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OXFORD

Special Issue: The FOXO3 Gene and Its Relation to Lifespan and Healthspan

Screening Health-Promoting Compounds for Their Capacity to Induce the Activity of FOXO3

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Abstract

Several chemical compounds including natural products have been suggested as being effective against age-related diseases or as beneficial for a healthy life. On the other hand, forkhead box O (FOXO) proteins are emerging as key cellular components associated with extreme human longevity. FOXO proteins are mainly regulated by posttranslational modifications and as these modifications are reversible, activation and inactivation of FOXO are attainable through pharmacological treatment. Here, we questioned whether a panel of compounds with known health-beneficial properties has the capacity to induce the activity of FOXO factors. We show that resveratrol, a phytoalexin present in grapes and other food products, the amide alkaloid piperlongumine found in the fruit of the long pepper, and the plant-derived β-carboline compound harmine induced nuclear translocation of FOXO3. We also show that piperlongumine and harmine but not resveratrol activate FOXO-dependent transcription. We determined the half maximal effective concentration (EC50) values for resveratrol, piperlongumine, and harmine for FOXO translocation, and analyzed their inhibitory impact on chromosomal maintenance 1 (CRM1)-mediated nuclear export and the production of reactive oxygen species (ROS). We also used chemical biology approach and Western blot analysis to explore the underlying molecular mechanisms. We show that harmine, piperlongumine, and resveratrol activate FOXO3 independently of phosphoinositide 3-kinase (PI3K)/AKT signaling and the CRM1-mediated nuclear export. The effect of harmine on FOXO3 activity is at least partially mediated through the inhibition of dual-specificity tyrosine (Y) phosphorylationregulated kinase 1A (DYRK1A) and can be reverted by the inhibition of sirtuins (SIRTs).

Keywords: Aging, FOXO3, High-content screening, Longevity, Natural products

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but not resveratrol activate FOXO-dependent transcription. We determined the half maximal effective concentration (EC_{50}) values for resveratrol, piperlongumine, and harmine for FOXO translocation, and analyzed their inhibitory impact on chromosomal maintenance 1 (CRM1)-mediated nuclear export and the production of reactive oxygen species (ROS). We also used chemical biology approach and Western blot analysis to explore the underlying molecular mechanisms. We show that harmine, piperlongumine, and resveratrol activate FOXO3 independently of phosphoinositide 3-kinase (PI3K)/AKT signaling and the CRM1-mediated nuclear export. The effect of harmine on FOXO3 activity is at least partially mediated through the inhibition of dual-specificity tyrosine (Y) phosphorylation-regulated kinase 1A (DYRK1A) and can be reverted by the inhibition of sirtuins (SIRTs).

The demographic shift toward an older population is a global issue that affects or will soon affect virtually every region around the world. However, this remarkable extension of life span has not been accompanied by an equivalent increase in healthspan. Aging is the single biggest risk factor for cancer and several other age-related diseases including neurodegenerative, cardiovascular, inflammatory, and metabolic diseases (1). Research over the last few decades revealed several mechanisms underlying human aging (2). The identification of cellular signaling pathways regulating the life span in animal models and genetic factors associated with human longevity has shaped our molecular understanding of aging. This scientific progress might eventually lead to a new era in aging research identifying actionable targets to promote healthy aging (3). FOXOs are thought to protect humans from age-related diseases including cancer by coordinating a response to environmental changes such as growth factor deprivation, metabolic stress, and oxidative stress, and thereby help maintain tissue homeostasis over time (4). Intriguingly, genetic manipulations of FOXO transcription factors are associated with longevity in worms, flies, and mammals (5). In the nematode Caenorhabditis *elegans*, activation of the FOXO homolog daf-16 can more than double life span and substantially increase organismal resistance to thermal and oxidative stress (6). FOXO3 was first demonstrated to be important for human longevity in 2008 in Americans of Japanese ancestry living in Hawaii (7). Protective alleles of *FOXO3* are the second most-replicated genetic factors found so far to be associated with long life in humans (8). Human *FOXO3* hosts about 40 common, noncoding single nucleotide polymorphisms (SNPs) that have been consistently associated with longevity (8). In addition, SNPs of *FOXO1* were found to be associated with longevity in women (9).

FOXO transcription factors belong to the family of Forkhead proteins, characterized by a ~100-residue forkhead (FKH) DNA-binding domain. FOXO proteins are a family of proteins that consists in mammals of FOXO1, FOXO3, FOXO4, and FOXO6 (10). FOXO transcription factors bind as monomers to consensus binding sites within the promoter of their target genes. The FOXO target genes encode proteins known to be involved in several cellular processes including cell cycle, apoptosis, autophagy, stress resistance, and metabolism (11). FOXOs are mainly regulated by reversible posttranslational modifications which generate a molecular code to sense external stimuli and determine the transcriptional programs mediated by these transcription factors (12). Under stress conditions or in the absence of growth or survival factors, FOXO proteins translocate to the cell nucleus, where their transcriptional functions can be executed. Conversely, in the presence of growth factors or in cancer cells where the PI3K/AKT pathway is constitutively activated, AKT phosphorylates FOXO transcription factors in the nucleus, creating a docking site for 14-3-3 protein dimer. The binding of 14-3-3 chaperone to nuclear FOXO reduces its affinity to DNA and facilitates its nuclear export, eventually leading to cytoplasmic sequestration and inactivation of FOXO. The activity of FOXO proteins is largely regulated by their subcellular localization, though several stimuli are known to induce FOXO transcriptional activity without affecting their translocation (11). Chemical compounds affecting enzymes that act upstream of FOXO proteins mediating posttranslational modifications or interacting directly with these proteins are expected to exert a regulatory effect on FOXOs (13). It has been suggested that activating FOXO proteins could be used as a strategy to treat age-related diseases and promote healthy aging. On the other hand, several agents have been reported as potentially being effective against age-related diseases or beneficial for healthy aging (14) including astaxanthin (15), epigallocatechin gallate, harmine, hydroxyurea, metformin, piperlongumine, phenformin, rapamycin, resveratrol, spermidine, and tetrahydrocurcumin. Here, we explore the possibility that these compounds act through activating FOXO proteins and characterize the underlying mechanisms.

Method

Compounds

Acetylcysteine, astaxanthin, epigallocatechin gallate, harmine, LY294002, metformin, moclobemide, phenformin, piperlongumine, rapamycin, resveratrol, sirtinol, spermidine, and tetrahydrocurcumin were purchased from Selleck Chemicals (Houston, TX, USA). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA); dihydroethidium, hydroxyurea, and phenformin hydrochloride were obtained from Sigma (St. Louis, MO, USA); and leptomycin B (LMB) was purchased from Alomone Labs (Jerusalem, Israel). AnnH75 was developed and synthesized in the Bracher Lab (Ludwig-Maximilians University, Munich, Germany) (16).

Cell Culture

The stable reporter cell lines U2foxRELOC (17,18), U2nesRELOC (19,20), and 293foxREP (21) have been generated previously. These cell lines as well as U2OS parental cells and HepG2 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Sigma, PT) and antibiotics (Gibco, Carlsbad, CA, USA). Cell cultures were maintained in a humified incubator at 37 °C with 5% CO_2 and passaged when confluent using trypsin/ethylenediaminetetraacetic acid (EDTA).

FOXO Translocation Assay

The U2foxRELOC cells were seeded at a density of 2×10^4 cells per well into black clear-bottom 96-well microplates (BD Biosciences, San Jose, CA, USA). After 24-hour incubation, 1.6 µL of each compound (100× concentrated, 10 mM and 0.0195 mM) was added in quadruplicated and cells were incubated for different time periods (1, 3, or 6 hours). Then cells were fixed with 4% paraformaldehyde. Finally, the plates were washed with 1× phosphate-buffered saline (PBS) and stored at 4 °C before analysis. Cell images were taken in a DMIL LED FLUO inverted microscope (Leica, Wetzlar, Germany) at 20-fold magnification.

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Luciferase Assay

For luciferase assay, 2 × 10^5 293foxREP cells were seeded per well in transparent round-bottom 96-well plates. Once attached, the cells were treated with 1.6 µL of each natural compound and incubated for 6 hours. All treatments were performed in triplicate. Automated luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Cell Viability

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, an index of early toxicity, was performed in HepG2 hepatic cell line, seeded at a density of 2 × 10^4 cells per well in round-bottom 96-well plates. After 24 hours of incubation, 1.6 µL of each 100× concentrated compound was added per well and cells were incubated for 12, 24, 48, or 72 hours. After the incubation period, the plates were washed with 1× PBS and incubated for 4 hours with 100 µL per well of 0.5 mg/mL MTT (Merck, Darmstadt, Germany). Then, formazan crystals were dissolved using dimethylsulfoxide (DMSO) (100 µL per well) with gentle rocking at room temperature for 1 hour. Absorbance was measured at 570 nm.

Oxidative Stress

ROS generation was detected with ethidium fluorescence assay in HepG2 cells. To this end, cells were seeded at a concentration of 10^5 cells per well in black clear-bottom 96-well microplates and cultured for 24 hours before exposing them to the selected compounds. Dihydroethidium, a membrane-permeable fluorescent DNA-binding dye, was added at the time of dosing. Treatment with DMSO was used as negative control and hydrogen peroxide at 0.5% was used as positive control. After 2 hours of treatment, the plates were washed with PBS and fixed with 4% paraformaldehyde. Images of the cells were taken in a DMIL LED FLUO inverted microscope (Leica) at 20-fold magnification. One hundred fifty cells were analyzed per condition using ImageJ software.

Data Analysis

Quantification of FOXO localization was performed by Definiens Developer v2.5 software (Definiens, Munich, Germany). Nucleus and cytoplasm segmentation was performed with a custom-made ruleset using the DAPI (4′,6-diamidino-2-phenylindole) stain to define the nucleus and then expanding the area to identify the cytoplasm. After the segmentation, the ratio of green intensity was measured in both nucleus and cytoplasm and then the ratio of nucleus versus cytoplasm was calculated to define a threshold for translocation.

Western Blot Analysis

For the preparation of whole cell lysate, cells were harvested and lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 nM calyculin A, and EDTA-free complete protease inhibitor cocktail [Sigma]). Sample buffer was added to 1× final, and samples were boiled at 95 °C for 5 minutes. The samples were resolved by 8%–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted according to the antibody manufacturer's instructions. Secondary antibodies were added (GE Healthcare, Chicago, IL, USA) at typically 1:10 000 dilution for 1 hour at room temperature. Visualization of signal was achieved using a ChemiDocXRS þ Imaging System (BioRad, Oxford, UK).

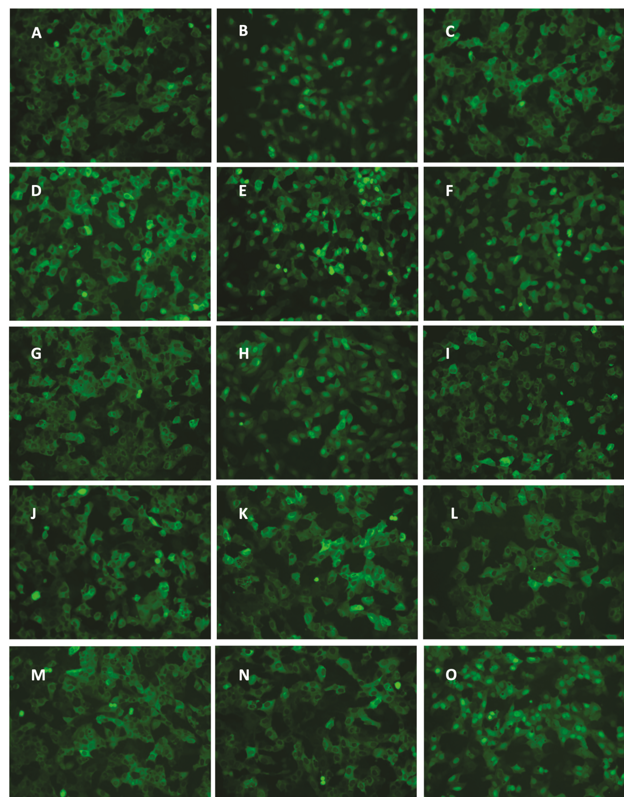

Figure 1. Images of reporter cells exposed to chemical compounds. U2foxRELOC cells treated with (**A**) DMSO, (**B**) 25 µM LY294002, (**C**) 8 µM astaxanthin, (**D**) 10 µM epigallocatechin gallate, (**E**) 30 µM harmine, (**F**) 20 µM piperlongumine, (**G**) 2 µM rapamycin, (**H**) 50 µM resveratrol, (**I**) 50 µM spermidine, (**J**) 10 µM tetrahydrocurcumin, (**K**) 100 µM metformin, (**L**) 100 µM phenformin, (**M**) 30 µM hydroxyurea, (**N**) nontreated, and (**O**) 500 nM BEZ235 (dactolisib). After 1 h of compound treatment, cells were fixed with paraformaldehyde and images were acquired by fluorescent microscopy as detailed in the "Method" section. DMSO = dimethylsulfoxide. Full color version is available within the online issue.

Results

Single-Point Translocation Assay

One of the major regulatory inputs into FOXO activity is its nuclear translocation. In order to explore if health-promoting agents induce the accumulation of FOXO proteins in the cell nucleus, we used a previously established reporter system (17). U2foxRELOC stably expresses a fluorescently labeled FOXO3 fusion protein in the osteosarcoma cell line U2OS. We used U2OS cells because they were derived from human tissue, exhibit an epithelial morphology, are well suited for cellular imaging, and lack mutations in canonical components of the PI3K/AKT pathway. Therefore, U2OS cells have been extensively used to identify chemical compounds capable of inducing the nuclear translocation of FOXO proteins. U2foxRELOC enables an image-based approach to monitor the subcellular localization of FOXO3, compatible with high-throughput evaluation of small molecule compounds. We exposed the U2foxRELOC reporter cell line to relevant concentrations of several natural products, including astaxanthin, epigallocatechin gallate, harmine, hydroxyurea, piperlongumine, rapamycin, resveratrol,

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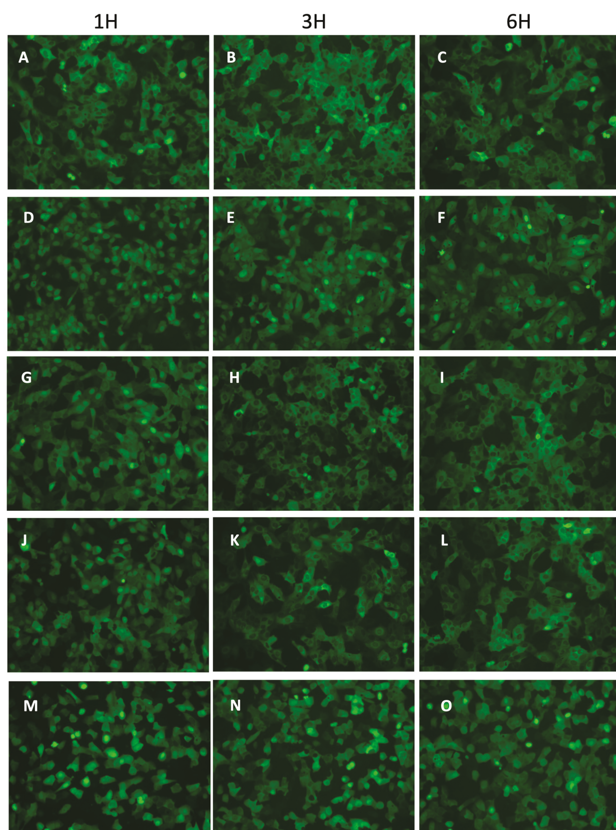

Figure 2. Time course of compound-induced nuclear translocation of FOXO. These reporter cells were treated for 1, 3, or 6 h with (**A–C**) DMSO, (**D–F**) 25 µM LY294002, (**G–I**) 50 µM resveratrol, (**J–L**) 30 µM harmine, and (**M–O**) 20 µM piperlongumine. After compound treatment, cells were fixed with paraformaldehyde and images were acquired by fluorescent microscopy as detailed in the "Method" section. DMSO = dimethylsulfoxide; FOXO = forkhead box O. Full color version is available within the online issue.

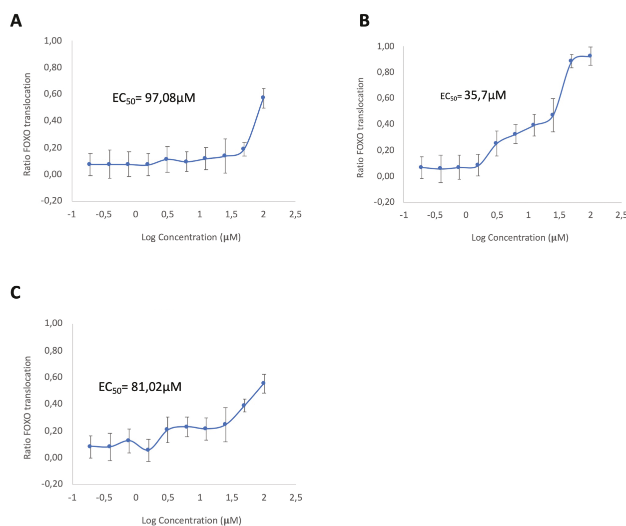

Figure 3. Dose response of compound-induced nuclear translocation of FOXO. Reporter cells were seeded in 384-well imaging plates incubated overnight before exposing them to the compounds for 1 h. (**A**) Resveratrol, (**B**) piperlongumine, and (**C**) harmine. After compound incubation, cells were fixed, and FOXO translocation was quantified by fluorescent microscopy. The ratio of FOXO translocations and EC_{50} was calculated compared to control cells treated with DMSO. DMSO = dimethylsulfoxide; EC_{50} = half maximal effective concentration; FOXO = forkhead box O. Full color version is available within the online issue.

spermidine, and tetrahydrocurcumin, and the synthetic chemical compounds metformin and phenformin in a single-point assay (Figure 1C–M). As negative and positive controls, the vehicle DMSO, and the PI3K inhibitors LY294002 (25 µM) and BEZ235 (500 nM; dactolisib) were used, respectively (Figure 1A, B, and O). We were primarily interested in identifying compounds acting directly on FOXO-modifying enzymes. Therefore, in order to limit the impact of secondary molecular mechanisms, we treated the reporter cells for only 1 hour before measuring translocation. Among the compounds tested, harmine, piperlongumine, and resveratrol elicited nuclear accumulation of FOXO in the cell nucleus after 1 hour (Figure 1E, F, and H). In order to determine if the active compounds of our panel can induce nuclear FOXO translocation after exposure during longer periods, we treated the reporter cells during 3 and 6 hours. While resveratrol and harmine lose their effect on FOXO after 3 hours of treatment, piperlongumine maintains its effect on the subcellular localization of FOXO after 6 hours (Figure 2M–O). Astaxanthin, epigallocatechin gallate, hydroxyurea, rapamycin, spermidine, tetrahydrocurcumin, metformin, and phenformin fail to affect the subcellular localization of FOXO reporter protein after longer exposure (Supplementary Figure 1). Then, we increased the concentration of these compounds to determine if higher nontoxic doses could drive FOXO into the cell nucleus. We observed that astaxanthin, epigallocatechin gallate, hydroxyurea, rapamycin, spermidine, tetrahydrocurcumin, metformin, and phenformin are not capable of inducing the nuclear translocation of FOXO (Supplementary Figure 2).

Dose–Response Translocation Assay

In order to establish a dose–response relationship, we treated the U2foxRELOC cells with different concentrations of harmine, piperlongumine, and resveratrol. We exposed the reporter cells to 10 serial dilutions of the compounds between 100 µM and 0.195 µM for 1 hour (Supplementary Figure 3A). Piperlongumine was the most potent compound and induced nuclear translocation of FOXO with an EC_{50} value of 37.7 µM (Figure 3B). Harmine was capable of driving FOXO into the cell nucleus with an EC_{50} value of 81.2 µM (Figure 3C). Conversely, the EC_{50} value for resveratrol was 97.9 µM (Figure 3A). As the chemical structures of these natural products are unrelated, they might affect FOXO through different molecular mechanisms.

Nuclear Export Assay

In order to determine if the active compounds trap FOXO inside the nucleus by inhibiting their nuclear export, we used an established high-content assay to monitor the inhibition of CRM1, the nuclear export receptor responsible for the export of many proteins that contain a nuclear localization sequence including FOXO proteins. We exposed U2nesRELOC cells to harmine, piperlongumine, and resveratrol for 1 hour using the known CRM1 inhibitor LMB as a positive control. We did not observe nuclear accumulation of the fluorescent signal for any of the tested compounds (data not shown).

FOXO3-Dependent Transcriptional Activity

FOXO3 is a transcription factor that binds to a TTGTTTAC FOXO responsive enhancer element, generally referred to as a DAF-16 family protein-binding element (DBE) (22). We used the previously established cell system (21), that stably expresses a luciferase reporter gene under the control of human DBE to monitor FOXO-dependent transcription upon compound treatment. We exposed the luciferase reporter cells to the different compounds for 6 hours and observed that piperlongumine and harmine significantly increase the expression of

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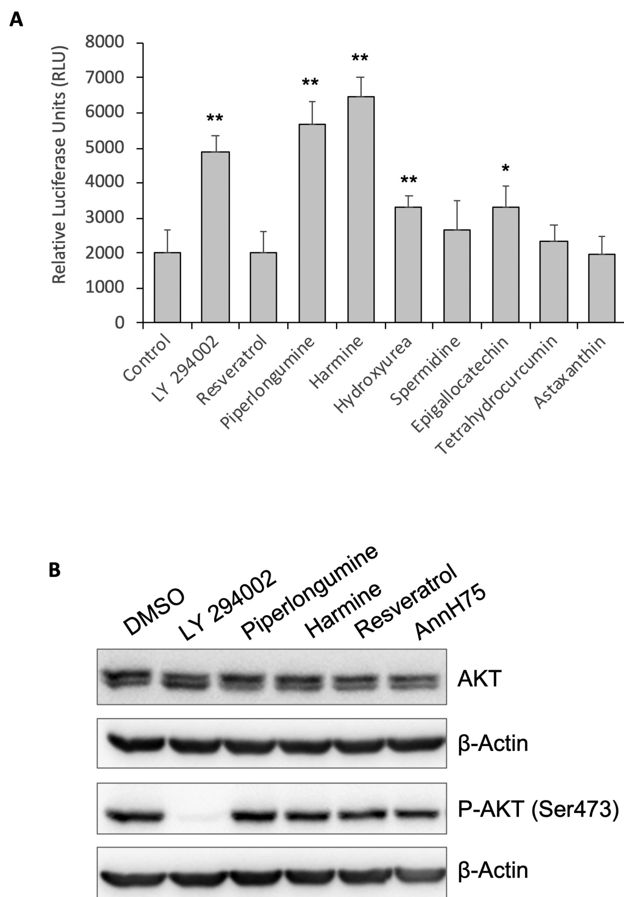

Figure 4. Effect on FOXO-dependent transcription and AKT activity. (**A**) Transcriptional activity of FOXO upon compound treatment as measured by FOXO-driven expression of luciferase in cotransfection experiments. Each construct was transiently cotransfected with plasmids encoding FOXO3a and Renilla luciferase into 293foxREP cells, and the luciferase activities were determined as described in the "Method" section. The data were normalized to the Renilla luciferase (phRG-TK vector) reporter construct. The statistical significance is represented with $^*p$ <.05 and $^{**}p$ <.01. (**B**) Western blot analysis of AKT and its phosphorylation at serine 473 (Ser473) after 1 h of treatment with selected compounds, using β-actin as normalization control. FOXO = forkhead box O.

luciferase from the FOXO-driven promoter. These two alkaloids increased the level of luciferase expression more than 3-fold, even more than the PI3K inhibitor LY294002, which was used as a positive control (Figure 4A). Surprisingly, resveratrol failed to affect FOXO-dependent transcription. Conversely, several compounds, including epigallocatechin gallate and hydroxyurea that were not capable of inducing nuclear translocation of FOXO, induced luciferase production in a FOXO-dependent manner (Figure 4A).

Monitoring the Activity of the PI3K/AKT Pathway

The phosphorylation of FOXO proteins by the serine/threonine kinase AKT at three different conserved residues is known as one of the major molecular events regulating the subcellular localization of FOXOs. Western blot analysis of the phosphorylation of AKT at serine 473 (S473) represents a standard method to monitor the activity of the PI3K/AKT pathway and in particular the kinase activity of AKT. In order to investigate if the active compounds exert their effect on FOXO through inhibition of AKT, we analyzed S473 phosphorylation after 1 hour of compound treatment by Western blot analysis. As shown in Figure 4B, while LY294002 greatly reduced the phosphorylation of AKT at S473, the active compounds failed to have an effect on the S473 phosphorylation. This observation suggests that harmine, piperlongumine, and resveratrol exert their effect independent of AKT-dependent phosphorylation of FOXOs.

Monitoring Oxidative Stress

While the PI3K/AKT pathway presents the major negative regulatory input of FOXOs, cellular stress signaling can drive FOXO nuclear translocation and FOXO-mediated transcription even in the presence of active PI3K/AKT signaling (23). In order to determine if compound-induced FOXO translocation is mediated by oxidative stress, we tested our panel of active compounds for their ability to induce cellular oxidative stress within a relevant time window. Human HepG2 liver cells were exposed to the compounds or hydrogen peroxide in the presence of dihydroethidium which is converted into the fluorescent dye ethidium in oxidatively stressed cells (24). We observed that compared to 0.1% hydrogen peroxide which produced

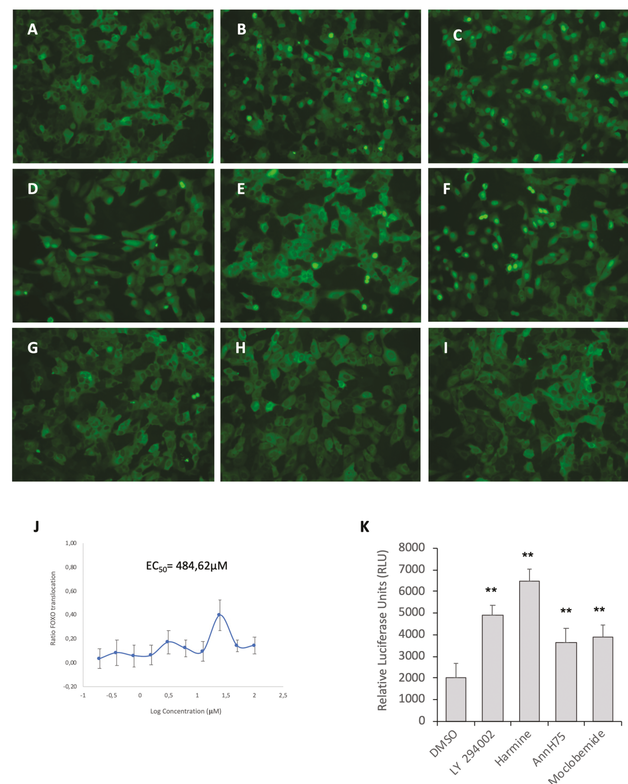

Figure 5. Determination of harmine's mode of action. U2foxRELOC cells were treated with (**A**) DMSO as negative control, (**B**) 25 µM LY294002 or (**C**) 500 nM BEZ235 as positive controls, (**D**) 30 µM harmine, (**E**) 20 µM moclobemide, and (**F**) 30 µM AnnH75 for 1 h. Larger exposure was tested in cells treated with (**G**) DMSO for 6 h and (**H, I**) AnnH75 for 3 and 6 h, respectively. After compound treatment, cells were fixed with paraformaldehyde and images were acquired by fluorescent microscopy. (**J**) AnnH75 dose–response assay and EC_{50} estimation after 1 h of treatment in U2foxRELOC cells compared to DMSO treatment. (**K**) Transcriptional activity of FOXO after 6 h of treatment with 30 µM harmine, 30 µM AnnH75, and 20 µM moclobemide measured with luciferase reporter assay. The statistical significance is represented with $^*p$ <.05 and $^{**}p$ <.01. DMSO = dimethylsulfoxide; EC_{50} = half maximal effective concentration; FOXO = forkhead box O. Full color version is available within the online issue.

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high levels of fluorescent signal, none of the test compounds seemed to generate significant oxidative stress, suggesting that their effect on FOXO does not rely on cellular stress oxidative signaling (Supplementary Figure 4).

Investigating Harmine's Mode of Action

Harmine is a naturally occurring β-carboline alkaloid known to reversibly inhibit monoamine oxidase A (MAO-A) (25) and the DYRK1A (26). In order to distinguish these two activities and to explore whether one of them is responsible for the effect of harmine on FOXO shuttling, we used specific chemical probes. Recently, distinctive changes at N-9 and C-1 have been introduced into the harmine molecule, resulting in AnnH75, a compound that preserves DYRK1A inhibition and eliminates MAO-A inhibition (16). We exposed U2foxRELOC cells to equal concentrations of harmine and AnnH75. In addition, we treated the reporter cells with the specific MAO-A inhibitor moclobemide. While AnnH75 induced the nuclear translocation of FOXO similar to harmine, moclobemide failed to do so (Figure 5D–F). In order to compare the potency of harmine and AnnH75, we performed dose–response experiments. We observed that harmine drives FOXO more potently into the nucleus than its analog AnnH75 (Figure 5J). These results suggest that harmine regulates the subcellular localization of FOXO partially through the inhibition of DYRK1A. An additional action seems to be triggered by harmine which is not the inhibition of MAO-A. This is also in agreement with the effect of AnnH75 on the FOXO-driven transcriptional activity as AnnH75 was capable of inducing luciferase expression in the reporter cells, but the effect was only about 50% of the harmine-mediated increase (Figure 5K). Surprisingly, while moclobemide treatment failed to affect the subcellular localization of FOXO, it significantly increased the FOXO-dependent transcription (Figure 5K).

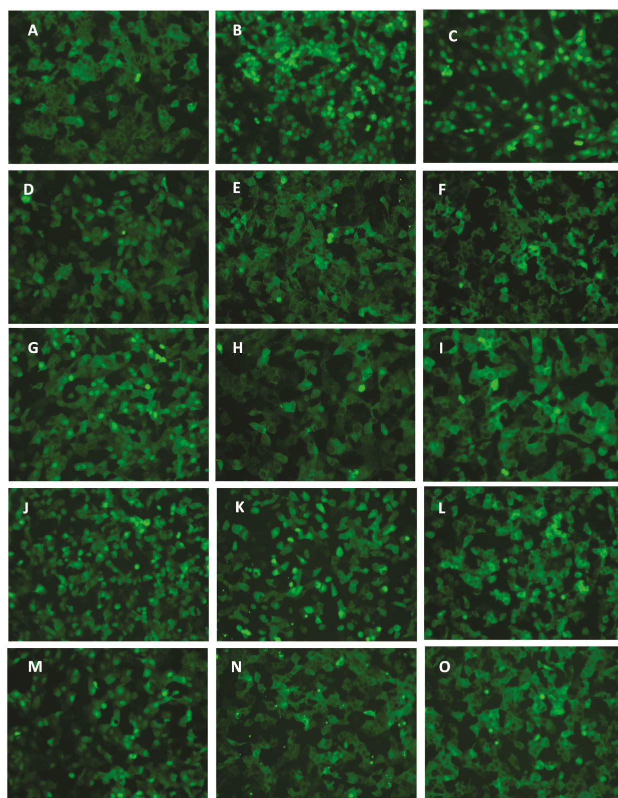
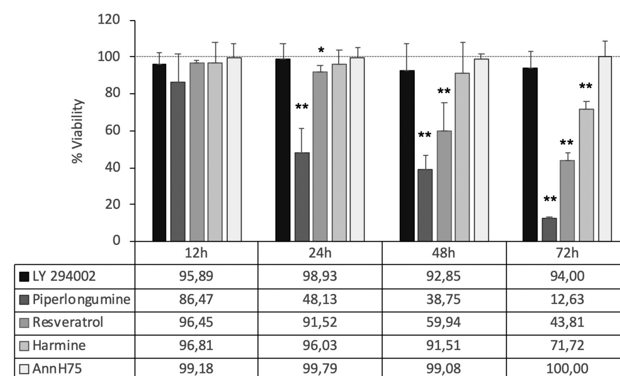


Figure 6. *N*-acetylcysteine and Sirtinol reverts FOXO translocation induced by Resveratrol and Harmine. FOXO translocation was analyzed in U2FOXreloc cells after 1 hour treatment with: (**A**) DMSO as negative control, (**B**) 25 μM LY294002, (**C**) 500 nM BEZ235 as positive controls, and 50 μM Resveratrol combined with (**D**) 10 mM Nicotinamide, (**E**) 50 μM Sirtinol or (**F**) 1 mM *N*-acetylcysteine; 30 μM Harmine combined with (**G**) 10 mM Nicotinamide, (**H**) 50 μM Sirtinol or (**I**) 1 mM *N*-acetylcysteine; 20 μM Piperlongumine combined with (**J**) 10 mM Nicotinamide, (**K**) 50 μM Sirtinol or (**L**) 1 mM *N*-acetylcysteine; 30 μM AnnH75 combined with (**M**) 10 mM Nicotinamide, (**N**) 50 μM Sirtinol or (**O**) 1 mM *N*-acetylcysteine. The cells images were acquired by fluorescent microscopy after fixing cells. Full color version is available within the online issue.

Further Analysis of Mode of Action

Resveratrol has been suggested to affect FOXO shuttling through the activation of SIRTs known to deacetylate FOXO proteins. We used the SIRT inhibitors nicotinamide (NAM) and sirtinol to investigate if these compounds were capable of reverting resveratrol-mediated FOXO translocation. U2foxRELOC cells were exposed to a combination of resveratrol and NAM or resveratrol and sirtinol for 1 hour. Surprisingly, NAM failed to affect resveratrol-mediated translocation (Figure 6D), whereas sirtinol completely prevents resveratrol from affecting FOXO localization (Figure 6E). Interestingly, sirtinol also affected harmine's effect on FOXO (Figure 6H). Conversely, sirtinol did not interfere with the effect of piperlongumine on FOXO (Figure 6K). These data confirm the mode of action of resveratrol and suggests that inhibition of SIRTs contributes to the effect of harmine and its analog AnnH75 on FOXO while piperlongumine acts independent of SIRTs. However, the mechanism by which harmine can affect the activity of SIRTs remains to be established. Importantly, we observed that *N*-acetylcysteine (NAC) a thiol-containing antioxidant reverted the piperlongumine-mediated FOXO translocation (Figure 6L). NAC treatment also reverts the effects of resveratrol, harmine, and AnnH75 on FOXO translocation (Figure 6F, I, and O). It has been reported that NAC activates the extracellular-signal-regulated kinase (ERK) pathway (27) consequently inhibiting FOXO activation and translocation (28), which suggest a possible mechanism for our observation.



	12h	24h	48h	72h
LY 294002	95,89	98,93	92,85	94,00
Piperlongumine	86,47	48,13	38,75	12,63
Resveratrol	96,45	91,52	59,94	43,81
Harmine	96,81	96,03	91,51	71,72
AnnH75	99,18	99,79	99,08	100,00

Figure 7. Effect of compound treatment on the viability of HepG2 cells. Human liver cancer cells HepG2 were treated with 20 μM piperlongumine, 50 μM resveratrol, 30 μM harmine, and 30 μM AnnH75 for 12, 24, 48, and 72 h, respectively. Viability index was measure with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay after compound incubation as described in the "Method" section. The statistical significance is represented with $^{*}p$ <.05 and $^{**}p$ <0.01.

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Effects on Cellular Viability

In order to investigate if some of the compounds affect cellular viability in long periods of treatment, we performed MTT assays in HepG2 cells exposed to resveratrol, piperlongumine, harmine, and AnnH75 for 12, 24, 48, and 72 hours, respectively (Figure 7). Piperlongumine shows the highest impact on cell survival, which significantly decreases after 24 hours. Additionally, resveratrol and harmine, but not AnnH75, also affect cell viability after 72-hour treatment compared to control cells. These data indicate that these compounds have a long-term cytotoxic effect although it is not due to oxidative stress (Supplementary Figure 4).

Discussion

FOXO proteins have emerged as promising targets to treat age-related diseases and slow aging. Here, we have investigated the effect of compounds reported to be beneficial for human health on the activity of FOXO proteins and found that the alkaloids piperlongumine and harmine as well as the polyphenol resveratrol drive FOXO3 into the cell nucleus, where it can act as a transcription factor. It is important to note that we limited the period of exposure to the compounds in order to focus on regulatory events directly related to the subcellular localization of FOXO proteins. FOXO proteins are transcription factors known to exert their gene regulatory function in the cell nucleus and their activity is mainly regulated by shuttling between the cytoplasm and the nucleus. Several small molecule compounds including nuclear export inhibitors, PI3K, mechanistic target of rapamycin (mTOR), AKT, phosphoinositide-dependent kinase 1 (PDK1), and calmodulin inhibitors as well as some chemotherapeutic drugs have been shown to affect the subcellular localization of FOXO protein (13). Interestingly, we recently found that piperlongumine and harmine are capable of reverting the downstream transcriptional effects of the FOXO repressor protein Tribbles homolog 2 (TRIB2) (29). Piperlongumine is an amide alkaloid extracted from the fruit of long pepper (*Piper longum*) with an ever-growing list of biological activities and in particular interesting as an anti-cancer agent (24). Mechanistically, piperlongumine was reported to modulate key regulatory proteins, including PI3K, AKT, mTOR, nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT), cyclin D1, and CRM1 (24–26). Though several of these activities could affect FOXO localization, we did not detect an effect of piperlongumine on AKT or the nuclear export receptor CRM1 in the relevant time frame to explain the induction of nuclear FOXO translocation within 1 hour. Similarly, piperlongumine failed to produce ROS within a short exposure of 2 hours. Taken together, these data suggest that the mechanism by which piperlongumine drives FOXO into the nucleus upon short-time exposure acts independent of the PI3K/AKT pathway and the CRM1-mediated nuclear export. However, piperlongumine was the only compound within our panel capable of maintaining nuclear FOXO localization after 6 hours of exposure. We cannot rule out that inhibition of AKT and/or CRM1 by piperlongumine upon extended treatment might contribute to FOXO translocation. Conversely, the effect of harmine and resveratrol treatment on FOXO translocation was limited to a short period and absent after 3 hours. A wide range of pharmacological properties have been reported for the beta-carboline alkaloid harmine which is present in several plant species, for example in the seeds of Syrian rue (*Peganum harmala*). Harmine has shown to be highly cell permeant and acts as an inhibitor of several enzymes including MAO-A, DYRK1A kinase, cdc-like kinases, and the 5-hydroxytryptamine 2A (5-HT2A) serotonin receptor. MAO-A is a flavoenzyme that degrades amine neurotransmitters by oxidative deamination. Inhibition of the enzyme MAO-A can also be achieved by reversible inhibitors chemically unrelated to harmine such as moclobemide. Using this compound, we ruled out the contribution of MAO-A-inhibitory activity in harmine-mediated FOXO regulation. In contrast, we determined that the inhibition of the DYRK1A by the harmine analogue AnnH75 induced the nuclear translocation of FOXO though with less potency than harmine. This observation is also reflected in the reduced capacity of AnnH75 to drive FOXO-mediated transcription compared to harmine. In line with these data, DYRK1A kinase is known to phosphorylate FOXO1 at serine 329 (30), and harmine and AnnH75 might also affect the phosphorylation of the FOXO3 reporter protein. While we show that DYRK1A inhibition by harmine affects FOXO localization, additional regulatory events exist. It remains to be determined whether our observation that specific inhibition of the NAD-dependent protein deacetylases SIRT1 and SIRT2 reverts harmine-mediated FOXO translocation reflects a direct action of harmine activating SIRT1 and/or SIRT2. Alternatively, SIRT inhibition might override regulatory events triggered by harmine treatment. Resveratrol is a polyphenol found in several plant species including grapes, mulberries, and peanuts and has been suggested to act as a calorie-restriction mimetic and to exert a broad range of health benefits (31). Though a variety of molecular targets of resveratrol have been studied, the molecular mechanisms underlying its therapeutic properties still remain elusive (32). Frescas et al. reported that resveratrol caused FOXO1-green fluorescent protein (GFP) translocation to the nucleus of hepatocytes via SIRT activation (33). The authors also showed that resveratrol treatment induced FOXO1-dependent expression of glucogenetic genes. In contrast, our data suggest that resveratrol-mediated FOXO3 translocation does not lead to an increase in the activity of a promoter under the control of FOXO proteins. Conversely, epigallocatechin gallate and hydroxyurea increased FOXO-mediated transcription without inducing nuclear translocation. A possible explanation is that our image-based assay system is based on a reporter protein that monitors subcellular localization of FOXO3 and does not detect isoform-specific induction of nuclear FOXO1 or FOXO4. Conversely, the luciferase reporter system is responsive to all FOXO isoforms. A more likely possibility is that subcellular localization and transcriptional activity are events that can be regulated independently. It is also important to note that the fact that several natural products failed to affect FOXO functions in our assay systems does not necessarily indicate their incapability to activate FOXO expression or activity in other cell systems or organisms. A recent review article mentions an unpublished pilot study in which the treatment of mice with the natural tetraterpenoid pigment astaxanthin increased FOXO3 expression in heart tissue by 90% and in blood, albeit more modestly (15). It is important to note that our experiments to monitor FOXO translocation and FOXO-dependent transcription have been conducted in osteosarcoma U2OS cells and HEK293 embryonic kidney cells engineered to overexpress FOXO3. Therefore, the data obtained in the current study does not represent the full spectrum of cellular context and it is reasonable to think that compounds found inactive here

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might induce the activation of other FOXO isoforms or activate FOXO3 in other cell types.

Further studies will be required to characterize the precise modes of action of the potentially active compounds and to explore their therapeutic potential against cancer, age-related diseases and for promotion of healthy aging.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

V.B. and W.L. are cofounders of Refoxy Pharmaceuticals GmbH, Berlin. W.L. is required by his institution to state so in his publications. The other authors declare no conflict of interest.

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Author Contributions

L.J., A.S., G.C., I.G., R. M., and V.M.-V. carried out the experiments, including drug treatments, translocation and luciferase assays, viability and proliferation studies, immunoblot analysis, statistical analysis, and analysis of data. C.B.-A. supervised biological assays. J.P. and F.B. supervised compound selection and handling. F.B. designed and provided compound AnnH75. D.M. carried out high-content screening and image analysis. B.I.F., V.B., and W.L. designed and supervised research. W.L. and L.J. drafted the manuscript. All authors contributed to the completion of the manuscript.

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