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Original Article

Systemic inhibition of neddylation by 3-day MLN4924 treatment regime does not impair autophagic flux in mouse hearts and brains

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Abstract: Beyond helping the cell survive from energy starvation via self-eating a portion of cytoplasm, macroautophagy is also capable of targeted removal of defective organelles or cytoplasmic aberrant protein aggregates, thereby playing an important role in quality control in the cell. Impaired or suppressed macroautophagy activity is associated with the progression from a large subset of heart diseases to heart failure and with the development of the vast majority of, if not all, neurodegenerative diseases, the leading causes of death and disability in humans. Hence, a better understanding of the impact of existing and upcoming pharmacotherapies on macroautophagy in the heart and brain will undoubtedly benefit the search for safer and more effective treatment to improve human health. Neddylation is a recently recognized posttranslational modification process that modifies a subset of cellular proteins and is, by virtue of regulating Cullin-RING ligases, essential to ~20% ubiquitin-proteasome system (UPS)-mediated protein degradation. MLN4924 (Pevonedistat), a specific inhibitor of neddylation that promises to become a new anti-malignancy agent, is capable of inhibiting UPS-mediated progression of the cell cycle and activating macroautophagy in cancer cells. However, no reported study has tested the impact of systemic inhibition of neddylation on autophagic activity in a post-mitotic organ such as the heart and brain. This study was conducted to fill this gap. Sixteen GFP-LC3 transgenic mice of mixed sexes were divided equally into either MLN4924-treated or vehicle-treated groups and were treated respectively with MLN4924 (30 mg/kg, *s.c.*, twice a day × 3 days) or equal volume of solvent. The resultant changes in myocardial levels of neddylated cullin 1 as well as autophagic flux in cardiac and brain tissues were assessed. The effectiveness of the MLN4924 regime was verified by myocardial accumulation of neddylated cullin 1. Myocardial LC3-II flux and free GFP levels were comparable between the MLN4924 and the vehicle groups whereas the protein level of p62, *a bona fide* substrate of macroautophagy, in the brain was significantly decreased by the MLN4924 treatment. Our data suggest that systemic inhibition of neddylation by a 3-day MLN4924 treatment regime does not suppress macroautophagy activities in the heart and brain.

Keywords: Neddylation, NEDD8 activating enzyme inhibitor, MLN4924, macroautophagy, p62/SQUSTM1, heart, brain, mice

Introduction

Posttranslational modifications and targeted protein degradation represent two major molecular mechanisms that regulate the function and fate of cellular proteins and thereby cell function and survival, touching virtually every corner of the cell. This is arguably best exemplified by the ubiquitin-proteasome system (UPS) which encompasses ubiquitination and proteasome-mediated protein degradation [1]. Ubiquitination is covalent attachment of the carboxyl terminus of a small protein ubiquitin (Ub) to the e-amino group of the side chain of a lysine (K) residue on the target protein molecule through an isopeptidyl bond. For poly-ubiquitination, subsequent rounds of this reaction similarly attach additional Ub to the preceding Ub to form a poly-Ub chain [1]. Both mono-ubiquitination and poly-ubiquitination can serve as post-translational modifications to alter the non-proteolytic fate of the ubiquitinated protein; however, poly-ubiquitinated proteins, especially K48-linked, are often targeted for degradation

by the proteasome [2]. UPS-mediated proteolysis is responsible for targeted degradation of the vast majority of cellular proteins. The UPS-mediated degradation of normal proteins that are no longer needed is regarded as regulatory degradation, pivotal to regulation of virtually all cellular processes, such as cell cycle control [3], DNA damage responses and DNA repair [4], cell signal transduction [5], and cell death pathways [6]. Meanwhile, targeted degradation of abnormal/misfolded proteins, representing the last resort of protein quality control, relies primarily on the UPS; thus, the UPS plays an indispensable role in protein quality control as well [2]. The latter functions to minimize the level and toxicity of misfolded proteins in the cell and is accomplished by intricate collaboration between molecular chaperones and targeted protein degradation pathways [7].

When misfolded proteins have failed to be repaired by chaperones and escaped from UPS-mediated degradation, they tend to form aggregates via hydrophobic interaction [7]. The removal of these aberrant protein aggregates appears to rely on macroautophagy [7]. Macroautophagy is the most studied form of autophagy, involving segregation of a portion of cytoplasm (e.g., defective organelles, protein aggregates, and lipid droplets) via formation of a double-membraned vacuole known as an autophagosome which delivers its cargo to lysosomes by fusion with the lysosomes where the autophagic cargoes are degraded by lysosomal enzymes and resultant small biomolecules (e.g., amino acids, fatty acids, etc.) are recycled [8]. Hence, macroautophagy (referred to as autophagy hereafter) plays an important role in the quality control of both organelles and cytoplasmic proteins, in addition to provision of fuel during starvation by self-digestion of a portion of the cytoplasm [8]. The interplay between the UPS and the autophagic-lysosomal pathway has attracted increasing attention from the protein quality control research community [9]. Proteasome inhibition has been shown to activate autophagy while defective autophagy may hinder UPS performance [10, 11].

Heart failure is the final common pathway for most, if not all, cardiovascular diseases [12, 13]; it remains the leading cause of death and disability for humans despite recent advances in pharmacological and surgical interventions [14, 15]. Meanwhile, neural degenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, and Huntington's disease represent another category of debilitating diseases constituting another leading cause of human mortality and morbidity. Increasing evidence suggests that proteotoxic stress (characterized by elevated levels and toxicity of misfolded proteins and aberrant protein aggregation) and inadequate protein quality control are associated with the progression from a large subset of cardiovascular diseases to heart failure [16, 17], as well as with neurodegeneration [18]. Hence, a better understanding of the impact of existing and upcoming therapies on the ability of cells to handle misfolded proteins would benefit the prevention and/or better treatment of human diseases.

Ubiquitination occurs through enzymatic reactions sequentially catalyzed by Ub activating enzyme (E1), Ub conjugating enzymes (E2), and Ub ligases (E3). Once the target is poly-ubiquitinated, selective degradation occurs [1, 19-21]. Since Ub E3s determine substrate specificity of ubiquitination, they are some of the most intensively studied targets in the UPS [19]. The largest family of Ub E3 ligases is cullin-RING ligases (CRLs) which were shown to be responsible for 20% of Ub-dependent protein degradation in cells [22, 23]. There is evidence that CRLs regulate autophagy and that CRL abnormalities are linked to a variety of developmental, neurological, and cardiac diseases [20].

The activity of CRLs is closely regulated by neddylation, a posttranslational modification similar to ubiquitination that attaches an Ub-like protein, NEDD8, to target proteins. The process of NEDD8 attachment involves an NEDD8 activating enzyme (NAE) (E1), NEDD8 conjugating enzyme (E2), and NEDD8 ligases (E3) [20, 24, 25]. NEDD8 and Ub share high sequence similarity (76%) [26], and high identity (58%) [25], suggesting cooperativity between ubiquitination and neddylation. This certainly is the case as various Ub E3 ligases have been shown to facilitate NEDD8 attachment [27, 28] and as NEDD8 is frequently incorporated into Ub chains [25]. It is well-proven that neddylation of cullins is required for CRLs to efficiently ubiquitinate their targets [23, 25, 29, 30]. Meanwhile, NEDD8 removal via a process known

as deneddylation is also essential to the proper functioning of CRLs [23, 25]. Cullin deneddylation is primarily performed by the COP9 signalosome (CSN), a zinc metalloprotease containing 8 subunits: COPS1 through COPS8 [25].

MLN4924 (Pevonedistat) is an adenosine sulfamate derivative and inhibitor of neddylation. It acts by irreversibly forming a NEDD8-MLN4924 adduct at the ATP-binding site of NEDD8, disrupting NEDD8-NAE conjugation [31]. Since many cell cycle regulators are degraded by CRLs-mediated ubiquitination [32], MLN4924 has shown great potential in cancer chemotherapy [33-35], with multiple Phase I/II clinical trials ongoing to test its effects on various malignancies [36, 37]. MLN4924 treatment has been shown to activate autophagy in cancer cells and tumor tissues. However, cancer cells are generally in a proliferating state, in sharp contrast to terminally differentiated cells such as cardiomyocytes and neurons. Thus, neddylation inhibition may impact on these cells differently. For this reason, it becomes important to study the effects of MLN4924-mediated neddylation inhibition on terminally differentiated cells. Cops8 deficiency, which disrupts CSN formation and thereby disables deneddylation of cullins, impairs autophagic flux in mouse hearts [38, 39]. As stated, both neddylation and CSN-mediated deneddylation are required for proper CRLs functioning; hence both CSN deficiency and neddylation inhibition impairs CRLs. However, no previous studies yet examined the effect of neddylation inhibition on autophagic flux in cardiac muscle. Thus, the present study was conducted to test the hypothesis that like Cops8 deficiency, neddylation inhibition impairs cardiac autophagic flux and may similarly affect the brain.

By examining the effect of systemic administration of MLN4924 on myocardial LC3-II flux and brain tissue p62 protein levels in mice in the present study, we have unveiled for the first time that neddylation inhibition by MLN4924 for three consecutive days does not decrease autophagic flux in the heart and brain, two vital post-mitotic organs in the body.

Material and methods

Animals and MLN4924 treatment

Transgenic (tg) mice with ubiquitous expression of a green fluorescence protein fused microtubule associated protein light chain 3 (GFP-LC3), which were originally created and described by Dr. Noboru Mizushima and colleague and has been extensively employed as a autophagosome reporter model [40], were used in the present study. The tg line was maintained in the FVB/N inbred background. DNA from toe clips was used for Polymerase Chain Reaction (PCR) to determine mouse genotypes. Mice heterozygous for tg GFP-LC3 were obtained and used for the experiment. The protocol for the care and use of animals in the present study had been approved by The Institutional Animal Care and Use Committee of the University of South Dakota.

Sixteen adult GFP-LC3 tg mice of mixed sexes were randomly divided into two groups (n = 8 each): an MLN4924 group and a vehicle control group. The MLN4924 group was treated with MLN4924 (obtained from ActiveBiochem.com) (30 mg/kg, s.c.) dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (HPBCD) twice a day (with an interval of 12 hours) for 3 consecutive days, totaling 6 injections for each mouse. This dosage and regime was based on a recent hepatic cancer study which showed that MLN4924 caused apoptosis and increased autophagy in proliferating hepatic cells [41]. The vehicle control group received an equivalent volume of 10% HPBCD (vehicle) in the same manner and at the same time as the MLN4924 group.

Autophagic flux assay

At 10 hours after last injection of MLN4924 or HPBCD, each group was randomly split into two subgroups: a BFA-treated subgroup and a DMSO-treated subgroup. The subgroups received two intraperitoneal injections of bafilomycin A1 (BFA, 3 μmol/kg) or DMSO (vehicle control), respectively, at the 10^{th} and 11^{th} hour after the final injection of MLN4924 or HPBCD. BFA was used as a vacuole proton-ATPase inhibitor to inhibit lysosomes and block the fusion between the autophagosome and the lysosome [42]. The use of BFA here was to block lysosomal degradation of autophagosomes, allowing the rate of autophagosome removal by the lysosome to be assessed when compared with the DMSO subgroup.

On the 12^{th} hour after the last injection of MLN4924 or HPBCD (i.e. 1 hour after the second BFA injection), each mouse was weighed

Table 1. Gravimetric characterization of mice used in this study

Groups	n (sex ratio)	BW (g)	HW/BW (mg/g)	VW/BW (mg/g)	LuW/BW (mg/g)	KW/BW (mg/g)	LiW/BW (mg/g)
HPBCD+DMSO	4 (1 m/3 f)	21.6±3.3	4.98±0.20	4.20±0.15	7.0±0.30	15.3±1.0	43.0±1.2
HPBCD+BFA	4 (2 m/2 f)	20.1±2.1	4.97±0.17	4.12±0.18	6.8±0.45	14.7±0.4	44.6±0.7
MLN4924+DMSO	4 (2 m/2 f)	19.3±1.9	5.07±0.27	4.33±0.24	6.7±0.45	14.9±0.4	45.3±1.0
MLN4924+BFA	4 (2 m/2 f)	23.2±3.4	5.32±0.15	4.12±0.12	6.5±0.45	15.7±0.7	46.2±1.3

Shown are mean ± SEM; difference in each parameter among the 4 groups is not statistically significant as tested with one way ANOVA. m, male; f, female; BW, body weight; HW, heart weight; VW, ventricular weight; LuW, lung weight; KW, kidney weight; LiW, liver weight.

and sacrificed by CO_2 inhalation. The heart, ventricular, lung, kidney, and liver weights were measured after organ collection, and are summarized in **Table 1**. After the organs were weighed and collected, they were snap-frozen in liquid nitrogen before transferred to and stored in a -80°C freezer until they were used for subsequent western blot analyses for the protein levels of LC3, p62, free GFP, GAPDH, and α-actinin.

The level of LC3-II in a tissue or cells reflects the abundance of autophagosomes. The difference of LC3-II protein levels in each group between BFA-treated and non-BFA-treated subgroups is referred to as LC3-II flux and widely used as an indicator of autophagic flux (Refs). The LC3-II flux presented here refers to the net amount of LC3-II accumulated by the BFA-mediated lysosomal inhibition. Mathematically, it is calculated by subtracting the α-actinin normalized LC3-II level of a BFA-treated sample with the mean value of the α-actinin normalized LC3-II levels of the DMSO treated samples of the same group.

The protein levels of p62, which is a substrate of autophagy [43], are used by many studies as an inverse indicator of autophagic activity. In GFP-LC3 expressing cells, GFP-LC3-II is incorporated into the autophagosome membrane as the autophagosomes are formed, thereby marking them. When fused with lysosomes, autophagosomes undergo degradation, during which a fraction of GFP-LC3 is first cleaved by lysosomal enzymes to form free GFP; hence, the level of free GFP proteins (shorter than GFP-LC3) is also regarded as an indicator of autophagic flux [44].

Western blot analysis

Snap-frozen tissues were individually and separately crushed to a powder form by smashing them in their frozen state, covered in tin foil, with a sterilized wrench. The powder was then aliquoted: one such aliquot of each tissue sample was placed into a tube containing 200 μL 1 × loading buffer; the remaining aliquots were stored at -80°C for future analysis. This process was replicated for each tissue. Samples were then sonicated 4 times for 2 seconds each, and cooled on ice (repeated twice). Following this process, the protein concentration of each sample was analyzed using bicinchoninic acid (BCA) assays, in which graded bovine serum albumin (BSA) concentrations were used as a control. Samples were then diluted as necessary to achieve uniform concentrations per sample set. Total protein extracts from the collected tissue samples were then fractionated via SDS-polyacrylimide gel electrophoresis (PAGE), transferred to PVDF membrane, and probed with antibodies against specific proteins: LC3, p62, and free GFP. Either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or α-Actinin was probed as a loading control. Also, to test the efficacy of MLN4924, western blot analysis of Cullin-1 (Cul1) and neddylated Cul1 was performed. Cul1 is a well-known target of neddylation [20, 43], thus we expected a decrease in neddylated Cullin-1 following MLN4924 treatment.

Statistical methods

All continuous variables are presented as mean ± SEM unless indicated otherwise. Differences between two groups were evaluated for statistical significance with a 2-tailed unpaired *t*-test. When appropriate, Welch's correction was employed for small sample size. When the difference among ≥3 groups was evaluated, 1-way ANOVA or, when appropriate, 2-way ANOVA followed by the Tukey test for pairwise comparisons was performed. A p value <0.05 is considered statistically significant.

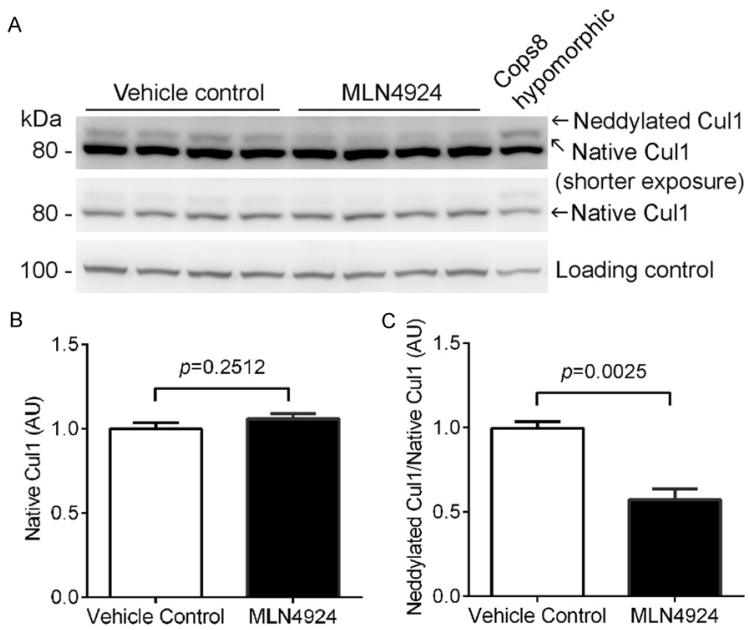

Figure 1. Western blot analysis for myocardial native and neddylated forms of cullin1 (Cul1) in mice treated with MLN4924 or vehicle control. Total protein extracts from mouse myocardium were fractionated via SDS-PAGE and transferred to a PVDF membrane before immuno-probing for Cul1. A non-specific band at the molecular weight of approximately 100 kDa is included as a loading control. A myocardial sample from a *Cops8* hypomorphic mouse, which is known to have increased neddylated Cul1 [52], was included as a positive control (the far right lane of A) to identify neddylated Cul1. Each lane represents a mouse. A shorter exposure (middle image of A) was used for the densitometry of the native Cul1 to avoid saturation that might have occurred in the longer exposure required to detect the neddylated Cul1. (A) Representative western blot images. (B) A summary of native Cul1 densitometry data. (C) A summary of the pooled data of the neddylated Cul1 to native Cul1 ratios. AU, arbitrary unit, with the mean of the ratios from the vehicle control group set as 1. Mean ± SEM are shown, n = 4 mice/group; the *p* values shown are derived from unpaired *t*-test with Welch's correction.

GraphPad Prism Version 6.04 (GraphPad Software, Inc., La Jolla, CA) was used for the analyses and making the graphs.

Results

Effect of MLN4924 treatment on mouse gravimetric characteristics

To test whether or not MLN4924 and BFA treatment had an effect on body weight (BW) and on the weight of major organs, we collected BW, heart weight (HW), cardiac ventricular weight (VW), lung weight (LuW), kidney weight (KW), and liver weight (LiW) of each mouse at the time of mouse sacrifice 12 hours after the last MLN4924 or vehicle control injections (i.e. 1 hour after second BFA or control injections). These gravimetric measurements and derived parameters including HW/BW, VW/BW, LuW/BW, LuW/BW, KW/BW, and LiW/BW ratios, are summarized in **Table 1**. Statistical analyses revealed no significant difference in any of the parameters among any of the groups, suggesting that the treatment did not induce cardiac hypertrophy or atrophy and that the treatment did not cause significant functional impairment to the left or right heart, as functional insufficiency of the left heart would lead to an increase in the LuW/BW ratio and right heart failure would increase both the KW/BW and LiW/BW ratios.

Effect of MLN4924 treatment on myocardial Cul1 neddylation

To test whether the MLN4924 treatment regime was effective or not in terms of inhibiting neddylation, we performed western blot analyses for detection of the level of neddylated form of Cul1 in myocardial tissues collected at end of the treatment. As expected, our results show that the myocardial native Cul1 protein levels were not discernibly different between the MLN4924 and the vehicle control groups ($p = 0.2512$, **Figure 1A**, **1B**) whereas neddylated Cul1 relative to native Cul1 were significantly lower in the MLN4924 treated group compared to the vehicle control group ($p = 0.0025$, **Figure 1A**, **1C**). These results indicate that MLN4924 treatment regime had effectively inhibited neddylation in the heart. Although these results are specific to the heart, our MLN4924 treatment likely had yielded similar effects on other tissues and can be assumed to have led to systemic inhibition of neddylation. This assumption is supported by the fact that in order for the subcutaneously injected MLN4924 to have reached the heart muscle, the drug first needed to travel through the cardiovascular system which connects to all organs including the brain; MLN4924 would not have been hindered

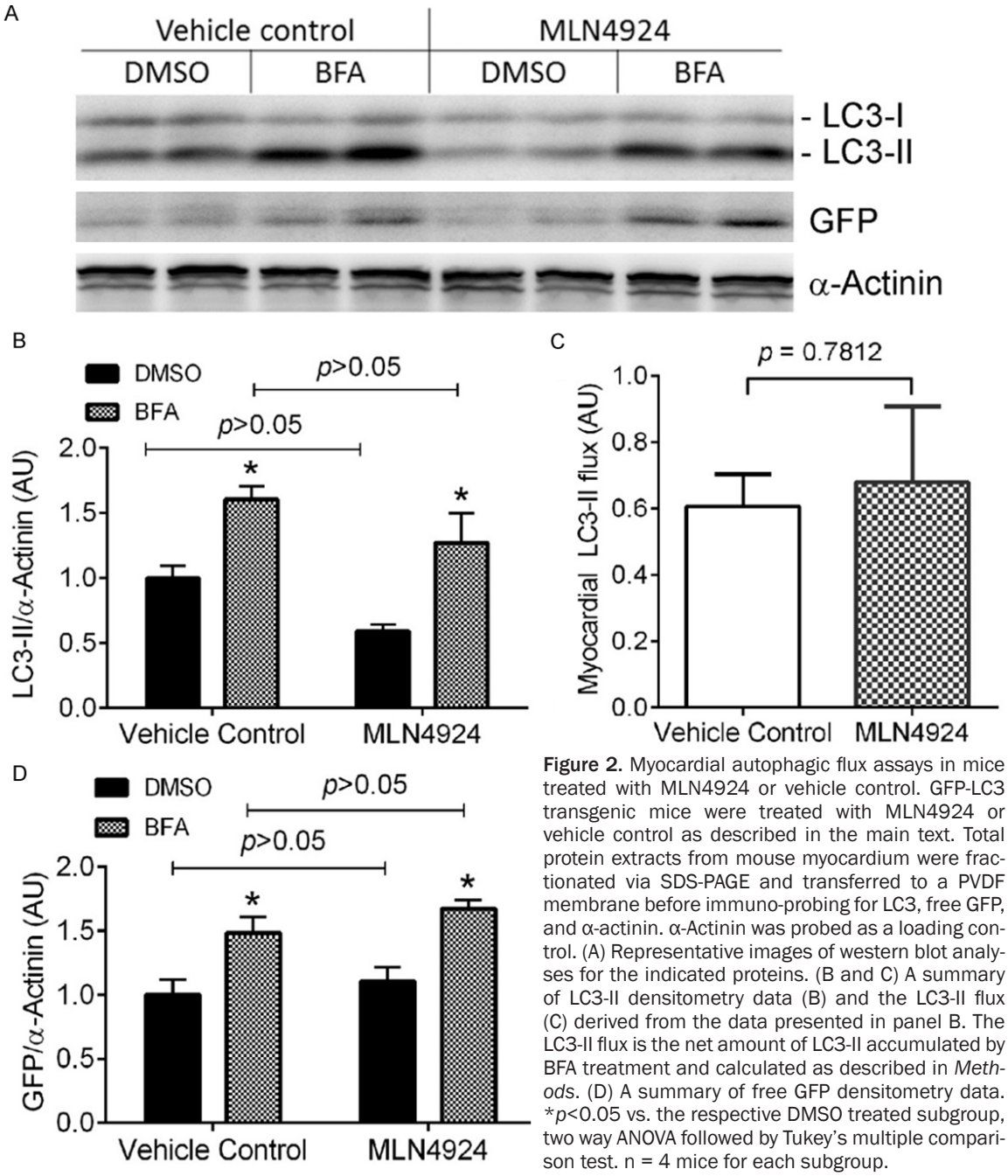

Figure 2. Myocardial autophagic flux assays in mice treated with MLN4924 or vehicle control. GFP-LC3 transgenic mice were treated with MLN4924 or vehicle control as described in the main text. Total protein extracts from mouse myocardium were fractionated via SDS-PAGE and transferred to a PVDF membrane before immuno-probing for LC3, free GFP, and α-actinin. α-Actinin was probed as a loading control. (A) Representative images of western blot analyses for the indicated proteins. (B and C) A summary of LC3-II densitometry data (B) and the LC3-II flux (C) derived from the data presented in panel B. The LC3-II flux is the net amount of LC3-II accumulated by BFA treatment and calculated as described in *Methods*. (D) A summary of free GFP densitometry data. **p*<0.05 vs. the respective DMSO treated subgroup, two way ANOVA followed by Tukey's multiple comparison test. n = 4 mice for each subgroup.

by the blood brain barrier [45]. Thus, although these results are specific to myocardium, they can be assumed to have effected in a similar manner as described in the aforementioned hepatic cancer model [41].

Effect of MLN4924 treatment on myocardial autophagic flux

Microtubule associated protein 1 light chain 3 (LC3) is a mammalian homolog of yeast Atg8 and plays an important role in autophagosome formation. With the help of ATG4, native LC3 is processed to LC3-I which is diffusely distributed in the cytosol. Through an ubiquitination-like process, LC3-I is conjugated with phosphatidylethanolamine. The resultant lipidated form of LC3-I is referred to as LC3-II. The lipidation allows LC3-II to incorporate into the phagophore during the elongation phase of autophagosome formation so that LC3-II stays in the autophagosome membrane throughout the lifetime of an autophagosome. The LC3-II in the inner membrane of autophagosomes is degrad-

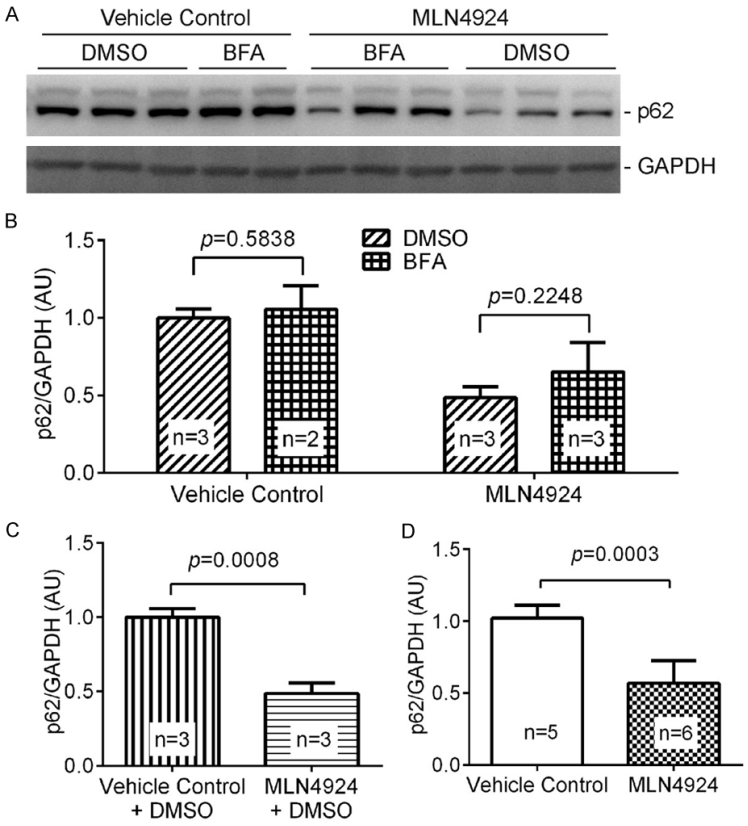

Figure 3. Changes in brain p62 in mice treated with MLN4924. Total protein extracts from mouse brain tissues were fractionated via SDS-PAGE and transferred to a PVDF membrane before immunoprobing for p62 and GAPDH. GAPDH was probed as a loading control. (A) Western blot images for the indicated proteins. (B) Pooled densitometry data of the western blot images shown in (A). The *p* values shown were derived from multiple t-tests corrected for multiple comparisons using the Holm-Sidak method. (C and D) Pooled densitometry data of the western blot images shown in (A) specifically comparing the DMSO treated MLN4924 subgroup with the DMSO treated vehicle control subgroup (C) and comparing the combined MLN4924 subgroups with the combined vehicle control subgroups (D). The *p* values shown in (C and D) were derived from unpaired t-test with Welch's correction.

ed along with the membrane and cargos [46]. Therefore, the protein level of LC3-II in a cell reflects generally the abundance of autophagosomes in the cell and the rate of LC3-II degradation by lysosomes (i.e., LC3-II flux) serves as an excellent indicator of the rate of autophagosome degradation by lysosomes (i.e., autophagic flux) [47]. In reduced denatured polyacrylamide gel electrophoresis (SDS-PAGE), LC3-II runs faster than LC3-I, making it easy to differentiate LC3-II from LC3-I.

To test whether or not MLN4924 treatment alters autophagic flux in mouse hearts, we performed western blot analyses for detection of myocardial levels of LC3-II and free GFP protein in mice subject to the BFA-mediated lysosomal inhibition or DMSO control treatment during the final two hours before sample collection (**Figure 2**). The densitometry data of LC3-II and free GFP were adjusted for potential loading variation by dividing LC3-II and GFP band densities by that of the in-lane loading control (α-actinin). The mean value of the vehicle control without lysosomal inhibition subgroup (i.e., Vehicle Control+DMSO) is designated as 1 arbitrary unit (AU). As shown in **Figure 2A** and **2B**, significant increases in LC3-II in both BFA subgroups compared to their respective DMSO subgroups were observed ($p < 0.05$), indicating that BFA did inhibit autophagosome removal as expected. However, the LC3-II differential (i.e., LC3-II flux) between the MLN4924 and vehicle control groups was not statistically significant ($p = 0.7812$, **Figure 2C**), suggesting that the MLN4924 treatment produced no significant change in autophagic flux in mouse myocardium.

In GFP-LC3 expressing cells, the free GFP protein levels are used by some to reflect autophagic flux [44]; hence, we also examined myocardial steady state free GFP protein levels in the animals subject to manipulation of lysosomal function. The free GFP levels were comparable between the MLN4924+DMSO and the Vehicle Control+DMSO subgroups (**Figure 2D**, $p>0.05$), consistent with the finding from the LC3-II flux assay (**Figure 2C**) that myocardial autophagic flux is not altered by MLN4924-mediated neddylation inhibition. A significant increase in free GFP in both BFA subgroups relative to their respective DMSO subgroups also indicates that BFA did inhibit lysosomal degradation, as expected. However, the GFP differential between the MLN4924 and vehicle control groups was in-

significant (**Figure 2D**), which also indicates that MLN4924 produced no significant change in autophagic flux in mouse myocardium. Thus results from detection of baseline free GFP protein levels and from the GFP flux assay corroborate very well those from myocardial LC3-II measurements.

Effect of MLN4924 treatment on p62 protein levels in mouse brains

To test whether or not MLN4924 treatment alters autophagic activity in mouse brains, we performed western blot analyses for p62, a known substrate of autophagy. We found that brain p62 protein levels in both MLN4924-treated and the vehicle control-treated mice tended to be increased at 2 hours after intraperitoneal injections of BFA but the increase is not statistically significant ($p>0.05$, **Figure 3A**, **3B**). This could be either because the rate of p62 degradation by autophagy was too slow for a short duration of lysosomal inhibition to discernibly accumulate p62 in the brain or because the BFA treatment regime was insufficient to inhibit lysosomes in the brain. Previously it was observed that autophagic inhibition could not cause a discernible increase of p62 proteins in cultured cardiomyocytes until 6 hours after initiation of the inhibition [11]. And it has also been suggested that BFA may have very limited ability to pass across the blood-brain barrier [48]. Regardless of the underlying cause, the p62 protein analyses allowed us to compare p62 levels between the DMSO subgroups (**Figure 3C**) and between the combined MLN4924 group and the combined vehicle control group (**Figure 3D**). Both means of comparison reveal a striking reduction of brain p62 protein levels by MLN4924 treatment ($p< 0.001$), suggesting that neddylation inhibition by MLN4924 might have increased autophagy in mouse brains as p62 is primarily degraded by autophagy.

Discussion

Since impaired quality control at either the protein or the organelle level can cause not only dysfunction of the cell but ultimately cell death as well, adequate quality control in the cell is essential to the wellbeing of organs, especially those (e.g., hearts and brains) composed primarily of post-mitotic cells (e.g., cardiomyocytes and neurons). This is because these organs have very limited, if any, regenerative capacity to deal with the loss of their primary cells [49]. Autophagic impairment has been implicated in the genesis or progression of common heart diseases and most neurodegenerative diseases. MLN4924, the first-in-class inhibitor of neddylation, has been shown to induce autophagy in tumor cells [34]; however, the present study demonstrates for the first time that there are no suppressive effects of MLN4924-mediated neddylation inhibition on the autophagic flux in the heart and brain, which suggests that a finite term of neddylation inhibition may not impair autophagy in the heart and brain, alleviating the concern that neddylation inhibition therapy would compromise autophagy-mediated intracellular quality control in these vital organs.

Cullin neddylation is required for the activation of CRLs, a family of Ub ligases crucial for propelling cell division through regulatory degradation of key cell cycle regulators, which is taken advantage of by tumor cells through increasing their neddylation activity [50, 51]; hence, the use of neddylation inhibition as a strategy to suppress tumor growth is to target the vital role of cullin neddylation in cell proliferation. However, recent studies have shown that CRLs also participate in UPS-mediated degradation of misfolded proteins whereas atypical neddylation hinders UPS-degradation of misfolded proteins in at least cardiomyocytes [52, 53], suggesting that neddylation inhibition may yield significant effects on protein quality control which, as discussed earlier, is more concerning to the terminally differentiated cells or post-mitotic organs such as the heart and brain. Hence, from the protein quality control point of view, neddylation inhibition might be a double-edged sword: on one hand, it may impair UPS-mediated degradation of misfolded proteins via inactivating CRLs; on the other hand, it might reduce atypical neddylation and thereby allow the UPS to more efficiently degrade misfolded proteins. However, the latter possibility actually depends on how neddylation is inhibited. For example, proteasomal degradation of misfolded proteins was promoted when atypical neddylation was inhibited by reduction of NEDD8 via overexpression of NEDD8 ultimate buster-1 long (NUB1L) [53]; however, neddylation inhibition by MLN4924, which specifically targets the NAE [20],

may not yield the same effect because the atypical neddylation does not seem to require NAE [54]. Therefore, the proper functioning of autophagy becomes more important to protein quality control during MLN4924 treatment because the UPS-mediated degradation of misfolded proteins will likely be suppressed as the result of the suppression of CRLs by MLN4924, underscoring the significance of findings reported here.

MLN4924 administration does not overtly impair myocardial autophagic flux and cardiac function

It is well demonstrated that both neddylation and deneddylation are required for proper functioning of CRLs. Perturbation of cullin deneddylation and thereby the inactivation of CRLs through genetic ablation of *Cops8*, an essential subunit of the CSN deneddylating holoenzyme impairs cardiac autophagic flux in both perinatal and adult mice through perturbation of autophagosome-lysosome fusion [38, 39, 55]. Deficiency of Atrogin-1, a major substrate receptor protein for CRLs in muscle tissues was also shown to impair cardiac autophagy in mice [56]. Hence, there was a good reason to hypothesize that neddylation inhibition via MLN4924 impairs cardiac autophagic flux. However, the findings of the present study seem to reject the hypothesis. Both the widely used LC3-II flux assay and the measurement of the free GFP proteins resulting from autophagic cleavage of the transgenic GFP-LC3 demonstrated that myocardial autophagic flux was not discernibly altered by the MLN4924 treatment regime (**Figure 2**) that was verified to have effectively inhibited neddylation as reflected by decreased myocardial levels of neddylated Cul1 (**Figure 1**). Similarly, LC3-II flux in liver was not discernibly affected by the MLN4924 treatment, either (*data not shown*).

Neddylation inhibition by MLN4924 increases autophagic flux in the brain

MLN4924 has the ability to cross the blood-brain barrier [45]. Hence, it is important to assess the impact of the MLN4924 regime on autophagy in brains. A striking finding of this study is that brain p62 protein levels of MLN4924 treated mice were remarkably lower than that in the vehicle control treated mice (**Figure 3**). Although we were not able to assess the alteration of p62 protein synthesis in the brain by MLN4924 treatment, it is unlikely that the decreased p62 proteins were caused by a reduction of p62 synthesis. This is because several lines of evidence support the notion that the synthesis of p62 should likely be increased by MLN4924 treatment. First, a recently reported study showed that p62 expression in vascular smooth muscle cells was upregulated by MLN4924 [57]; and second, neddylation inhibition by MLN4924, as discussed above, is expected to compromise UPS performance while increased p62 had been associated with UPS impairment resulting from proteinopathy [58]. Thus, the decreases in brain p62 protein levels induced by the MLN4924 treatment are very likely caused by enhanced degradation, indicating that neddylation inhibition by MLN4924 increases autophagic activity in the brain. Indeed, the tissue levels of steady state p62 proteins have been used frequently as an inverse indicator of autophagic flux because p62 is primarily degraded by autophagy [44].

In summary, our data suggest that systemic neddylation inhibition by MLN4924 for three consecutive days does not significantly affect autophagic flux in mouse hearts and livers but may increase autophagic activity in the brain in normal animals. It should be pointed out that the effect of repeated episodes of the 3-day MLN4924 treatment regime remains to be tested in the future.

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Disclosure of conflict of interest

None.

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