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The cytotoxicity of fatty acid/α-lactalbumin complexes depends on the amount and type of fatty acid

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Abbreviations: **α-LA**: Alpha lactalbumin, **BAMLET**: Bovine alpha lactalbumin made lethal to tumor cells, **EA**: Elaidic acid, **FA**: Fatty acids, **FCS**: Fetal calf serum, **HAMLET**: Human alpha lactalbumin made lethal to tumor cells, **LA**: Linoleic acid, **OA**: Oleic acid, **PA**: Palmitoleic acid, **PS**: Phosphatidylserine **SA**: Stearic acid, **VA**: Vaccenic acid.

Summary

Complexes of the milk protein, α-lactalbumin, and the fatty acid, oleic acid, have previously been shown to be cytotoxic. Complexes of α-lactalbumin and five different fatty acids (vaccenic, linoleic, palmitoleic, stearic and elaidic acid) were prepared and compared to those formed with oleic acid. All complexes were cytotoxic to human promyelocytic leukemia-derived (HL-60) cells but to different degrees depending on the fatty acid. The amount of fatty acid per α-lactalbumin molecule was found to correlate with the cytotoxicity; the higher the number of fatty acids per protein, the more cytotoxic the complex. Importantly, all the tested fatty acids were also found to be cytotoxic on their own in a concentration dependent manner. The cytotoxic effect of complexes between α-lactalbumin and linoleic acid, vaccenic acid or oleic acid was further investigated using flow cytometry and found to induce cell death resembling apoptosis on Jurkat cells.

Practical applications

Cytotoxic complexes of α-lactalbumin and several different fatty acids could be produced. The cytotoxicity of all the variants is similar to that previously determined for α-lactalbumin/oleic acid complexes.

Introduction

During the last decade, researchers have studied a cytotoxic complex consisting of the milk protein, alpha lactalbumin (α-LA), and the fatty acid, oleic acid (OA). Such a compound, called HAMLET or BAMLET (Human/Bovine alpha lactalbumin made lethal to tumor cells), can be generated by addition of purified α-LA to an anion exchange column pretreated with OA, followed by elution of the α-LA:OA complex with a high salt buffer [1]. In order to generate the cytotoxic complex, the fatty acid was shown to be a required factor and for optimal cytotoxic effect, the fatty acid had to be either OA or vaccenic acid [2], suggesting that the apoptotic effect of the complex was sterio selective.

HAMLET showed promising healing results *in vivo* when tested as topical application on human skin papillomas [3], on human glioblastoma xenografts in a rat model [4] and by intravesical HAMLET instillations in both mice and humans with bladder cancer [5, 6].

In addition to human and bovine α-LA, equine, caprine, and porcine α-LA can also form cytotoxic complexes with OA [7]. Later investigations showed that the loss of tertiary structure in α-LA denatured by heat-treatment or a recombinant variant of α-LA in which all four cysteines have been exchanged with alanine did not prevent it from forming cytotoxic complexes using an OA conditioned column [8, 9]. Furthermore, equine lysozyme, a structural homolog to α-LA, can also be converted to a cytotoxic complex on an OA pretreated anion exchange column [10, 11]. Complexes between bovine β-lactoglobulin and OA have also been formed [12, 13]. In addition it was found that it is possible to make BAMLET-like complexes without the need for a pretreated anion-exchange column; Knyazeva et al. formed complexes

of α-LA and OA by titrating the protein solution with OA under alkali conditions [14]. Furthermore, complexes of peptide fragments of α-LA have been formed by mixing the protein with OA in ethanol dispersed in a buffer [15] and BAMLET has also been made by mixing α-LA with the more soluble sodium salt of OA, sodium oleate [16]. The α-LA:OA complexes are cytotoxic to a wide range of cancer cells [17-19] as well as some types of primary cells [17]. The structure of the H/BAMLET has been a subject of multiple studies, often with conflicting results. The number of OA molecules bound to α-LA has reported values between 1 and 13 [2, 9, 14, 20].

A recent study showed that OA plays an essential role for the cytotoxicity of α-LA:OA complexes and that the cytotoxicity of OA alone is comparable to that of the α-LA:OA complex in a concentration dependent manner [17]. Another study showed that the cytotoxicity of complex appears to depend entirely on the fatty acid component [12]. Fatty acids are cytotoxic on their own, but the sensitivity of different cell types to treatment with fatty acids differs with both the concentration and the type of fatty acid in question [21, 22]. Several mechanisms involved in the toxicity of fatty acids have been suggested. Examples are changes in mitochondrial transmembrane potential and intracellular neutral lipid accumulation [21], activation of caspase 3 and production of reactive oxygen species [23], as well as endpoints such as loss of membrane integrity and DNA fragmentation [24].

In the present study, complexes of α-LA and fatty acids (α-LA:FA complexes) were prepared as well as complexes with different amounts of fatty acids per protein in order to investigate the cytotoxicity of α-LA in combination with different fatty acids. The fatty acids used were OA (18:1 cis Δ9), elaidic acid (EA, 18:1 trans Δ9), vaccenic acid (VA, 18:1 cis Δ11), palmitoleic acid (PA, 16:1 cis Δ9), linoleic acid (LA, 18:2

cis $\Delta 9$, $\Delta 12$) and stearic acid (SA, 18:0). The influence of the number of fatty acids per α-LA molecule on the cytotoxicity was examined. In addition, the cytotoxicity of the fatty acids in the absence of α-LA was investigated. Lastly, flow cytometric analysis was performed to assess the phosphatidylserine (PS) exposure and membrane permeability in response to treatment of Jurkat cells with the α-LA:FA complexes.

Materials and Methods

All buffers were prepared from analytical grade chemicals purchased from Sigma-Aldrich (Brøndby, Denmark) or Merck (Hellerup, Denmark). Milk used for the purification of α-LA to make BAMLET was sourced from a local farm (Aarhus, Denmark). For the formation of the α-LA:FA complexes in the single-phase system (see below), α-LA (95% purity, 3% β-lactoglobulin content) was purchased from Davisco Foods International, Inc (Eden Prairie, MN, USA). The fatty acids and sodium derivates of the fatty acids were purchased from Nu-Chek Prep (Elysian, MN, USA) with the exception of sodium stearate and sodium oleate, which were purchased from Sigma Aldrich (Dublin, Ireland). Cell lines HL-60 (human promyelocytic leukemia-derived cell line, code ACC3) and Jurkat (human acute T cell leukemia-derived cell line, code ACC 282) from Deutsche Sammlung von Mikroorganismen und Zellkulturen were used.

Preparation of α-LA:OA complexes by use of ion-exchange column

α-LA was purified from bovine milk and used for the preparation of BAMLET as previously described. [20]. In brief, α-LA was purified by subjecting the whey fraction of bovine milk to two consecutive DEAE-Sepharose fast flow columns in the presence or absence of Ca^{2+} on the first and second column, respectively. The α-LA-containing fractions were pooled, lyophilized and stored at -20°C until use.

The purified α-LA was used for the preparation of BAMLET [20]; a DEAE-Sepharose fast flow column was pretreated with OA and unbound OA was removed from the column by washing with a high salt buffer (1 M NaCl, 10 mM Tris, pH 8).

α-LA dissolved in buffer (10 mM Tris, 0.08 mM EDTA, pH 8.5) was added to the OA pretreated column. After removal of pure α-LA with a low salt buffer (0.2 M NaCl, 10 mM Tris, pH 8.5), the α-LA:OA complex was eluted using the high salt buffer (1 M NaCl, 10 mM Tris, pH 8). The eluate was dialyzed against 10 L phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and concentrated to approximately 2 mg/mL using an Amicon stirred cell with an Ultracell Amicon Ultrafiltration Disc membrane (MW cut-off = 10,000 Da). The samples were frozen and kept at -20°C until use. The protein content of the samples was determined by acid hydrolysis followed by quantitative amino acid analysis. The given concentrations of BAMLET are thus based on the α-LA component alone.

Preparation of α-LA:FA complexes by single-phase system

α-LA purchased from Davisco Foods International, Inc was further purified by ion-exchange chromatography. Briefly, the powder was solubilized in 10 mM Tris-HCl, pH 7.0 and applied to a Q-Sepharose (GE Healthcare, Dublin, Ireland) ion-exchange column. The protein was eluted using a gradient from 0 to 1 M NaCl in 10 mM Tris-HCl, pH 7.0. Fractions were collected and those containing only α-LA were pooled and dialyzed against distilled water before freeze-drying. SDS-PAGE analysis indicated the absence of any β-lactoglobulin impurity, therefore the final purity of α-LA was greater than 98%. For preparation of the complexes, α-LA was solubilized in PBS overnight. The α-LA concentration was determined using absorbance at 280 nm and an extinction coefficient $E_{1\%, 280nm}$ of 20.1. The α-LA solution was diluted to a concentration of 3 mg/mL (0.21 mM) and one of the salts of various fatty acids was

added: sodium oleate, sodium vaccinate, sodium linoleate, sodium elaidate, sodium palmitoleate, or sodium stearate. Three fatty acid concentrations were used: 0.42 mM, 1.06 mM, and 2.11 mM corresponding to an initial molar ratio of 2, 5, and 10 fatty acid molecules per α-LA molecule. The mixtures (20 mL) were heated at 60°C for 1 hour. The heated solutions were cooled on ice and dialyzed against distilled water at room temperature for 36 hours with three changes of water to remove unbound fatty acid and salt. The dialyzed solutions were freeze-dried.

Dynamic light scattering

The hydrodynamic radius of the aggregates formed in solutions containing the sodium salts of the fatty acids and in α-LA:sodium salt mixtures were measured using a Nano-Zetasizer system (Malvern Instruments Inc., Worcestershire, UK). The measurements were made at 25°C at a scattering angle of 12º. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution. Given the low concentration of protein, the viscosity of the solution was assumed to be equal to that of water.

Measurement of fatty acid content

The amount of fatty acid added to the cells was quantified using a Free Fatty Acid Quantification assay (Biovison, Mountain View, CA, USA) according to the manufacturer's instructions. In brief, the samples with unknown fatty acid content were added to the wells of a 96 well plate in several dilutions to make sure the fatty acid content falls within the standard curve prepared with known amounts of palmitic

acid. Fatty acid assay buffer was added to all wells to reach a final volume of 50 µL. In addition, 2 µL Acyl CoA synthetase were added to each well followed by incubation at 37°C for 30 minutes to allow conversion of the fatty acids to their CoA derivatives. A mix of enzymes, enhancer and a fatty acid probe was added to each well resulting in oxidation of the CoA derivatives and generation of hydrogen peroxide as a side product. The fatty acid probe then reacts with the hydrogen peroxide and generates a red color during 30 minutes incubation at 37°C in the dark. The color was detected at 570 nm using an ELISA plate reader.

Cell culture

HL60 and Jurkat cells were grown in the culture medium RPMI-1640 with glutamine (Gibco, Invitrogen, Taastrup, Denmark) supplemented with 10 mM HEPES, 23.8 mM NaHCO$_3$, 10% heat treated fetal calf serum (FCS) (Gibco), 2 mM Glutamax (Gibco), 60 µg/mL penicillin (Benzylpenicillin, Panpharma, Luitré, France) and 0.1 mg/mL streptomycin (Sigma). The cells were kept between $1x10^5$–$1.5x10^6$ cells/mL.

Measurement of Cytotoxic Activity

To analyze the cytotoxic activity of the various compounds, i.e., fatty acids, α-LA and α-LA:FA complexes, an assay estimating cell viability by measuring the ATP content of the cells (CellTiter-Glo Luminescent cell viability assay, Promega) was used.

HL-60 cells were harvested by centrifugation at 150g for 5 minutes, resuspended at 2×10^5 cells/mL in serum-free culture medium and seeded into 96-well plates with 50

µL cell suspension per well (1×10^4 cells/well). HL-60 cells were chosen for the cytotoxicity experiments as they have been used as a reference cell line in an earlier study [17].

Lyophilized α-LA or α-LA:FA complexes to be used in the assays were dissolved in PBS before use. For investigation of the cytotoxicity of fatty acids alone, free fatty acids were dissolved in 96% ethanol and diluted three-fold in ethanol covering 100 mg/mL to 0.14 mg/mL (350 mM - 500 µM). Each FA/ethanol dilution was subsequently diluted 100 times in PBS and the resulting dilutions were added to the cells (50 µL FA + 50 µL cells in serum free culture medium, final concentration 1777, 592, 197, 65.8, 21.9, 7.31, 2.44 µM). Immediately before addition to the cells, the FA dilutions were sonicated for 5 minutes, which allowed for better dispersion of the FA and more reproducibility when aliquoting the FA. As controls 50 µL, of PBS and PBS + 5 µL of lysis solution (Cytotoxicity Detection Kit^{PLUS} (LDH) from Roche) were added to cells in 50 µL culture medium. In all experiments, the cells and proteins or FA were incubated for 1 hour in cell culture medium without FCS in a humidified 5% CO_2 atmosphere at 37°C. After 1 hour, heat-inactivated FCS was added to all wells to reach 5% FCS followed by 3.5 hour incubation. After a total incubation time of 4.5 hours the experiments were evaluated using the ATP assay as recommended by the manufacturer (CellTiter-Glo Luminescent cell viability assay) and the resulting luminescence was detected on a VICTOR^3 1420 Multilabel Counter (PerkinElmer).

The results presented are the average and standard deviation of two independent replicates with measurements on each replicate made in triplet. The LC_{50} was determined by fitting the data to a sigmoidal dose response curve using GraphPad Prism ® software (version 3.03, GraphPad Software Inc, La Jolla, CA, USA).

Analysis of variance (ANOVA) was carried out using SigmaStat (version3.0, Jandel Scientific, Corte Madera, CA, USA); the Student-Newman_Keuls method, pairwise multiple comparison procedures were used to determine differences between the LC50 of the different complexes and fatty acids.

Flow Cytometry

Cell death induced by α-LA:FA complexes was investigated using Annexin V and 7-Amino-actinomycin (7AAD) modified from the Annexin V/PI assay [25] as described previously [17]. Jurkat cells were harvested by centrifugation at 150g for 5 minutes followed by resuspension in serum free cell culture medium. A cell suspension of 3.75×10^5 cells/mL was prepared and 1 mL cell suspension was added to the wells of 6 well plates (TPP tissue culture test plate 92006). Then 500 µL PBS or α-LA:FA complex were added to the appropriate wells followed by incubation in a humidified 5% CO_2 atmosphere at 37°C. After one hour, 75 µL heat inactivated FCS was added to reach a final concentration of 5% serum. Positive controls for necrosis and apoptosis were attained by heat-treatment for 30 minutes at 56°C or by treatment with 10 µM camptothecin for 4.5 hours, respectively. After a total incubation time of 4.5 hours, the cells were washed two times in PBS and resuspended in Hanks Balanced Salt Solution ($+Ca^{2+}$, $+Mg^{2+}$). The cells were stained with Phycoerythrin (PE)-conjugated Annexin V and 7-AAD (BD Biosciences, Annexin V-PE Apoptosis Detection kit I, 55976) using 5 µL Annexin V or 7AAD per 1×10^5 cells in 100 µL buffer followed by 15 minutes incubation in the dark at room temperature before immediate analysis. The binding of Annexin V and 7AAD was investigated using a Cytomics FC 500 flow cytometer (Beckmann Coulter, FC 500 MCL System with

CXP Software) and a 488 nm argon laser for excitation. Annexin V-PE was detected in FL-2 by a 575 nm band pass filter, while 7AAD was detected in FL4 by a 675 nm band pass filter. Standard compensation using unstained and single stained cells was conducted in FlowJo Flow Cytometry Analysis Software (Tree Star, Inc.). For each sample, 7,500 cells were analyzed and the double negative (Annexin V⁻, 7AAD⁻), single positive cells (Annexin V⁺, 7AAD⁻) and double positive cells (Annexin V⁺, 7AAD⁺) were expressed as a percentage of the total cell number.

Results

Complexes between α-LA and the following six fatty acids; OA, EA, SA, VA, PA and LA were prepared according to a previously established method, using a single phase system [16]. The low solubility of the fatty acids in aqueous systems can limit its application. However, this problem could be overcome by heating the more soluble fatty acid salts in the presence of the protein (60°C).

Dynamic light scattering measurements at 25°C prior to heating the mixtures, showed that the presence of α-LA broke-up the large aggregates formed by the fatty acid salts in solution. The size of the particles present in the fatty acid solution was distributed in a trimodal manner in the absence of α-LA (Fig. 1A, C, E, G). In the presence of α-LA, the mixture took on a monomodal size distribution (Fig. 2B, D, F, H). The size of the particles present was slightly larger than that of an α-LA solution, 3.5 nm (not shown). The disappearance of the large fatty acid aggregates indicates the possible formation of a complex between α-LA and fatty acids. The sodium elaidate and sodium stearate were insoluble at room temperature making any meaningful dynamic light scattering measurements impossible; however, the addition of the protein greatly increased the solubility of these fatty acids.

The results outlined here show that the previously used single-phase system used for complex preparation was successfully employed to form complexes with a variety of fatty acids. While ratios of 2, 5, and 10 fatty acids per α-LA were used when preparing the complexes, not all the fatty acid bound to α-LA. The extensive dialysis step in the preparation removed the unbound fatty acid [16]. The binding stoichiometry was dependant on the initial quantity of fatty acid present. The variation in the quantities of the various fatty acids bound was low, on average approximately 50 % of the fatty acid added was bound to the protein after the dialysis step. The values for stoichiometry of complexes are shown in table 1.

Cytotoxicity of α-LA/FA complexes

All six complexes were found to be cytotoxic to HL-60 cells with a 50% loss of viability (LC_{50}) at 17 ± 2, 25 ± 1, 26 ± 1, 28 ± 2, 239 ± 4 and 294 ± 11 μM fatty acid in complexes of α-LA and VA, PA, LA, OA, EA and SA respectively, thus resulting in a difference between the most cytotoxic (α-LA:VA) and the least cytotoxic complex (α-LA:SA) of approximately 17 fold (Fig. 2). The differences between the VA, PA, LA and OA complexes were not significant however, the SA and EA complexes were significantly less cytotoxic that the others (P<0.05).

Influence of the amount of fatty acid on the cytotoxicity of the α-LA:FA complexes

The influence of the number of fatty acid per α-LA molecule on the cytotoxicity was investigated for complexes between α-LA and LA, VA and OA. A correlation was seen between the cytotoxicity and the α-LA:FA ratio, i.e., more fatty acid per α-LA results in higher cytotoxicity (Fig. 3). The cytotoxicity of BAMLET prepared on the

OA-pretreated ion chromatography column with a final α-LA:OA ratio of 1:6.1 was very similar to that of the α-LA:OA complex with a final ratio of 1:5.7 prepared by heating mixtures of sodium oleate and α-LA followed by heating (Fig. 3A).

Cytotoxicity of various fatty acids

The cytotoxicity of the various fatty acids alone was investigated using HL-60 cells and the ATP assay. All tested fatty acids were cytotoxic as illustrated in Fig. 4. LA, PA, VA and OA had very similar cytotoxicities. EA showed the least cytotoxic activity. It was not possible to investigate the cytotoxicity of SA under the experimental conditions used here, as the melting point for stearic acid is 69-70°C [26]. The measured fatty acid concentration in the cell media was lower than theoretically calculated, probably due to loss of FA to plastic surfaces of tubes (eppendorpf tubes) and pipette tips. The amount of FA used in this assay was measured using a fatty acid quantification kit and the measured FA concentration is depicted on the x-axis in Fig. 4. The LC_{50} of the fatty acids was 5 ± 1, 5 ± 1, 8 ± 1, 9 ± 1, and 896 ± 1 μM for LA, PA, VA, OA and EA respectively. EA is significantly (P<0.05) less cytotoxic than the other fatty acids.

Flow cytometry analysis of cell death induced by α-LA:FA complexes

To further investigate cell death induced by the various α-LA:FA complexes, Jurkat cells treated with complexes between α-LA and VA, LA, or OA were analyzed by flow cytometry using Annexin V and 7AAD. Jurkat cells were chosen over HL-60 cells in these experiments as the incubation time with camptothecin required to obtain

a positive control for apoptosis for HL-60 cells (>12 hours) exceeded the duration of the experiment (4.5 hours). Exposure of PS on the outer leaflet of the plasma membrane indicative of cell death resembling apoptosis was detected by Annexin V binding. Membrane damage indicative of necrosis or late apoptosis was detected by staining with the DNA intercalating dye, 7AAD. As illustrated in Fig. 5., the three types of complexes tested (α-LA:LA, α-LA:VA, and α-LA:OA) all resulted in an increase in Annexin V positive and 7AAD negative cells (Annexin V$^+$, 7AAD$^-$) from 1% for untreated cells to 12.9%, 21.2% and 21.5% for cell treated with the three complex-types, respectively. A slight increase in double positive cells (Annexin V$^+$, 7AAD$^+$) was also seen for the complexes from 0.57% for untreated cells to 2.44%, 3.04% and 6.76% for cell treated with α-LA:LA, α-LA:VA and α-LA:OA, respectively (Fig. 5D-F). As seen in Fig. 5G and 5I, cells treated with 75 µg/mL α-LA:LA complex closely resembled untreated cells, whereas treatment with 125 µg/mL α-LA:LA complex resulted in loss of cells manifested as a low number of events counted during flow cytometry.

Discussion

Two different methods were used for the preparation of complexes for this study. BAMLET complex was prepared using the well-established chromatographic method [1]. While this method has some shortcomings in terms of lab-to-lab reproducibility it is the method that has been used for the majority of studies involving H/BAMLET in the literature. Therefore it is beneficial to use BAMLET for comparative purposes.

In the present study, complexes containing OA, EA, VA, PA, LA and SA were produced by mixing the sodium salts of the fatty acids with α-LA followed by heating at 60°C. Several variations of this approach had been employed previously to make BAMLET-like complexes [12-15]. Excess fatty acid was removed by extensive dialysis of the complex. The formation of BAMLET either by a chromatographic or mixing method is not fully understood. Recent studies have shown that large quantities of oleate can associate with α-LA [27]; however, the quantities of oleate which have been reported in BAMLET complexes are much lower. This suggests that some of the FA may be only loosely associated with α-LA and may be removed by dialysis. If there is an equilibrium between bound and free FA in the complex, it could be expected that if the dialysis is sufficient, all bound FA could be removed. In light of these unknown binding mechanisms, the dialysis was carefully carried-out to standardize the way each preparation was treated and to eliminate any differences due to the treatment during the dialysis step.

We show here that complexes between α-LA and different fatty acids with varying degrees of saturation and double bond conformation are also cytotoxic (Fig. 2). The order of cytotoxicity was α-LA:VA = α-LA:LA = α–LA:OA = α-LA:PA > α-LA:EA > α-LA:SA with a difference in LC_{50} of approximately 17 fold between the most (α-

LA:VA and α-LA:PA LC_{50} = 17 µM fatty acid) and the least (α-LA:SA LC_{50} = 294 µM fatty acid) cytotoxic complex.

Earlier reports have suggested that only OA and VA are optimal for formation of a cytotoxic complex on a column pretreated with the fatty acid in question [2]. While other unsaturated cis fatty acids were shown to form complexes with α-LA, the yield of complex formation was lower and the complexes had suboptimal cytotoxicity [2]. There may be some possible explanations for the difference in results observed here compared to this previous study. It was suggested that the interaction between the fatty acids and α-LA was stereo-specific favoring the cis conformation. However, in the present study, complexes with EA and SA, which are both trans fatty acids, were successfully formed. The suggested stereo-specific interactions may play a role in how the fatty acids bind to the column matrix and how they are "presented" on the matrix to be available for the protein. As an ion-exchange resin was used for the preparation in the previous study, the pKa of the fatty acid may affect its interaction with the column. As both chain length and conformation of the fatty acids influence the pKa, the interaction with the column and possible association with α-LA may differ between various fatty acids. The method employed for preparation of the complexes in this study is more straightforward as any influence of the interaction of the fatty acids with the column is removed, and the complex formation is solely dependent on the interactions between the fatty acid and the protein. The equilibrium between a-LA and fatty acid is further complicated by the equilibrium between the fatty acid and column matrix. As the binding to α-LA has been reported to be quite weak [28], the absorption onto the column matrix may in fact limit the amount of fatty acid taken up by the protein. The variations in the affinity of different fatty acids for the column matrix and/or α-LA could be significant in the formation of the complexes

using the column method. However, as can be seen in Figure 1, the fatty acid readily associates with the protein when they are in solution together. The earlier study [2] did not determine the amount of fatty acid bound to the column matrix. It is thus possible that this is where the variation in the yields of the different complexes could have arisen. The different preparation methods may offer an explanation for the discrepancies between the results obtained here and the previous study.

In previous studies, the quantification of the concentration of complex used for cytotoxicity assays was based solely on the protein concentration, whereas the crucial measure of the fatty acid component of the complex was not made. Recent studies have shown that the fatty acid (OA) portion is the cytotoxic component of the complex [12], meaning that using a fixed concentration based on protein content may not be an adequate approach when determining cytotoxicity. The steep dose response curves observed here (Fig. 2.) imply that a small difference in the amount of fatty acid bound to α-LA could induce a difference between ~100% and ~0% viability of the cells in the presence of a fixed protein concentration. Thus it is crucial to measure the fatty acid content in order to compare the cytotoxicity of different complexes.

The dynamic light scattering measurements show that PA, VA and LA salts behave in a manner similar to sodium oleate when in solution with α-LA; a further indication that the approach described here is suitable for the formation of complexes for a variety of different fatty acids. The results show that there is an interaction between the fatty acid and the protein even prior to heating the solution. This agrees with previous studies where a decrease in particle size and/or turbidity was observed when OA was mixed with α-LA [15, 29]. While dynamic light scattering data for SA and EA salts was not included because of their insolubility, it is clear from the results for

the other fatty acids that α-LA greatly increases the solubility of the fatty acid salts. Thus, it is likely that α-LA had the same effect on the SA and EA salts. Visually, the solubility of the two salts seemed to increase greatly in the presence of α-LA. HAMLET has been shown to translocate across the plasma membrane and into the nucleus of cancer cells [4, 30], where it interacts with the histones and perturbs the chromatin structure [30]. HAMLET has also been shown to affect mitochondria [31] and induce apoptosis-like cell death with caspase activation, PS externalization and chromatin condensation irrespective of the p53 and bcl-2 status of the cells [32] and to cause changes in proteasome structure and function [33]. In addition, it has been shown by Aits et al. that HAMLET induces autophagy [34], while Rammer et al. see activation of the lysosomal death pathway in response to BAMLET treatment, but that the cytotoxicity of BAMLET does not depend on autophagy [18]. However, apart from these quite specific effects of HAMLET, there is also evidence of a more crude effect connected to membrane interactions and alterations. α-LA binds to the membrane, but HAMLET causes loss of membrane integrity and leakage of lipid membranes [35] and ELOA, the cytotoxic complex between equine lysozyme and OA, has also been shown to induce loss of membrane integrity with ELOA accumulating at the cell membrane until the cells rupture and ELOA fills the cells [11, 36].

Several reports show that fatty acids cause loss of membrane integrity in higher concentrations [21, 23, 24], which could indicate that the fatty acid in itself is responsible at least for the membrane-altering effects of α-LA:FA complexes.

When investigating the cytotoxicity of the α-LA:FA complexes, it was found that the concentration range between no measurable response and 100% cell death was very

narrow (Fig. 1 and 3). The ATP assay used in this report does not discriminate between direct lysis of the cells and induction of cell death followed later by loss of membrane integrity. However, the results shown in Fig. 5 demonstrate the existence of cells with PS exposure but without loss of membrane integrity, indicative of induction of cell death rather than direct lysis. Nevertheless, as Fig. 5 illustrates, there is a very narrow concentration range (e.g., 75–125 µM for α-LA:LA) between no detectable effect and the loss of membrane integrity or even loss of intact cells as seen by the low cell numbers detected by the flow cytometer (Fig. 5G to I). This narrow concentration range was also seen for α-LA:VA and α-LA:OA complexes (data not shown).

By preparing complexes of α-LA and LA, VA or OA in different α-LA:FA ratios, it was found that the cytotoxicity of the complexes was correlated to the number of fatty acid per protein with more fatty acids per protein being more cytotoxic (Fig. 3).

When BAMLET, prepared on the OA pretreated ion exchange column as described in this paper, is compared to HAMLET and BAMLET prepared according to Svanborg et al. [1], the complexes show similar cytotoxicity [17]. As illustrated in Fig. 3, the cytotoxicity of BAMLET (α-LA:OA = 1:6.2) was also comparable to that of the α-LA:OA complex prepared with the single-phase system resulting in a α-LA:OA ratio of 1:5.7 (Fig. 3A). Together this indicates that the fatty acids play a crucial role in the cytotoxicity of HAMLET and BAMLET-like complexes. Furthermore, the comparability of the various complexes underlines that the cytotoxic complexes can be prepared using different methods as also shown by Permyakov [37].

Some of the free fatty acids seem to be more cytotoxic than their corresponding complexes. Earlier reports on H/BAMLET and other α-LA:FA complexes did not

find fatty acids to be cytotoxic in the concentrations present in the complexes [1, 2, 15]. However a recent study suggested that the amount of fatty acid delivered to the cells may be greatly reduced due to adsorption of the fatty acids on the surfaces of plastics meaning that the reported LC_{50} of the fatty acids may have been over-estimated previously; this may explain the apparent discrepancies in the literature regarding the cytotoxicity of OA [17]. In the present study great care was taken in solubilising the fatty acids by dissolving them in ethanol and subjecting them to sonication; this may aid in increasing their cytotoxicity. It is worth noting that the cytoxicity of elaidic acid increased greatly when it was in complex, there is a nearly 6 fold reduction in the LC_{50} of the fatty acid when in complex compared to the free fatty acid. Elaidic acid was one of the least soluble fatty acids used for this study so it is hardly surprising that its cytotoxic activity showed the greatest increase when in complex with α-LA. From both the results with the free fatty acid and the complexes, it can be seen that the type of fatty acid may be significant in terms of the cytotoxic effect. The cis fatty acids were all grouped together with little or no differences in their cytotoxicty, the trans fatty acids (elaidic and stearic) were significantly less cytotoxic that the cis fatty acids. The poor solubility of the trans fatty acids could be a contributing factor here.

These results raise questions about the benefit of delivering the fatty acids in a complex with α-LA. Further studies are required to evaluate the stability and cytotoxicity of the complexes over time versus the solubilised fatty acids over time. The molar concentration of fatty acid present is the critical factor in determining the cytotoxicity, however, how the fatty acid is solubilised will also play a role. It could be expected that too high concentrations of the fatty acids would be less soluble and may not be as effective. To this end the CMC of the fatty acids may also be an

important factor in determining cytotoxicity, further studies into the behaviour of the fatty acids in cell culture media is required to determine whether this is the case. Forming a complex between the fatty acids and α-LA may be a more efficient means of solubilising the fatty acid and thus lead to more reproducible results. The strength of any association of fatty acid with the protein has to be sufficiently weak to allow the fatty acid be released from the protein to have a cytotoxic effect. Previous values reported in the literature would indicate that the association constant of OA to α-LA is relatively weak [28].

It is becoming increasingly accepted that the cytotoxic component of the H/BAMLET complex is the oleic acid portion, but the present study shows that other fatty acids may be just as adequate. Importantly the cytotoxicity is related to the number of fatty acid molecules bound to the protein.

Conflict of interest

The authors have declared no conflict of interest

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Figure legends

Table 1. The quantity of fatty acid remaining in complex with α-lactalbumin after dialysis of the heated mixtures.

Figure 1. Dynamic light scattering measurements on the sodium salts of fatty acids.

Measurements of 1.06 mM Oleic acid (A, B), Vaccenic acid (C, D), Palmitoleic acid (E, F) or Linoleic acid (G, H) sodium salts in PBS solution. The distributions shown in panels B, D, F, and H were in the presence of 30 g/L α-LA. Measurements were carried out at 25°C. The x-axis shows the hydrodynamic radii of the particles. The y-axis shows the distribution of the particles as a percentage based on their volume.

Figure 2: α-LA/FA complexes cytotoxixity

The effect of the complexes between α-LA and the six different fatty acids on HL-60 viability was measured by the ATP assay. The FA acid concentration, measured using assay, applied to the cells is on the x-axis. The values on the y-axis are given as a percentage of the ATP signal of control cells treated with PBS. The experiment was performed in duplicate and the mean and standard deviation is shown for each concentration. vaccenic acid ■, linoleic acid ▲, palmitoleic acid ○, oleic acid ●, elaidic acid ◆, stearic acid ×. The data shown represents the mean and standard deviation of three independent replicates.

Figure 3: Cytotoxicity of α-LA/FA complexes containing differing ratios of FAs

The cytotoxicity towards HL-60 cells by α-LA:LA, α-LA:VA and α-LA:OA complexes with different α-LA:FA ratios were measured with the ATP assay after incubation for 4.5 h. A) α-LA:OA complexes 1:0.9 ●, 1:1.4 ■ & 1:5.7 ▲ and BAMLET 1:6.2 □; B) α-LA:VA complexes 1:0.84 ●, 1:3.1 ■ & 1:6.2 ▲; C) α-LA:LA complexes 1:0.8 ●, 1:2.05 ■ & 1:6.3 ▲. Each experiment was performed in triplicate and mean and standard deviation is shown for each point.

Figure 4 Cytotoxicity of the fatty acids used to prepare the complexes

The effect of elaidic □, vacceinc ○,, linoleic ▲, palmitoleic × and oleic ■ acids on HL-60 viability was measured by the ATP assay. The actual fatty acid concentration applied to the cells was determined using a fatty acid quantification assay to quantify the fatty acid concentration present in the cell culture media. The values on the y-axis are given as a percentage of the ATP signal of control cells treated with PBS. The experiment was performed in duplicate and the mean and standard deviation is shown for each concentration.

Figure 5: Flow cytometry analysis of the cytotoxicity of complexes between α-LA and VA, LA or OA

Flow cytometry analysis of PS exposure and membrane permeability using Annexin V-PE and 7AAD. Jurkat cells were incubated with camptothecin, PBS or complexes between α-LA and LA, VA or OA, for 4.5 hours before flow cytometry analysis. A: Untreated cells (PBS). B: 10 μM camptothecin (positive control for PS exposure). C: heat treatment of the cells (56°C for 30 minutes) (positive control for membrane

permeability). D: α-LA:LA treated cells (100 μg/mL). E: α-LA:VA treated cells (75 μg/mL). F: α-LA:OA treated cells (200 μg/mL). G-I: Jurkat cells treated with increasing concentrations of α-LA:LA. The experiment was repeated three times and the results shown are representative. For each sample, 7,500 cells were analyzed, except for (I) where only 403 cells were analyzed due cell loss.

Table 1, Brinkmann et al.

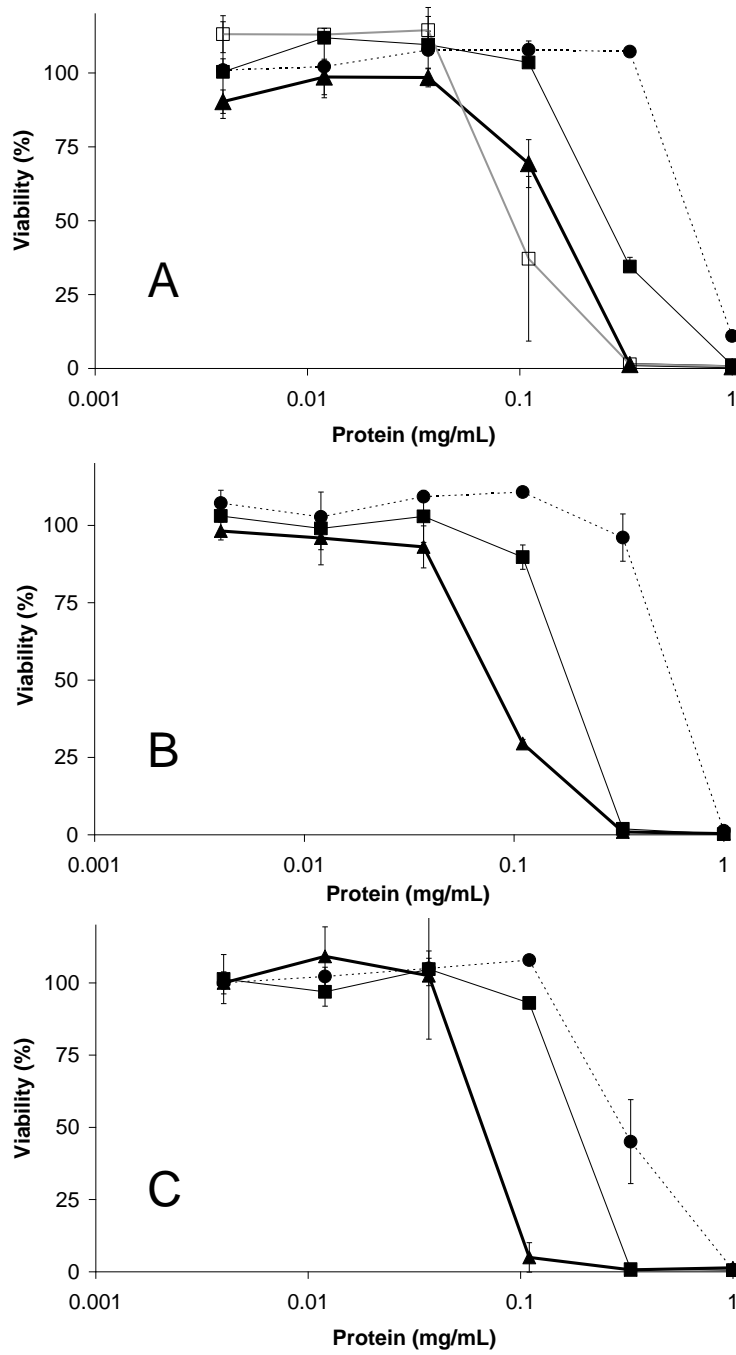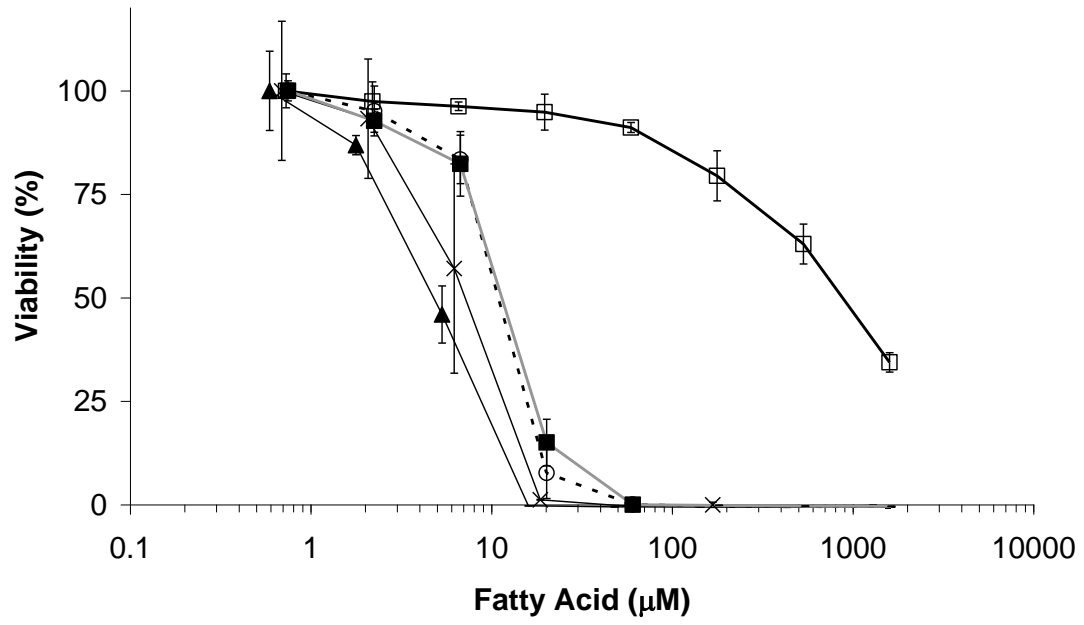| Initial molar ratio	Molar ratio after dialysis	Binding efficiency
Oleic Acid		
2	0.9	45 %
5	1.4	28 %
10	5.7	57 %
BAMLET	6.2	N.A.
Vaccinic acid		
2	0.84	42 %
5	3.1	62 %
10	6.2	62 %
Linoleic acid		
2	0.8	40 %
5	2.05	41 %
10	6.3	63 %

Figure 1, Brinkmann et al.
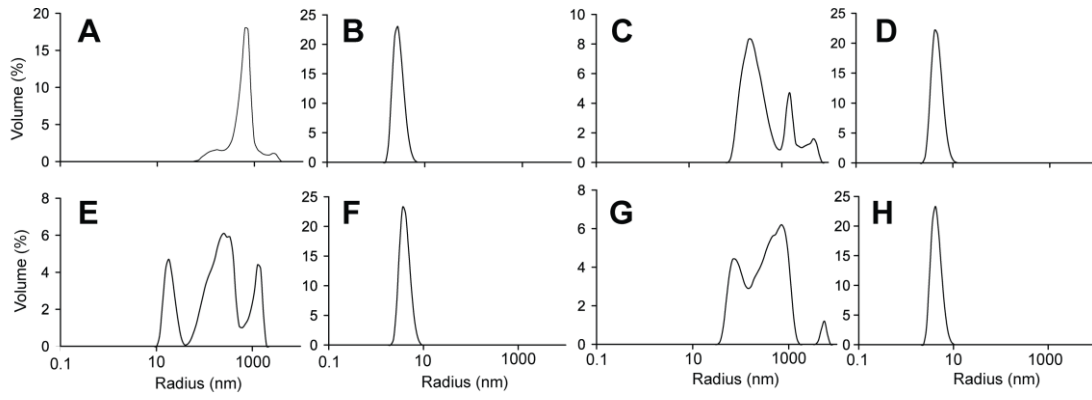
Figure 2, Brinkmann et al.
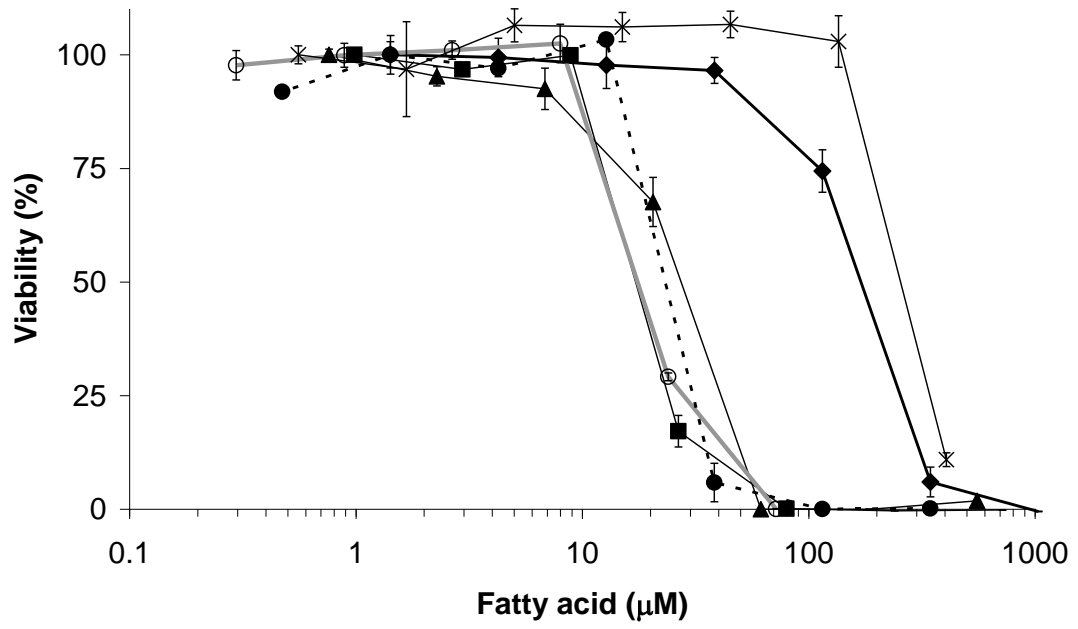
Figure 4, Brinkmann et al.

Figure 5, Brinkmann et al.