ARSB), were also checked. As shown in Figure 2E, Hdj-23 induced the transcription of LAMP1, CTSB, CTSA, ATP6V0E1 and ARSB, whereas its analog Hdj-26 neither induced TFEB/TFE3 nuclear translocation nor induced the transcription of lysosomal genes. To assess TFEB phosphorylation, we tested endogenous TFEB in cells treated with Hdj-23. Hdj-23 reduced the phosphorylation of the 14-3-3-binding motif (Ser211) in TFEB (Figure 2F), which binds to 14-3-3 proteins to sequester TFEB in the cytoplasm. All these data suggested that Hdj-23 induced lysosomal biogenesis via the TFEB/TFE3 axis.

3.4. Compound Hdj-23 activates autophagic flux by inhibiting mTOR

To investigate whether Hdj-23 can promote autophagy, the formation of autophagosomes was checked by LC3-positive dots. Consistently, Hdj-23 efficiently promoted autophagosome formation, while Hdj-26 did not show significant induction of autophagosomes (Figure 3A and B). Western blot assays showed that Hdj-23 increased the protein levels of LC3-II in a time-dependent manner (Figure 3C). As a highly conserved kinase important for autophagy regulation, target of rapamycin complex 1 (mTORC1) signaling is a critical molecule for the origin of autophagy and serves as a major negative regulatory axis of autophagy. The activity of mTORC1 signaling was checked by western blot assay. Hdy-7 decreased the levels of phosphorylated S6K in HeLa cells (Figure 3C). The fluorescent probe RFP-GFP-LC3 can be used to assess autophagic flux. Here, we transfected HeLa cells with the RFP-GFP-LC3 plasmid and monitored autophagic flux by confocal microscopy. After 9 h of

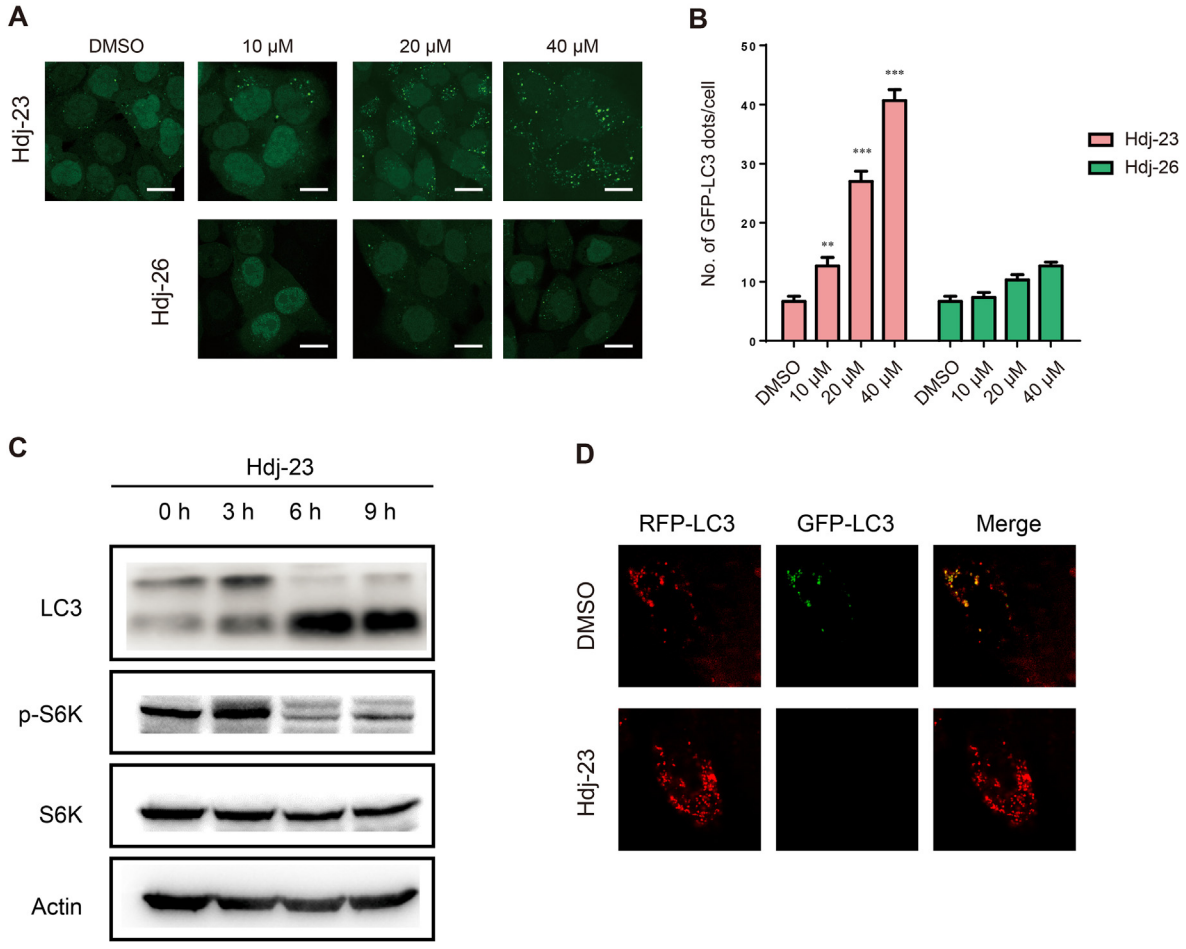

Figure 3. Hdj-23 activates autophagic flux by inhibiting mTOR. (A&B) Hdj-23 promoted the formation of autophagosomes in a dose-dependent manner. (A) Representative images of autophagosomes labeled with GFP-LC3 dots. (B) Quantification of the number of GFP-LC3-positive dots per cell. (C) Cells were treated with Hdj-23 at 20 μM. LC3, p-S6K, and S6K were detected by western blot assay. (D) Hdj-23 induced functional autolysosome formation. HeLa cells were transduced with RFP-GFP-LC3, treated with Hdj-23 (40 μM) for 9 h and examined by confocal laser scanning microscopy. The punctate pattern of LC3 (GFP positive) indicated an autophagosome, and LC3 (RFP positive only) indicated an autolysosome. Data are presented as the mean ± SD, and all the data were collected from 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

treatment, Hdj-23 activated autophagic flux. As shown in Figure 3D, upon Hdj-23 treatment, the green fluorescent protein (GFP) puncta decreased, while the red fluorescent protein (RFP) puncta increased. These results indicated that Hdj-23 inhibited mTOR signaling, which contributes to autophagy. According to the structures of Hdj-23 and its analog Hdj-26, we found that the α,β-unsaturated ketone in ring A might be the key active group for inducing lysosomal biogenesis.

mTORC1-TFEB/TFE3 axis regulates lysosomal homeostasis under different nutrient conditions [28]. When the cells are in nutrient-rich status, mTORC1 is stimulated and phosphorylates transcription factors TFEB and TFE3, which prevent them from nuclear translocation to activate the lysosomal genes. Under starvation, mTORC1 does not phosphorylate TFEB and TFE3. Instead, TFEB is dephosphorylated by calcineurin and translocates to the nucleus to initiate lysosomal biogenesis. Under conditions other than nutrient conditions, besides the canonical mTORC1-TFEB axis, PKC-TFEB axis has been reported as well [17]. The identification of PKC-TFEB axis using a natural compound highlights the potential applications of natural products in revealing previous unrecognized mechanisms in lysosomal homeostasis.

Dysfunction or disturbance of homeostasis of lysosomes results in diseases, such as neurodegenerative diseases [29]. Neurodegenerative diseases are a diverse group of age-dependent neurodegenerative diseases that are associated with decreased antioxidants and increased oxidative damage. It has been proposed that the autophagy-lysosomal pathway plays an effective role in eliminating damaged proteins and organelles generated by oxidative stresses in neurodegenerative diseases. Understanding lysosome acidification, autophagy, lysophagy and how lysosomes are involved in cellular homeostasis maintenance contributes to the elucidation of the functions of lysosomes as well as the molecular pathology of neurodegenerative diseases and other diseases closely related to lysosome functions, such as lysosomal storage diseases (LSDs) [30]. Chemical biology provides unique approaches for investigating lysosome-mediated signaling pathways and cellular processes. Natural products, especially active ingredients isolated and identified in plant extracts that are used in traditional Chinese medicines, have been an important and indispensable source of phenotypic drug discovery.

4. Conclusions

With the advantages of microscopy techniques for subsequent lysosomal biogenesis in real time, we used LysoTracker to identify natural compounds that could induce lysosomal biogenesis. By using this lysosome-based screening strategy, we screened more than 200 compounds of plant origin and found that Hdj-23 could efficiently induce lysosomal biogenesis. Evaluation of the subcellular localizations of the master transcription factors TFEB, TFE3 and ZKSCAN3 provided clues for further investigation on the mechanisms of action of those active natural-derived compounds. We found that Hdj-23 induced lysosomal biogenesis via activation of the TFEB/TFE3 axis. Additional studies on how active natural products induce lysosomal biogenesis using biochemical techniques will expand the exploration of novel mechanisms of lysosomal biogenesis regulation. Active natural small molecule compounds might help reveal potential candidates and novel strategies for treating lysosome-related diseases.

Declarations

Author contribution statement

Xiao Ding: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xu Yang, Yueqin Zhao, Yinyuan Wang, Zhenpeng Niu: Performed the experiments.

Jimin Fei, Xianxiang Dong, Xuenan Wang: Analyzed and interpreted the data.

Biao Liu, Hongmei Li: Contributed reagents, materials, analysis tools or data.

Xiaojiang Hao: Conceived and designed the experiments.

Yuhan Zhao: Conceived and designed the experiments; Wrote the paper.

Funding statement

Dr Yuhan Zhao was supported by National Natural Science Foundation of China [32000548], Natural Science Foundation of Yunnan Province [2019FY003003 & 202001AT070055], State Key Laboratory of Phytochemistry and Plant Resources in West China [P2019-ZZ06 & P2021-ZZ04].

Dr Xiao Ding was supported by National Natural Science Foundation of China [82073740 & 31730053], Natural Science Foundation of Yunnan Province [202001AT070053], State Key Laboratory of Phytochemistry and Plant Resources in West China [P2018-ZZ05].

Dr Xiaojiang Hao was supported by CAMS Innovation Fund for Medical Sciences [CIFMS, 2021-I2M-5-004].

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e11179.

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