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Unfolding the Therapeutic Potential of Chemical Chaperones for Age-related Macular Degeneration

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SUMMARY

Recent studies suggest that pathological processes involved in age-related macular degeneration (AMD) might induce endoplasmic reticulum (ER) stress. Growing evidence demonstrates the ability of chemical chaperones to decrease ER stress and ameliorate ER stress-related disease phenotypes, suggesting that the field of chaperone therapy might hold novel treatments for AMD. In this review, we examine the evidence suggesting a role for ER stress in AMD. Furthermore, we discuss the use of chaperone therapy for the treatment of ER stress-associated diseases, including other neurodegenerative diseases and retinopathies. Finally, we examine strategies for identifying potential chaperone compounds and for experimentally demonstrating chaperone activity in *in vitro* and *in vivo* models of human disease.

Keywords

AMD; chemical chaperone; pharmacological chaperone; endoplasmic reticulum stress; protein misfolding; therapy

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in the developed world. In the United States alone there are over 1.75 million individuals affected by AMD and that number is expected to increase to over 3 million by 2020 [1].

AMD is a neurodegenerative disease characterized by progressive loss of photoreceptors in the macula and surrounding retina [2]. Clinical manifestations of the condition begin with abnormalities in the retinal pigment epithelium (RPE); formation of lipoproteinaceous deposits, referred to as drusen in Bruch's membrane, beneath the RPE and above the choroid; focal geographic chorioretinal scarring; and loss of photoreceptors in the macula. Advanced AMD involves an increase in the number and size of drusen deposits, choroidal neovascularization (CNV), and geographic atrophy of RPE and photoreceptors [2-4].

Therapeutic options for early AMD are limited. The most effective treatments currently available for advanced AMD are inhibitors and antibodies of the neovascular endothelial derived growth factor (VEGF) that causes CNV [5]. Several studies have found that diet influences AMD development and progression. Foods rich in carotenoids, beta carotene, vitamin C, vitamin E, zinc were found by van Leeuwen et al. to decrease AMD risk [6]. The large multi-center Age-Related Eye Disease Study (AREDS) also found vitamins C and E, beta

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carotene, zinc, and omega-3 long-chain polyunsaturated fatty acids to be beneficial in attenuating vision loss in advanced AMD patients [7-9].

The elucidation of novel, more effective therapies for AMD depends largely upon a deeper understanding of the molecular pathogenesis of AMD. Possible mechanisms of AMD development include abnormal extracellular matrix, inflammation, altered RPE-choriocapillary behavior, CNV development due to hypoxia-induced biochemical changes, and oxidative stress [2,10]. AMD might also be the result of RPE dysfunction resulting from the overwhelming oxidative stress, which may lead to endoplasmic reticular (ER)-stress and eventually compromised ER-stress response and cell health [2,4,11,12].

Recent studies suggest a role for ER stress in neurodegenerative diseases, like AMD. ER stress and protein misfolding and subsequent aggregation have been implicated in a variety of other neurodegenerative diseases that share pathological features with AMD [13,14]. Proteome analysis of human retinas with AMD and murine models of retinal degeneration found altered expression of molecular chaperones --- a diverse group of proteins found in high concentration in ER, which help prevent protein aggregation by encouraging unfolded proteins to a folding-competent state and also function as a component of the ER-specific protein-degrading apparatus to eliminate misfolded proteins [15-18]. These and other data discussed in this review indicate that the pathogenesis of AMD may be mediated, at least in part, through ER stress and protein misfolding.

If ER stress, protein misfolding, or altered chaperone expression, in particular, are implicated in AMD pathogenesis, it is likely that strategies that facilitate protein folding might be effective against AMD. One such strategy involves the use of chemical chaperones to augment the native action of molecular chaperones or recover specific misfolded proteins, prevent aggregation, and hence restore overall cell health. Indeed, studies have reported promising results regarding the use of chemical chaperones to treat disorders caused by protein misfolding [19-21]. In this review, we examine the evidence of ER stress, the role of chaperones, and the pharmacological potential of chaperone compounds for study in AMD. We also discuss strategies of screening for potential chaperones, as well as general principles to determine the effectiveness of chaperone treatments.

MOLECULAR CHAPERONES & ER QUALITY CONTROL

For an unfolded protein to reach its native conformation it must pass through several different free energy confirmations from the primary amino acid sequence to the complex tertiary and quaternary structures [19]. Mutated proteins often become stuck along this pathway in low free energy intermediate or mutant confirmations. Chaperones are a diverse group of molecules that can act at different stages of protein maturation in the cytosol and ER to promote acquisition of the desired conformation by decreasing the energy barriers between these different confirmations [15]. Defined by function rather than chemical or molecular structure, chaperones range from the endogenous molecular chaperones, such as the well-studied heat shock proteins (Hsp), to the small chemical molecules known as “chemical chaperones”, like glycerol and trimethylamine-N-oxide (TMAO). Much like molecular chaperones they promote the folding of a range of proteins. A subset of the chemical chaperones is that of the pharmacological chaperones, which act with specificity for a particular protein and are frequently protein antagonists [15,20-22].

Newly synthesized proteins are bound by ER or cytoplasmic chaperones that facilitate proper protein folding. These chaperones stabilize partially folded protein intermediates, bind exposed surfaces of misfolded proteins to prevent protein aggregation, catalyze the unfolding of misfolded proteins, and target terminally misfolded proteins to ER-associated degradation pathways [19]. The expression of chaperones plays a fundamental role in the ER quality control

system, whereby protein folding is facilitated and terminally misfolded proteins are degraded before they can form aggregates or otherwise interfere with cellular function [13]. At times, however, this stringent quality control system fails and allows misfolded proteins to accumulate within the cell. This occurs in situations of ER stress, defined as the accumulation of misfolded protein or the dysregulation of the ER environment required for proper protein folding by a variety of stresses including oxidative injury, hypoxia, and alterations in calcium homeostasis. In the presence of ER stress, the cell initiates a compensatory response called the unfolded protein response (UPR) [23]. The UPR involves initial inhibition of translation to prevent further accumulation of misfolded proteins, upregulation of chaperone genes to further facilitate protein folding, and activation of the ER-associated degradation system, which retro-translocates misfolded proteins from the ER for proteasome-dependent degradation. If the level of ER stress is particularly high, the UPR can promote apoptosis to safely dispose of the ER-stress injured cell [15,24,25].

Several neurodegenerative diseases discussed in this review result from misfolded aggregates and/or ER stress [13,14,24,25]. We believe that ER stress might be implicated in the pathogenesis of AMD and thus, that enhancing chaperone activity might be one therapeutic strategy for the treatment of AMD. Altering the cell's molecular chaperone levels through direct gene regulation or protein supplementation has proven successful in ameliorating ER stress and preventing cell death in a variety of disease states. For example, application of heat shock proteins can prevent axotomy-induced sciatic nerve cell death [26].

CHEMICAL CHAPERONE APPLICATIONS

Protein folding can also be enhanced by small, membrane-permeable protein-interacting molecules. Chemical chaperones can have this effect on a diverse group of proteins, both native and mutant. Like the cell's own molecular chaperones they are able to interact non-covalently with immature or misfolded proteins to promote proper folding, localization, or degradation [22].

Pharmacological chaperones, also referred to as “pharmacoperones”, act more specifically [27]. Like other chemical chaperones they are small molecules capable of traversing the cell membrane and encouraging protein folding through non-covalent interactions [21]. Pharmacological chaperones can be protein ligands that bind the nascent protein or recover a late-folded mutant protein [28]. However, since they interact, presumably, only with their target protein they generally effective at a lower concentration. For instance, known chemical chaperones glycerol and trimethylamine-N-oxide (TMAO) were both found to restore activity of a mutant chloride channel in mice, yet effective serum concentrations were lethal in nearly half the mice [29]. Thus, because some chemical chaperones prove toxic *in vivo*, pharmacological chaperones are suggested to have great potential for human therapy [21]. Additionally, several authors have suggested that pharmacological chaperones effectiveness could further be improved through the application of multiple chaperones acting at different sites of the protein or along different parts of the protein folding or localization pathways [27,30]. Overall, the area of chemical and pharmacological chaperone therapy is growing rapidly and the applications spreading to new and not exclusively protein folding disorder fields. Chemical and pharmacological chaperones used in some ER-stress related neurodegenerative and protein folding disorders have been summarized in Table 1.

4-Phenylbutyric Acid Sodium Salt (PBA), an FDA approved drug for urea-cycle disorders, is a chemical chaperone with wide application that is known to stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins. The oral administration of PBA to a murine model of type 2 diabetes alleviated ER stress, reduced diabetes symptoms, and lowered systemic inflammation in the mouse model [31]. De Almeida

et al. found that PBA given to transfected HFE C282Y cells decreased aggregates of the HFE C282Y mutant protein involved in hereditary hemochromatosis. It was hypothesized that PBA contributed to a more efficient disposal of the mutant by chemically enhancing the ER capacity to cope with the expression of misfolded HFE [32]. Recently, Yam et al. tested the effect of PBA on myocilin aggregates. Misfolding of myocilin, a secretion of the trabecular meshwork cells and a constituent of aqueous humor, is a potential cause of juvenile-onset primary open-angle glaucoma. Treatment with PBA reduced the amount of myocilin aggregates in the ER and decreased apoptosis of mutant myocilin transfected HEK293 cells [33]. Since PBA not only has proven protein folding interactions, but a positive systemic effect in cell and animal models of diseases involving ER stress, it could also have some effect in AMD models.

Tauroursodeoxycholic acid (TUDCA), an endogenous bile acid and putative chemical chaperone, has also been shown to reduce ER-stress and inflammatory responses in models of diabetes and liver disease and to be cytoprotective and anti-apoptotic in animal models of Huntington, Parkinson's and stroke [34,35]. Injection of TUDCA into both a retinal degeneration mouse model (rd10) and a light-induced retinal degeneration mouse model prevented apoptosis and preserved function and morphology of the photoreceptor cells [35].

The utility of these chaperone compounds in AMD, in which there is no single mutated protein to target is strongly supported, in particular, by studies that demonstrate that chaperone compounds can improve folding of even non-mutated proteins. Indeed, at baseline, a substantial proportion of proteins never achieve their native form and are thus degraded by proteasome. One study reported only 40% of opioid receptors fold properly and localize to the cell membrane. Application of pharmacological chaperones consisting of non-peptidic opioid ligand agonists and antagonists stabilized nascent opioid receptors in the ER and augmented the maturation and cell surface expression to nearly 100% of the potential opioid receptor precursors [36]. Another example is nicotine, a membrane-permeable ligand of human neuronal nicotinic acetylcholine receptors (AChR), which can act as a pharmacological chaperone to promote AChR assembly in the ER. Less permeable quaternary amine cholinergic ligands were also found to have chaperone-like function [37]. The role of chaperone therapy in the treatment of diseases characterized by ER stress but not a single, mutated and misfolded protein is further supported by *in vivo* studies demonstrating the effectiveness of chemical chaperones in the treatment of diabetes and hypoxic injury [31,34,35,38].

ER STRESS IN AMD

While definitive studies demonstrating increased ER stress in AMD are warranted, several processes documented in AMD have also been shown to produce ER stress in other neurodegenerative diseases, suggesting a possible role for ER stress in AMD. The potential mechanisms for ER-stress in AMD and a hypothetical role for chaperone therapy are summarized in Figure 1. For example, ER stress can be caused by ROS and perturbations in the redox state [23,24]. Prior studies have demonstrated that the retina and RPE are constantly under oxidative stress due to intense light exposure, and high metabolic activity, oxygen consumption, and concentration of polyunsaturated fatty acids in the outer photoreceptor segments [11]. What is more, the age-related thickening of Bruch's membrane and accumulation of drusen likely impair the choroidal-RPE hemodynamics and lead to potential hypoxic conditions [4]. Recently, Shen et al. reported a direct demonstration of increased oxidative stress in the retinas of AMD eyes with geographic RPE atrophy [39]. Additionally, changes in select RPE redox proteins have been reported in AMD eyes and oxidized phospholipids in the macula have been reported to increase with age and in eyes with AMD [40,41]. Moreover, isolation of microgram quantities of drusen identified a high concentration of oxidatively-modified proteins in AMD eyes [42]. A component of drusen, amyloid β protein, is particularly known to generate ROS [43]. Clinical studies have also found that antioxidant

treatment (vitamins A and E, and β -carotene) is effective in decreasing risk of AMD progression to advanced stages [9]. In addition, smoking, which can generate ROS, is strongly associated with an increased risk of AMD [44]. AMD is therefore linked closely to oxidative stress, and given the demonstrated ability of oxidative stress and redox changes to induce ER stress, it is quite possible that ER stress mediates at least some of the pathology observed in AMD.

Accumulation of lipofuscin, a yellow pigment granule, in the RPE is a hallmark of the aging retina and also a potential cause of ER stress [45]. High levels of lipofuscin are correlated to degeneration of RPE and adjacent photoreceptors in atrophic AMD [46]. At least 90% of lipofuscin in the RPE is the result of phagocytosis of the rod outer segments disc membranes, an important function of the healthy RPE [47]. The primary component of RPE lipofuscin is the autofluorescent pigment N-retinylidene-N-retinylethanolamine (A2E), which can create light-induced oxidative stress, exhibit detergent-like effects on cell membranes at high physiological concentrations, and activate the complement pathway [45,46]. At the high concentration found in AMD RPE, A2E can cause lysosomal bodies to leak. If the highly crosslinked lipofuscin components escape they could occupy cytosolic chaperone proteins and distract them from their proper protein folding interactions. Additionally, the age-related accumulation of lipofuscin in the lysosome may prevent healthy autophagy of old mitochondria. Retention of old mitochondria can lead to inefficient ATP production, which many chaperones are dependent upon, and increased oxidative stress because of the mitochondrial natural production of H_2O_2 . As a result of the stymied mitochondrial recycling, the increased oxidative stress further promotes lipofuscin accumulation and lysosomal occupation, a positive feedback cycle known as the “mitochondrial-lysosomal axis theory of postmitotic cellular ageing.” [48]. The interruption of cell dynamics has implications for a range of disease pathogenesis, but the general unbalance and increased oxidative stress may also impact ER homeostasis.

Another important consideration is the continual balance between ER stress and the UPR. Just as increased ER stress itself through the mechanisms described above can produce disease phenotypes, impaired UPR can also tip the scale toward ER stress-induced diseases. Importantly, recent data in both humans and an animal model suggests that AMD might be mediated, in part, through impaired UPR. In a proteomic analysis of human eyes at progressive stages of AMD, Ethen et al. found differential expression of 26 proteins in the central and peripheral retina during AMD progression. Among these were several molecular chaperones whose expression decreased with AMD progression, including Hsp60 and HOP [16]. Additionally, several antioxidant enzymes and chaperones that facilitate refolding or degradation of oxidatively damaged proteins decreased in AMD eyes: copper-zinc superoxide dismutase, manganese superoxide dismutase, catalase, and Hsp27, Hsp 90, and proteasome [40]. Moreover, human AMD lesions and retina from the *Ccl2/Cx3cr1* deficient murine model of AMD were reported to exhibit decreased mRNA and protein expression of ERp29, a protein escort and folding chaperone [18]. Thus, it is possible that the age-related decline in the expression levels or functionality of chaperone molecules might impair the retinal and RPE cells' ability to cope with ER stress, rendering them susceptible to ER stress-induced cell injury. On the other hand, these expression changes may reflect the cell's natural response mechanism, further elucidating the importance of chaperone exploration in AMD.

Recently, Yang et al. also reported that the rd1 mouse model for photoreceptor degeneration has elevated levels of ER stress related proteins GRP78, caspase-12, phospho-eIF2 α , and phospho-PERK. Expression peaked with photoreceptor apoptosis, leading the authors to conclude that ER stress modulators could be potential therapeutic targets for retinal degenerative diseases [49].

In summary, our present knowledge of AMD suggests that ER stress due to factors such as oxidative injury that are known to induce ER stress and/or impaired UPR might underlie the pathogenesis of AMD. Logically, then, interventions that shift the ER stress–UPR balance towards decreased ER stress might prove useful in the treatment of AMD. A variety of data have demonstrated that chemical and pharmacological chaperones can decrease signs of ER stress [31–33]. Taken together, this suggests a potential for such compounds in the treatment of AMD.

SIMILARITIES BETWEEN AMD AND OTHER NEURODEGENERATIVE DISEASES ASSOCIATED WITH PROTEIN MISFOLDING

A variety of diseases characterized by the misfolding and aggregation of a particular protein have been investigated as targets for chemical and pharmacological chaperones. Both *in vivo* and *in vitro* studies of these diseases have elucidated the role of particular chaperone compounds in decreasing misfolded protein aggregation, enhancing proper trafficking of the protein(s) in question to target sites, and ameliorating the overall disease phenotype. The success of chaperones in other disorders, such as nephrogenic diabetes insipidus and Parkinson's disease, is encouraging [21,31,32,38,50]. However, to date, no published studies have examined the use of chemical chaperones as a treatment for AMD in humans or in AMD models.

Although there is no particular misfolded protein correlated with AMD, histologically, AMD shares a common link with other neurodegenerative protein misfolding diseases, such as Alzheimer's disease and Parkinson's disease: extracellular deposits of misfolded or aggregated protein oligomers. In Alzheimer's, these plaques are composed of aggregated beta-amyloid; and in Parkinson's, alpha-synuclein protein bound to ubiquitin accumulates in cytoplasmic inclusions known as Lewy bodies because the alpha-synuclein-ubiquitin complex cannot be directed to the proteasome [13,51]. Drusen, commonly associated with AMD progression, is an extracellular deposit in Bruch's membrane between the RPE and choroid that consists of glycoprotein, lipid, complement components, and amyloid. Interestingly, drusen contains several protein components found in other amyloid diseases, including vitronectin, amyloid P, apolipoprotein E (ApoE), amyloid-beta oligomers, and oxidatively modified proteins. These component similarities between drusen and the extracellular deposits of protein folding disorders suggest that the lesions in AMD may also be due to protein misfolding and/or aggregation [51,52]. In fact, the amyloid beta oligomers in drusen were characterized as nonfibrillar oligomers (i.e. aggregates) that are toxic to human RPE cells *in vitro* [53]. In other words, drusen, which are key features of AMD pathology, share several features with misfolded protein aggregates seen in other neurodegenerative conditions. Thus, as chaperone therapy has been demonstrated to ameliorate disease phenotypes in these neurodegenerative diseases, it might also be useful in AMD.

The treatment of other neuronal and age-related diseases with chemical and pharmacological chaperones provides the foundation for research into their applications in AMD [38,50]. Although, there is no clear target for chaperone treatment in AMD, increasing the expression of native molecular chaperones would likely help the cells respond to the stressed environment of the aging AMD macula. Moreover, chemical chaperones that help the RPE handle the accumulation of drusen components and lipofuscin or help photoreceptors prevent opsin aggregation may ameliorate ER-stress and stem AMD progression [54]. Less specifically, chemical chaperones with general protein folding interactions could help the AMD eye accommodate the range of stresses imposed on it. With more efficient chemical chaperone screens and a growing understanding of the molecular pathogenesis of AMD, there is growing potential for AMD chaperone therapy.

PROTEIN CONFORMATIONAL DISORDERS SIMILAR TO AMD

The plausible role of ER-stress in AMD is also supported by several AMD-like maculopathies, which are characterized largely by protein misfolding. Malattia Leventinese, also referred to as Doyme honeycomb retinal dystrophy, is a hereditary form of macular degeneration caused by a missense mutation (R345W) in FBLN3, the gene encoding protein fibulin-3 (EFEMP1). The disease is characterized by drusen formation in early adulthood that progresses to form a honeycomb pattern. Inefficient secretion and accumulation of EFEMP1 leads to protein aggregation in the ER and retinal cell death [55]. Interestingly, a subset of AMD patients is known to have mutations in the fibulin-5 and fibulin-6 genes; and just as mutations in fibulin-3 produce protein misfolding, the fibulin-5/6 mutants found in these AMD patients may also be prone to misfolding [56,57].

Late-onset macular degeneration is an autosomal dominant condition resembling AMD that features a thick extracellular sub-RPE deposit. The disease is caused by a mutation in C1QTNF5, a short-chain collagen gene, which results in accumulation of the protein in the ER, preferential degradation by proteasomes, and interactions with the membrane-type frizzled related protein [58].

The autosomal dominant Stargardt-like macular dystrophy is yet another AMD-like disease with protein misfolding implications. The disease is attributed to a mutation in the ELOVL4 gene, a member of the fatty acid elongase family. Karan et al. found that HEK293 cells transfected with mutant ELOVL4 resulted in altered ELOVL4 protein localization, aggregate formation, and an elevated UPR stress response compared to wild-type ELOVL4 cells. They also found that mutant ELOVL4 could suppress wild-type ELOVL4 expression and hypothesized that the mutant proteins effectively inactivated wild-type expression by sequestration to a non-ER compartment [59].

Pharmacological chaperones have been reported to promote protein folding associated with another photoreceptor degeneration disease, retinitis pigmentosa (RP). In RP, as in AMD, photoreceptor degeneration is the ultimate cause of blindness. Nearly 70% of RP patients are associated with a mutation in the rhodopsin gene that makes the protein prone to aggregation and a target for degradation by the ubiquitin proteasome system [60]. The most common of these RP-causing proteins is the P23H mutant of opsin. Recently, Kaushal and colleagues demonstrated that the pharmacological molecule 11-cis-7-ring retinal, the inverse agonist to opsin, rescues the P23H mutant. They found that pharmacological chaperone therapy enabled the mutant protein to form pigment, acquire mature glycosylation, and localize to the cell surface [54]. Noorwez et al. then showed that 9- and 11-cis-retinals could also act as pharmacological chaperones for P23H rhodopsin mutants expressed in HEK-293 cells. The group proposed that these retinals act as pharmacological chaperones by binding the native P23H opsin and stabilizing it or by binding non-native folding intermediates and acting as scaffold to increase the rate of conversion to subsequent folding states. These properly folded P23H opsin proteins thereby avoid ER-associated degradation and are adequately transported to the Golgi apparatus for proper localization [61]. Another study reported that the P23H mutant accumulates in perinuclear inclusion bodies in transfected P661W photoreceptor cells. However, cotransfection with plasmid constructs of Hsp70, β -synuclein, or γ -synuclein chaperones decrease the number of inclusion bodies [62].

In short, the presence of several inherited AMD-like maculopathies attributable to misfolding and accumulation of mutated proteins indicate that protein accumulation and/or ER stress are capable of causing AMD-like pathology. Moreover, the demonstrated ability of chaperone therapy to decrease protein accumulation in another photoreceptor degenerative disease, retinitis pigmentosa, suggests that chaperones should also be investigated in AMD.

IDENTIFYING CHEMICAL AND PHARMACEUTICAL CHAPERONES

We have discussed thus far that protein misfolding, protein aggregation and ER stress are implicated in a multitude of disease states, including possibly AMD, and that chemical and/or pharmacologic chaperones enhance the ability of cells to handle such stress. This leads to the important question of how investigators might go about identifying putative chemical or pharmaceutical chaperones. As we discuss screening and testing for chaperones, we will identify challenges and potential solutions for identifying putative chaperone therapies for diseases such as AMD where the pathology involves ER-stress, rather than a specific, targetable misfolded protein.

Protein folding is a dynamic process whereby a nascent protein passes through several partially folded stages during and after translation, until it (hopefully) achieves its native conformation. Naturally, some of these partially folded intermediates are more or less like the native, i.e. properly folded, protein. It is thought then, that chaperones might operate by preferentially binding the native-like intermediates to stabilize them and shift the equilibrium away from unfolded intermediates and toward the functional conformation [19]. In fact, most pharmacological chaperones identified to date are competitive inhibitors/antagonists of the protein in question. Logically, since an appropriately formed active site generally requires a properly folded protein, inhibitors/antagonists at these active sites are likely, in the ER, to preferentially stabilize native proteins and native-like intermediates [20,21]. This potential for competitive antagonists/inhibitors to act as chaperones creates a role for high-throughput analysis to rapidly screen large libraries of compounds for inhibitors that could be potential pharmacological chaperones. Such screening has been successful in identifying pharmacological chaperones in lysosomal disorder and Gaucher disease studies [63,64].

Unfortunately, this high-throughput screen for pharmacological chaperones described above assays for inhibitors of a target protein and would therefore not be suitable for AMD, since no single misfolded protein has been identified in AMD. Strategies for identifying chaperones for AMD therapy should focus instead, upon chemical chaperones which have the ability to facilitate folding of a broad range of proteins, rather than pharmacological chaperones which promote folding of a specific protein. To date, there is one report of a high-throughput screen for chaperone compounds that does not seek inhibitors of a particular protein and thus might be modified to search for chemical chaperones. Since aggregation of Htt-103Q-EGFP (*huntingtin* gene with extended polyglutamine tract) in yeast cells results in cytotoxicity, poor cell growth, and maintenance of the fusion protein at low levels, Zhang and his colleagues assayed a library of 16,000 compounds for the ability to increase yeast growth or increase Htt-103Q-EGFP expression. They then tested whether the hit compounds decreased Htt-103Q aggregation in brain slices from transgenic Huntington's disease mice, and then tested one compound in a *Drosophila* model of Huntington's disease [65]. We hypothesize that a similar high-throughput screen might also identify chaperones for testing in ER-stress diseases such as AMD. Of course, while some of the compounds that initially show increased yeast growth or increased Htt-103Q-EGFP expression may be specific to the huntingtin protein, it is possible that a few of the compounds identified will be generalized chemical chaperones applicable to diseases such as AMD where there is no specific mutated protein to target. It may even be possible to decrease the number of protein-specific chaperones and increase the number of general, chemical chaperones identified by performing a sequential study using two or more different proteins, the Htt and some other aggregate-prone protein. Only those compounds that show chaperone-like behavior for both proteins might be considered for further study. Moreover, the small number of hit compounds identified might be assayed by an AMD-specific assay, such as evaluating for decreased ER stress in RPE and neuronal cell cultures or tissue from animal models of AMD.

NATURALLY-OCCURRING CHEMICAL AND PHARMACOLOGICAL CHAPERONES

Another interesting finding relevant to the discovery of novel chemical and pharmacological chaperones is the abundance of these substances in nature. Each organism, based upon the environmental conditions in which it resides, predator-prey relationships, life cycle, and a host of other factors, is subject to unique stressors. These stresses may, at times, produce ER stress-like conditions, providing evolutionary pressure for the susceptible species to develop chemical or other means to counter such ER stress. Studies to date have, in fact, found several chemical chaperones amongst the chemical substances that particular organisms use to prevent generalized protein misfolding and aggregation during times of stress. TMAO is one example. TMAO, an osmolyte that accumulates in tissues of marine organisms such as the shark that concentrate urea to balance osmolality and provide buoyancy in seawater, has been shown to protect these organisms from the destabilizing effects of urea on proteins [66]. Interestingly, this naturally occurring compound has demonstrated chaperone activities for protein misfolding disease [67,68].

The literature suggests that many of the naturally occurring compounds shown to hinder progression of AMD might operate through chaperone-like activities. As an example, consider the natural plant carotenoid, lutein/zeaxanthin. The Age-Related Eye Disease Study Group found that dietary intake of lutein/zeaxanthin decreases risk of neovascular AMD, geographic atrophy, and drusen [69]. Lutein/zeaxanthin's antioxidant effects might be implicated in its therapeutic effects. In addition, however, the benefits of lutein/zeaxanthin might be attributable to their chaperone-like functions: lutein/zeaxanthin are naturally occurring plant carotenoids, have been shown to be critical for proper folding of light-harvesting complex proteins of photosynthetic plants [70]. Next, consider the green tea component, epigallocatechin gallate (EGCG), which has been shown to attenuate retinal degeneration induced by oxidative stress [71]. As discussed above, accumulated damage from oxidative stress is a source of ER stress and has long been considered an important player in the pathogenesis of AMD and other aging processes [11]. One might suggest, then, that EGCG ameliorates retinal degeneration by decreasing ER stress, perhaps by promoting proper folding of proteins. Though this may seem like quite a leap in logic, a study by Ehrnhoefer et al recently demonstrated that EGCG blocks the misfolding and aggregation of polyglutamine-containing huntingtin protein *in vitro*, as well as huntingtin aggregation and cytotoxicity in yeast expressing polyglutamine-containing Huntingtin protein [11,72]. EGCG may therefore exert its beneficial effects in photoreceptor degeneration by ameliorating oxidative-stress induced ER stress. Similarly, vitamin E and omega-3 fatty acids, both of which are natural products that have been reported to have beneficial effects on AMD, decreases amyloid beta protein aggregation, thereby indicating that it might perform a similar anti-ER stress function in macular degeneration [7,73,74].

Taken together, these data suggest that several of the natural compounds already considered protective against AMD progression have been shown in studies to be chemical chaperones or, at the least, to ameliorate protein aggregation and ER-stress induced diseases. This reaffirms our hypothesis that AMD may be an ER-stress mediated disease and that chaperone compounds might prove useful therapeutically. More generally, the abundance of chemical and pharmacological chaperones in nature is useful in the discovery of new chaperone compounds. Indeed, the fidelity of protein folding is paramount to the survival of any biological system, and thus, it is not surprising that the chemical substances that living species utilize to prevent protein destabilization in times of stress might be employed towards human diseases characterized by ER stress and/or protein misfolding. In fact, molecular chaperones are some of the most highly conserved proteins across the Eukaryotic kingdom [15]. Those beings that live in extreme environments with unusual stressors may be especially likely to harbor such chemical chaperones. Moreover, the prevalence of natural compounds in already identified

chemical and pharmacological chaperones for disease underlines the importance of including natural substances in libraries screened for chaperone compounds.

EXPERIMENTAL END POINTS FOR DEMONSTRATING CHAPERONE ACTIVITY

Whether potential chemical or pharmacological chaperones are identified from literature, from nature, from high-throughput screening, or some other source, studies must confirm the chaperone activity of these molecules. A survey of the literature reports a broad spectrum of useful methods that most researchers employ in varying combinations to demonstrate chaperone activity of compounds.

Given that many of the diseases for which chemical and pharmacological chaperones are being investigated involve a mutated, misfolded protein, many studies evaluate whether a putative chaperone compound can increase activity of the mutated protein [75]. Since misfolded protein commonly is retained in the ER, forms aggregates, and/or is targeted for ER-associated degradation, some studies test for chaperone activity by determining whether a test compound improves proper cellular localization of a mutated protein. For example, De Almeida et al. evaluated for decreased ER retention of the HFE C2824 mutant by double confocal microscopy using HFE-C2824-GFP and immunofluorescence using antibody to KDEL, an ER-retention signal found in ER-resident proteins [32]. Other groups have utilized methods such as double immunocytochemistry to show proper cellular localization of mutant proteins. Okumiya et al. showed proper lysosomal localization of mutated alpha-glucosidase by double immunocytochemistry for alpha-glucosidase and wild-type beta-hexosaminidase, a lysosomal protein [76]. For plasma membrane proteins, proper localization can be assessed simply by confocal microscopy or flow cytometry [77].

Many investigators employ protein maturation studies to determine the effectiveness of putative chemical or pharmacological chaperones. These studies are based upon known differences between immature and mature processed proteins, which in many cases involve differences in the molecular weights of immature and mature proteins. For instance, since the immature, intermediate, and mature alpha-glucosidase are 110, 95, and 76 kD in size, respectively, Okiyuma et al. assayed by immunoblotting for increased levels of 76 kD alpha glucosidase after treatment with the test compound [76]. Some investigators also employ pulse chase studies to monitor protein maturation [78].

In addition to protein maturation studies, researchers have also employed existing knowledge about differential chemical properties of normal versus mutant, misfolded protein. For example, since myocilin mutant is less soluble in Triton than wild-type Triton, Yam et al. tested whether their test chaperones increased solubility of the Y473H mutant myocilin in Triton [33]. Similarly, since the delta-F508 cystic fibrosis transmembrane receptor (CFTR) mutants are more susceptible to trypsin digestion than wild-type CFTR, Maitra et al. used decreased trypsin digestion as a surrogate for improved folding of the delta-F508 CFTR after treatment with a putative chemical chaperone [79].

Given that misfolded proteins are more likely to be sequestered by chaperone proteins, some studies assess for a decrease in the physical association of the mutant protein with the endogenous chaperone proteins after treatment with a test compound. This has been accomplished by confocal immunofluorescent microscopy with antibodies against the mutant protein and against chaperone proteins, as well as by immunoprecipitating for the mutant protein and then western blotting the precipitate for a chaperone protein (or vice versa) [33].

All of the experimental methods described above require that a mutated, misfolded protein that can be assayed for improved localization, function, decreased aggregation, etc. has been identified. So the question arises, then, of how one might evaluate potential chaperones for diseases, such as AMD, which potential involves ER stress, but not by any singular misfolding protein. Indeed, if most wild type proteins are well managed by the ER, it might be difficult to show chemical/pharmacological chaperone-mediated improvements in a protein that was already processed in the ER with high efficacy. But what if we can find certain proteins that, even in their wild type form, exhibit inefficient ER processing – might it be easier to show increased expression or improvements in the function and localization of these proteins after chaperone treatment? Schubert et al. reported that up to 30% of newly synthesized proteins in a variety of cell types never attain their properly folded, native structure [80]. They go on to speculate that some proteins may be predisposed to misfolding, perhaps due to their structure and inherent difficulties in folding. Indeed, there are reports of several proteins that, even in their normal forms, are particularly less adept at achieving proper folding and trafficking. These include the rat luteinizing hormone receptor and human delta opioid receptor and several other G-protein coupled receptors [81,82]. If this is the case, it might be possible to examine whether treatment with a potential chaperone compound improves protein folding, maturation, and trafficking and/or inhibits protein aggregation or degradation of one or more of these generally poorly processed proteins. Moreover, with regard to AMD, if inefficient trafficking is common amongst G-protein coupled receptors, it is possible that opsin, a membrane bound G-protein coupled receptor, also achieves native conformation and appropriate cell surface localization at low rates. If so, since opsin is highly expressed in the retina, it may be particularly well suited for the evaluation of potential chaperone compounds for AMD. In fact, in their study of retinal derivative chaperones for the P23H opsin mutant, Noorwez et al. found that addition of the these chaperone molecules resulted in a 1.2 to 1.3-fold increase in the total amount of wild-type opsin in cells [61]. Additionally, proteome comparison of AMD and non-AMD eyes identified 26 proteins with significant expression change [16]. These also might serve as targets to measure the effectiveness of chaperone treatment on protein expression. To date, our review of the literature did not find any such experimental approaches for evaluating chaperone compounds specifically for AMD.

In general however, studies of chemical and pharmacological chaperones for diseases characterized by ER stress but no single misfolded protein – such as, ischemic injury, diabetes, and perhaps AMD – can demonstrate chaperone activity of test compounds indirectly. Studies of chaperone compounds for diabetes and ischemia rely largely on demonstration of improvement in the clinical endpoint – for example, smaller infarcts and improved neurological status after ischemic brain injury or improved glucose regulation and insulin sensitivity in mice predisposed to diabetes [31,83,84]. Another commonly, often concurrently, employed approach is demonstration of decreased ER stress after treatment with a chaperone compound. The rationale here is that by improving protein folding, chaperones may alleviate ER stress. This method is particularly attractive, again, for ER stress diseases such as AMD where there is no mutated, misfolded protein to assay for improved protein folding, maturation, localization, and function.

Since the UPR responds to the level of ER stress, one way to determine whether a potential chaperone decreases ER stress is to determine whether the UPR is attenuated. One way that this has been done is by Western blotting for p-PERK or p-IRE1, both of which are phosphorylated in the early stages of UPR induction. Other possibilities include western blotting and/or RT-PCR for proteins activated or upregulated by the ER stress response, such as GRP78, GRP94, CHOP, and p-eIRFalpha [31-33,83,84]. Since the ER stress response induces ubiquitin-proteosomal degradation of misfolded proteins, Maitra et al. demonstrated by coimmunoprecipitation of delta-F508 CFTR and ubiquitin, that doxorubicin decreases delta-F508 CFTR proteasomal degradation [79]. Still others have employed nuclear

fragmentation assays such as Hoechst 33258 stain and TUNEL assay to demonstrate decreased apoptosis after treatment with a chemical chaperone [33].

Another parameter that is common assessed, especially in diseases such as AMD that are characterized by increased ER stress without a single identifiable mutated protein is tolerance to known ER stress-inducing agents. In the evaluation of 4-PBA for cerebral ischemic injury, Qi and Hosoi et al. supplemented *in vivo* studies with *in vitro* studies demonstrating decreased cell death with 4-PBA treatment in neuroblastoma cell lines exposed to known causes of ER stress such as hypoxia/reoxygenation and tunicamycin [83,84]. Similarly, Ozcan et al. evaluated whether TUDCA or 4-PBA attenuate ER stress response activation in hepatoma cells treated with tunicamycin [31].

EXPERT COMMENTARY & FIVE-YEAR VIEW

A common pathogenic mechanism shared by age-related disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and AMD may be altered protein homeostasis by ER stress, which leads to protein misfolding and aggregation. We recently reported the altered chaperone protein profile in an AMD animal model and hypothesize that enhancing chaperone activity might be one therapeutic strategy for the treatment of AMD. Although the study of the role of ER stress in AMD has only recently begun, the concept of AMD as an ER-stress related disease is quite plausible. Indeed, AMD is a multifactorial disease in which several of the known pathological processes induce ER stress. With the increasingly rapid progress in the identification of AMD-associated genes and of the proteins in AMD lesions, we predict that in the next five years the aggregated proteins in AMD lesions will be identified more specifically and that the pathways involved in the aggregation will be unveiled. If those molecules are discovered, high throughput screening using chemical libraries will provide a pipeline of potential pharmacological chaperones for testing in animal models. Otherwise, screens for chemical chaperones that promote folding of proteins in general might be employed to identify potential therapeutic compounds. Naturally occurring compounds will draw particular attention due to the requirement of long-term management of AMD. Further understanding of the biochemical mechanisms involved in protein misfolding and aggregation may optimize the planning of common therapeutic approaches for protein conformation diseases. The strategies would focus on reversal of protein misfolding, blockage of protein oligomerization, interference with the action of toxic proteins, and amelioration of ER stress. A remaining obstacle is the lack of ideal animal models with proteomics profiles similar to those in human AMD lesions. Selection of specific endpoints reflecting protein misfolding is another key issue to resolve.

KEY ISSUES

- AMD pathology and proteomics suggest a potential role for ER stress in AMD pathogenesis.
- AMD shares features with ER-stress and protein folding disorders, including other neurodegenerative diseases and retinopathies
- Molecular chaperones represent one of the cell's tools for countering ER stress
- Enhancing chaperone activity might be one therapeutic strategy for AMD.
- Chemical chaperones are effective treatments in some ER stress and protein folding disorders.
- Natural compounds have proven effective chemical chaperones.
- Specific *in vivo* experimental end-points to determine chaperone activity are needed.

- Chemical chaperones warrant investigation in AMD.

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Figure 1.