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Interaction of Ceramides and Tear Lipocalin

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Abstract

The distribution of lipids in tears is critical to their function. Lipids in human tears may retard evaporation by forming a surface barrier at the air interface. Lipids complexed with the major lipid binding protein in tears, tear lipocalin, reside in the bulk (aqueous) and may have functions unrelated to the surface. Many new lipids species have been revealed through recent mass spectrometric studies. Their association with lipid binding proteins has not been studied. Squalene, (O-acyl) omega-hydroxy fatty acids (OAHFA) and ceramides are examples. Even well known lipids such as wax and cholesteryl esters are only presumed to be unbound because extracts of protein fractions of tears were devoid of these lipids. Our purpose was to determine by direct binding assays if the aforementioned lipids can bind tear lipocalin. Lipids were screened for ability to displace DAUDA from tear lipocalin in a fluorescence displacement assay. Di- and tri-glycerides, squalene, OAHFA, wax and cholesterol esters did not displace DAUDA from tear lipocalin. However, ceramides displaced DAUDA. Apparent dissociation constants for ceramide-tear lipocalin complexes using fluorescent analogs were measured consistently in the submicromolar range with 3 methods, linear spectral summation, high speed centrifugal precipitation and standard fluorescence assays. At the relatively small concentrations in tears, all ceramides were complexed to tear lipocalin. The lack of binding of di- and tri- glycerides, squalene, OAHFA, as well as wax and cholesterol esters to tear lipocalin is consonant with residence of these lipids near the air interface.

Graphical Abstract

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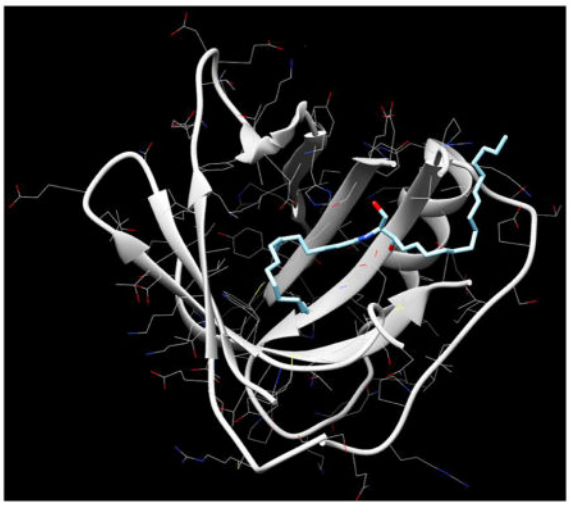

Keywords

Tear lipocalin; Lipocalin-1; LCN1; Lipid binding; ceramide; linear spectral summation; DAUDA; squalene; tears; dry eye; wax esters; cholesterol esters; (*O*-acyl) omega-hydroxy fatty acids (OAHFA); di- and tri- acylglycerols

1.0 Introduction

Numerous studies have elucidated the lipid components in human tears, Table 1.[1–23] These studies show that classes and species of lipids in tears are surprisingly consistent. The most abundant tear lipids are wax and cholesteryl esters. Phospholipids including sphingomyelin, as well as fatty acids and alcohols are present. Squalene has been recently identified in tears. [23,24] Ten species of ceramides (sphingolipids) have been identified.[18] Quantitative differences in lipid composition evident in Table 1 may be due to many factors such as variations in collection technique, samples, spectrometric methods, ion suppression, and instrument response function.[8,20,21,25]

The tear film has been classically modeled by components that populate each of 3 distinct layers. The components are 1. ocular surface membrane spanning mucins 2. aqueous soluble proteins including mucins and 3. lipids at the air-aqueous interface. The presumed location of lipids is based on interferometry that discern a surface layer with a different index of refraction than the bulk.[26] The mean thicknesses accounting for interference fringes as measured by a number of techniques are about 40–90 nm but with a dynamic range between ~15–160 nm.[25,27–32] Schemes for modeling the position of individual components within the layer are generally predicated on differences of polarity of classes of lipids. In such schemes non-polar lipids such as wax and cholesterol esters have been modeled at the air-interface. Polar lipids, such as fatty acids and alcohols and even phospholipids, are proffered to reside near the aqueous-lipid interface.[19,25] The assumptions for these models have been aptly challenged.[33]

Most of the tear film lipids feature long alkyl chains which are suited for retardation of evaporation.[34] However, many tear film lipids are completely insoluble in water. Molecular associations of insoluble lipids with proteins no doubt affect their position and add a dimension poorly accounted by the tear film models. For example many tear lipids, including fatty acids and alcohols, glycolipids, phospholipids, cholesterol and even diacylglycerols, have been found complexed to tear lipocalin and some have been tested with binding assays.[7,8,12,35–38] Tear lipocalin resides in the bulk. In native human tears, tear lipocalin is not completely saturated with lipid. Most lipids associated with tear lipocalin are expected to dwell in the aqueous portion of the tears not in the putative lipid layer. Other lipid binding proteins in tears are insignificant in amount compared to tear lipocalin. [39–43]

Wax and cholesterol esters are absent in extracts from native or expressed tear lipocalin as determined by thin layer chromatograms.[7,44] These lipids are presumed to reside at the air-lipid interface of the tear film based on their chemical properties. The location(s) of recently identified tear lipids, squalene, OAHFA and ceramides are unknown. These lipids were not specifically studied in earlier analysis of extracts from lipocalin.[7,12] Knowledge of their binding to tear lipocalin is critical to being able to localise these lipids to the aqueous or the lipid layer of the tear film and consequently contribute to better modelling of the lipid layer. Similarly, the binding of other lipids such as diacylglycerols (diglyceride)to tear lipocalin is controversial. Some groups presumably identified diacylglycerol in extracts of lipocalin fractions of tears [12], but not other investigators.[7] Our purpose is to fill this gap in knowledge by investigating the binding of examples of these lipid classes to tear lipocalin.

2 Materials and Methods

2.1 Reagents

C18-ceramide (ceramide- N-(octadecanoyl) sphing-4-enine, also known as N-stearoyl-D-erythro-sphingosine), was obtained from ACROS Organic (Pittsburg, PA). C6-NBD ceramide (N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine) and C12-NBD ceramide (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine) were obtained from Avanti Polar Lipids (Alabaster, AL). Squalene and the wax ester, stearyl behenate were purchased from MP Biomedicals, LLC (Solon, OH). Behenyl stearate and behenyl oleate were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Cholesteryl oleate was purchased from Alfa Aesar (Tewksbury, MA). Stearic acid, cholesteryl stearate, oleoyl chloride, 16-hydroxypalmitic acid, 1,3-distearin, tristearin and NBD-Cl (4-chloro-7-nitrobenzofurazan) were obtained from Sigma (St. Louis, MO). DAUDA or 11-(((5-(dimethlyamino)-1-naphthalenyl)sulfonyl)amino) undecanoic acid was obtained from Molecular Probes (Eugene, OR). 1,2-distearoyl-sn-glycerol (1,2-distearin) was purchased from Chem-Impex Int'l Inc. (Wood Dale, IL). The structures of the key lipids in this study are shown in Figure 1 in [45]. Egg white lysozyme was obtained from ICN Biomedicals Inc. (Aurora, OH). (*O*-oleoyl)-16- hydroxypalmitic acid was synthesized with minor modifications from previous published methods.[46,47] The details of synthesis and mass spectrometric validation are provided (Figure 2 in [45]).

2.2 Spectroscopy

Fluorescence measurements were made at 20°C in a Jobin Yvon-SPEX Fluorolog-3 spectrofluorimeter; bandwidth for excitation was 2 nm and for emission, 4 nm. Raman and background scattering by the solvent were corrected where necessary using appropriate blank solutions.

Ultraviolet-visible absorption spectrophotometric measurements were obtained with a Shimadzu UV-2401PC instrument, (Kyoto, Japan). The concentrations of proteins and fluorescent lipids were calculated from molar extinction coefficients that have been published or derived herein (Table 2).[48–52]

2.3 Collection of Human Tears for Protein Purification

Stimulated human tears were collected from healthy volunteers in accordance with the tenets of the Declaration of Helsinki and approved by the institutional review board. Informed consent was obtained from donors after explanation of the nature and possible consequences. Collection was performed as previously published [8,53,54] with polished glass tips and glass transfer pipettes. Tears were pooled in polytetrafluoroethylene-lined glass vials and stored under nitrogen at −80°C until use.

2.4 Purification of tear lipocalin

Fractionation of the tear proteins included: gel filtration liquid chromatography using the AKTA purifier Versatile FPLC with Superdex 75 preparative column, (GE Healthcare, Piscataway, NJ) followed by pooling of peak tear lipocalin fractions, anion exchange chromatography using DEAE-Sephadex A-25 and analysis by tricine polyacrylamide gel electrophoresis as previously published.[7,8,54] The amount tear lipocalin was determined from the molar extinction coefficient (Table 2). This purification scheme results in six isoforms that have been previously characterized by mass spectrometry and sequenced. The major isoform has 157 amino acids and a mass of 17,446.5 Da.[54,55] Even though native ligands remain complexed to lipocalin after purification, additional capacity for binding partners has been repeatedly demonstrated.[35,38,44,53,56,57] Notwithstanding slightly reduced binding, use of native tear lipocalin purified in this way avoids structural perturbations seen after partial delipidation with organic solvents.[51,58,59] An inescapable consequence is that the dissociation constants and stoichiometry derived from the use of tear lipocalin are apparent and reflect only the native protein’s additional capacity for complexing ligand.

2.5 DAUDA displacement fluorescence assay

In order to test the binding of tear lipocalin to key tear lipids, cholesteryl stearate, cholesteryl oleate, behenyl stearate, behenyl oleate, stearyl behenate, (*O*-oleoyl)-16-hydroxypalmitic acid, squalene, C18-ceramide, di- and tri- glycerides were screened for the displacement of DAUDA from the binding site of tear lipocalin. Displacement of DAUDA from the hydrophobic environment of the binding site results in a decrease in fluorescence (λ_{em} =490 nm, λ_{ex} =345 nm). Stearic acid, which was shown previously to bind tear lipocalin, served as a positive control.[56]

In general the ligand complex resulting from a mixture of tear lipocalin and DAUDA (5 μM each) in 10 mM sodium phosphate (pH 7.3) was titrated by successive addition of 0.2μL the test lipid. Each addition increased the final concentration of test lipid by 2 μM. The final concentration was about 18 μM. The lipids in their respective solvents were: DAUDA, squalene, 1,2 distearin, 1,3 distearin, tristearin, and *(O*-oleoyl)-16- hydroxypalmitic acid (ethanol), cholesteryl stearate, cholesteryl oleate, behenyl stearate, stearyl behenate, and behenyl oleate (chloroform), as well as C18-ceramide (methanol). At the end of each titration experiment, the total organic solvent concentration did not exceed 2%. To ensure that the various solvents did not impact the fluorescence spectra, solvent alone was added to the solution prior to the addition of the lipid and the maximum amount of solvent used was added at the end of the experiment. The effects on the spectra were negligible.

2.6 High speed centrifugal precipitation assay for ceramide-tear lipocalin binding complexes

This binding assay is based on separating unbound lipids from those bound to tear lipocalin or other proteins by capitalizing on centrifugal precipitation of the insoluble lipid in water. [60] Long chain lipids such as ceramides are insoluble in buffer and particularly suited to this technique. C6 and C12 -NBD ceramides showed complete precipitation in 10 mM Na-phosphate pH 7.3. NBD labeled lipids have a large extinction coefficient in methanol or bound to protein (Table 2). Measurements by ultra violet- visible absorbance spectrophotometry are accurate for both unbound ceramide in methanol and protein bound ceramides provided the lipids bear an NBD label. Tear lipocalin, 10 or 20 μM, 500 μL in 10 mM sodium phosphate (pH 7.3), was incubated with successively increasing concentrations of C6 or C12 -NBD ceramides, respectively in DMSO. In experiments with C6-NBD ceramide 0.5 μL increments of 1 mM C6-NBD ceramide in DMSO were added to obtain total final concentrations of ligand from 1 to 20 μM. In experiments with C12-NBD ceramide 0.5 μL increments of 2 mM of C12-NBD ceramide were added to 500 μL 20 μM tear lipocalin in 10 mM sodium phosphate (pH 7.3) in DMSO to obtain total concentrations from 2 to 40 μM. The maximum concentration of DMSO in any sample with 20 μM of ligand was 2%. Mixtures of tear lipocalin and ligand were incubated at either 25°C for 30 min or 34°C for 16 hours, followed by centrifugation in a Sorvall Discovery M150 (Thermo Fisher Scientific, Fairlawn, NJ, USA) with an S150AT rotor at ~196,000 × g for 1 h at 25°C or 34°C. The concentrations of insoluble unbound ligand and bound ligands were determined from the absorbance spectra in methanol and buffer using appropriate extinction coefficients (Table 2). Molar extinction coefficients of ligand bound tear lipocalin were calculated from the amount of bound ligand at saturation by subtracting the free ligand from the total. Control experiments with C6 or C12 -NBD ceramides without tear lipocalin showed no absorbance in spectra of the supernatant at 400–500 nm after centrifugation. Each experiment was repeated 3 times and the means were fit to the hyperbola curve and Hill equation (Microcal Origin, Northhampton, MA, USA). To exclude non-specific binding to lipocalin (a negative control) 10 μM lysozyme was used instead of lipocalin.

2.7 Testing C6-NBD ceramide for linear spectral summation

Increasing concentrations of suspensions of C6-NBD ceramide, 1 to 20 μM, in water or buffer were obtained by addition of 1 mM C6-NBD ceramide in DMS0 to seek a range

where absorbance obeys a linear relationship with concentration according to Beer's law. Tear lipocalin, 500 μL in 10 mM sodium phosphate at pH 7.3 was titrated by successive addition of 0.5–10 μL of 1 mM C6-NBD ceramide in DMSO and the absorbance spectra were measured. Following each addition of C6-NBD ceramide, the solution was mixed and allowed to equilibrate for 5 min. At the end of the titration experiment, the DMSO concentration did not exceed 2%. Bound C6-NBD ceramide spectra were obtained from spectra of the mixture of 2 μM C6-NBD ceramide and 10 μM tear lipocalin in 10 mM sodium phosphate at pH 7.3. The molar excess of tear lipocalin ensures that essentially all C6-NBD ceramide molecules are bound to the protein. The unbound spectra were obtained from 2 μM C6-NBD ceramide (without any protein) suspended in buffer. Composite spectra were recomposed by fitting the scaled spectra of bound (2 μM) and unbound (2 μM) C6-NBD ceramide using a program created in LabView (National Instruments, Austin, TX). In addition, a baseline component was added for better quality fit. The program uses the "General linear Fit VI" subVI to fit each experimental spectrum to the linear summation of the components and the bound and unbound lipid concentrations were determined as previously described.[61] The experiment was repeated 3 times and the means were calculated.

2.8 Fluorescence Binding Assays with NBD-labeled lipids

In separate experiments C6 and C12 -NBD ceramides in DMSO were added successively to 1 μM tear lipocalin in 10 mM sodium phosphate pH 7.3. After equilibration, fluorescence spectra were obtained, λ_{ex} =420 nm. The relative intensity at 523 nm was used to compute each bound label. Experiments were repeated in triplicate and the means calculated. The total solvent concentration did not exceed 0.6%.

2.9 Data Analysis

Plots were fit to a single rectangular hyperbola or the Hill equation, $B/P= nL/(K_d+L)= L^x/(L^x+K_d)$, respectively by standard nonlinear regression techniques (Microcal Origin, Northhampton, MA, USA): where B,P and L are concentrations of bound lipid, total protein and unbound lipid, respectively to determine apparent number of binding sites (n), apparent dissociation constant K_d and Hill coefficient (x) for tear lipocalin and ligand.

2.10 Docking Analysis

Molecular Docking studies were performed in DockingServer and SwissDock. The X-ray crystallographic structure of tear lipocalin (1XKI) was retrieved from Protein Data Bank (PDB), and used as a target protein. Structures of C18 ceramide (18C), stearic acid (ZINC04978673), and sphingosine (ZINC08195650) were obtained from PubChem and ZINC databases. The docking analysis and visualization was performed with default parameters implemented by the SwissDock site.[62,63]

3 Results

3.1 DAUDA displacement of native lipids from tear lipocalin

DAUDA loses fluorescence when displaced from the hydrophobic environment of the binding cavity of tear lipocalin.[56] DAUDA was displaced from the binding complex of

tear lipocalin by the tear lipids, stearic acid (positive control) and C18-ceramide (Figure 1). The drop in fluorescence from displacement of DAUDA appeared at lower concentrations of ceramide than stearic acid. However, the residual fluorescence was twice as great with ceramide as that observed with stearic acid. There was negligible displacement of DAUDA by squalene, wax and cholesterol esters, (*O*-oleoyl)-16- hydroxypalmitic acid, as well as di- and tri-acylglycerols.

3.2 Testing binding of NBD fluorophores (pure label and lipids) to tear lipocalin

In order to determine apparent binding constants and explain the residual fluorescence seen with C-18 ceramide, NBD labeled lipids were tested. The results for the control NBD-CL alone are shown (Figure 3 in [45]).

Fluorescent assays showed NBD labeled ceramides were amenable to binding experiments with tear lipocalin (Figure 2). C12 and C6 -NBD ceramides showed enhanced fluorescence in the presence of tear lipocalin compared to the unbound label. Interestingly, bound C6-NBD ceramide showed more fluorescence than C12-NBD ceramide.

3.3 C6-NBD ceramide binding by linear summation, high speed centrifugal precipitation and fluorescence

In order to determine apparent kinetic parameters for binding of the C6-NBD ceramide several techniques were investigated. The insolubility of unbound C6-NBD ceramide availed the opportunity to use centrifugal separation. The technique of centrifugal separation has the advantage of clearing any lipid that aggregates in suspension. The C6-NBD ceramide-tear lipocalin complex remains in solution and a binding curve could be generated (Figure 3 and Figure 4 in [45]). To ensure that the binding reaction equilibrated, the reactions were run at 34° C (Figure 4). The apparent K_d was noted to be sub-micromolar, n≈ 0.5 (Table 3). To rigorously verify these results, we tested the binding without centrifugal separation using the technique of linear spectral summation. This technique is predicated on an acceptable spectral separation of the bound and free ligand (Figure 4 in [45]). Previously we found that the spectral peaks of bound and free ligand must be offset by at least 7–10 nm.[61] Because unbound ceramides are suspended, this technique necessitates that the suspended fluorophore obeys Beer's law in the range of the assay (Figure 5 inset). The apparent dissociation constant was even smaller with linear spectral summation but a similar stoichiometric relationship of the tear lipocalin-ligand complex was observed (Table 3, Figure 5). Finally, we tested the binding relationship with a standard fluorescence assay which capitalizes on the fluorescence of the bound fluorophore compared to unbound (Figure 6). The dissociation constants and stoichiometry of the fluorescence assay were similar to that of linear spectral summation (Table 3).

3.4 C12-NBD ceramide binding to tear lipocalin

In order to ensure that the results are not due a unique characteristic of the NBD labeled ceramide, comparison with a second ceramide, C12-NBD ceramide, was performed. Binding was tested by high speed centrifugal precipitation of the insoluble unbound precipitate from the bound soluble ligand (Figure 3). The apparent dissociation constant (K_d=1.2 μM) suggested strong binding but less affinity for this ligand compared to C6-NBD

ceramide. Spectral fitting using linear summation was obviated because the peaks of the bound and unbound spectra were not well separated (Figure 4, inset in [45]). The binding curve was also performed using fluorescence of the bound ligand (Figure 6).

3.5 Docking studies of ceramide to tear lipocalin

The docking studies of the tear lipocalin ceramide complex showed a full fitness score −1328.48 kcal/mole with a free energy minima (ΔG =−8.38 kcal/mole) Table 4. The predominant pose featured the alkyl chain, fatty acid side, slightly folded but fully contained in the cavity and the sphingosine portion positioned at the mouth (Figure 7). This was the case for several of the the highest rated docking poses of ceramide. The controls for the docking studies are shown in supplementary materials and include, stearic acid posed with the alkyl chain extended in the cavity (ΔG =−7.30 kcal/mole). Sphingosine alone was posed near the mouth (ΔG =−8.21 kcal/mole) (Figures 5 and 6 in [45]).

4 Discussion

The key findings of this study are that 1) tear lipocalin binds ceramides 2) tear lipocalin does not appear to bind squalene, OAHFA's, diacylglycerols, triacylglycerols, wax and cholesteryl esters 3) assays using high speed centrifugal precipitation match those using fluorescence and linear spectral summation, providing validation for these techniques and for their results and 4) docking studies suggest a predominant orientation with the alkyl chain of the fatty acid moiety of ceramide extending into the cavity and the sphingosine moiety positioned near the mouth of the cavity of tear lipocalin. Both the positive and negative results presented here have implications for the potential distribution and functions of lipids in the tear film. This study marshals strong evidence that native and fluorescent labeled ceramide analogs bind tear lipocalin. For the NBD labeled analogs the apparent binding constants are in the submicromolar to micromolar range according to 3 different binding assays. Interpretation of the precise stoichiometry and K_d are vitiated because lipocalin as purified is always complexed to other lipid products.[7,8,64] The dissociation constants may therefore be overestimated. Squalene, wax and cholesteryl esters have no discernible binding. Since tear lipocalin generally is believed to be restricted to the aqueous portion of tears, one would expect ceramides to reside in bulk tears. Squalene, wax esters and cholesteryl esters are insoluble in aqueous and therefore very likely to be integrated with lipids close to the air-surface interface. If (*O*-oleoyl)-16- hydroxypalmitic acid properly represents the class of OAHFA's, one would not expect it to be soluble in the aqueous of tears without protein binding.

There are other lipid binding proteins in tears, such as lipophilin, phospholipid transfer protein and apolipoprotein D. However these proteins are estimated to be at least 10–100 times less abundant. [39–43]. Furthermore, the lipid binding profiles of these less abundant proteins do not include any of the lipids explored in this study. Therefore, it is probably safe to assume that lipids explored in this study that do not bind to tear lipocalin are absent from the aqueous.

4.1 Novel techniques for binding studies of insoluble lipids

In determining apparent binding with insoluble lipids, we have generally used a fluorescence approach. The method accrues the key advantages that often the unbound lipid shows no appreciable fluorescence and the sensitivity of fluorescence permits very low concentrations of reagents in the assays. Two approaches advanced in the current work capitalize on the absorbance of the unbound lipid in order to directly measure free and bound ligand. The results invite comparison to the results of fluorescence. In the first approach, separation by high speed centrifugation clears insoluble ligand and aggregates. We have used this technique before to study rifalazil binding to lipocalin.[60] This technique has the advantage that unbound insoluble ligand, and bound soluble ligand are both separated and measured directly by spectrophotometry in solvents appropriate for the mixtures. No assumptions are made regarding particles in suspension. Further, a fluorimeter is not required. The disadvantages include the laborious nature of the experiment, the need for higher concentrations than fluorescence, and incidental, although usually inconsequential losses when removing the supernatant. The mathematical approach for analyzing kinetics is simplified to the calculation of the solubility product (K_{sp}), where the concentration of free compound in solution is negligible, and the protein bound fraction and precipitated compound are easily separated and directly measurable.

Linear spectral summation was described for fluorescence previously, but altered here for absorbance spectrophotometry. This technique has 2 key prerequisites. First, the ligand, soluble or insoluble, must obey Beer's law in the concentration range of the experiment (Figure 4, inset). Beer's law is generally applied to solvated particles. While deviations are seen for large particles in solution, smaller particles show a linear absorbance plot that coincides with that of a condensed homogeneous film of the same material.[65] The second prerequisite is that the spectral peaks of the unbound lipid must be separated by at least 7 nm from the bound lipid.[61] That observation was supported by the experiments here. The bound and unbound peaks for C6-NBD ceramide were 27 nm apart and therefore amenable to the technique (Figure 4 in [45]). However, the spectral peak for unbound C12-NBD ceramide was broad and overlapped the sharper bound peak, obviating the technique in this case (Figure 4, inset in [45]). The advantages of linear spectra summation are rapidity, the use of routine laboratory equipment (spectrophotometer) and easily obtainable software. For C6-NBD ceramide, linear spectral summation closely matched the results for fluorescence assays for apparent K_d. Interestingly, centrifugal separation showed almost an order of magnitude larger K_d's compared to fluorescence and linear summation giving the appearance of less binding affinity. One explanation, albeit speculative, is that during prolonged centrifugation, precipitation of unbound ligand removes suspended particles that would otherwise be accounted as solvated ligand. This reduction would convey slightly less binding for high speed centrifugal precipitation than the other techniques where the free ligand remains in suspension. The impact would be expected to be greater on the K_d than on the stoichiometry (n) since the former is determined mainly in the steeper portion of the binding curve.

4.2 Clinical Relevance of Ceramide Binding with Tear Lipocalin

Ceramides have long hydrocarbon tails and functional groups that confer both hydrophobic and polar qualities (amphiphiles). Albeit scarce in meibum of normal subjects, 0.03 mole % of total lipids,[66] ceramides are slightly more abundant in tears (Table 1). Ceramides are known to be rigid and have a high melting point. If ceramides are added in small quantities to meibum, hysteresis is dramatically elevated in Langmuir troughs experiments. Surface pressure rises with compression. Ceramides induce eventual collapse of the lipid film.[67] Ceramides comprise about 7% (average of 3 samples) of total lipids in chalazia.[68] Elevation of ceramides has been noted in moderate dry eye compared to normal subjects (although apparently not in severe dry eye).[66] Given these detrimental circumstances, sequestration of ceramides away from the lipid film in tears would appear functionally advantageous. Our data suggest that tear lipocalin binds the 3 different ceramides tested including the most abundant species in tears, C18-ceramide (Cer d18:1/18.0).[66] The low apparent dissociation constants in the submicromolar range are reflective of strong binding affinity and imply very little unbound ceramide can be expected in tears at natural concentrations. In fact, NBD ceramides have the lowest dissociation constant of any at least 20 ligands studied for tear lipocalin.[7,56,58,60,69–73]

4.3 Mechanistic features of binding

The stronger displacement of native ceramide compared to stearic acid matches the best full fitness pose provided by docking studies (Figure 7). All the poses place the alkyl chains of either the fatty acid (predominant) or sphingosine in the cavity. In each case the other chain is positioned at the mouth of the cavity. The poses vary in the degree of folding of the fatty acid or sphingosine alkyl chains in the cavity. Docking studies are known to have false positives. This study is limited because the PDB file for tear lipocalin was based on crystallographic studies that did not resolve loop structures.[74] The loop structures that overhang the cavity could limit folding of alkyl chains near the cavity mouth.[75,76] As a control, docking studies done with stearic acid show an extended fit over the full length of the cavity, which by crystallographic estimates are about 15 Å (Figure 5 in [45]).[74,77] C6 and C12- NBD ceramides molecules were not available in the proper format for docking studies. However, the NBD moiety fluoresces only in a hydrophobic environment and therefore it is safe to assume it complexes within the lipocalin cavity. NBD has an estimated maximum diameter of about 6.38 Å,[78], less than the known diameter (10 Å) of the calyx of tear lipocalin by crystallography. This is also consistent with functional studies that show the tear lipocalin cavity permits molecules up to about 6.7 Å in diameter and reject those of 9.1 Å and greater. [56]

Binding free energy differences between C6 and C12 -NBD ceramide association complexes with tear lipocalin are very small by fluorescence assays 0.4–1 kjoules/mole. At first, the minor differences initially appear incongruous to the longer alkyl chain length of C12-NBD ceramide on one arm of the molecule. However, the cavity is only 15 Å in length and therefore only a portion of the fatty acid side C12-NBD ceramide could enter the cavity. If the distance between carbon atoms (C-C distance) is assumed to be about 1.25 Å, then the cavity would be filled by NBD plus 6 carbon units reaching the limit of the hydrophobic effect. C6 and C12-NBD ceramides (Figure 1 in [45]) would be expected penetrate to similar

depths with similar binding constants. As indicated in the docking studies of native ceramide the sphingosine tail could also participate in binding at the mouth of the NBD-ceramides. Alternatively, another binding mechanism involving charged interactions between the ligands and tear lipocalin cannot be completely excluded. The amphiphilic nature of ceramides might also permit a charged interaction with a trigonal cluster in tear lipocalin. Ion pairing between Lys 114 of lipocalin and the sulfonate group of ANS has been posited.[79] C12-NBD ceramide would be expected to have more exposure of the hydroxyl group at the center of the molecule than C6-NBD ceramide. A charge effect alone with tear lipocalin is unlikely to account for dissociation constants in the micromolar range.[80,81] The docking experiments do not support this mode of binding.

4.4 Squalene

Squalene, a terpenoid, is abundant in skin secretions and may arise from cells in both apocrine and sebaceous glands. Squalene is known to be an anti-oxidant in tears.[82,83] Squalene exists in meibum and ranges in mole percentage from 1–7. [84–89] Terpenoids have been identified in tears by mass spectrometry that presumably originate from the eyelid.[9,10,84] The negative results of the DAUDA displacement assay precludes significant bind of squalene to tear lipocalin. Comparison of tear lipocalin to squalene binding proteins is revealing. A protein involved in cholesterol synthesis called supernatant protein factor can bind squalene in vitro if a constraining C terminal jelly-role shaped domain is altered.[90] Supernatant protein factor has a lipid binding core domain that promotes squalene to assume a horseshoe shape in the cavity. The dimensions of tear lipocalin with overhanging loops and a restricted cavity would not permit this ligand configuration.[75] The implication is that squalene is likely to be distributed in the lipid layer of the tear film above the protein-aqueous layer. This notion is congruous with recent Langmuir trough experiments that squalene reduces the phase transition of meibum by 5 °C, and promotes disordered lipid configurations with gauche rotamers resulting in lipid expansion.[23] The lack of change achieved in maximum surface pressure by squalene was taken as evidence against its surfactant properties at the aqueous interface with meibum.[23] Our results are consistent with the findings that squalene may be active at the air interface in Langmuir-Blodgett films.

4.5 Wax and Cholesterol Esters

By most estimates the most abundant lipid components of the tear film are wax and cholesteryl esters (Table 1). Previous studies showed these components were conspicuously absent in extracts made from purified tear lipocalin despite the positive identification in tears.[7] The current study affirms these prior findings. Tear lipocalin is structurally distinct from known cholesteryl ester binding proteins. For example cholesteryl ester transfer protein, 53 kDa, is 3 times larger than tear lipocalin 17.4 kDa and sports a 60Å long hydrophobic tunnel with a highly flexible distal B barrel.[91,92] By comparison, tear lipocalin's smaller cavity would place the long alkyl chains of cholesteryl stearate as well as wax esters in energetically unfavorable states exposed to solvent.

4.6 OAHFA

Synthetic (*O*-oleoyl)-16- hydroxypalmitic acid has been used as the standard to represent the class of OAHFA in tears.[46,47] By necessity experiments were limited to the synthetic compound as neither the native OAHFA's nor their precursors that have been identified by mass spectrometry in tears are commercially available. The amphiphilic nature of these lipids have prompted some to proffer that OAHFA may be responsible for stabilizing the interface between nonpolar lipids and aqueous tears. This function was previously assigned to phospholipids.[46,93] Our data are consistent in that unlike phospholipids,[8] OAHFA is not sequestered by tear lipocalin and therefore is not likely to reside in the aqueous bulk. Therefore these lipids are key candidates to be interposed between the aqueous and surface lipids such as wax and cholesteryl esters.

4.7 Diacyl and triacylglycerides

Our prior studies failed to show di- or tri-acylglycerols in binding complexes with tear lipocalin by thin layer chromatography.[7] In contrast, a more recent work identified 1-palmitoyl-2-stearoyl-glycerol and 1,2-distearoyl-glycerol based on the fragmentation patterns of peaks with *m/z* values of 607 and 579 in extracts of tear lipocalin.[12]However, a contaminant, an oxidized form of Irgafos 168 from plasticware may mimic diacylglycerols, was also identified. In the current study DAUDA is not displaced by authentic class members. The conclusion is that the binding of diacyl and triacylglycerides to tear lipocalin is far less than that of DAUDA and probably functionally negligible.

5 Conclusions

The data shown here support a recurrent theme with regard to a major function of tear lipocalin. Tear lipocalin acts as a scavenger of a wide assortment of lipids.[7,35,36,53,70,94] and ceramide can now be added to that list. The protein has a relatively flexible cavity which binds a broad array of lipids including potential harmful substances such as the plasticizer, dioctyl phthalate, as well as proinflammatory mediators. The promiscuous binding properties have invited exploitation of lipocalin constructs for use pharmaceutical applications to scavenge drugs such as digitalis for use in cases of digitalis toxicity.[95] Alteration of the lipocalin loops alters binding and has lead to potential agents to even interrupt angiogenic pathways in cancer.[96,97] Native tear lipocalin has been proposed for delivery of anti-tuberculous drugs that show off-target toxicity in the unbound state.[60] The binding of ceramide in tears extends the scavenger theme to sequester potentially destabilizing lipids from the surface of the tear film.

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Highlights

- Recently discovered tear lipids were tested for binding to tear lipocalin.
- Novel spectroscopic applications were applied for these insoluble lipids.
- Lipids bound to lipocalin are unlikely to function at the air-tear interface.
- Ceramides bind to and implicate a scavenger role for tear lipocalin.

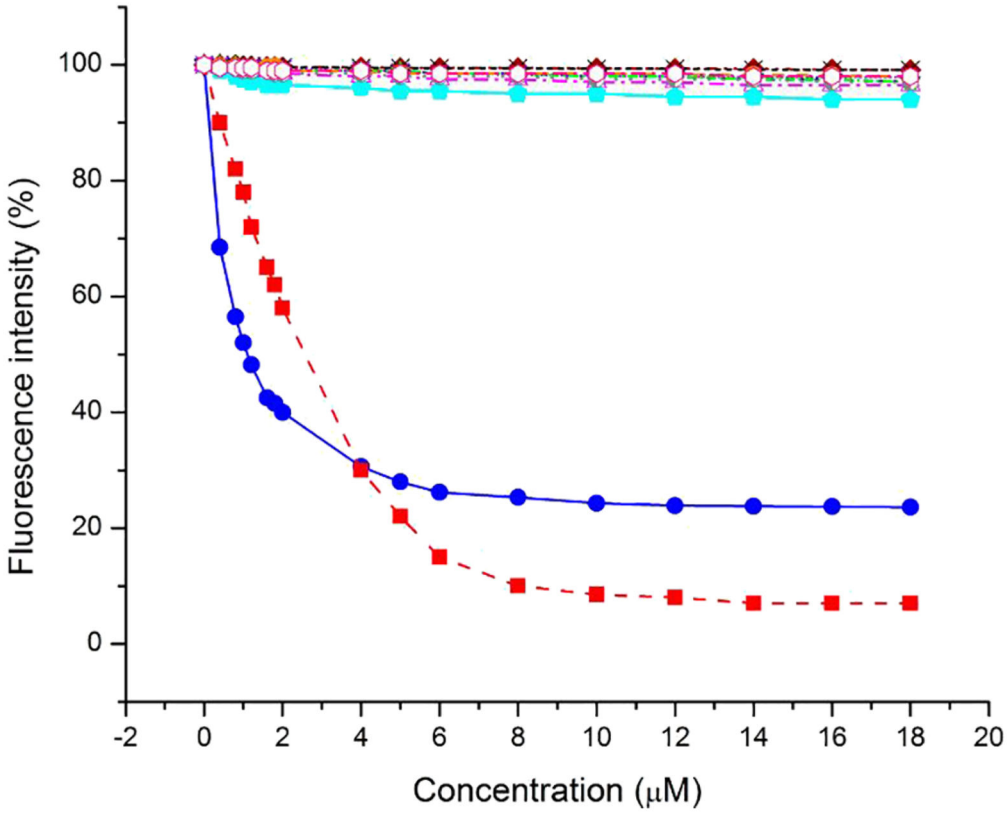

Figure 1.

DAUDA displacement assay for tear lipids. DAUDA bound to tear lipocalin displays fluorescence. Unbound DAUDA has exiguous fluorescence. Decreased fluorescence indicates displacement of DAUDA from the tear lipocalin binding site by the tested lipids. Fluorescence of tear lipocalin and DAUDA after addition of ceramide (-●-), stearic acid (-■-), cholesteryl stearate (-▼-), cholesteryl oleate (-△-), stearyl behenate (-◆-), behenyl stearate (-x-), behenyl oleate (-✻-), squalene (-★-), (O-oleoyl)-16-hydroxypalmitic acid (-⬢-) 1,2-distearin (-⬭-), 1,3-distearin (-◐-), tristearin (-○-).

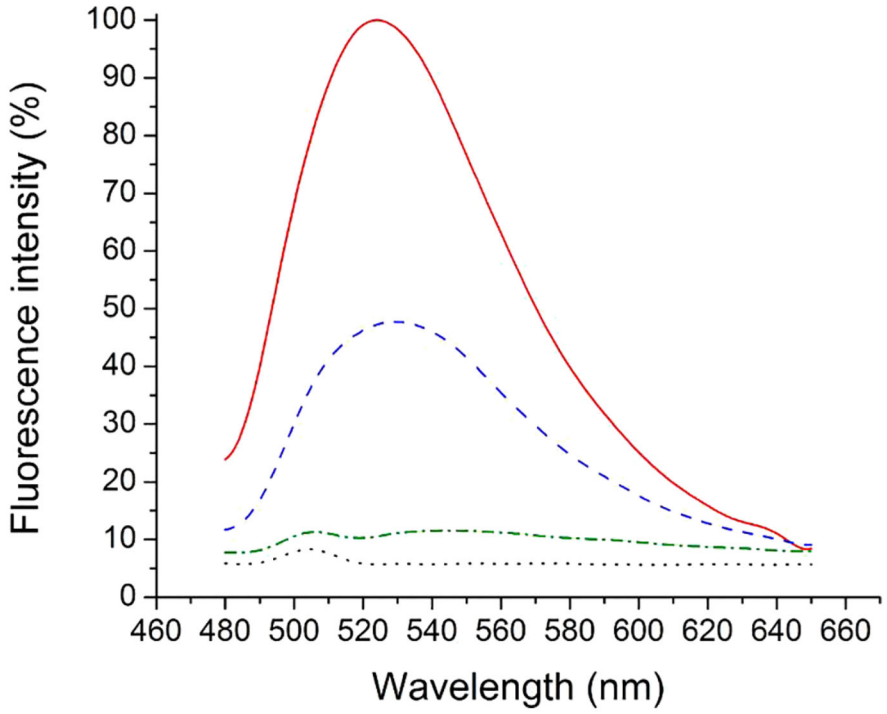

Figure 2.

NBD6-Ceramide and NBD12-Ceramide show fluorescence only when bound to tear lipocalin. Fluorescence spectra of free 1 μM C6-NBD ceramide (·····), 1 μM C12-NBD ceramide (- - -), 1 μM C6-NBD ceramide bound to 1 μM tear lipocalin(—) and 1 μM C12-NBD ceramide 1 μM tear lipocalin (- - -).

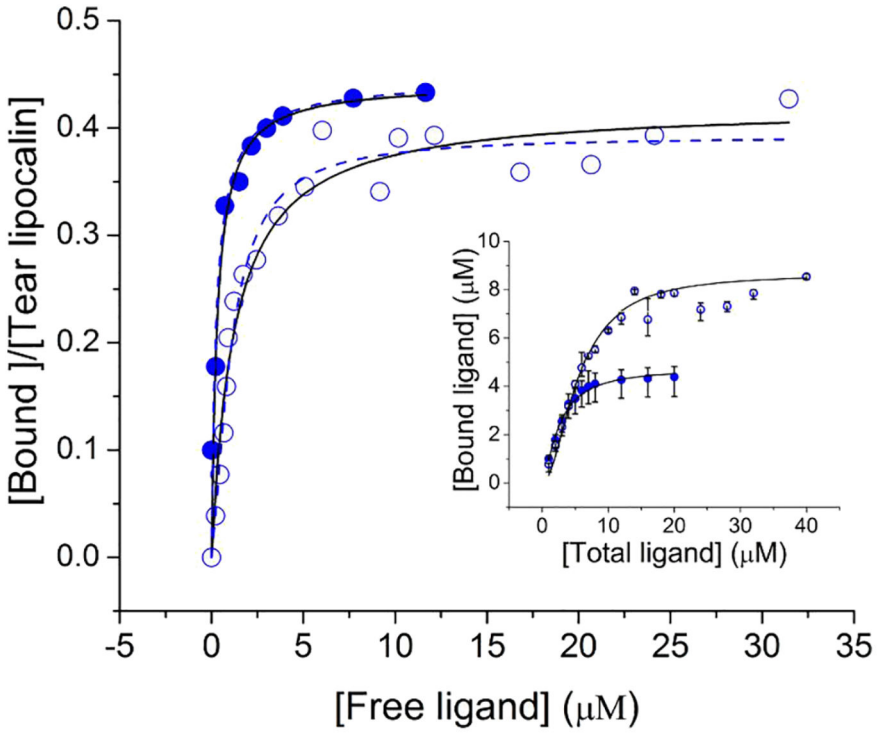

Figure 3.

High speed centrifugal precipitation assay to test binding of C6-NBD ceramide (●) and C12-NBD ceramide (○) to 10 μM or 20 μM tear lipocalin, respectively. Concentration of bound ligand-protein complex (supernatant) was determined by absorption spectra after centrifugal separation from unbound insoluble precipitant. Curve fit to a hyperbola (—) K_d= 0.32 μM, n=0.44 for C6-NBD ceramide and K_d= 1.23 μM and n=0.42 for C12-NBD ceramide; and the Hill equation (- - -) K_d= 0.29 μM, n=0.45 for C6-NBD ceramide and K_d= 1.06 μM and n=0.39 for C12-NBD ceramide. Inset: concentration of total versus bound ligand concentration. Error bars shows the range from 3 experiments.

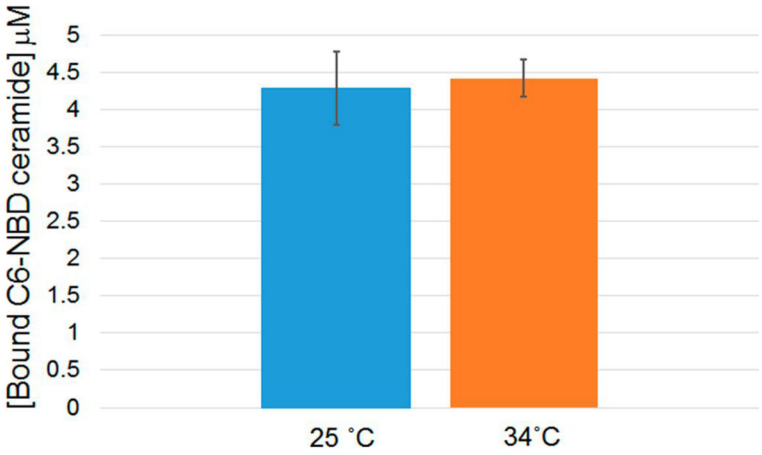

Figure 4.

Comparison of high speed centrifugal precipitation of C6- NBD ceramide at 25° C and 34° C for a single concentration. Bars show the range from 3 experiments.

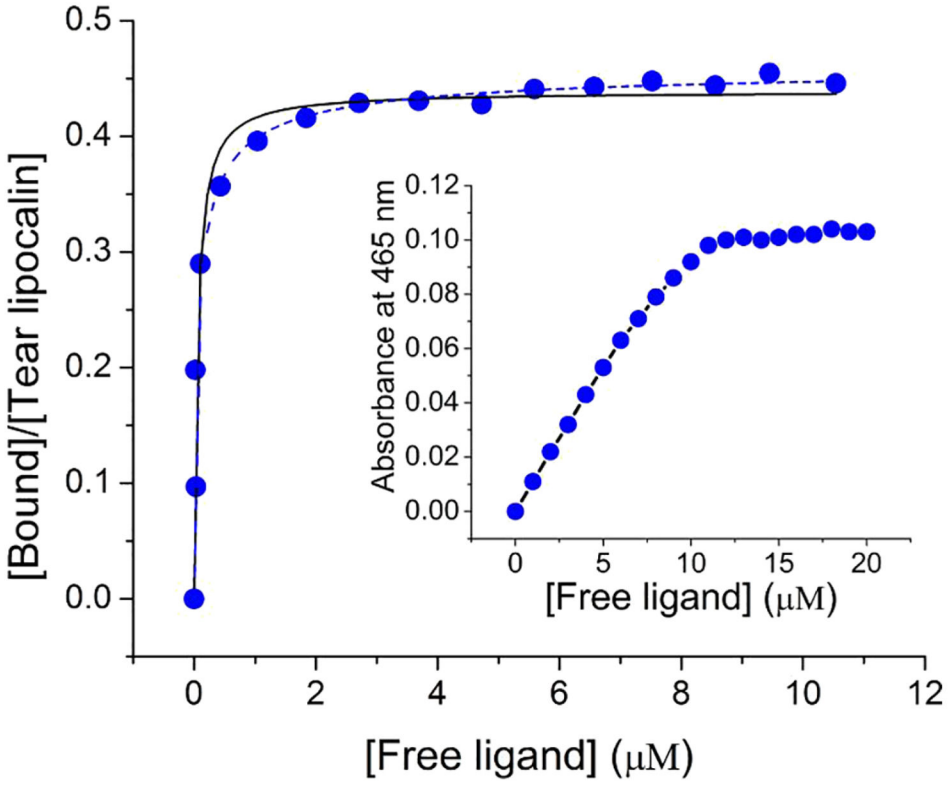

Figure 5.

Binding curve of C6-NBD ceramide to tear lipocalin 10 μM by linear spectral summation (●). Absorbance composite spectra of mixtures were fit to the sum of varying pure free and pure bound spectra. Curve was fit to hyperbola (—) K_d= 0.06 μM, n=0.45, and to Hill equation (- - -) K_d= 0.07 μM, n=0.47. Inset, concentration dependent absorption of C6-NBD ceramide suspended in 10 mM sodium phosphate, pH 7.3.

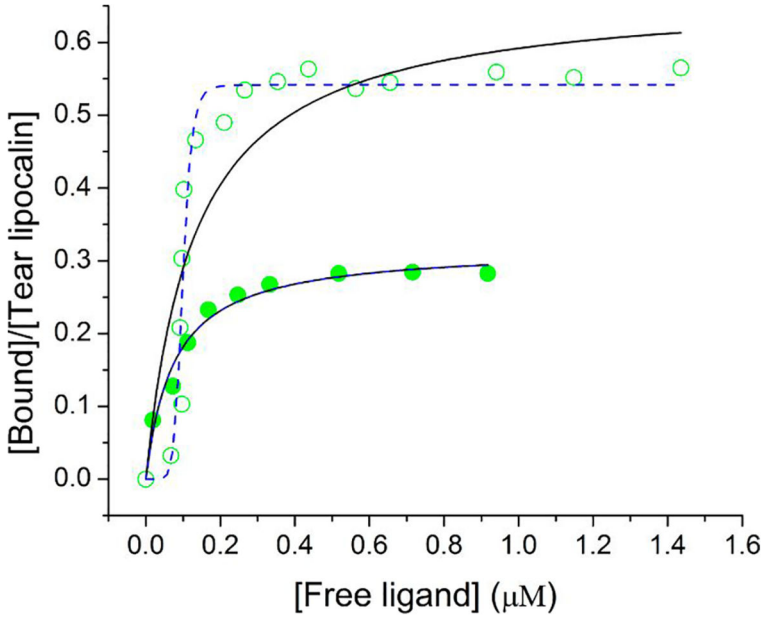

Figure 6.

Binding curve of C6-NBD ceramide (●) and C12-NBD ceramide (○) to tear lipocalin by fluorescence. Curve fit to hyperbola (—) K_d= 0.08 μM and n=0.32 for C6-NBD ceramide binding and K_d= 0.13 μM and n=0.67 for C12-NBD ceramide, and to the Hill equation (- - -) K_d= 0.08 μM and n=0.32 for C6-NBD ceramide and K_d= 0.1 μM and n=0.21 for c12-NBD ceramide.

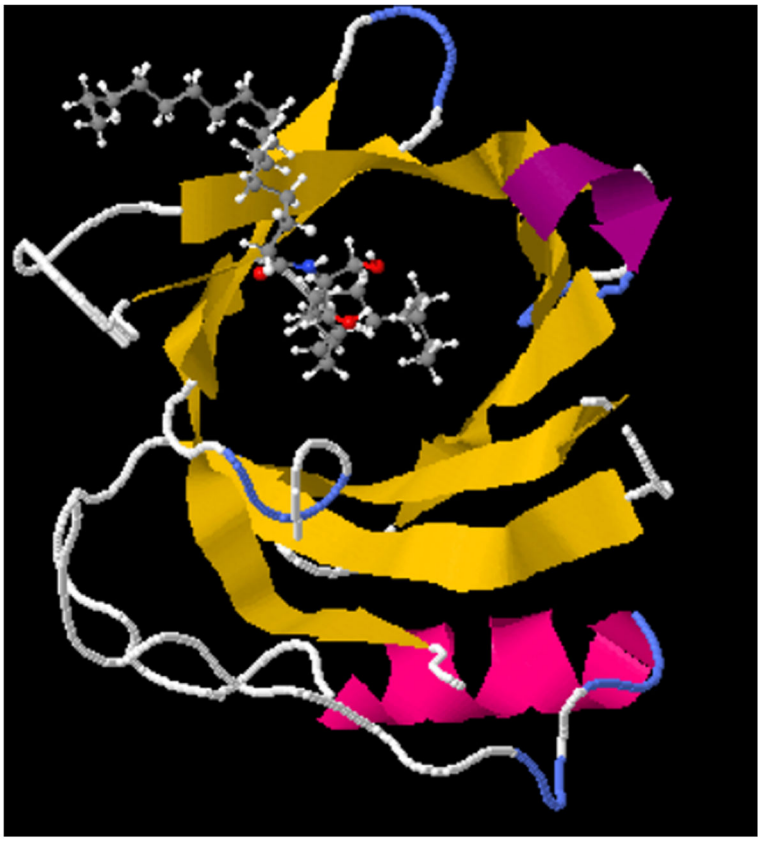

Figure 7.
Swiss Dock image of C18 ceramide complexed to tear lipocalin shows pose with the highest full fitness score (see Table 4).

TABLE 1

Studies of the Lipid Composition in Human Tears

Lipid Author	DAG	TAG	WE	Ch	CE	Foh	FA	OAHFA	PL	Squ	GL	CER
[1]	+	+	+	+	+	+	+					
[2]				5mM								
[3]				0.8mM								
[4]		6.9%		7.1%	9.7%		18.3%		0.9%		55%	
[5]				1.45mM								
[6]				15%			<15%		15%			
[7,8]		+	+	+	+	+	+		+		+	
[9,10]	+	+	+		+				+			
[11]							+		+		+	
[12,13]	+	+	+	0.5–1%	+				neg/small	+		
[14,15]									+			
[16]	+	+			+				−			
[17]		0.01mM			0.02mM				0.048mM			0.0009mM
[18,19]	0.3%	2.8%	35.2%	5.4%	44.9%				3%		0.4%	0.26%
[20–22]		1–2%	18.6–45%	8–18.6%	35.7–54.8%			1–4.4%	5.4–12%			
[23]		10%	45%		38%					7%		

% is the calculated mole percent as calculated by the various authors.

Abbreviations: DAG- diacylglycerol, TAG- triacylglycerol, WE-wax ester, Ch-cholesterol, CE-cholesteryl ester, Foh-fatty alcohol, FA-fatty acid, OAHFA-(o-acyl) omega-hydroxy fatty acid, PL-phospholipid, Squ-squalene, GL-glycolipid, CER-ceramide

TABLE 2

Molar Extinction Coefficients in Solvents of Tested Compounds

Chromophore	**solvent**	**ε (M^{-1} cm^{-1})**	**λ_{abs} (nm)**	**Reference**
DAUDA	ethanol	4400	335	[48]
C6-NBD ceramide	methanol	22000	465	[49]
C6-NBD ceramide-tear lipocalin complex	NaP, pH 7.3	22000	475	current work
C12-NBD ceramide	methanol	22000	465	[49]
NBD-Cl	methanol	9800	336	[50]
Tear lipocalin	NaP, pH 7.3	13760	280	[51]
Lysozyme	NaP, pH 7.3	37970	280	[52]

ε is the molar extinction coefficient; λ_{abs} is the peak wavelength of absorbance.

TABLE 3

Binding Parameters Calculated from Fitting of Ligand Binding to Tear Lipocalin

Ligand	Assay used	Fitting Equation	Kd μM	n
C6-NBD ceramide	High speed centrifugal precipitation	Hyperbola	0.32	0.44
		Hill	0.29	0.45
	Spectral linear summation	Hyperbola	0.06	0.45
		Hill	0.07	0.47
	Fluorescence	Hyperbola	0.08	0.32
		Hill	0.08	0.32
C12-NBD ceramide	High speed centrifugal precipitation	Hyperbola	1.23	0.42
		Hill	1.06	0.39
	Fluorescence	Hyperbola	0.13	0.67
		Hill	0.10	0.21

TABLE 4

Clustering results from the docking of Ceramide 18 into Tear Lipocalin by SwissDock

Cluster rank for ligand poses based on fullfitness	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
1	−1328.48	−8.38
2	−1324.35	−8.86
3	−1322.12	−9.69
4	−1318.96	−8.28
5	−1319.64	−9.06

The 5 top SwissDock clusters of 30 (250 runs) are shown.