

Function, Structure and Stability of Human Gamma D Crystallins: A Review

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Abstract The human γ D crystallins is one of the most abundant protein of the lens nucleus and it is believed that their main function is to help maintaining the optical properties of the lens during the life span. Human γ -D crystallins is a 173 residues protein that fold into two homologous domains (N-terminal and C-terminal), each containing two Greek key motifs. Although, this protein is extremely stable, over the years the protection mechanism loses efficiency and the protein accumulates damages, resulting in protein aggregation which is associated with cataracts formation. At present, cataracts is the main cause of blindness in the world and surgical removal of the lens remains the only treatment. This review summarizes the current knowledge on cataracts risk factors, *in vivo* studies, unfolding and inhibition associated with human γ D crystallins.

Keywords Human γ D crystallin • Cataracts • Blindness • Protein unfolding • Aggregates • Inhibition • Greek key motifs • Protein damage • Blindness • *In vivo* studies

Introduction

The main function of the eye lens is to produce the refractive power to focus the light onto the retina [1]. Lens is an avascular tissue composed of a single layer of epithelial cells, which has to maintain the transparency to allow the passage of light [2]. During the lens formation, the epithelial cells elongate to become fiber cells.

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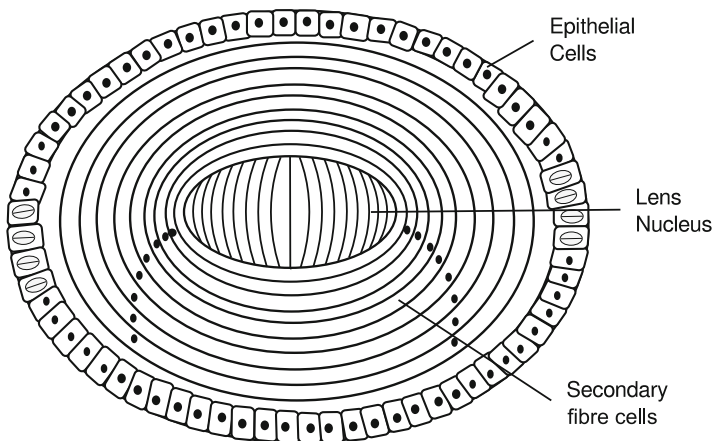


Fig. 1 Lens morphology. Lens nucleus is formed by the primary fiber cells, which differentiated from epithelial cells during embryogenesis. After the elongation, the organelles (*black dots*) are eliminated from the fiber cells to avoid light scattering

In this process of differentiation, the cells produce large quantities of proteins. After this elongation, in order to avoid any potential light scattering structure, the organelles are removed from the fiber cells by a programmed proteolytic process (Fig. 1) [3, 4]. Even though this process continues throughout life, it does at a minimal pace, and the older fibers are pushed to the center of the lens by the newer fiber cells (Fig. 1) [2]. Therefore, fully differentiated lens cells consist of a high concentration of proteins without nuclei or any other organelles and do not produce or degrade proteins [1]. Human lens has the highest protein content within the body [5] and, as a direct consequence of the missing repair mechanisms, the proteins in the lens have to be stable and soluble for the organism lifetime.

Most of the refractive power of the lens is conferred by high concentrations of three types of proteins: α , β and γ -crystallins, which count for more than 90 % of the proteins in the lens [6, 7]. In normal lenses, these proteins are found in concentrations higher than 300 mg/mL in order to have a high refractive index [6], and while they still scatter light, they do it in a specific manner. The correlation in the position of pairs of proteins reduces the scattering; this is due to the fact that the fluctuation in the number of protein molecules over a short space is small, resulting in transparency [8]. This is known as short-range spatial order. Any microscopic fluctuation will induce light scattering, these fluctuations could be produced by molecules of large molecular weight formed, for example, by protein aggregation or polymerization [8]. Such protein aggregation leads to the development of opacities known as cataracts.

Cataracts Risk Factors

The cataracts are an opacification of the lens of the eyes due to the accumulation of aggregates of crystallins. At present, cataracts formation is the main cause of blindness in the world [9]. The formation of cataracts is highly related to aging, and it is generally accepted that proteins modifications have to occur to promote the aggregation and then the cataracts. Some of the modifications that have been observed with aging, include oxidation of the tryptophans, cysteines and methionines [10], deamidation of arginines and glutamines [11, 12], glycosylation of lysine residues [13], and peptide fragmentation [14]. Most of these modifications are believed to be induced by environmental factors such as ultraviolet light exposure [15, 16], and oxidative stress [10, 17, 18]; the consequence for all of them is the formation of insoluble aggregates resulting in lens opacity and then loss of vision for cataracts (Table 1).

In the human eye, the cornea absorbs most of the UV radiation (240–400 nm) [27], and there is also low-molecular weight UV-filter molecules in the human lens, the most abundant are kynurenine, 3-hydroxykynurenine glucoside (3OHKG), and 4-(2-amino-3-hydroxyphenyl)-4-oxybutanoic acid O-glucoside, which absorb in the UV-B (280–330 nm) and UV-C ranges (100–280 nm) [28]. However, throughout the years, significant radiation reaches the lens, producing proteins UV radiation damage, which has been correlated mainly to structural changes in different residues that destabilize the proteins and accelerate their unfolding [16].

Table 1 Some of the risk factor and modification associated with cataracts

Risk factor	Modification	Damage	Reference
Aging	Deamidation	Introduce carboxylate anion, which induce ionic interactions that may lead to conformational changes in the protein	Stacy et al. [19]
	Methylation	Avoids disulfide bond formation preventing aggregation	Lapko et al. [20]
	Disulphide bonds	Formation of intra-chain disulfide bonds causing protein conformational changes Multimer formation by inter-chain disulfide bonds	Stacy et al. [19]
UV radiation	Photodamage reaction of tryptophan residues	Destabilization of the native structure of the protein	Borkman [21]
Mutations	R14C	Altered protein surface properties	Stephan et al. [22]
	P23T	Reduced solubility	Mackay et al. [23]
	R36S	Crystallisation	Kmoch et al. [24]
	R58H	Impaired protein folding	He et al. [25]
	W158X	Fourth Greek key motif	Gopinath et al. [26]

On the other hand, the oxidative stress becomes more important with aging, due to the decline of the levels of glutathione (GSH) and antioxidant enzymes as the superoxide dismutase and catalase activity [29, 30]. The decrease of GSH levels lead to crosslinking, aggregation, insolubility, and fragmentation of crystallin, which then result in the formation of cataracts [31]. Moreover, aging may also reduce the functionality of the chaperon α -crystallins to rescue the unfold β and γ -crystallins [32].

As well as aging, diabetes, malnutrition, hypertension and ionic imbalance have been correlated with cataracts formation. For instance, in diabetes, the hyperglycemia produced the diffusion of extracellular glucose to lens which produced then post-translational modification in the proteins of the lenses [33]. Also, the glucose is metabolized by the sorbitol-aldose reductase pathway and, as a result, the lens accumulates sorbitol. Then the sorbitol produces osmotic swelling, leakage of glutathione, myo-inositol changes in the membrane permeability, and generation of free radicals [34, 35]. Another factor, associated with cataracts is the ionic imbalance in the lens. An increase in the concentrations of calcium produces the activation of calpain serine-proteases that will degrade crystallins [36, 37], while a magnesium concentration decrease has been associated with an increase in the oxidative stress [38].

Finally, an intrinsically different group of cataracts are the hereditary cataracts which, unlike the other types, occur at early ages. The congenital cataracts are caused by a single-gene disorder that induces the substitution of one amino-acid, and the estimated frequency is between 1 and 6 per 10,000 of new born babies [39]. Some of these mutations are listed in Table 1; the mutation can cause protein insolubility, crystallization, changes in charge, or destabilization.

Even though there are several factors related to cataract formation, the only effective treatment is still surgical removal of the lens. This procedure has become routine in many clinics, but nevertheless cataracts remain as the main cause of blindness in the world. This is due to the fact that this surgical procedure is expensive and is often inaccessible in low and middle-income countries. Also, it has been argued that the artificial lens doesn't have all the optical properties of the "normal" lens [40, 41].

***In Vivo* Studies**

The use of *in vivo* studies allows to conduct essential investigations in the characteristic physiological environment of the disease, providing a deeper understanding of the mechanisms involved in the appearance and progression of cataracts. Therefore, several different animals models have been developed, capable of reproducing the characteristics of the disease seen in humans, mainly to study the different factors that give rise to cataracts and some potential treatment molecules. These studies would contribute decisively to the possible development of future preventive treatments.

Some of the earliest studies focused on radiation damage induced in lenses. Rabbit lenses were subjected to X-rays, and by electronic microscopy, it was possible to observe very high molecular aggregates in irradiated cortex while in normal cortex only relatively small aggregates were visible [119]. Later, some studies of UV radiation effects in rodents lenses were done, demonstrating by histological analysis that the first target tissue of UV radiation was the epithelium [42]. Also it was found that the level of glutathione (GSH) suffers modification [43]. With these types of experiments, it has been generally accepted that the long term effect of UV-radiation is cataract formation probably by protein damage [44].

Studies focusing in oxidative stress have made extensive use of specific mouse models with known alterations for oxidative damage, and were then able to reproduce some of the features of human aged related cataracts [45, 46]. For instance, the OXXYS strain exhibits premature aging and significantly shortened life span associated with oxidative damage, and therefore the status of the lens can be characterized due to the inherent over-generation of ROS (Radical Oxygen Species) [47]. In a different methodology, oxidative stress can be induced in rats through a galactose diet, and then induce the depletion of levels of GSH [48].

Cataract has also been studied in animal model using other inducers. Selenite has been commonly used, causing alteration of the epithelial metabolism, calcium accumulation, calpain-induced proteolysis, crystallins precipitation and cytoskeletal [49, 50]. Galactose-induced cataracts produce the accumulation of osmolytes causing ocular lens swelling of fiber cells and then disruption of the native state of lens proteins [51]. Cataracts have also been studied by using genetics mouse models [45, 52, 53].

In a different type of studies, the specific aim of using animal models has been to test routes of administration of compounds, like intraperitoneal [29, 30, 54], subcutaneous [55] or as a supplementary diet [56, 57].

Crystallins Family

Crystallins are the main constituent of the lens and are separated into three types in vertebrates: α , β and γ -crystallins [6, 7]. α -Crystallin is the predominant and biggest type of crystallins, with a molecular weight around 800 kDa [6]. It is formed by the non-covalent association of two subunits (A and B), in a molar ratio around 3:1 to 2:1 [58, 59]. Subunits A and B are homologue and form part of the small heat shock proteins family (sHSPs). Their secondary structure is primarily β -sheets [60], and the subunits seem to be in equilibrium between dissociating and re-associating constantly, which makes the protein a dynamic oligomer [61]. The main function of the native α -crystallin complex in the lens is its chaperone function [60, 62–65], but also, some experimental evidence suggests that α -crystallin is involved in remodeling and protection of the cytoskeleton, inhibition of apoptosis and the resistance to stress [66].

The β and γ -crystallins are members of the same family; they have 30 % sequence identity [67, 68], consisting mainly of β -sheets that form two domains each formed for two Greek Key motif. The β -crystallins are multimeric and contain N-terminal extensions, while the γ -crystallins are monomeric in solution [6]. The β -crystallins family is constituted by four acidic proteins (β A1, β A2, β A3, and β A4) and three basic (β B1, β B2, β B3) [69, 70]. The β B1, β B3 and β A4 crystallins are homo-oligomers [71, 72] while the β B2-crystallin forms dimers by domain swapping, and hetero-oligomers with β A1, β A2 and β A3-crystallin. The domain swapped dimer is thermodynamically unstable [67, 73], but has been proposed to act as a chaperon to stabilize or to assemble other β -crystallins [74]. The γ -crystallins are encoded by seven different genes, γ A, γ B, γ C, γ D, γ E, γ F, and γ S, all encoding proteins of about 20 kDa. Nevertheless, only the γ D, γ C and γ S crystallins are expressed at an appreciable concentration in human lenses [66].

Human γ D-Crystallin Structure and Function

The human γ D-crystallin (H γ D) is the second most abundant protein of the lens nucleus (Ji 2013), and it is believed that their main function is to help maintaining the optical properties of the lens during the life span. Consequently, the γ D-crystallins have evolved to be stable, soluble at elevated concentrations and damage tolerant.

H γ D is a monomeric protein with 173 amino acids. It is composed of two domains: N and C-terminal domain connected by a short linker (Fig. 2). Its structure has been resolved by X-ray diffraction [75]. It folds into two Greek key motifs with eight β -strands characterized by an unusual folded β -hairpin (Fig. 2).

Some of the features that seem to confer stability to the protein include six pair of aromatic residues distributed all over the protein (F11/Y6, Y16/Y28, Y50/Y45, Y92/Y97, Y138/y133, F117/F115) [76], a group of hydrophobic and polar residues in the inter-face of the two domains [77] and two “tyrosine corners”, a classic characteristic of the Greek key motifs [78]. This motif is formed in the N-terminal domain with tyrosine 62 and in the C-terminal domain with tyrosine 150, and it connects two β -strands by a hydrogen bond between the hydroxyl group of the tyrosine and a carboxyl group [79]. Interestingly, H γ D have six cysteines, more than any of the α and β -crystallins, but does not have any disulphide bond [80].

While the UV-damage protection is conferred by four buried tryptophan: W42 and W68 in the N-terminal domain, and W130 and W156 in the C-terminal domain, these tryptophan are highly conserved among vertebrates [6] and are important in the stability of the protein by dispersing the UV radiation [16, 81]. The conformation of the tryptophans seems to enable the lens to be a very effective UV filter, and their efficient quenching of the radiation provides an excellent mechanism to prevent photochemical degradation [82].

Although, this protein is extremely stable, over the years the protection mechanism loses efficiency and the protein accumulates damages. Some of the post-translational modifications of γ D-crystallins include deamidation of Q12, N49 and

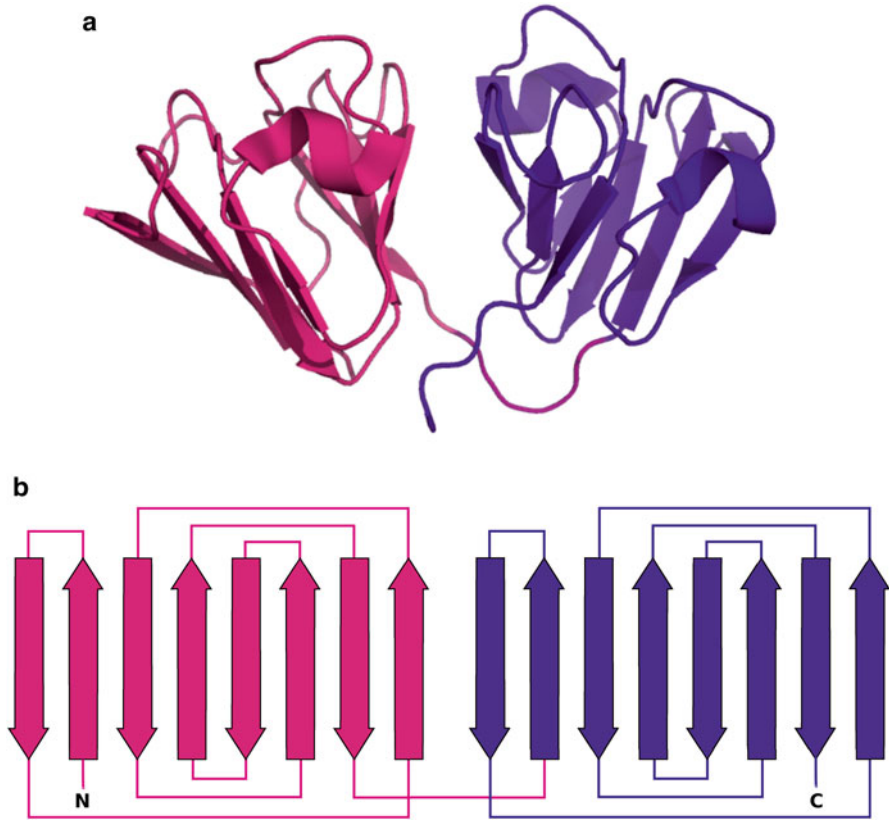


Fig. 2 HyD-crystallin structure. (a) Three-dimensional structure of HyD-crystallin (2KFB), drawn in Pymol. (b) Topology of the N-terminal domain (*pink*), and C-terminal domain (*purple*)

N160 [83, 84], Y45 and W156 oxidation [83, 85], C110 methylation [20], G1 carbamylation [20, 84], and C18-C32 and C108-C110 disulfide binding [11]. All of these major modifications become more important with aging, and are considered to be the first step in cataract development [12].

Unfolding: Aggregation

As in many other protein folding diseases, it is believed that a partially unfolded intermediate produces the formation of the aggregates that lead to cataracts. Consequently, the aggregation pathway for the HyD-crystallin has been extensively studied. By using equilibrium temperature and chemical unfolding, it was found that the HyD-crystallin is stable to 8 M urea and has a T_m close to 80 °C. In order to unfold the protein it was necessary to use GuHCl at 37 °C [86]. A very useful

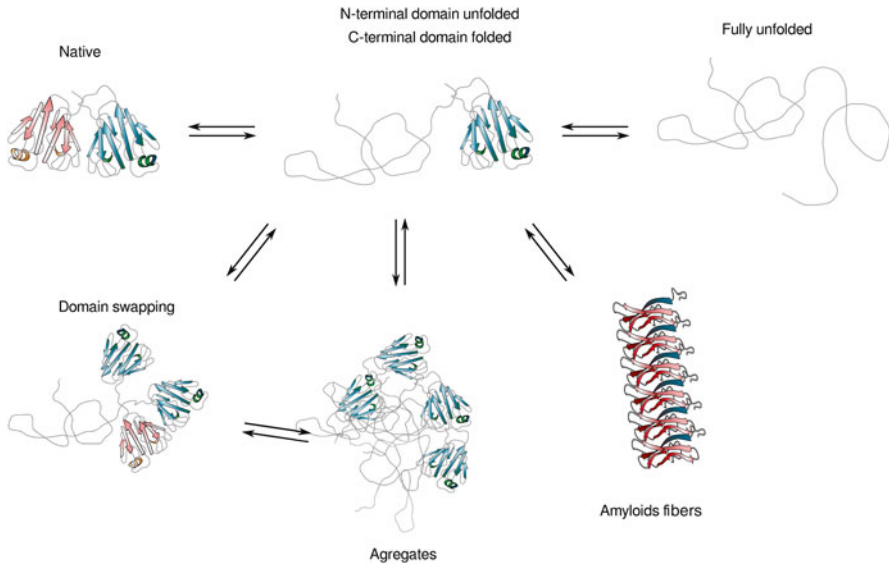


Fig. 3 Schematic of the HyD-crystallin unfolding and aggregation models

feature of the HyD is that the fluorescence is shifted between the native fold at 325 nm and the unfolded state at 348 nm. Mostly, the unfolded experiments were done going from a native conditions to a high level of denaturant, usually 5.5 M GuHCl at 37 °C. For the native protein the unfolding curve showed, apparently, a single major unfolding transition [86]; later it was found by kinetic analysis in triple mutants the existence of a partially unfolded intermediate [87], and it was confirmed that the native unfolding curve was better adjusted by a three state model [88]. The first unfolding event consists in the unfolding of the N-terminal domain, intermediately followed by the unfolding of the C-terminal domain. Even though the state with one domain structured and the other one unfolded is a short live state in the native, it has been proposed as a good candidate for the aggregation intermediate in the cataract formation (Fig. 3).

There has been several stability/unfolding studies of mutants proteins, mainly divided in two types: mutations to change the stability elements of the protein and mutations that mimic some protein damage observed in *in vivo* experiments. In the first group, there are reports for the mutations of the conserved aromatic pairs in the protein F11/Y6, Y16/Y28, Y50/Y45, Y92/Y97, Y138/Y133, F117/F115 [76], mutations of the four internal tryptophans W42, W68/W130/W156, mutations of residues in the protein interface (Q54, Q143, and R79, M147) [88] among others. Meanwhile, in the second group, most of the mutations evaluated have been a single mutation that has been reported in cataracts studies. For instance, the genetic studies of families exhibiting juvenile-onset cataracts R114, R38, and R36 [24, 25], possible glutamine deamidation sites Q54, Q143 [89], deletion mutant W156X [90], R140, A36 [91], among others.

Most of the mutations can be classified according to where the mutation occurred: the N-domain, the interface or the C-domain. In general, N-terminal domain mutations

shifted the first transition to lower denaturation concentration without affecting the C-terminal domain. The lower stability of the first unfolding event, in many cases, populated more this intermediate state and clearly presented a three unfolding curve [76], whereas mutation in the C-domain did not usually affect the first unfolding event or the shape of the curve. The mutations seem to destabilize the two domains in a more cooperative way [76].

Finally, the mutations in the interface produce a destabilization of the N-terminal and reveal a clear plateau in the unfolding curves [88, 89]. And, at the same time, they affect the N-terminal domain folding but not the C-terminal domain, suggesting that the interface could function as a scaffold for the folding of the N-terminal domain [88]. Many of these mutations studied would, consequently, effectively increase the life span of the partially unfolded conformer. Interestingly, many of the observed cataract point mutations *in vivo* cluster in the N-terminal domain.

It has been also studied the unfolding of the protein due to external factors. For instance, by using UV radiation, depending on the exposition time, protein aggregation has been observed [1, 92], as well as several damages from affecting specific residues to complete cleavage of the backbone [93]. It is worth to notice that out of the four internal tryptophan, it was reported that W42 is the more easily affected by UV radiations [94]. As a different external factor, the protein has been exposed to acidic pHs, and the formation of amyloid fibril was observed in these conditions (Fig. 3) [95].

Ultimately, the unfolding processes have been studied by molecular simulation *in silico*. In this type of studies, it has also been observed the preferential unfolding of the N-terminal domain, due to urea, temperature or mutations [96, 97]. Also it was proposed that the N-terminal domain stability is regulated by the inter-domain interaction. In simulations where the four internal tryptophan were changed to kynurenine simulating UV damage, it was found that both domain unfold faster and not sequentially. This suggests that, if the UV radiation is such that it makes this damage to any of the four tryptophans, the domain is going to unfold [16]. Also by simulation, the formation of a swap dimer was observed (Fig. 3) [96].

Inhibition

As mentioned before, cataracts is the main cause of blindness in the world [9], and while surgical removal of lens is effective, it is also expensive and is not available for all persons in developing countries. Hence, a search of a preventive treatment remains a key aspect in the research related to this disease.

Many different compounds have been studied in order to measure their anti-cataractogenic ability (Fig. 4). Several small molecules have been tested to prevent cataract formation due to external factors. For instance, caffeine was reported as an effective compound to prevent damage of lens by UV radiation [48]. The synthesis of bifunctional antioxidant with radical scavenging and chelation ability was found to delay the opacity of lenses exposed to gamma radiation and delay reactive oxygen species [56].

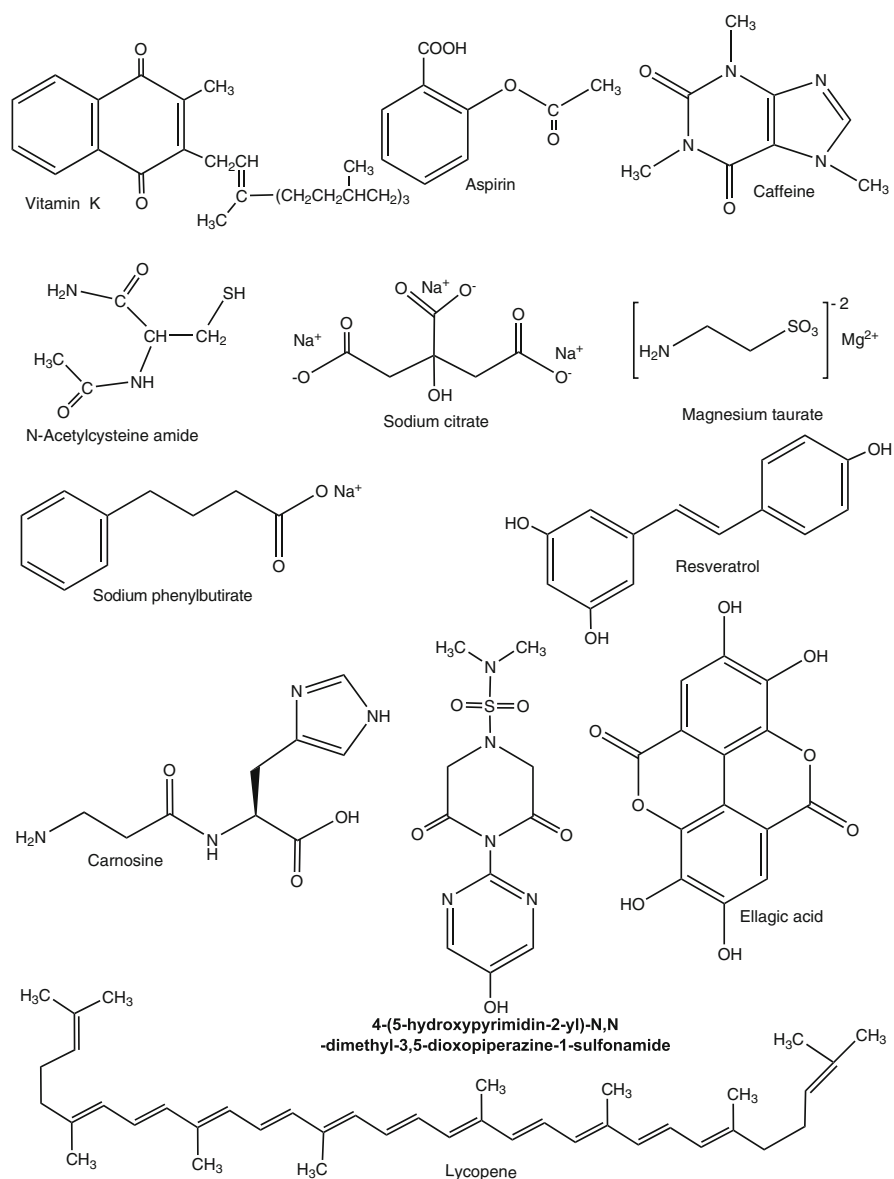


Fig. 4 Inhibitors. Chemical structure of some of the molecules that have been tested as cataract inhibitors

Nevertheless, most of the studies have focused in the inhibition of the factor responsible of producing cataracts. For instance, it has been reported that byakangelicin, the principal component of roots of *Angelica dahurica*, suppress sorbitol accumulation and galactose-induced cataract [98], citric acid inhibits the progression of cataracts in diabetics rat [99], and caffeine inhibits the oxidative stress in the lens [48].

In diabetic mouse models, there has been several reports of molecules studied to prevent cataracts. It was first tested the inhibitory effect of aspirin [100], then better results were obtained by combining aspirin and carnosine eye drops [101]. Vitamin K was showed to be effective to keep Ca^{2+} ATPase activity and lens calcium homeostasis resulting in an inhibition of diabetic cataractogenesis [57]. Similarly, magnesium taurate treatment to galactose diabetic rats showed its ability for correcting the lenticular Ca^{2+}/Mg^{2+} ratio [55]. Also, the aldose reductase has been a target for anti-cataract drug [31, 33].

There have been studies on inhibition of cataract formation by using several natural products, which are known to contain anti-oxidants (Table 2). The crude extract of plants *Ocimum sanctum* [110], *Ginkgo biloba* [112], and *Adhatoda vasica* [102], have been tested to inhibit the polyol accumulations, oxidative stress and aldose reductase activity in animal models. Flavonoid fractions of *Vitex negundo* present an increase effect in the enzymatic activity of superoxide dismutase, glutathione peroxidase and catalase [29]. Other molecules tested include flavonoids isolated from honey [113] and gallic acid [30].

Table 2 Plants tested to treat cataracts

Name of plants	Part of plant	Fraction	Mechanism of action	Reference
<i>Morinda cetrifolia</i> (Rubiaceae)	Leaves	Aqueous extract	Inhibition of aldose reductase	Gacche and Dhole [102]
<i>Foeniculum vulgare</i> (Apiaceae)	Fruit	Aqueous extract	Inhibition of aldose reductase	Dongare et al. [103]
<i>Momordica charantia</i> (Cucurbitaceae)	Fruit	Aqueous extract Ethanol extract	Antihyperglycemic effects in experimental diabetics	Rathi et al. [104]
<i>Cheilanthes glauca</i> (Adiantaceae)	Stems	Aqueous	Inhibits generated oxidative stress	Pastene et al. [105]
<i>Brassica oleracea</i> (Brassicaceae)	Flower	Flavonoids fraction	Inhibits selenite induced-cataracts	Vibin et al. [106]
<i>Emblica officinalis</i> (Phyllanthaceae)	Fruit	Aqueous extracts	Inhibits aldose reductase activity	Suryanarayana and Petrash [107]
<i>Angelica dahurica</i> (Apiaceae)	Roots	Ether extracts	Inhibits aldose reductase activity	Shin et al. [108]
<i>Vitex negundo</i> (Verbenaceae)	Leaves	Flavonoids	Inhibits oxidative stress	Rooban et al. [29]
<i>Emilia sonchifolia</i> (Asteraceae)	Leaves	Flavonoids fraction	Inhibits lens opacification and oxidative stress	Lija et al. [109]
<i>Ocimum sanctum</i> (Lamiaceae)	Plant materials	Aqueous extract	Inhibits aldose reductase	Halder et al. [110]
<i>Cochlospermum religiosum</i> (Cochlospermaceae)	Leaves	Isorhamnetin-3-glucoside fraction	Inhibits selenite induced-cataracts	Devi et al. [111]

Meanwhile, in studies *in vitro*, the search of cataract inhibition focuses in molecules that present an effect in the native γ -crystallin or the partially folded intermediaries. Making use of spectroscopic techniques, the ability of sodium citrate to interact with human wild type and mutants γ D-crystallin was tested, showing that the molecule interacts with unfolding intermediates, and promotes stabilizing interactions [114]. While sodium 4-phenyl butyrate increments the solubility of a truncated γ D-crystallin mutant [115] and pantethine stops the aggregation in solution and reduces the phase separation temperature [116]. For aggregation induced by UV light, resveratrol inhibits the aggregation [117] and carnosine inhibits the induced degradation [118].

Perspectives

Crystallins are not regenerated in the lens during the organism lifetime, therefore, they have to remain unchanged during aging in order to not affect the lens transparency. When different factors affect the protein, cataracts are formed. The understanding of the conformational changes that γ -crystallins suffer during the cataracts development is necessary in order to get structural information that would allow the development of treatment strategies different than the surgical removal. In spite of the multiple compounds that have been studied as potential drugs for cataracts, there is not a potential preventive molecule for the inhibition of aggregation of crystallins. The study of inhibitors previously related to or associated with other misfolding protein aggregation diseases may be a (valuable) option for testing new compounds.

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