Melatonin-Based Therapeutics for Atherosclerotic Lesions and Beyond: Focusing on Macrophage Mitophagy

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Abstract

Atherosclerosis refers to a unique form of chronic inflammatory anomaly of the vasculature, presented as rupture-prone or occlusive lesions in arteries. In advanced stages, atherosclerosis leads to the onset and development of multiple cardiovascular diseases with lethal consequences. Inflammatory cytokines in atherosclerotic lesions contribute to the exacerbation of atherosclerosis. Pharmacotherapies targeting dyslipidemia, hypercholesterolemia and neutralizing inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17, and IL-12/23) have displayed some promising although contradictory results. Moreover, adjuvants such as melatonin, a pluripotent agent with proven anti-inflammatory, anti-oxidative and neuroprotective properties, also display promises in alleviating cytokine secretion in macrophages through mitophagy activation. Here, we share our perspectives on this concept and present melatonin-based therapeutics as a means to modulate mitophagy in macrophages and, thereby, ameliorate atherosclerosis.

REVIEW ARTICLE

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Abstract

Atherosclerosis refers to a unique form of chronic inflammatory anomaly of the vasculature, presented as rupture-prone or occlusive lesions in arteries. In advanced stages, atherosclerosis leads to the onset and development of multiple cardiovascular diseases with lethal consequences. Inflammatory cytokines in atherosclerotic lesions contribute to the exacerbation of atherosclerosis. Pharmacotherapies targeting dyslipidemia, hypercholesterolemia and neutralizing inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17, and IL-12/23) have displayed some promising although contradictory results. Moreover, adjuvants such as melatonin, a pluripotent agent with proven anti-inflammatory, anti-oxidative and neuroprotective properties, also display promises in alleviating cytokine secretion in macrophages through mitophagy activation. Here, we share our perspectives on this concept and present melatonin-based therapeutics as a means to modulate mitophagy in macrophages and, thereby, ameliorate atherosclerosis.

Keywords: Atherosclerosis, Macrophages, Melatonin, Mitophagy, Therapeutics

Abbreviations:

AJUBA (ajuba LIM protein), AMP (adenosine monophosphate), AMPK (5' AMP-activated protein kinase), ATG (autophagy related), BECN1 (beclin 1), BNIP3 (BCL2 interacting protein 3), BNIP3L (BCL2 interacting protein 3 like), COX4I1 (cytochrome c oxidase subunit 4I1), DNM1L (dynamin 1 like), FOXO3 (forkhead box O3), FUNDC1 (FUN14 domain containing 1), GABARAP (GABA type A receptor-associated protein), HAS3 (hyaluronan synthase 3), HSPA1L (heat shock protein family A [Hsp70] member 1 like), MAP1LC3/LC3 (microtubule associated protein 1 light chain 3), MTORC1 (mechanistic target of rapamycin kinase complex 1), NBR1 (NBR1 autophagy cargo receptor), NFE2L2 (nuclear factor, erythroid 2 like 2), NFKB1 (nuclear factor kappa B subunit 1), NLRP3 (NLR family pyrin domain containing 3), OPA1 (OPA1 mitochondrial dynamin like GTPase), PARL (presenilin associated rhomboid like), PGAM5 (PGAM family member 5, mitochondrial serine/threonine protein phosphatase), PINK1 (PTEN induced kinase 1), PRKN (parkin RBR E3 ubiquitin protein ligase), ROS (reactive oxygen species), SIRT1 (sirtuin 1), SLNs (solid lipid nanoparticles), SQSTM1 (sequestosome 1), TRAF3 (TNF receptor associated factor 3), ULK1 (unc-51 like autophagy activating kinase 1), VDAC1 (voltage dependent anion channel 1).

1. Introduction: Atherosclerosis and inflammation

Atherosclerosis displays a wide spectrum of pathological clinical presentations. However, patients with atherosclerosis might be asymptomatic despite bearing atherosclerotic plaques for years or even decades within their vasculature. The initial presentation of atherosclerosis is shown by silent lesions that grow slowly and are termed "stable plaques" whereas the secondary clinical presentation is shown by overtly increased unstable plaques (Fuster, Badimon, Badimon & Chesebro, 1992).

Atheroma denotes the formation of adhering materials such as cholesterol, fat, and calcium within arteries (Lee & Libby, 1997). Clinical manifestations of atheroma typically include thrombosis in adults. The risk of developing thromboembolism and thrombosis as major complications of atherosclerosis is attributed to an

atheroma's instability rather than duration of the disease (Little et al., 1988). Furthermore, reactive oxygen species (ROS), hypoxia, and nitric oxide (NO) in expanding atheroma further accelerate proinflammatory responses and progression of atherosclerosis.

In all types of atherosclerotic plaques, inflammation constitutes a major component. Plaque rupture and thrombosis are accompanied by profound inflammatory infiltration (Van Der Wal, Becker, Van der Loos & Das, 1994). Among all stakeholders for inflammation, macrophages play a cardinal role, for example, in the pathogenesis of acute myocardial infarction (MI) at the cap rupture site. Ample evidence has revealed that T lymphocytes and activated macrophages usually triggers plaque destabilization (Hansson, 2005; Rocha & Libby, 2009). Improper assembly of macrophages and lymphocytes in plaques contributes to the generation and secretion of lytic enzymes and cytokines in fibrous cap, leading to rupture of the lesion, and ultimately, exacerbation of atherosclerosis (Hansson, 2005; Rocha & Libby, 2009).

With respect to inflammation in atherosclerosis, various strategies have displayed promises to cease secretion of inflammatory cytokine from macrophages and atherogenesis. One of the natural-occurring cellular defensive processes upon inflammatory insult is induction of mitophagy (Minton, 2016). Mitophagy refers to selective engulfment and removal of damaged/depolarized or superfluous mitochondria, thus preserving mitochondrial homeostasis (Ajoolabady et al., 2020; Ajoolabady, Aslkhodapasandhokmabad, Aghanejad, Zhang & Ren, 2020; Ajoolabady et al., 2021a; Ajoolabady et al., 2021b). Impairment of mitochondria triggers ROS generation, inflammasome activation, and cytokine secretion from macrophages (Ma et al., 2018). Recycling of damaged mitochondria by way of mitophagy retards inflammation in atherosclerotic lesions.

Melatonin is a commonly employed over-the-counter therapeutic agent with diverse biological activities, one of which is "mitophagy induction" (Zhou et al., 2018). Recent reports have delineated the role of melatonin in mitophagy regulation in many mammalian cells. Here we will decipher the role of melatonin in mitophagy regulation in macrophages based on the lessons acquired from mammalian cells. We wish to propose melatonin therapy as an adjuvant and alternative approach to cease inflammation in atherosclerotic lesions via macrophage mitophagy regulation. Our ultimate goal is to share perspectives on the accessory therapeutic strategies that accompany melatonin therapy, pharmaceutical, and natural mitophagy modulators as well as targeted delivery of melatonin and therapeutic agents to macrophages.

2. Mitophagy: Definition and molecular mechanisms

Mitophagy, also known as mitochondrial autophagy, governs the removal of long-lived, damaged/depolarized mitochondria, making it cardinal to mitochondrial and cellular homeostasis (Zhou, Zhu, Wang, Zhu, Ren & Chen, 2018). Mitophagy process also plays a key role in development, for example removal of unnecessary mitochondria during erythropoiesis (Barde et al., 2013), or adaptation to changing nutrient conditions. Mitophagy and mitochondrial dynamics including fission and fusion dominate mitochondria reconstruction (Hernandez-Resendiz, Prunier, Girao, Dorn, Hausenloy & Action, 2020). Mitophagy also wards off mitochondrial apoptosis by engulfing/neutralizing long-lived or damaged mitochondria. Therefore, defective mitophagy culminates in accumulation of impaired mitochondria, thus evoking onset of chronic diseases such as cancer, neurodegenerative, liver, and cardiovascular diseases.

Mechanistically, macroautophagy/autophagy involves the formation of a transient double-membraned structure, termed a "phagophore" that sequesters cytoplasm, including mitochondrial compartments (Wu, Zhang & Ren, 2019). Upon completion, phagophores close to generate "autophagosomes" or, in the case of mitophagy, "mitophagosomes". Eventually, autophagosomes or mitophagosomes fuse with lysosomes, leading to degradation and recycling of the cargo content (Fig. 1).

2.1. Mitophagy receptors

Mammalian cells would mandate a sophisticated quality control system, mitophagy, to sustain mitochondrial homeostasis. Once mitochondria are depolarized, mitophagy receptors are recruited to the mitochondrial outer membrane (MOM) and recognize the depolarized/damaged mitochondria (Ajoolabady, Aslkhodapasandhokmabad, Aghanejad, Zhang & Ren, 2020). Mitophagy receptors are integrated into MOM via a C-

terminal transmembrane domain, and also bind to mammalian Atg8-family proteins localized on phagophore membrane, including components of the MAP1LC3 (microtubule associated protein 1 light chain 3) and GABARAP (GABA type A receptor-associated protein) subfamilies via a specific LC3-interacting region (LIR) motif. This interaction connects the cargo with the autophagy machinery to promote sequestration of the mitochondria within autophagosomes (Fig. 1) (Yamaguchi, Murakawa, Nishida & Otsu, 2016).

BNIP3 (BCL2 interacting protein 3) is an essential mitophagy receptor that participates in mitochondrial turnover upon hypoxia (Zhang & Ney, 2009). Mutations in the BNIP3 monomer or its LIR motif can contribute to mitophagy defects (Hanna, Quinsay, Orogo, Giang, Rikka & Gustafsson, 2012). Also, BNIP3L/NIX (BCL2 interacting protein 3 like), another mitophagy receptor, exhibits a 53-56% homology to BNIP3 (Matsushima et al., 1998), and is activated upon reticulocyte maturation and ROS accumulation (Melser et al., 2013; Schweers et al., 2007). LIR motif of BNIP3L binds MAP1LC3B, GABARAPL2 (GABA type A receptor associated protein like 2), GABARAP, and GABARAPL1 (GABA type A receptor associated protein like 1) (Marinković, Šprung & Novak, 2020).

Upon hypoxia challenge, FUNDC1 (FUN14 domain containing 1) also functions as a mitophagy receptor. Mechanistically, PGAM5 (PGAM family member 5, mitochondrial serine/threonine protein phosphatase) phosphatase dephosphorylates FUNDC1 at serine 13 residue and mediates its binding to MAP1LC3B to favor mitophagy induction (Ma et al., 2020; Ren et al., 2020). Furthermore, BCL2L13 (BCL2 like 13) and FKBP8 (FKBP prolyl isomerase 8) are two other MOM integral proteins that serve as mitophagy receptors in mammalian cells (Fig. 1) (Bhujabal et al., 2017; Fujiwara et al., 2019).

2.2. PINK1-PRKN-mediated mitophagy

PINK1 (PTEN induced kinase 1), a serine/threonine kinase, and PRKN (parkin RBR E3 ubiquitin protein ligase), an E3 ubiquitin ligase, are causal factors that drive hereditary recessive Parkinson disease (Truban, Hou, Caulfield, Fiesel & Springer, 2017). Under physiological conditions, PINK1 is recruited to the mitochondrial inner membrane and is cleaved/inactivated by mitochondria-residing proteasomes. Conversely, upon mitochondrial depolarization/impairment, PINK1 begins to accumulate in MOM, recruiting PRKN to this location for phosphorylation (activation). Phosphorylated PRKN ubiquitinates specific MOM proteins such as VDAC1 (voltage dependent anion channel 1), RHOT1 (ras homolog family member T1), MFN1 (mitofusin 1), and certain mitophagy receptors recognized by an Atg8-family protein on phagophores (Fig. 1) (Eiyama & Okamoto, 2015; Springer & Kahle, 2011).

PINK1 stabilization on MOM is an essential initial step in mitophagy. Under normal circumstances, the serine protease PARL (presenilin associated rhomboid like) relocates to the mitochondrial inner membrane and degrades PINK1 and PGAM5. Once mitophagy is activated, PHB2 (prohibitin 2) deactivates PARL, leading to PINK1 stabilization and PGAM5 activation. Then, PGAM5 interacts with PINK1 and further increases its stabilization on the mitochondria (Yan et al., 2020). Also, HSPA/HSP70 participates in PINK1 stabilization by retarding its degradation (Zheng et al., 2018). In addition, overexpression of TAMM41 (TAM41 mitochondrial translocator assembly and maintenance homolog; a mitochondrial protein) accelerates PRKN recruitment and enhances PINK1 stabilization (Yang et al., 2019).

PINK1 also phosphorylates ubiquitin chains, which accelerates PRKN recruitment to the mitochondria, leading to polyubiquitination of MOM proteins (Gao, Yu, Lv, Liang, Sun & Zhang, 2021). Ultimately, autophagy cargo receptors such as OPTN (optineurin), SQSTM1 (sequestosome 1), TAX1BP1 (Tax1 binding protein 1), CALCOCO2 (calcium binding and coiled-coil domain 2), and NBR1 (NBR1 autophagy cargo receptor), which contain a ubiquitin-binding domain (UBD), recognize polyubiquitinated proteins, and also interact with MAP1LC3 on phagophores via their LIR motif (Onishi, Yamano, Sato, Matsuda & Okamoto, 2021).

As aforementioned, PRKN polyubiquitinates certain mitophagy receptors such as BNIP3L, which is recognized by the UBD domain of the autophagy receptor NBR1 (Gao et al., 2015). NBR1 also binds to MAP1LC3B and GABARAP on the phagophore membrane to promote engulfment of mitochondria (Gao et al., 2015).

CALCOCO2, another receptor, is also recruited to MOM, where, it binds polyubiquitinated proteins, resulting in the activation and recruitment of the ULK1 (unc-51 like autophagy activating kinase 1) complex to the MOM, resulting in amplified PINK1-PRKN-mediated mitophagy (Padman, Nguyen, Uoselis, Skulsuppaisarn, Nguyen & Lazarou, 2019). ULK1 phosphorylates serine 14 residue of BECN1 (beclin 1, an essential protein in autophagy), and thereby, promotes BECN1-PRKN interactions, which enhances PRKN recruitment to mitochondria (Fig. 1) (Kumar & Shaha, 2018).

Furthermore, the interaction between BNIP3 and PINK1 facilitates PINK1 accumulation in MOM, and consequently, fosters PRKN recruitment to MOM (Zhang et al., 2016). It is thought that autophagy receptors known thus far can participate in PINK1-PRKN-mediated mitophagy. This process ultimately leads to the bridge formation between phagophores and depolarized mitochondria. Eventually, mitochondria-encapsulating autophagosome/mitophagosomes fuse with lysosomes for ultimate degradation of sequestered mitochondria into macromolecules that are released into the cytosol for reuse (Ding & Yin, 2012).

Other molecules have been discovered that mediate mitophagy independent of PINK1 and PRKN including cardiolipin, DNM1L (dynamin 1 like), HUWE1 (HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1), MARCHF5 (membrane associated ring-CH-type finger 5), ARIH1 (ariadne RBR E3 ubiquitin protein ligase 1), SQSTM1-KEAP1 (kelch like ECH associated protein 1)-RBX1 (ring-box 1), and MUL1 (mitochondrial E3 ubiquitin protein ligase 1) (Ambivero, Cilenti, Main & Zervos, 2014; Di Rita et al., 2018; Kageyama et al., 2014; Villa et al., 2017).

3. Mitophagy and inflammation

Mitophagy effectively alleviates inflammation, while defective mitophagy usually elicits inflammatory responses in mammalian cells. In*Atg5* (autophagy related 5)-deficient C57BL/6 J mice administered with angiotensin II, impaired mitophagy results in enhanced ROS production, and, subsequently, NFKB1 (nuclear factor kappa B subunit 1) activation, causing inflammation and cardiac damage (Vincent et al., 2020). Therefore, mitophagy anomaly culminates in inflammation, suggesting a role for mitophagy in alleviation of inflammation. Also, impaired mitophagy can lead to sterile inflammation as mutation in DNM1L results in mitophagy defect, mitochondrial depolarization, loss of ATP, and myocardial inflammation in a murine model of dilated cardiomyopathy (Cahill et al., 2015).

It is noteworthy that *Mir103* (microRNA 103) inhibits mitophagy through suppressing TRAF3 (TNF receptor associated factor 3; a target molecule of *Mir103*), thus reinforcing inflammation in both*in vitro* and *in vivo* models of adipose inflammation (Zhang, Zhang, Feng, Huang, Xia & Sun, 2019). In addition, ARF3 (ADP ribosylation factor 3) inhibits *Mir103* , resulting in TRAF3 upregulation, mitophagy activation, NFKB inhibition, and NLRP3 (NLR family pyrin domain containing 3) inflammasome suppression (Zhang, Zhang, Feng, Huang, Xia & Sun, 2019). Thus, the ARF3-*Mir103* -TRAF3 signaling cascade depicts a new paradigm in the regulation of mitophagy and its link with inflammation suppression upon obesity disorders.

Moreover, FUNDC1-mediated mitophagy inhibits activation of the NLRP3 inflammasome, resulting in inhibition of inflammation secondary to intracerebral hemorrhage in murine models (Zheng, Jian, Gan, Wang, Zhao & Zhai, 2021). This study suggests that FUNDC1 is a potential target for inhibition of inflammation. Apart from this, the AJUBA (ajuba LIM protein) molecule participates in PINK1-mediated mitophagy through translocation to depolarized mitochondria (Ponia et al., 2021). In this respect, AJUBA deficiency causes mitophagy impairment and contributes to systemic inflammation upon viral infection in mice (Ponia et al., 2021). Therefore, AJUBA acts as an essential molecule to maintain PINK1-mediated mitophagy and prevent inflammation.

In contrast, some pieces of evidence also suggests that excessive mitophagy can further exacerbate inflammation. For instance, KMT2B (lysine methyltransferase 2B) deficiency culminates in excessive mitophagy, inflammation, and lipolysis in murine white adipose tissues (He et al., 2020). Scrutiny for a role of mitophagy in mitochondrial DNA release demonstrated that PINK1-PRKN-mediated mitophagy evokes mitochondrial DNA release, which in part triggers TLR9 activation (Jing et al., 2020). Subsequently, TLR9 activates MYD88 (MYD88 innate immune signal transduction adaptor)-NFKB1 axis, leading to inflammation in*in*

vivo models of lung injury (Jing et al., 2020). This study suggests that hyperactive mitophagy evokes inflammation through the TLR9-MYD88-NFKB1 pathway. In summary, mild induction of mitophagy is an anti-inflammatory process, indicating that mild induction of mitophagy in macrophages might cease inflammation in atherosclerotic lesions.

4. Melatonin and mitophagy regulation in mammalian cells

Myriad studies have delineated the preventive role of melatonin against atherosclerosis. To begin with, our group for the first time, reported that melatonin administration impedes activation of NLRP3 inflammasome and consequently ceases secretion of inflammatory factors from macrophages in atherosclerotic lesions in a murine model of atherosclerosis (Ma et al., 2018). The underlying mechanism seems to be related to melatonin-mediated activation of mitophagy via a SIRT3-FOXO3 (forkhead box O3)-PRKN signaling cascade, resulting in ROS scavenging and inhibition of ROS-activated NLRP3 inflammasomes (Ma et al., 2018).

Similar results were noted from examination of melatonin in subarachnoid hemorrhage (Cao et al., 2017). This study revealed that melatonin treatment significantly upregulates autophagy-associated molecules such as ATG5 and MAP1LC3A, and mitophagy molecules such as PRKN and PINK1 in subarachnoid hemorrhage, resulting in ROS scavenging, remarkable inactivation of the NLRP3 inflammasomes, and attenuation of cytokine secretion (Cao et al., 2017). Furthermore, examination of a rat model of radiculopathy showed that melatonin alleviates apoptosis and NLRP3 inflammasome activation through instigating PRKN-dependent mitophagy (Xie et al., 2021).

Conversely, melatonin was shown to induce OPA1 (OPA1 mitochondrial dynamin like GTPase)-mediated mitophagy and mitochondrial fusion via AMP-activated protein kinase (AMPK) under ischemia-reperfusion (I/R) injury *in vivo* and *in vitro* , prompting the role of AMPK-OPA1 axis as a new paradigm for melatonin-evoked mitophagy induction (Zhang et al., 2019). Likewise, melatonin significantly attenuates calcium deposition in vascular smooth muscle cells in a pattern dependent on mitophagy via AMPK-OPA1 axis (Chen, Zhou, Yang, Liu, Wu & Sha, 2020). Besides, melatonin downregulates cleaved CASP3 (caspase 3) and RUNX2 (RUNX family transcription factor 2), upregulates MAP1LC3B and MFN2 (mitofusin 2), reduces mitochondrial superoxide, and activates mitophagy via the AMPK-OPA1 signaling axis (Chen, Zhou, Yang, Liu, Wu & Sha, 2020).

In addition, melatonin treatment reactivates mitophagy and boosts mitochondrial function via upregulation of HSPA1L (heat shock protein family A [Hsp70] member 1 like) in senescent mesenchymal stem cells. Mechanistically, HSPA1L forms a complex with cellular PRNP (prion protein) then recruits PRNP to the mitochondria. Afterward, the HSPA1L-PRNP complex binds to COX4I1 (cytochrome c oxidase subunit 4I1), resulting in elevated mitochondrial membrane potential, induced antioxidant enzymes, and mitophagy, validating the role of the HSPA1L-PRNP-COX4I1 axis in melatonin-induced mitophagy induction (Lee, Yoon, Song, Noh & Lee, 2020). Likewise, supplementation of human mesenchymal stem cells with melatonin, causes HSPA1L upregulation, and, enhances HSPA1L-mediated recruitment of PRKN to mitochondria, to favor mitophagy and cell survival (Yoon, Kim, Lee & Lee, 2019). Therefore, the HSPA1L-PRKN axis is modulated by melatonin and promotes mitophagy.

A number of studies have noted PINK1 and PRKN modulation by melatonin. Melatonin supplementation culminates in upregulation of PINK1, PRKN, PPARGC1A (PPARG coactivator 1 alpha), NRF1 (nuclear respiratory factor 1), and TFAM (transcription factor A, mitochondrial) proteins with a role in mitophagy and mitochondrial integrity in rats with liver fibrosis (Kang, Hong & Lee, 2016). Melatonin-mediated upregulation of PRKN is also observed in nucleus pulposus cells in a time- and dose-dependent manner (Chen et al., 2019). In addition, melatonin enhances PRKN mitochondrial translocation via inhibition of MST1 (macrophage stimulating 1) phosphorylation, compromises mitophagy in diabetic cardiomyopathy (Wang et al., 2018).

Furthermore, ample evidence has shown that melatonin modulates NFE2L2 (nuclear factor, erythroid 2 like 2), HAS3 (hyaluronan synthase 3), and MTOR (mechanistic target of rapamycin kinase) complex 1

(MTORC1) to induce mitophagy. Melatonin upregulates NFE2L2 to induce NFE2L2-dependent mitophagy to heal brain injury in a murine model of subarachnoid hemorrhage (Sun, Yang, Li & Hang, 2018). Also, melatonin activates HAS3 and associated mitophagy, in a neuroblastoma N2a cell line (Lee et al., 2019). Furthermore, melatonin activates mitophagy through MTORC1 modulation, which in turn, ceases inflammation by suppressing IL1B (interleukin 1 beta) secretion upon immunopathology of traumatic brain injury (Lin et al., 2016).

Melatonin also modulates other signaling pathways to activate mitophagy. Interestingly, our group revealed that melatonin backs up the CGAS (cyclic GMP-AMP synthase)-STING1 (stimulator of interferon response cGAMP interactor 1)-TBK1 (TANK binding kinase 1) signaling pathway, leading to mitophagy reactivation involving ALDH2 (aldehyde dehydrogenase 2 family member) activation in APP- (amyloid beta precursor protein) and PSEN1 (presenilin 1)-mutant mice (Wang et al., 2020). Our study suggested the role of melatonin in rescuing myopathic changes in the heart via reinstating mitophagy. Moreover, melatonin therapy inhibits mitochondrial fission and boosts mitophagy through inhibiting NR4A1 (nuclear receptor subfamily 4 group A member 1)-PRKDC (protein kinase, DNA-activated, catalytic subunit)-TP53 (tumor protein p53) signaling pathway in nonalcoholic fatty liver disease (Zhou et al., 2018).

Despite mitophagy activation, ample studies have revealed other mechanisms of melatonin regarding mitophagy regulation in mammalian cells. It was suggested that melatonin maintains mild induction of mitophagy through preventing excessive mitophagy induction. This notion was observed in a murine model of microvascular I/R injury, where melatonin activates PRKAA1 (protein kinase AMP-activated catalytic subunit alpha 1) to inhibit DNM1L-based mitochondrial fission. Subsequently, the VDAC1-HK2 (hexokinase 2) interaction is recovered, which results in inhibition of the mitochondrial permeability transition pore opening, and attenuation of PINK1-PRKN-dependent mitophagy (Zhou et al., 2017). It is perceived that melatonin-evoked PRKAA1-DNM1L-VDAC1-HK2-mitochondrial permeability transition pore signaling pathway is a cytoprotective mechanism that blunts excessive mitophagy-evoked cell death ensuing microvascular I/R damage.

More cell signaling pathways are reported to be modulated by melatonin to govern mitophagy including the MT2A (metallothionein 2A)-SIRT3-FOXO3 pathway, which inhibits mitophagy in H9c2 cells (Wu, Yang, Gao, Wang & Ma, 2020), and MAPK8 (mitogen-activated protein kinase 8)-PRKN pathway, which is negatively modulated by melatonin, and causes a cessation of excessive mitophagy induction in human HeLa cells (Chen, Liu, Li & Gao, 2018). In addition, melatonin maintains mild levels of mitophagy-associated proteins such as PRKN, BECN1, SIRT3, FOXO3, and BNIP3L, thus keeping mitophagy in check (Wu, Yang, Gao, Wang & Ma, 2020). Melatonin can also upregulate SIRT1 (sirtuin 1), as a result suppressing excessive PINK1-PRKN mitophagy (Yi, Zheng, Zhu, Cai, Sun & Zhou, 2020). Collectively, these data suggest that melatonin regulates mitophagy by two dogmas: (i) induction of a low-level activation and (ii) prevention of excessive activation, both of which, can be beneficial to cell homeostasis and inflammation suppression.

5. Combinational therapy: Melatonin therapy with accessory therapeutic agents

Based on the aforementioned studies we propose melatonin therapy to inhibit inflammatory cytokine secretion and alleviate atherosclerosis by regulating macrophage mitophagy. However, for optimal management of mitophagy, accessory therapeutic agents could be used alongside with melatonin therapy, forming combinational therapeutic systems.

5.1. Therapeutic agents targeting the SIRT3-FOXO3-PRKN pathway

As mentioned above (Ma et al., 2018; Reiter, Ma & Sharma, 2020; Reiter, Tan, Rosales-Corral, Galano, Jou & Acuna-Castroviejo, 2018), melatonin activates the SIRT3-FOXO3-PRKN pathway to trigger mitophagy and block inflammation. In this regard, applying pharmaceutical or natural therapeutic agents to modulate SIRT3 and FOXO3 may aid melatonin to reinstate mitophagy in macrophages.

A natural biphenolic compound, honokiol, was reported to exert anti-oxidative and anti-inflammatory properties and reverse cardiac hypertrophy due to its capacity to mediate SIRT3 upregulation both *in vivo* and *in*

vitro (Pillai et al., 2015). Honokiol also binds with SIRT3 in mitochondria to enhance SIRT3 activity (Pillai et al., 2015).

Furthermore, resveratrol is a natural phenol abundant in peanuts and grape skins, which significantly attenuate mitochondrial ROS by enhancing SIRT3 levels in the mitochondria and evoke FOXO3 upregulation in human vascular endothelial cells (Zhou et al., 2014). In addition, resveratrol activates the SIRT1-FOXO3 pathway, upregulates PINK1, BNIP3, RAB7A (RAB7A, member RAS oncogene family), and BECN1, and therefore, resulting mitophagy induction (Kuno et al., 2018; Ren & Zhang, 2018). Resveratrol is also capable of inhibiting AKT1 (AKT serine/threonine kinase 1), resulting in FOXO3 activation and consequently activation of antioxidant enzymes (Franco et al., 2014).

Troxerutin is also a natural flavonol extracted from flavonoid rutin, and its administration remarkably upregulates SIRT3 and SIRT1, leading to suppression of oxidative stress, apoptosis, and acute neuroinflammation in Wistar rats following lipopolysaccharide challenge (Jamali-Raeufy, Kardgar, Baluchnejadmojarad, Roghani & Goudarzi, 2019).

Metformin is perhaps one of the most widely used medication clinically that can enhance FOXO3 activation through AMPK activation and may enhance mitophagy due to the activation of the AMPK-FOXO3 axis (Sato et al., 2012). Auranofin is an approved therapeutic agent with diverse biological affects, one of which is the activation of FOXO3 and promotion of its nuclear localization (Park, Lee, Berek & Hu, 2014).

6,8-diprenylorobol, extracted from *Glycyrrhiza uralensis* Fisch roots, is a phytochemical compound with anti-cancer properties, which are attributed to FOXO3 upregulation (Lee et al., 2020).

Taken together, a combinational therapeutic system comprising melatonin and SIRT3 or FOXO3 modulators might be a potential package for provoking mitophagy in macrophages. However, some of these modulators have not yet been clinically approved and much effort should be engaged for their optimization to meet clinical expectations.

Besides, a growing trend shows that miRNAs play a crucial role in the regulation of SIRT3 and FOXO3, and thus mitophagy induction. For instance, *Mir214* blocks SIRT3 expression as its target molecule, and its knockdown restores SIRT3 expression, as well as mitochondrial activity and morphology, in a murine model of angiotensin II-induced cardiomyopathy (Ding et al., 2020). Furthermore, *MIR708-5p*targets and blocks SIRT3 expression in cancer cells (Huang, Guo, Cao & Xiong, 2019), suggesting that *MIR708-5p* knockdown might induce SIRT3 upregulation. Similarly, *MIR494* suppresses SIRT3 expression in hepatoma cell lines and its inhibition might induce SIRT3 upregulation (Zhang, Zhu, Hu, Yan & Chen, 2019).

In the case of FOXO3, *Mir182* transfection into rat muscle cells targets *Foxo3* mRNA and suppresses its expression (Hudson, Rahnert, Zheng, Woodworth-Hobbs, Franch & Russ Price, 2014). Further,*MIR96* binds to the seed region in *FOXO3* mRNA, and remarkably reduces its expression, whereas *MIR96* downregulation induces FOXO3 upregulation (Li et al., 2015). Moreover, *MIR629*negatively regulates FOXO3 at the post-transcriptional stage and suppresses its expression (Yan et al., 2017), indicating that inhibitory targeting of *MIR629* may reverse its effect on FOXO3 expression. Overall, miRNAs regulate the expression of FOXO3 and SIRT3, and, thereby, their modulation could be a part of melatonin-based combinational therapies.

Apart from microRNAs, two major lifestyle medication factors, exercise and diet, mediate SIRT3 and FOXO3 upregulation. Exercise training, caloric restriction, and fasting upregulate SIRT3, whereas a high-fat intake downregulates SIRT3 in skeletal muscles (Palacios et al., 2009). Mechanistically, caloric restriction activates SIRT3, which in turn deacetylates lysine residues on SOD2 (superoxide dismutase 2; an antioxidant enzyme residing in mitochondria), leading to removal of ROS (Qiu, Brown, Hirschey, Verdin & Chen, 2010). In addition, caloric restriction activates mammalian SIRT2, which in turn deacetylates FOXO3 and enhances its function (Wang, Nguyen, Qin & Tong, 2007). Calorie restriction in aged rats also preserves the melatonin rhythm which probably helps to maintains SIRT3 activity (Stokkan, Reiter, Nonaka, Lerchl, Yu & Vaughan, 1991). Thus, caloric restriction can also boost mitophagy through upregulation of FOXO3 and SIRT3.

5.2. Therapeutic agents targeting the AMPK-OPA1 pathway

As several studies have noted a role for the AMPK-OPA1 pathway in mitophagy activation upon melatonin treatment (Zhang et al., 2019), targeting components of AMPK-OPA1 should help to strengthen melatonin-regulated mitophagy.

AMPK activators induce intracellular accumulation of either adenosine monophosphate (AMP) or Ca^{2+} and are thus termed "indirect activators", whereas, some activators directly interact with AMPK to foster its kinase activity, thus being termed "direct activators" (Samant et al., 2014).

Substantial evidence indicates that metformin antidiabetic actions are attributed to AMPK activation. Mechanistically, metformin blocks complex I of the mitochondria, thus increasing AMP levels and activating AMPK (Owen, Doran & Halestrap, 2000). Thiazolidinediones are a class of pharmaceutical drugs including rosiglitazone, pioglitazone, and troglitazone, which activate AMPK via AMP accumulation due to the inhibition of mitochondrial respiratory complex I (Brunmair et al., 2004).

In addition to pharmaceutical agents, some naturally occurring agents can activate AMPK. Polyphenols including curcumin, quercetin, genistein, berberine, epigallocatechin-3-gallate, and resveratrol, which exist in fruits, vegetables, and plants, have been reported to switch on AMPK (Sharma & Kumar, 2017). Epigallocatechin-3-gallate, curcumin, quercetin, and resveratrol increase AMP levels by targeting the mitochondrial ATP synthase (Gledhill, Montgomery, Leslie & Walker, 2007; Zheng & Ramirez, 2000), whereas berberine increases AMP levels by targeting mitochondrial respiratory complex I (Turner et al., 2008).

Ginsenosides are extracted from ginseng and exhibit a favorable impact on type 2 diabetes, largely attributable to AMPK activation (Jeong, Kim & Chung, 2014). A naturally available dithiol agent, α-lipoic acid, generated from octanoic acid, can also activate AMPK likely by inducing Ca^{2+} accumulation, and, thereby, activation of the CAMKK1 (calcium/calmodulin dependent protein kinase kinase 1)-AMPK axis.

Several AMPK activators directly interact with AMPK complex and cause conformational changes, leading to its enzymatic activation. For example, 5-aminoimidazole-4-carboxamide riboside, thienopyridine, salicylate, compound-13, PT-1, and MT 63–78 are AMPK activators (Cool et al., 2006; Corton, Gillespie, Hawley & Hardie, 1995; Gómez-Galeno et al., 2010; Hawley et al., 2012; Pang et al., 2008; Zadra et al., 2014). Overall, a combinational therapeutic system comprising melatonin plus AMPK modulators might boost mitophagy activation and regulation in macrophages. Given that mitochondrial SIRT3 can deacetylate OPA1 and enhance its activity (Samant et al., 2014), SIRT3 modulators may confer OPA1 activation as well.

5.3. Therapeutic agents targeting the MTORC1 pathway

Regarding the role of MTORC1 in mitophagy and the effect of melatonin on MTORC1 regulation, it can be assumed that inhibiting MTORC1 may upregulate mitophagy. Besides, a combinational therapeutic system including melatonin and MTORC1 inhibitors might show satisfactory results.

Rapamycin is an FDA-approved MTORC1 inhibitor employed in clinics for suppression of organ rejection following tissue transplantation and cancer treatment (Kennedy & Lamming, 2016). In various disease models, rapamycin protects mitochondria via enhancing mitophagy (Li et al., 2018; Li et al., 2014). Of note, melatonin reduces transplant rejection probably due to its antioxidant property to reduce rapamycin toxicity, denoting the value of combining melatonin with rapamycin (Vairetti et al., 2005). A derivative of rapamycin, Everolimus serves as an immunosuppressant, now in phase II of clinical trials. Everolimus has been shown to block MTORC1 with greater specificity (Goutagny et al., 2015). Also, temsirolimus is a preclinical therapeutic agent with the potential to inhibit MTORC1 through allosteric regulation, leading to mitophagy activation (Chiarini et al., 2011). Ridaforolimus/deforolimus is another novel MTORC1 inhibitor which went through several phases I/II and even phase III clinical trials for the treatment of certain tumors (Mita, Sankhala, Abdel-Karim, Mita & Giles, 2008).

5.4. New perspective on targeting PINK1-PRKN-mediated mitophagy

PINK1 and PRKN activators were proposed to drive PINK1-PRKN-mediated mitophagy. However, PINK1 and PRKN are also parts of other signaling pathways in biological processes; thus, targeting these molecules

may yield off-target effects (Miller & Muqit, 2019). Thus, we propose new trends to reinvigorate PINK1-PRKN-mediated mitophagy. To do this, we divide this process into three major stages including (i) the initiation stage, which is commenced by mitochondrial depolarization or damage, (ii) the maintenance stage, which requires PINK1 stabilization, and (iii) the amplification stage, which mainly involves ULK1 activation.

We propose that inducing mild mitochondrial depolarization along with manipulating other stages might boost PINK1-PRKN-mediated mitophagy (Georgakopoulos, Wells & Campanella, 2017). Mild induction of mitochondrial depolarization mainly influences already depolarized mitochondria. Such an approach might avert unwanted depolarization of plasma membrane and intact/healthy mitochondria. For this purpose, two polymethoxylated flavones including nobiletin and tangeretin have shown promising effects (Wu et al., 2013). Nonetheless, new compounds should be developed to be applicable in clinical settings.

As discussed above, for PINK1 stabilization at the MOM, the PHB2-PARL-PGAM5 pathway, TAMM41, and HSPA2 (heat shock protein family A [Hsp70] member 2) are essential components. Therefore, future attempts should be directed towards pharmaceutical/natural agents to modulate these components and strengthen PINK1-PRKN-mediated mitophagy in macrophages. Of note, before drug development, to limit off-target effects and avert excessive mitophagy, altered expression, dysfunction, and activation of these components in macrophages of atherosclerotic lesions should be explored. Table 1 lists the mitophagy modulators under clinical investigation.

5.5. New mitophagy targets, new therapeutic agents

As discussed in section 4, melatonin modulates various signaling pathways to turn on mitophagy. For instance, melatonin modulates HSPA1L-PRNP-COX4I1, CGAS-STING1-TBK1, HSPA1L-PRKN, HSPA1L-PRNP-COX4I1, and NR4A1-PRKDC-TP53 pathways, leading to mitophagy activation and regulation in healthy mammalian cells. Thus, molecular components of these pathways are new targets for mitophagy regulation. Given that therapeutic modulation of these pathways has largely remained unexplored, new drug-developing approaches should be geared towards novel pharmaceutical or natural agents for specific targeting of these pathways.

5.6. Caution merited to avoid excessive mitophagy induction

As mentioned above, mild mitophagy induction is a benign process in mammalian cells, whereas excessive mitophagy may have adverse effects. In line with this, caution should be exercised when implementing combinational therapeutic systems. Currently, there is no available method to restrain mitophagy overactivation upon using mitophagy inducers. Thus, melatonin therapy and accessory therapeutic agents might lead to mitophagy overactivation. However, executing pre-clinical studies may show us a safe regimen for applying these agents. Thus, we propose dose-finding and duration-finding studies to identify optimal drug concentrations and treatment durations to avoid excessive mitophagy activation, while maintaining mild induction. Of note, some drugs also exhibit different effects in terms of intensity at different times of the day, which is referred to as chronopharmacology.

6. Optimization of combinational therapeutic systems

6.1. Encapsulation techniques

The beneficial effects of therapeutic agents discussed earlier will only be realized if they can reach intended site of action within human body in a bioactive form. There are a number of issues currently limiting the bioavailability and bioactivity of therapeutic agents deemed effective against atherosclerosis. Initially, it is important to be able to formulate a delivery vehicle that contains a sufficiently high dose of the therapeutic in a chemically stable and bioavailable form. For oral administration, this formulation may be in the form of a pill, capsule, fluid, or functional food. It is important that this formulation is designed to inhibit any chemical degradation of the therapeutic agent during production, transport and storage but that it then releases the therapeutic agent in a bioavailable form in the human gastrointestinal tract. The design of an effective formulation is highly dependent on the nature of the therapeutic agent and the delivery format and must be established on a case-by-case basis (McClements, 2018). Some of the most important factors to

take into account when designing an efficacious formulation are the polarity (LogP), water solubility, melting point, charge, and chemical reactivity of the therapeutic agent.

Under physiological conditions, resveratrol is a strongly hydrophobic molecule (LogP = 3.4) with a low water solubility (0.14 mg/L) and poor chemical stability (especially under alkaline conditions) (Zhou, Zheng & McClements, 2021a), which reduces its bioavailability and bioactivity and therefore limits its application as a therapeutic agent in the pharmaceutical industry (De La Lastra & Villegas, 2007; Erlank, Elmann, Kohen & Kanner, 2011; Salehi et al., 2018). Similarly, melatonin is a modestly hydrophobic molecule (LogP=1.15) with a relatively low water solubility (1.0 mg/mL) (chemicalize.com). Melatonin has also been reported to chemically degrade when dispersed in aqueous solutions, with the rate of degradation increasing with increasing pH, temperature, and light exposure (Daya, Walker, Glass & Anoopkumar-Dukie, 2001; Pranil, Moongngarm & Loypimai, 2020). The limited water solubility and chemical stability of melatonin reduces its bioavailability and bioactivity, which again limit its efficacy as an effective therapeutic agent (Molska, Nyman, Sofias, Kristiansen, Hak & Widerøe, 2020). For these reasons, there has been considerable interest in the utilization of nanotechnology-based encapsulation methods to improve the efficacy of resveratrol and melatonin as therapeutic agents in drugs, supplements, and functional foods (Chuffa et al., 2021; Schaffazick, Pohlmann & Guterres, 2007; Zhou, Zheng & McClements, 2021a; Zhou, Zheng & McClements, 2021b).

Nanoenabled-encapsulation typically involves trapping the therapeutic agent within small (colloidal) particles, which typically have dimensions somewhere between around 10 and 1000 nm (McClements, 2020a; McClements, 2020b). Having said this, larger particles are sometimes utilized for certain applications. The colloidal particles may be solid, semi-solid, or liquid and may be fabricated from a range of different natural and synthetic ingredients, including proteins, polysaccharides, lipids, phospholipids, surfactants, and synthetic polymers. In general, these particles may vary in their size, shape, composition, charge, physical state, internal structure, and aggregation state, which means that their properties can be tailored for specific applications. It should be noted that these properties may change once the particles are incorporated into a formulation or after they enter the human gut, which needs to be taken into account for drug design purpose.

The entrapped ingredients (in this case therapeutic agents) are often referred to as the "core material" whereas the surrounding matrix is referred to as the "encapsulant" or "shell material" (Nedovic, Kalusevic, Manojlovic, Levic & Bugarski, 2011). Encapsulation has been shown to enhance the dispersibility of therapeutic agents in aqueous solutions, to protect them against chemical degradation by environmental factors, and to promote their absorption in gastrointestinal tract (Davidov-Pardo & McClements, 2014). Emulsions and nanoemulsions, microemulsions, liposomes, and cyclodextrins are among the most commonly used encapsulation technologies for this purpose.

Oil-in-water emulsions and nanoemulsions are composed of oil, water, and emulsifiers, which exist as numerous small emulsifier-coated spherical oil droplets suspended in water (McClements & Rao, 2011). By definition, droplets in nanoemulsions present diameters below 200 nm whereas those in emulsions have diameters above this value. The smaller dimensions of oil droplets in nanoemulsions offer benefits for certain encapsulation applications, including increased optical clarity, greater storage stability, and higher bioavailability of therapeutic agents. Emulsions and nanoemulsions can be used to encapsulate lipophilic bioactive compounds such as resveratrol and melatonin within their hydrophobic cores (Donsì, Sessa, Mediouni, Mgaidi & Ferrari, 2011; Rondanelli et al., 2012). Studies have shown the encapsulating melatonin within oil-in-water nanoemulsions significantly increases its physicochemical stability and solubility (Molska, Nyman, Sofias, Kristiansen, Hak & Widerøe, 2020). Nevertheless, it is important to carefully select the oil phase of emulsions and nanoemulsions so that it can inhibit any chemical degradation of the therapeutic agents during storage, as well as to ensure that it promotes their bioavailability after ingestion. For instance, it has been shown that long chain triglycerides are more effective than medium chain triglycerides at increasing the bioavailability of strongly hydrophobic therapeutical agents, which is attributed to their ability to form large mixed micelles that can trap the bioactive agents inside (McClements, 2021). It is also important to carefully selected the type of emulsifier used to ensure that small droplets can be formed during homogenization, the systems remain stable during storage, and the droplets do not undergo extensive aggregation within

the gastrointestinal tract (as this can reduce bioavailability by restricting the access of digestive enzymes to the lipids). It may also be important to include other additives, such as antioxidants, to preserve the therapeutic agents during storage. Emulsions and nanoemulsions are typically produced using mechanical devices known as homogenizers, such as high shear mixers, colloid mills, high pressure valve homogenizers, sonicators, and microfluidizers (McClements, 2011; McClements & Rao, 2011). Emulsions and nanoemulsions are thermodynamically unstable and may be broken down through a variety of physical and chemical instability mechanisms, including creaming, sedimentation, flocculation, coalescence, and Ostwald ripening (McClements, 2011; McClements & Rao, 2011). Consequently, they must be carefully formulated to avoid these problems. After formation, emulsion-based systems are typically in a fluid form. They can be converted into gels by adding gelling agents or promoting aggregation of the oil droplets. They can be converted into powders through dehydration, which is usually carried out commercially using spray drying technologies. The functional performance of emulsions and nanoemulsions can be improved by using structural design methods to generate more sophisticated morphologies, such as multilayer emulsions, multiple emulsions, Pickering emulsions, or filled microgels (Tan & McClements, 2021). However, these advanced emulsion technologies are more costly to prepare and therefore they should only be utilized when required.

Nanostructured lipid carriers (NLCs) and solid lipid nanoparticles (SLNs) are oil-in-water emulsions or nanoemulsions where the lipid has been partially or fully solidified, respectively (McClements & Li, 2010). They are typically prepared using the same homogenization methods as used to produce emulsions and nanoemulsions (Weiss, Decker, McClements, Kristbergsson, Helgason & Awad, 2008). However, the lipid phase is comprised of a high melting point lipid. Typically, an emulsion or nanoemulsion is first formed at a high temperature (above the melting point of the lipid), then it is rapidly cooled to form NLCs or SLNs. However, the lipid phase must be carefully selected to ensure that the lipophilic therapeutic agent is not expelled and the lipid droplets do not aggregate after the lipid phase is solidified (Weiss, Decker, McClements, Kristbergsson, Helgason & Awad, 2008). Well-designed SLNs and NLCs are often more effective at preserving encapsulated lipophilic compounds against chemical degradation during storage, which is attributed to the ability of the solidified lipid phase to inhibit molecular diffusion (Weiss, Decker, McClements, Kristbergsson, Helgason & Awad, 2008). Encapsulation of melatonin in SLNs has also been shown to lead to a more sustained release profile after oral ingestion (Priano et al., 2007), to increase its bioavailability after oral administration to humans (Mistraletti et al., 2019), and to increase its antioxidant activity after dermal application (Mirhoseini et al., 2019). Stearic acid-based SLNs have been used to encapsulate resveratrol and increase its bioavailability after oral administration to Wistar rats (Pandita, Kumar, Poonia & Lather, 2014). Furthermore, NLCs have been shown to increase the bioactivity of melatonin in *in vitro* fertilization media (Siahdasht, Farhadian, Karimi & Hafizi, 2020).

Microemulsions contain small spheroidal particles consisting of a hydrophobic core and a hydrophilic shell, which are primarily made up from surfactants (McClements, 2020a). The non-polar tails of the surfactants cluster together through hydrophobic attraction and form a hydrophobic core, while the polar heads of the surfactants form a hydrophilic shell that ensures their water-dispersibility. Lipophilic therapeutic agents, like resveratrol or melatonin, can be incorporated into the hydrophobic core of microemulsions (Nemen & Lemos-Senna, 2011). These colloidal systems are thermodynamically stable and can often be formed by simply mixing the ingredients together, once the optimum composition has been established. They tend to be optically clear because the size of the particles (< 50 nm) is typically much less than the wavelength of light. Encapsulation of resveratrol in microemulsions has been shown to increase its water-dispersibility, photostability, and antioxidant activity (Juskaite, Ramanauskiene & Briedis, 2017; Lv et al., 2018). The encapsulation of melatonin in microemulsions has been shown to increase its bioavailability after being applied to the skin of human patients (Mistraletti et al., 2019). The main drawback of microemulsions is that they usually have to be formed from relatively high concentrations of non-ionic surfactants, which can cause taste, cost, or toxicity problems.

Liposomes ($d > 200$ nm) and nanoliposomes ($d < 200$ nm) are spheroidal particles that typically have an onion-like (vesicular) structure, which are formulated from phospholipids (McClements, 2020a). These liposomal systems are comprised of one or more concentric phospholipid bilayers surrounding an aqueous core.

The bilayer structures tend to form spontaneously when the phospholipids are mixed with water due to the hydrophobic effects. However, some form of processing is need to create a dispersion of relatively small and uniform liposomes or nanoliposomes, such as microfluidization. Liposomal systems can encapsulate hydrophilic and hydrophobic therapeutic agents because they have both polar and non-polar regions inside them. Hydrophobic molecules like melatonin and resveratrol are usually located within the non-polar regions formed by the tails of the phospholipids in the bilayer membranes. Encapsulating resveratrol within a liposomal formulation has been shown to prolong its release and increase its therapeutic effects in rat models of nerve injury (Feng, He, Mao, Shui & Cai, 2019). Metformin-encapsulated liposomes enhance therapeutic efficacy and clinical use of metformin against breast cancer cells (Khiavi, Safary, Barar, Ajoolabady, Somi & Omidi, 2020; Shukla et al., 2019).

Cyclic oligosaccharides, known as cyclodextrins, consist of glucopyranose units with α (1-4) bonds (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero & Simal-Gandara, 2009). In aqueous solutions, cyclodextrins have a hydrophobic cavity than can incorporate non-polar therapeutic agents and a hydrophilic exterior that ensures their water-dispersibility. Inclusion complexes formed between α-/β-/γ-cyclodextrins and resveratrol have been shown to significantly improve the water-dispersibility of resveratrol (Bertacche, Lorenzi, Nava, Pini & Sinico, 2006; Silva et al., 2021). Researchers have also shown that cyclodextrins can also be used to increase the water-dispersibility of melatonin (Grygorova et al., 2019). Studies have confirmed that cyclodextrins and resveratrol form 1:1 complexes with the therapeutic molecule trapped in the hydrophobic cavity (Lucas-Abellán, Fortea, Gabaldón & Núñez-Delicado, 2008). An *in vivo* study showed that encapsulation of resveratrol in cyclodextrins increased its anticancer activity against cervical cancer (Hao et al., 2021), which was probably because the delivery system increased the amount of the therapeutic agent reaching the site of action in an active form.

6.2. Targeted drug delivery to macrophages

For an effective therapeutic intervention, either melatonin or accessory therapeutic agents should be delivered specifically to macrophages in the atherosclerotic lesions to avoid off-target effects on other tissues. However, it is noteworthy that melatonin off-target effects are beneficial and caution should be directed towards other therapeutics (Reiter, Tan, Paredes & Fuentes-Broto, 2010). Targeted drug delivery refers to the specific delivery of a therapeutic agent or a combination of agents using various carriers (basically nano-scale carriers) such as nanoparticles, liposomes, niosomes, carbon nanotubes, and dendrimers to a certain cell or tissue. Targeted drug delivery to the macrophages in the atherosclerotic lesions seems to optimize the efficacy of therapeutic agents enwrapped in drug-delivery systems (Fig. 2) (Jain, Mishra & Mehra, 2013).

The basis of targeted drug delivery is to decorate drug carriers with ligands that can specifically recognize the target (cell or tissue of interest). For example, macrophages express high levels of sugar-binding receptors such as mannose receptors on their surface. Therefore, decorating drug-delivery systems with ligands to bind mannose receptors can create a targeted drug-delivery system for specific delivery of the therapeutic agents to macrophages (Ezekowitz et al., 1991; Taylor, Bezouska & Drickamer, 1992).

In line with this, functionalization of liposomes with mannosyl ligand (mannosylated liposomes) specifically targets rat alveolar macrophages and elicits increased uptake of liposomes both *in vivo* and*in vitro* (Chono, Tanino, Seki & Morimoto, 2007). Functionalization of gelatin nanoparticles with mannose ligands successfully delivers amphotericin B to macrophages to ameliorate visceral leishmaniasis (Nahar, Dubey, Mishra, Mishra, Dube & Jain, 2010). Mannosylated SLNs (Nimje et al., 2009), and mannose-engineered polyethylene glycol/PLGA show similar results (Tomoda & Makino, 2007). Also, encapsulating therapeutic agents in chitosan microparticles has shown enormous success in the specific delivery of the agents to macrophages (Kunjachan, Gupta, Dwivedi, Dube & Chourasia, 2011).

In addition to mannose receptors, other macrophage cell surface molecules could be a specific target, against which new ligands can be designed on drug carriers. We propose that further experiments should be carried out to find out the cell surface molecules that are specifically expressed/overexpressed in activated macrophages in the atherosclerotic regions. Discovering such molecules would enable us to deliver melatonin and

other accessory agents to macrophages that specifically reside in atherosclerotic regions.

7. Concluding remarks

Melatonin serves as a potential anti-inflammatory agent through regulation of mitophagy in macrophages in atherosclerotic regions. Thus, melatonin along with other mitophagy regulators and inducers can form a multiplex therapeutic package that can be delivered to macrophages to confer desired effects on the amelioration of atherosclerosis. Nanomedicine and drug delivery systems can be applied to guarantee the specificity of this delivery and avoid off-target effects of the therapeutic package.

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9. Conflict of interest statement

None of the authors declare any potential conflict of interest.

10- Competing Interests' Statement

None to be declared.

11. Data availability statement

For this work, no original data were used that would require data repository, however, the corresponding authors are committed to respond to any reasonable request for this publication.

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Table 1: Other mitophagy modulators.

Compounds	Full name/source	Mechanisms of action
MA-5	Mitochonic acid 5	PRKN-dependent mitophagy via activation of AMPK-S
Puerarin	Derived from Pueraria with antioxidant capacity	Regulates mitochondrial fission and fusion, reactivates P
TMP	2,3,5,6-Tetramethylpyrazine	Blocks CCL2 (C-C motif chemokine ligand 2)-CCR2 (C-
Tributyrin	A natural triglyceride that exists in butter	Enhances PRKN, ameliorates mitochondrial dysfunctio
Undecanone	2-Methyl nonyl ketone derived from *H. cordata*	Mitophagy activation via inhibition of AKT1-MTORC1

Figures:

Fig. 1. PINK1-PRKN-mediated mitophagy and initial signaling cascade of mitophagy.

Once mitochondria are damaged or depolarized, they evoke accumulation of ROS that trigger initial mitophagy induction. Mitochondrial injury also induces AMP and Ca^{2+} accumulation in the cytosol. ROS along with Ca^{2+} and AMP accumulation in the cytosol can activate AMPK, which, in turn, phosphorylates and activates a spectrum of mitophagy and autophagy regulators. Mainly, the AMPK-MTORC1-ULK1 axis represents the first complex that mediates phagophore, and subsequently autophagosome, formation. Mitochondrial changes also involve biochemical modulation inside mitochondria. Mitochondrial depolarization triggers PINK1 stabilization on the MOM, which recruits PRKN, and PRKN also mediates ubiquitination of MOM proteins. Further PRKN accumulation on the MOM results in polyubiquitination of MOM proteins. Ultimately, polyubiquitinated MOM proteins are recognized by autophagy cargo receptors such as OPTN, NBR1 and SQSTM1, which connect them with MAP1LC3B/LC3B and the phagophore. ULK1 not only is involved in the formation of phagophores and autophagosomes but also activates BECN1, which interacts with PRKN and amplifies its recruitment to the MOM. Following the sequestration of damaged mitochondria within autophagosomes and the subsequent fusion of the latter with a lysosome, ROS is dramatically scavenged from the cytosol. It is also noteworthy that Golgi-derived membrane can participate in the formation of phagophores.

Fig. 2. Targeted drug delivery to macrophages.

Using mannose-coated nanocarriers such as liposomes and SLNS, melatonin and other therapeutic drugs can be delivered specifically to macrophages. Once nanoparticles arrive, mannose receptors recognize them and endocytosis occurs, culminating in the release of the drugs within the macrophage cytosol.