REVIEW

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An Insight on α-crystallin Interactions with Various Proteins in Systemic Disorders

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αA- and αB- crystallins are the two principal components of the α-crystallin family of heat shock proteins which exhibit chaperone activity as well as cyto-protective function. It is well known that α-crystallin binds to misfolded or unfolded proteins and prevents their aggregation. The interactions of various proteins, such as methionine sulfoxide reductase A (MsrA), galectin-related interfiber protein (GRIFIN), histones and creatine kinase enzymes with α- crystallin may be deduced from their changes in abundance in the cell. The alterations in the abundance of histone proteins with a loss of normal chaperone function of α-crystallin suggest their importance in the biochemical mechanisms of hereditary cataract formation. Various proteomic and mass spectrometric methods have been utilised to elucidate the relationships between α-crystallin chaperone function, substrate binding and retinal disorders such as hereditary cataract, retinal neurodegenerative diseases and other systemic disorders and inflammation. A special emphasis on such interactions and in vivo protective roles of α-crystallin, under normal and pathological conditions, may highlight the potential of crystallins as therapeutic agents.

Key words: a-crystallin, misfolded protein aggregation, protein interactions, chaperone, systemic disorder

α-crystallins are one of the prominent members of the heat shock protein family and composed of two subunits αA and αB which are not only expressed in the retinal epithelial cells (αA) but also in the liver, spleen, muscular tissues (αB) (Kannan *et al.*, 2016). Misfolded protein aggregation is a leading cause of blindness worldwide. α-crystallins bind to misfolded or unfolded proteins and prevent their aggregation, thereby exhibiting a protective role in eye disorders (Chebotareva *et al.*, 2015, Budnar *et al.*, 2022). Such aggregated proteins are also responsible for causing a number of retinal neurodegenerative diseases that may induce retinal cell death (Mueller *et al.*, 2015, Piri *et al.*, 2016). The overexpression of both αA- or αB- crystallin subunits in the lens epithelial cells confers resistance against various forms of stress such as oxidative, photochemical and thermal stresses (Shin *et al.*, 2009, Hejtmancik *et al.*, 2015, Piri *et al.*, 2016, Kim *et al.*, 2020). Mutations in α-crystallin genes such as Crystallin Alpha A (CRYAA) or Crystallin Alpha B (CRYAB), expressing αA and αB are responsible for hereditary cataracts in humans (Khoshaman *et al.*, 2017, Caporossi *et al.*, 2021, Yu *et al.*, 2021).

The presence of a protein responsible for cataract, apart from α-crystallin is methionine sulfoxide reductase A (MsrA), which repairs protein methionine sulfoxide oxidised (PMSO) proteins. *In vitro* membrane filtration assay detected the interactions between galectin-related interfiber protein (GRIFIN) and α- crystallin which was enhanced by physiological concentrations of ATP (Barton *et al.*, 2009). The ability of α-crystallin to interact with a wide variety of proteins involved in signalling and cytoskeletal structure has been demonstrated as well. In a recent study, the presence of a certain peptide αA66-80 in the chaperone site of αA- crystallin was found to interact with α-crystallin sequences and promoted the aggregation of proteins by formation of insoluble protein peptide complexes through transient intermediates (Kannan *et al.*, 2013); however the mechanisms underlying the generation of the peptide in lens and how it causes aggregation of the protein are yet to be elucidated. Similarly, the interaction of α-crystallin with a number of substrates such as actin, filensin, creatine

kinase B, phosphoglycerate mutase, spectrin, βA3/A1-crystallin, γD-crystallin, gelsolin, calpain etc. were found to be related to hereditary human cataract formation, as determined from their respective increased or decreased associations (Wang and Spector, 1996, Barton *et al.*, 2009, Banerjee *et al.*, 2011, Andley *et al.*, 2014, Hamilton *et al.*, 2020). An increased abundance of histones or hemoglobin with a loss of chaperone activity of crystallins suggested the roles of these proteins in biochemical mechanisms of hereditary cataract formation (Feser *et al.*, 2010, Andley *et al.*, 2020, Hamilton and Andley, 2018). In some disorders such as Parkinson's disease, Lewy bodies are known to be a pathological hallmark. αB- crystallin, a small heat shock protein was found to be co-localised with α-synuclein in Lewy bodies and thus acted as an inhibitor of α-Syn amyloid fibril formation in an ATP-dependent manner *in vitro* (Waudby *et al.*, 2010, Guo *et al.*, 2022). An analysis of various protein interactions of α-crystallin with cytoskeletal proteins or those responsible for signalling may pave the way for drug targeting. α-crystallins and their functional peptides have shown significant favourable effects against several ocular diseases and thus their targeted delivery to the tissues would have a great therapeutic benefit (Nagaraj *et al.*, 2016, Phadte *et al.*, 2021). However, in some cases, crystallins were found to function as disease-causing proteins; these contradictory functions could be considered carefully prior to their therapeutic approach.

Role of interaction of α-crystallin and Methionine sulfoxide reductase (MsrA) in restoration of chaperone activity

Apart from α-crystallin, methionine sulfoxide reductase A (MsrA) is also responsible for lens cataract formation, along with the reparative role of protein methionine sulfoxide (PMSO). The overexpression of MsrA in lens epithelial and fibre cells protects the epithelial cells against oxidative stress, while its deletion makes the lens more susceptible to oxidative stress (Brennan *et al.*, 2009). The accumulation of MsrA increases with age and is responsible for age-related cataracts (Brennan *et al.*, 2009, Fort and Lampi, 2011, Koteiche *et al.*, 2015). As methionine is easily susceptible to oxidation by almost all forms of reactive

oxygen species (ROS), it leads to PMSO formation (Brennan *et al.*, 2009, Sreekumar *et al.*, 2011). PMSO formation is responsible for significant changes in protein structure, loss of regulatory function or chaperone activity. The loss of chaperone activity contributes to protein aggregation, resulting in cataract development and other associated diseases. MsrA protein consists of two specific enzyme activities and four separate enzymes, MsrA, MsrB1, MsrB2, MsrB3 (Kim and Gladyshev, 2004, Lim *et al.*, 2022). MsrA is selective for the reduction of S-epimers of PMSO while MsrB is specific for R-epimers arising from random symmetrical oxidation of methionine (Brennan *et al.*, 2009).

In case of rat hereditary cataracts, the substitution of methionine 68 in αB- crystallin with a less hydrophobic residue (Thr) leads to a loss of chaperone activity, thus providing evidence that methionine oxidation plays a key role in cataract formation (Kim *et al.*, 2007). The interaction of MsrA with α-crystallin in lens cells *in vivo* was also examined. The deletion of MsrA in mice leads to the oxidation of methionine 68 to PMSO in the eye lens, thereby highlighting the essential role of MsrA in the maintenance of lens α-crystallin chaperone function (Kim *et al.*, 2007, Brennan *et al.*, 2010).

Cyanogen bromide (CNBr) cleavage experiments showed that oxidation of α-crystallin with HOCl oxidised methionine 138 of αA- crystallin and methionine 68 of αB- crystallin, which was confirmed by mass spectroscopy analysis (Brennan *et al.*, 2009). Methionine oxidation reduced the ability of α-crystallin to protect lysozyme against chemical denaturation. The MsrA-mediated α-crystallin PMSO repair was independent of DTT reducing system (Sreekumar *et al.*, 2011). But each method demonstrated a different range of repair, ranging from approximately 50% by CNBr cleavage experiments to 60% by chaperone measurement to almost greater than 90%, which is determined by mass spectra analysis. Though the exact reason for such a disparity was unknown, differences in sensitivity of all three methods may have been a possible reason, which thus confirmed MsrA repair (Brennan et al, 2010). The interactions of α-crystallin with MsrA suggested that crystallin could be a major

target for MsrA in lens, though α-crystallin interacts with many other proteins in the cell in vivo.

Therefore, MsrA possesses the ability to interact and repair the chaperone activity of α-crystallin, which is lost upon methionine oxidation. A loss of MsrA activity could lead to cataract formation and other age-related oxidative stress associated disorders such as desmin-related myopathy, Lewy body disease, Parkinson's disease, Alzheimer's disease (Renkawek *et al.*, 1994, Andley *et al.*, 2008, Andley *et al.*, 2018).

Interactions between GRIFIN and α-crystallin

A number of lens structural proteins had been demonstrated to interact with α-crystallin with the aid of *in vitro* membrane filtration assay experiments. The unique protein, GRIFIN, a 32kDA homodimer of 16kDa subunits, has been identified in the eye lens (Ogden *et al.*, 1998, Barton *et al.*, 2009). GRIFIN is related to the galectin superfamily of proteins and lacks lactose binding activity due to sequence divergence at two positions (N48K and R72V), a requirement for β-galactoside binding (Hirabayashi *et al.*, 1991). Galectins are basically known to play important roles in mediating the cellular interactions with extracellular matrix elements and are crucial for the proper cell elongation and suture formation during lens development (Bassnet *et al.*, 1999, Hughes, 2001).

Interactions between α-crystallin and GRIFIN were found to be dependent on physiological concentrations of ATP; an approximately 5-fold increase in the level of binding of GRIFIN to αA- crystallin was observed in the presence of 3mM concentration of ATP (Biswas *et al.*, 2004, Barton *et al.*, 2009). The ATP levels are of significant importance in modulating the α-crystallin-GRIFIN interactions, as it induces the exposure of hydrophobic sites and stabilises the α-crystallin structure (Palmisano *et al.*, 1995, Muchowski *et al.*, 1999). Since GRIFIN comprises about 0.5% of the water-soluble lens proteins, not much is known about its physiological role in the lens. It may be hypothesised that interactions between α-crystallin and GRIFIN are useful for facilitating efficient protein packing in the concentrated lens cytoplasm (Delaye and Tardieu, 1983, Ogden *et al.*,

1998). It has been observed that lens growth and suture formation were considerably attenuated in α-crystallin deficient mice (Boyle *et al.*, 2003, Barton *et al.*, 2009). Further *in vivo* studies are required to elucidate whether α-crystallin can interact with other members of the galectin family, along with and their biological relevance.

In vivo protein-protein interactions of α-crystallin subunits

Numerous studies have examined the chaperone activities of both subunits of α-crystallin (Figure 1), but very little is known about their *in vivo* protein-interactions and their protective mechanisms. Figure 1 elaborates the summary of α-crystallin interactions with other proteins in human physiology, emphasising the role of α-crystallin as a molecular chaperone.

A number of proteomic and mass spectrometric methods have been utilised to elucidate the interactive relationships of α-crystallin with substrate binding, chaperone activity and structural proteins (Andley *et al.*, 2014). A 2D-DIGE analysis of 2-day old wild type lens of mice, αA-R49C heterozygous mutant and αA-R49C homozygous mutant, identified a number of proteins which showed an altered abundance in the mouse lens. The mutant heterozygous lens showed a 15-fold higher abundance of crosslinked αA- crystallin, a 3-fold increased amount of more acidic αA- crystallin along with a 2.6-fold higher abundance of degraded αA-crystallin (Andley *et al.*, 2014). A significant decrease in abundance of actin (15.6-fold) (Wang and Spector, 1996), GRIFIN (1.74-fold) (Barton *et al.*, 2009), γD-crystallin (6 fold) (Banerjee *et al.*, 2011), filensin (17.5 fold) (Djabali *et al.*, 2007, Andley *et al.*, 2014), βA3/A1-crystallin (6-fold) (Andley *et al.*, 2014, Frankfater *et al.*, 2020) established the fact that these proteins were likely to be *in vivo* substrates of α-crystallin. This was evident from the studies regarding their respective changes in their abundance at a young age even in the heterozygous lens, with no such change in lens morphology. These proteins may be structurally labile and interact with αA- or αB- crystallins for the conformational maintenance during the early stages of cell growth, but a mutation in the chaperone results in a greater association between the respective proteins

(Chang *et al.*, 2022, Khoshaman *et al.*, 2022). In the mutated αB-R120G lens, an altered abundance of β- or γ- crystallins and a degradation of phosphoglycerate mutase, a glycolytic enzyme important in metabolism, had been observed (Liu *et al.*, 2006).

At the same time, αA-R49C mutant knock-in lens showed an increased amount of αA-crystallin along with an increased amount of cytoskeletal proteins, encompassing α-spectrin, filensin, phakinin, tubulin, vimentin. The knock-in study suggested the role of these cytoskeletal proteins as *in vivo* substrates (Andley *et al.*, 2014, Haslbeck *et al.*, 2016). In 2-week old mutant lens, there had been an increase in the association of annexin proteins and αA-crystallins, elaborating a possible role in apoptosis. In neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, oxidation of some enzymes such as α-enolase, phosphoglycerate mutase, peptidyl-prolyl-cis-trans isomerase was observed with reduced enzyme activities (Renkawek *et al.*, 1999). The fact that mutations make the protein less stable may be suggested from the degradation of both αA- and αB- crystallins in αA-R49C and αB-R120G mutant lens. The changes in protein abundance and associations of crystallin proteins with such substrates may help to develop interventional strategies to prevent opacities of the future lens.

Interactions of α-crystallin with creatine kinase

The interaction of some enzymes such as creatine kinase B (CKB), phosphoglycerate mutase, may be significant for conformational maintenance during the early stages of lens growth, but become more stably associated with α-crystallin protein when it is mutated (Hamilton *et al.*, 2020). An isothermal titration calorimetry (ITC) analysis revealed that creatine kinase (CK) and α-crystallin form a stable complex *in vitro* and their interaction is of significant importance, owing to the upregulation of CK during cardiomyopathy (Diguet *et al.*, 2011). The interactions between CK and α-crystallin are likely to be hydrophobic and thus an abnormally high level of CK suggests a relationship with cataracts or other skeletal disorders (Hamilton *et al.*, 2016, Hamilton *et al.*, 2020). Various proteomic and immunoblot

analyses performed with CRYAA-R49C heterozygous mutant adult mice lens reported an increased *in vivo* CKB enzyme activity, suggesting that CKB might be an important early player in cataract development and other human diseases (Schlattner *et al.*, 2006, Andley *et al.*, 2014). Therefore, it may be assumed that CK is a useful parameter in the assessment of human disorders such as cataract and skeletal muscular ailments.
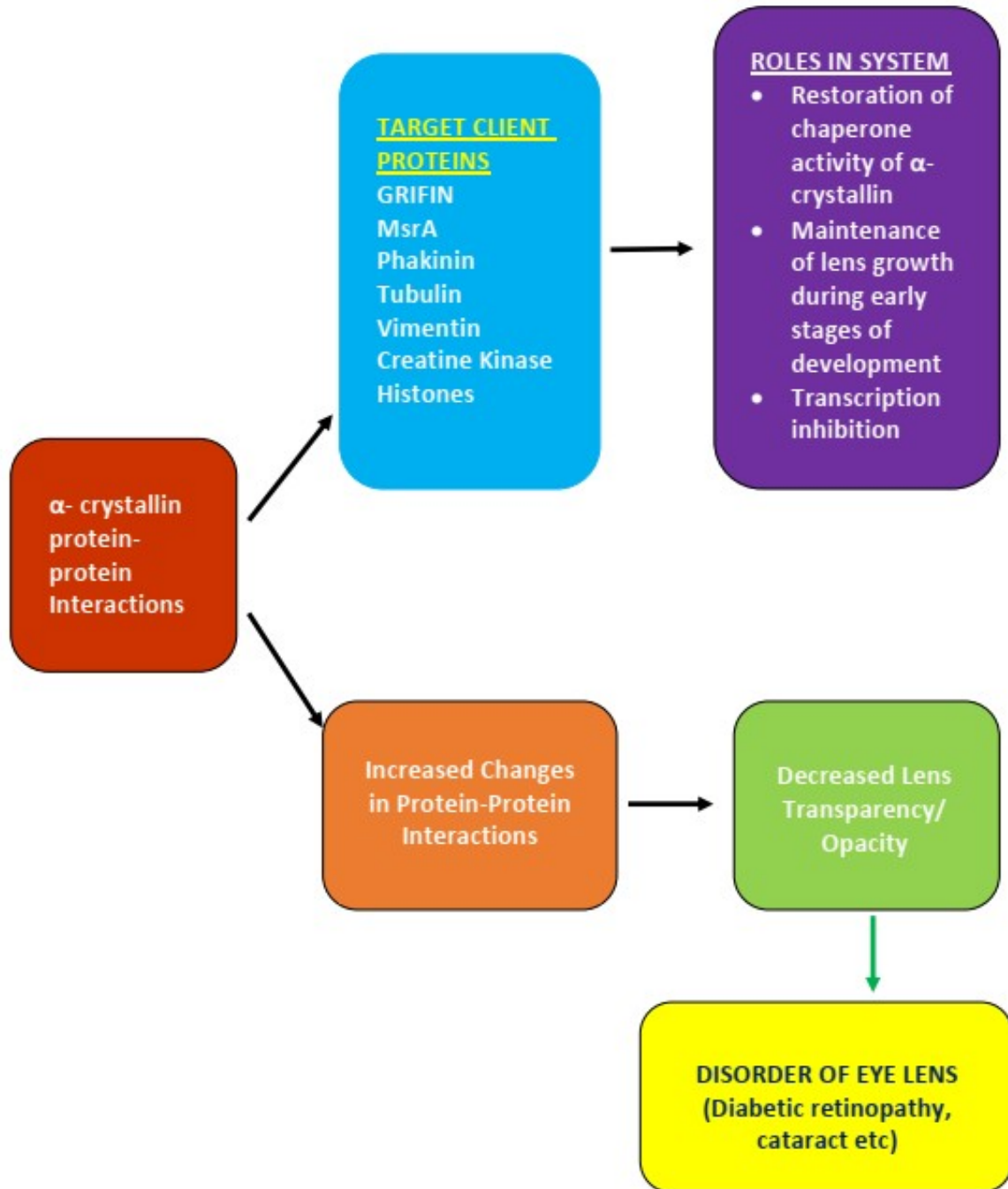

Figure 1. α-crystallin interacts with other proteins in the system which reflects its role as a molecular chaperone. Subsequent changes in such protein interactions leads to a decrease in lens transparency and disorders of the eye as well as of human body system.

Table 1. Interactions of α-crystallin with various proteins of the eye/ system. Such protein interactions hold a vital role in restoring the chaperone activity of crystallin or maintaining the lens conformation and thus taking control over the formation of many types of retinal or other systemic disorders of the living system.

Name of Protein Interacting with α-crystallin	Its Role In The Human System	Disorder of Eye/System caused due to changes in protein interactions	Reference
Methionine sulfoxide reductase A (MsrA)	Interacts with α-crystallin and restores its chaperone activity	Cataract caused due to protein oxidation from protein methionine sulfoxide (PMSO)	Kim & Gladyshev, 2004, Kim & Gladyshev, 2007,Brennan *et al.*, 2009,
Actin, GRIFIN, γD- crystallin, filensin, βA3/A1- crystallin.	Regulation of chaperone activity and maintenance of lens conformation	Hereditary cataract	Andley *et al.*, 2008, Andley *et al.*, 2014
Creatine kinase B, phosphoglycerate mutase	Interactions with crystallins maintain lens conformation during early stages of growth	Cataract, cardiomyopathy	Andley *et al.*, 2014, Hamilton *et al.*, 2020
Histone proteins in cell cytoplasm	Association with α-crystallin may serve as a inhibitor of transcription in cataract	Cataract, Inflammation, cancer or other related pathologies	Hamilton and Andley, 2020

Table 2. α-crystallin interacts with other proteins/molecules where its corresponding upregulation/downregulation may help to keep many retinal/systemic disorders under control.

α-crystallin Interactions	Disease in the System	Role of α-Crystallin In Disease Control	Reference
αA- crystallin with Toll-like receptor 4(TLR4)	Autoimmune uveitis	Upregulation of αA-	Rao *et al.*, 2008
αA- crystallin with VEGFR-1	Diabetic retinopathy	Upregulation of αA-	Zhu *et al.*, 2012
αB- crystallin with pro-inflammatory molecules	Multiple sclerosis/ischemia	Upregulation of αB-	Rothbard *et al.*, 2012, Fosu-Mensah *et al.*, 2019
αB- crystallin with VEGF-A	Cancer	Upregulation of αB- enhances cancer, so its expression is inhibited	Kase *et al.*, 2010, Qin *et al.*, 2014
αA-, αB- with caspase-3, Bax, Bcl-X(S)	UVA-induced apoptosis	Upregulation of both αA and αB, αA has better anti-apoptotic function than αB	Kamradt et.al, 2001, Kamradt *et al.*, 2005

In vitro Interactions of α-crystallin with Histones

In case of human cataractogenesis studies, an increased association of histone proteins with αA-crystallin was reported in mutant lens, revealing the possibility that histones were protected by both subunits of α- crystallin (Hamilton and Andley, 2018). However, little is known about the interactions between lens crystallins and histones.

Histones are best known for their primary function of packaging DNA into nucleosomes- the building blocks of chromatin, and may be involved in cancer, inflammation or other pathologies (Hamilton and Andley, 2018, Andley *et al.*, 2020). Recent studies suggested the functional relationship of α-crystallin with histones, though the specific role of histones in the lens is still under observation (Wolf *et al.*, 2013, Hamilton and Andley, 2018). The binding of histones with α-crystallins in mice lens expressing a mutant crystallin revealed that the

interaction occurs with a higher affinity. The stoichiometry of these two proteins suggested a greater interaction between the acidic groups of α-crystallin and the basic groups, such as lysines or histones (Wolf *et al.*, 2013). These interactions were not substantially altered under a high salt concentration, which suggested that hydrophobic, but not ionic interactions, might have a role in their association. Furthermore, ATP was found to affect the core domain of αB- crystallin which revealed the effect of ATP concentration on their interactions (Maki *et al.*, 2013).

A greater amount of histone transcripts in CRYAA-R49C mice suggested that an increased gene expression of histones might play an important role in cataractogenesis, though the role of α-crystallin as a modulator of histone expression is yet to be investigated (Burgess *et al.*, 2013, Maki *et al.*, 2013, Chen *et al.*, 2014). It might be possible that α-crystallin might interact with histones and serve as a transcriptional inhibitor. An overview of the various proteins interacting with α-crystallin and the role played by such interactions in the body system has been highlighted in Table 1.

Therapeutic potential of α-crystallin and its role in drug targeting

αA- and αB- crystallins possess chaperone activity i.e. they prevent non-specific protein aggregation of 'substrate proteins' *in vitro* (Nakata *et al.*, 2005). The expression of both subunits had been found to correlate with increased cellular survival in the presence of external stressors including etoposide, statosporine, hydrogen peroxide, TNF-α, or serum starvation/nutrient deprivation (Bova *et al.*, 1999, Rao *et al.*, 2008, Mueller *et al.*, 2015). An upregulation of αA- subunit may hold a vital role during retinal disorders, such as autoimmune uveitis or diabetic retinopathy (Table 2).

αB-crystallin inhibited the activation of microglia, suppressing its autophagy and in turn, reducing endotoxin-induced neuro-inflammation (Steeg, 2006, Chis *et al.*, 2012, Nollen *et al.*, 2017). Thus, it is a promising option for affecting microglial autophagy and reducing symptoms of certain ocular inflammatory diseases.

Recent studies have emphasised the use of timolol, a non-selective beta-adrenergic receptor antagonist drug administered for the treatment of glaucoma, hypertension and myocardial infarction. Timolol was found to possess amyloidogenic property, which was further evaluated by heat-induced denaturation studies of α-crystallin with timolol at different concentrations (Fosu-Mensah *et al.*, 2019, Prokai *et al.*, 2020). More than 50% induction of α-crystallin aggregation was observed at 60mM timolol, whereas Thioflavin T fluorescence assay exhibited a significant increase from ~100 to ~250, a characteristic of enhanced amyloid aggregation (Prokosch *et al.*, 2013, Nikbakht *et al.*, 2014, Fosu-Mensah *et al.*, 2019). Thus, the timolol-crystallin interaction was estimated to promote or trigger *in vivo* amyloid aggregation of α-crystallins. The following interpretation may be more useful in providing mechanistic insights to develop potential strategies in drug development against amyloid-related cataracts (Liao *et al.*, 2021, Yu *et al.*, 2021).

A recent study had even shown that an administration of 'mini α-crystallin' (MAC) was able to inhibit selenite-induced cataract in rats, along with lens epithelial cell apoptosis (Raju *et al.*, 2016, Islam *et al.*, 2022, Reddy and Reddy, 2022).

Since α-crystallins protect cells against undesirable consequences of cellular stress and protein denaturation, it is reasonable to highlight the fact that they can be used therapeutically (Horwitz 1992, Nagaraj *et al.*, 2016). An intravenous injection of α-crystallin was found to protect retinal ganglion cells against apoptosis. Such a therapeutic strategy showed an improved protection against heat and oxidative stress in lens epithelial cells. Detailed insights exploring the therapeutic use of α-crystallins are yet to be unleashed in near future.

Concluding remarks

α-crystallin has a chaperone-like ability to recognise and interact with denatured or unfolded proteins and prevent their aggregation. The interaction of α-crystallin with diversity of proteins under native *in vitro* conditions is of primary importance as they may serve as baselines for studying the effects of gene mutations or the cause of any ocular or systemic disorder. The functional

associations of α-crystallins with these substrate proteins are primarily non-covalent in nature and hydrophobic interactions require only a minor alteration on the protein surface of target proteins. Such hydrophobic interactions are common because proteins are mainly dynamic systems. MsrA plays a pivotal role in cataract formation as it may prevent the loss of chaperone activity of α-crystallin. Furthermore, an augmented concentration of CK or histone proteins may be indicative of the fact that CK is essential in cataract development, due to an increased demand for ATP, signifying the existence of a functional relationship between α-crystallin and CK. The molecular chaperone and anti-apoptotic activities of α-crystallin may unleash its therapeutic potential. The importance of α-crystallin interactions with other proteins may be relevant in eye disorders and even in drug discovery, which would facilitate the target of more specific sites on protein interface and disrupt such interactions. Such specific targets would assist novel pharmaceutical designing and unleash the significance of such protein- protein interactions, excavating new arena in human physiology.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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