Factor H inhibits complement activation induced by liposomal and micellar drugs and the therapeutic antibody rituximab in vitro

Tamás Mészáros[1,2], Ádám I. Csincsi[3], Barbara Uzonyi[4], Mario Hebecker[5], Tamás G. Fülöp[1,2], Anna Erdei[4,6], János Szebeni[1,2,*], Mihály Józsi[3,*]

[1]Nanomedicine Research and Education Center, Semmelweis University, Budapest, Hungary
[2]SeroScience Ltd., Budapest, Hungary
[3]MTA-ELTE "Lendület" Complement Research Group, Department of Immunology, Eötvös Loránd University, Budapest, Hungary
[4]MTA-ELTE Immunology Research Group, Department of Immunology, Eötvös Loránd University, Budapest, Hungary
[5]Junior Research Group for Cellular Immunobiology, Leibniz Institute for Natural Product Research and Infection Biology, Jena, Germany
[6]Department of Immunology, Eötvös Loránd University, Budapest, Hungary
*equal contribution

Corresponding author:
Mihály Józsi, MTA-ELTE "Lendület" Complement Research Group, Department of Immunology, Eötvös Loránd University, Budapest, Hungary; Tel.: +36 1 3812175, Fax: +36 1 3812176; E-mail: mihaly.jozsi@gmx.net

Conflict of interest statement: The authors declare no conflict of interest.

Authorship Contributions:
T.M., Á.I.C., B.U. and T.G.F. performed complement activation experiments; Á.I.C., B.U. and M.H. generated and purified recombinant proteins; A.E., J.S. and M.J. conceived and supervised the project; T.M., J.S. and M.J. wrote the paper with the help of the other authors.

Abstract

Hypersensitivity reactions to particulate drugs can partly be caused by complement activation and represent a major complication during intravenous application of nanomedicines. Several liposomal and micellar drugs and carriers, as well as therapeutic antibodies, were shown to activate complement and induce complement activation-related pseudoallergy (CARPA) in model animals. To explore the possible use of the natural complement inhibitor factor H (FH) against CARPA, we examined the effect of FH on complement activation induced by CARPAgenic drugs. Exogenous FH inhibited complement activation induced by the antifungal liposomal Amphotericin-B (AmBisome), the widely used solvent of anticancer drugs Cremophor EL, and the anticancer monoclonal antibody rituximab in vitro. An engineered form of FH (mini-FH) was more potent inhibitor of Ambisome-, Cremophor EL- and rituximab-induced complement activation than FH. The FH-related protein CFHR1 had no inhibitory effect. Our data suggest that FH or its derivatives may be considered in the pharmacological prevention of CARPA.

Abbreviations used in this paper:

AP, alternative pathway of complement; CARPA, complement activation related pseudoallergy; CCP, complement control protein domain; CFHR1, factor H-related protein 1; CrEL, Cremophor EL; FH, factor H; FH1-4, CCPs 1-4 of FH; FH15-20; CCPs 15-20 of FH; mAb, monoclonal antibody; NHS, normal human serum

Introduction

Hypersensitivity reactions, which may have various underlying mechanisms, represent a substantial medical problem during intravenous (i.v.) application of certain nanoparticle-containing medicines.[1-3] Premedication with steroids and other anti-allergic medicines can reduce the risk and severity of such adverse reactions; however, prevention by inhibiting the triggering mechanisms, e.g. complement activation, is expected to offer more specific and effective measure against this adverse immune effect.[3-6]

Complement activation can underlie acute, non-IgE-mediated allergic (infusion) reactions to particulate drugs, which reactions can occasionally be severe, or even lethal.[1,3] Thus, this phenomenon represents a major immune barrier to the therapeutic use of many state-of-the-art "nanomedicines", such as liposomal and micellar drugs and therapeutic antibodies.[3,7] This so-called complement activation-related pseudoallergy (CARPA) is characterized by severe cardiopulmonary changes including arrhythmia, pulmonary edema, hypotension, airway occlusion, respiratory distress and cardiac arrest that in serious cases can cause anaphylactic cardiogenic shock and death.[3] Activation of the complement system leads to the formation of the anaphylatoxins C3a and C5a that can trigger mast cells and basophils to release a variety of secondary mediators with potent pathophysiological actions.[3,8] Among these mediators, histamine, thromboxane A2 and platelet-activating factor have been shown to be causally involved in CARPA due to liposomes.[3]

Complement is a main humoral defense system of innate immunity against infectious microbes. It also plays an important role in the removal of immune complexes and dying cells, and modulates the activation of cells of the innate and adaptive immune systems.[8] Complement constantly probes surfaces and can be activated via three major pathways, the classical, the lectin and the alternative pathway (**Figure 1A**). The alternative pathway (AP) is continuously activated at a low level and, independent of the initial activation route, it

provides strong amplification of the system, because the central C3b molecule, generated by any pathway, forms the C3bBb AP C3 convertase that cleaves additional C3 molecules into the anaphylatoxin C3a and the opsonin C3b. C3b binding to the C3 convertases shifts the ligand specificity of these enzymes that then gain the ability to cleave C5 into C5a and C5b. C5a is a potent anaphylatoxin and inflammatory mediator. C5b initiates the terminal pathway, leading to the formation of the C5b-9 terminal complement complex, which when formed in cell membranes (then termed membrane attack complex, MAC) may cause cell lysis.

Adverse effects of complement in the host, due to overactivation or activation on inappropiate surfaces and ligands, are prevented by cell membrane-bound and soluble complement inhibitory molecules, which control this system at several check points. Factor H (FH) is one of the main soluble complement regulators.[9-11] This 155-kDa glycoprotein has an average plasma concentration of approximately 260 µg/ml. It is composed of 20 complement control protein (CCP) domains, of which CCPs 1-4 mediate the complement inhibiting function of FH and CCPs 19-20 mediate the docking of FH to certain host surfaces, such as basement membranes and endothelial cells (**Figure 1B**). Thus, FH inhibits complement activation not only in the body fluids but on host surfaces, too.[11] FH regulates complement at the AP C3 convertase level by preventing the formation of the C3bBb convertase, by facilitating its dissociation if already formed, and by assisting the protease factor I in the enzymatic inactivation of C3b. These activities render FH a potent down-regulator of complement activation (**Figure 1A**).

The antifungal drug, liposomal Amphotericin-B (AmBisome) and paclitaxel (Taxol), a widely used anticancer drug solubilized by Cremophor EL (CrEL) micelles, were shown to activate complement partly via the AP, and to induce CARPA in pigs with symptoms mimicking the human anaphylactic reaction to these drugs.[12,13] Various therapeutic antibodies also cause hypersensitivity reactions.[3,6] Thus, it was hypothesized that inhibition of AP

4

complement activation might interfere with the CARPAgenic activity of these drugs. Therefore, our aim was to assess the capacity of the natural AP regulator FH and its engineered derivative mini-FH to inhibit complement activation induced by AmBisome, CrEL and the therapeutic antibody rituximab in vitro.

Methods

Proteins, reagents and sera

Purified human FH was from Merck (Budapest, Hungary). Recombinant constructs covering FH CCP1-4 (FH1-4), CCP15-20 (FH15-20), CCP1-4 and CCP19-20 (Mini-FH), and CFHR1 were produced in insect cells as described.[14,15]

CrEL and zymosan (Zymosan-A) were from Sigma-Aldrich Ltd. (Budapest, Hungary), AmBisome was purchased from Gilead Sciences Ltd. (Paris, France), and rituximab (Rituxan; Roche, UK) was obtained from a local pharmacy.

Blood withdrawal and experimentation were performed with approval of the respective research ethics committee and in accordance with the Declaration of Helsinki. Blood donors were healthy Caucasian volunteers and gave informed consent. Peripheral blood was collected in BD Vacutainer® (BD, Franklin Lakes, NJ) serum tubes, allowed to clot at 4°C for 10 min, and after centrifugation aliquots of normal human serum (NHS) were stored at -80°C.

Preparation of liposomes

The liposomes were prepared using the extrusion method. Cholesterol (Solvay Pharmaceuticals, Brussels, Belgium), hydrogenated soy phosphatidyl choline (HSPC) and 1,2-distearoy l-phosphatidyl ethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-mPEG2000) - all from Lipoid GmbH (Ludwigshafen, Germany) - were dissolved in

5

alcohol, mixed in molar ratios as described in Table 1, then 250 mM ammonium sulfate (Sigma-Aldrich) was added to the mix. The multilamellar vesicle (MLV) particles were extruded to uniform particle size distribution on membrane filter (Thermo Fisher Scientific Inc., Waltham, MA, USA) by means of a Lipex™ Extruder (Northern Lipids Inc., Burnaby, B.C. Canada). The liposomes were dialyzed against 10% sucrose/10 mM His, pH 7.4 buffer (Sigma-Aldrich) and diluted (volume ratio of 1:1) in the same buffer after dialysis. The size was determined by Malvern Zetasizer Nano S (Malvern Instruments Ltd, Malvern, UK). Liposomes containing cholesterol-anchored PEG2000 (Chol-2P)[16] was obtained from Utrecht Institute for Pharmaceutical Sciences (Utrecht University, The Netherlands).

Complement activation assays

CrEL was diluted to 105.4 mg/ml and zymosan to 1.5 mg/ml with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). AmBisome was diluted to 20 mg/ml with sterile water. FH or the recombinant complement inhibitors, diluted in PBS in 5 µl, were mixed with 15 µl NHS, then 5 µl of the AmBisome, zymosan or CrEL dilutions were added, and incubated for 45 min at 37°C in a heated shaker. Rituximab (in 75 µg/ml or 300 µg/ml final concentration) and the complement inhibitors FH and mini-FH (both in 1.3 µM final concentration) in PBS were added to whole blood containing lepirudin as anticoagulant in microcentrifuge tubes (in a final volume of 300 µl, containing 50% blood), vortexed and incubated for up to 300 minutes at 37°C. SC5b-9 in the supernatant after centrifugation for 10 min at 1750 *g* was measured using a commercial kit (TECO*medical* AG, Sissach, Switzerland), according to the protocol provided by the manufacturer.

B cell survival assay

Human tonsil B cells were isolated as previously described.[17] BL-41 lymphoma cells were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. 2×10^6 B cells per sample were incubated in veronal buffered saline (VBS; 5 mM veronal, 145 mM NaCl, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.3) or 50% NHS diluted in VBS in 100 μl final volume. Rituximab was added at 10 μg/ml. In preliminary experiments, this concentration showed saturation of binding to B cells (measured by flow cytometry), and there was no increase in complement-mediated cell lysis when higher concentrations were used. The complement inhibitors FH and FH1-4 were added at 1.3 μM concentration. After incubation at 37°C for 30 min, dead cells were stained with propidium iodide (PI). 10,000 cells were analyzed in each sample using a FACSCalibur flow cytometer and CellQuest software for data acquisition (BD Biosciences, Heidelberg, Germany). Data analysis was performed using FlowJo (Tree Star, Ashland, OR) software.

Results

FH and mini-FH inhibit AmBisome- and CrEL-induced complement activation

To assess the efficacy of FH to inhibit liposome-induced complement activation, human serum samples were incubated with AmBisome with or without the addition of exogenous FH at the concentration of 200 μg/ml (~1.3 μM). This amount of FH approximately doubles the physiological FH concentration. SC5b-9 was detected because it is a quite stable end-product of complement activation, can be reliably measured, and is generated in parallel with C5a that is involved in the CARPAgenic effect. Serum or buffer alone resulted in baseline SC5b-9 formation, whereas AmBisome significantly increased the amounts of soluble SC5b-9. Exogenous FH strongly and significantly reduced AmBisome-induced formation of SC5b-9 (**Figure 2A**). Zymosan, a yeast cell wall extract and well-known activator of the complement

AP, was used as a positive control. Zymosan at 0.3 mg/ml concentration strongly activated complement and enhanced the formation of SC5b-9 in serum, which was slightly, but not significantly, inhibited by FH under the studied conditions (**Figure 2A**).

To further analyze the capacity of FH to inhibit liposome-induced complement activation, liposomes of various composition were prepared, which differed in the amounts of PEG incorporated and, accordingly, caused various degrees of SC5b-9 increase when added to human serum. In all cases, addition of 1.3 µM FH reduced SC5b-9 to the background level (**Figure 2B**). This is likely due to the lower level of SC5b-9 induced compared with AmBisome (**Figure 2A**).

Previously, we described mini-FH, an artificial complement inhibitor that combines the N-terminal complement regulatory domains (CCPs 1-4) and the C-terminal C3b- and polyanion binding domains (CCPs 19-20) of FH (**Figure 1**).[15] In our experiments, mini-FH also inhibited complement activation induced by AmBisome, and its effect was more pronounced in comparison with FH (**Figure 3A**). Similar to this, FH and mini-FH inhibited complement activation induced by CrEL-micelles (**Figure 3B**). The inhibitory effect was dose-dependent (**Figure 3C**), and the extent of inhibition suggested that mini-FH is on a molar basis approximately twofold more potent inhibitor of complement activation under these conditions than FH (compare **Figures 3A and 3C**), because 0.65 µM mini-FH reduced SC5b-9 to similar level as did 1.3 µM FH.

Evaluation of functional domains of FH and the FH-related protein CFHR1

While CCPs 1-4 of FH (FH1-4) are necessary and sufficient to control complement activation in fluid phase, CCPs 19-20 of FH are responsible for recognition of surface-deposited C3b and surface polyanionic molecules, and can direct FH to specific host surfaces.[18-21] Therefore, we tested if a FH1-4 construct can inhibit complement activation induced by liposomes and

CrEL micelles similar to FH. To this end, complement activation in human sera was induced by AmBisome or CrEL, in the presence of equimolar amounts (1.3 μM) of FH, FH1-4 or FH15-20. FH1-4 was less potent in inhibiting AmBisome- (**Figure 4A**) and CrEL-induced (**Figure 4B**) increase of serum SC5b-9 than FH, indicating that the full-length molecule is more active. Thus, the native conformation of full-length FH and/or its binding to the activating surface may be important to optimally reduce complement activation. FH15-20, which lacks the complement regulatory domains of FH, did not inhibit either AmBisome- or CrEL-induced complement activation.

In addition, we investigated whether the FH-related CFHR1 protein, which had been described as a terminal complement pathway inhibitor,[22] influenced the formation of SC5b-9 in our assays. CFHR1 when added at 1.3 μM concentration (~50 μg/ml; estimated CFHR1 plasma concentrations are 40 μg/ml[23] and 70-100 μg/ml[22]) did not inhibit SC5b-9 generation induced by either AmBisome (**Figure 4A**) or CrEL (**Figure 4B**).

FH and mini-FH inhibit rituximab-induced complement activation in vitro
A significant proportion of patients suffer adverse reactions caused by therapeutic antibodies, even if premedication is provided.[3,6] Rituximab, a widely used anti-CD20 mAb caused complement activation in anti-coagulated whole blood, more so when lepirudin was used as anticoagulant, compared to heparin (**Figure 5A**). FH inhibited complement activation induced by rituximab (**Figure 5B**). At higher, 300 μg/ml rituximab concentration, however, 1.3 μM FH was not effective. Mini-FH strongly inhibited rituximab-induced complement activation, even at 300 μg/ml rituximab (**Figure 5B**).

When the mechanism of action of a therapeutic antibody involves complement-dependent cytotoxicity, such as in the case of rituximab, it is important not to interfere with complement activation on the target cell curface. We therefore compared the effect of the same amounts of FH and the FH1-4 construct, which does not bind to cells, on survival of

human B cells in the presence of rituximab and serum. FH strongly, whereas FH1-4 only marginally increased the number of surviving primary tonsil B cells (**Figure 6A**) and BL-41 lymphoma cells (**Figure 6B**) when incubated with rituximab in the presence of human serum. Thus, FH1-4 may be considered to inhibit fluid-phase (i.e. serum) complement activation without interfering with the complement-mediated lytic effect of the therapeutic mAb on target B cells.

Discussion

After the discovery of the CARPA phenomenon, the application of complement inhibitors to ameliorate this adverse reaction was suggested,[24] and strategies to anchor complement inhibitor proteins or their active fragment to membranes, e.g. via a myristoyl group carrying the CCP1-3 of complement receptor type 1, were reported.[25,26] Nanomedicines can cause complement activation via various pathways, and theoretically each of these activation pathways could specifically be inhibited by a suitable inhibitor, e.g. C1 inhibitor for the classical/lectin pathways. We aimed to investigate an inhibitor that could likely be more broadly used. Because FH is a major inhibitor at the level of the central C3 complement component and that of the AP amplification loop (**Figure 1A**), it is an ideal regulator to reduce or even prevent generation of the C3a and C5a anaphylatoxins, and also that of C5b-9, particularly, because complement activation by any pathway would be amplified through this loop.[8-11,27] In addition, it was reported that FH could inhibit the classical pathway on ligands where FH can compete for binding with C1q,[28] which may also play a role in the case of certain nanomedicines. The results of our current study demonstrate that FH is a potent inhibitor of complement activation induced by liposomes, CrEL micelles and rituximab in serum in vitro.

FH does not only act in fluid phase but is also an effective and important complement inhibitor on surfaces where FH is able to bind, such as host basement membranes and cells, but in many cases also foreign structures such as pathogens, that sequester host FH to evade the action of complement.[11] We observed that the full-length FH molecule was a better inhibitor of complement activation induced by both AmBisome and CrEL than the FH fragment (FH1-4) containing only the regulatory domains CCPs 1-4 of the molecule (**Figure 4**). This suggests that the native conformation of the full-length molecule is important to optimally inhibit complement, and/or that FH is able to bind to the surface of liposomes and micelles and locally downregulate complement activation. Indeed, recently FH binding to liposomal membranes was demonstrated.[29] Naturally, the complement regulatory CCPs 1-4 are important for the inhibitory effect, and CCPs 19-20 harbour binding sites for both surface-bound C3b/C3d and glycosaminoglycans (or some other ligands).[11] The latter domains may mediate binding of FH, depending on the amount and nature of C3b fragments (C3b, iC3b, C3d) present on the surface. However, inhibition of complement activation in our assays does not necessarily require surface binding of FH, because significant inhibition was obtained with FH1-4.

Derivatives of FH are being evaluated as potential therapeutic complement inhibitors, including the mini-FH class of molecules.[15,27,30] The two reported, slightly differing mini-FH molecules have FH CCPs 1-4 fused with CCPs 19-20, and were shown to have enhanced complement inhibiting capacity compared to the full-length FH.[15,30] A third mini-FH version contains CCPs 1-5 and CCPs 18-20, has also superior complement inhibiting activity over FH, and showed its efficacy in reducing C3 turnover also in vivo.[31] The enhanced activity of mini-FH is likely due to the increased availability of the C-terminal glycosaminoglycan/C3d binding sites in the shorter molecules, which are probably partly hidden in the full-length FH. In agreement with these previous results using different types of complement activation

assays, on a molar basis mini-FH was more active than FH in preventing liposome- and rituximab-induced complement activation (**Figures 3 and 5**). Thus, engineered FH-derivatives may prove useful to effectively down-regulate such adverse complement reactions. While mini-FH in its current form, owing to its small size, is rapidly cleared from the circulation, it still could be useful especially to inhibit CARPA, which is a relatively quickly arising adverse reaction and thus the short-term inhibitory effect of mini-FH, when co-administered with or even coupled to the potentially CARPA-genic drug, could be satisfactory to prevent or reduce CARPA.

The background mechanism and the extent of complement activation differ due to the various composition, surface charge, size and physical properties etc. of the liposomes, micelles and other nanocarriers.[3] The amount and nature of deposited C3 fragments, as well as the physico-chemical properties of the surfaces, also influence the potential interaction of FH with these surfaces. These at least in part explain why the same amount of tested FH resulted in different degree of inhibition; for example, the stronger complement activation and thus higher SC5b-9 levels induced by AmBisome could only partially be inhibited by FH and completely by mini-FH, whereas in the case of CrEL, the less amount of SC5b-9 was fully inhibited by both FH and mini-FH, both applied in 1.3 µM in the assays (**Figure 3**). Altogether these results suggest that an engineered mini-FH-like derivative of FH could be more useful for inhibitory purposes than the native molecule, because it is a more potent complement inhibitor and can also be produced in a more controlled way.[15]

The FH-related CFHR1 protein was previously described as a novel complement inhibitor that binds to C5 and down-regulates the terminal pathway,[22] which effect could not be confirmed by other investigators.[32-35] We tested recombinant CFHR1 for its capacity to influence SC5b-9 formation in the liposome- and CrEL-induced complement activation assays in vitro and found no inhibitory effect of this FH-related protein (**Figure 4**). While this

does not exclude a potential of CFHR1 to regulate cell membrane integrated C5b-9, these results question a general terminal pathway inhibiting function of the protein.

Hypersensitivity or infusion reactions, mediated in part by complement activation, can be observed in a substantial number of patients when administering therapeutic mAbs.[6] Although such reactions do not affect every patient, reactions to various extent were reported in the case of the different mAbs (from <1% to over 50%), and even fatal reactions occur.[36] For rituximab, adverse reactions were reported in up to 85% of patients, with 2-10% being severe reactions and 0.04-0.07% being fatal.[37] In our assays, FH and mini-FH were able to reduce or inhibit rituximab-induced complement activation in serum in vitro (**Figure 5**). This is most likely due to fluid-phase inhibition of complement activation by FH and mini-FH.

The mechanism of action of rituximab on the target B cells involves complement-dependent lysis, antibody-mediated cellular cytotoxicity and induction of apoptosis; however, it is still unknown which of these is the most important mechanism.[38] FH when bound from serum to the cells can reduce the efficacy of complement-mediated B cell killing due to its inhibitory nature.[39] We found, however, that FH1-4, which can be used to inhibit complement activation in serum (**Figure 4**) but cannot bind to the cell surface, does not substantially decrease the efficiency of surface-bound rituximab to induce complement-mediated killing of B cells (**Figure 6**).

There are differences among individuals in the rate of complement activation, which is influenced by serum levels and functional variants of complement proteins and regulatory molecules, such as those of FH. For example, the FH level varies substantially among individuals (124.4-402 μg/ml).[40] The specific set of complement gene variants, termed complotype, determines how quickly and to what extent complement activation occurs under given circumstances in serum of a person, and influences the risk to inflammatory and infectious diseases.[41] This likely explains the observed differences in AmBisome- and CrEL-

induced SC5b-9 production in the sera of the various donors (**Figures 3 and 4**). While genetic analysis to determine the complotype and measurement of protein levels are time-consuming and costly, the capacity of given nanomedicines to induce complement activation in serum or blood of a patient can be tested relatively easily in a functional assay, as also shown here.[42,43]

In summary, our data suggest that FH or its derivatives like mini-FH could be a potentially useful approach to prevent complement activation and thus CARPA caused by a variety of nanomedicines, monoclonal antibodies and other agents. When the mechanism of action of a therapeutic antibody involves complement-dependent cytotoxicity, such as in the case of rituximab, a FH construct that does not bind to cells, e.g. FH1-4, may be considered. Importantly, with a relatively simple test patients can be pre-screened for proneness for CARPA and thus a complement inhibitor may be applied when indeed necessary.[42,43]

References

1. Adkinson NF Jr, Essayan D, Gruchalla R, Haggerty H, Kawabata T, Sandler JD, et al. Task force report: future research needs for the prevention and management of immune-mediated drug hypersensitivity reactions. J Allergy Clin Immunol. 2002 Mar;109(3):S461-78.

2. Demoly P, Lebel B, Messaad D, Sahla H, Rongier M, Daurès JP, et al. Predictive capacity of histamine release for the diagnosis of drug allergy. Allergy. 1999 May;54(5):500-6.

3. Szebeni J. Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. Mol. Immunol. 2014;**61**(2):163-173.

4. Lasser EC, Lang JH, Lyon SG, Hamblin AE. Complement and contrast material reactors. J Allergy Clin Immunol. 1979 Aug;64(2):105-12.

5. Lenz HJ. Management and preparedness for infusion and hypersensitivity reactions. Oncologist. 2007 May;12(5):601-9.

6. Cheifetz A, Mayer L. Monoclonal antibodies, immunogenicity, and associated infusion reactions. Mt. Sinai J. Med. 2005;72(4):250-256.

7. Szebeni J. Complement activation-related pseudoallergy caused by liposomes, micellar carriers of intravenous drugs, and radiocontrast agents. Crit Rev Ther Drug Carrier Syst. 2001;18(6):567-606.

8. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 2010;11(9):785-797.

9. Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. Mol. Immunol. 2004;41(4):355-367.

10. Ferreira VP, Pangburn MK, Cortés C. Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol. 2010 Aug;47(13):2187-97.

11. Kopp A, Hebecker M, Svobodová E, Józsi M. Factor H: a complement regulator in health and disease, and a mediator of cellular interactions. Biomolecules. 2012;2(1):46-75.

12. Szebeni J, Muggia FM, Alving CR. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. J. Natl. Cancer Inst. 1998;90(4):300-306.

13. Szebeni J, Bedőcs P, Rozsnyay Z, Weiszhár Z, Urbanics R, Rosivall L, et al. Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. Nanomedicine. 2012;8(2):176-184.

14. Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, et al. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. J. Infect. Dis. 2012;205(6):995-1004.

15. Hebecker M, Alba-Domínguez M, Roumenina LT, Reuter S, Hyvärinen S, Dragon-Durey MA, et al. An engineered construct combining complement regulatory and surface-recognition domains represents a minimal-size functional factor H. J. Immunol. 2013;191(2):912-921.

16. van den Hoven JM1, Nemes R, Metselaar JM, Nuijen B, Beijnen JH, Storm G, Szebeni J. Complement activation by PEGylated liposomes containing prednisolone. Eur J Pharm Sci. 2013;49(2):265-271.

17. Józsi M, Prechl J, Bajtay Z, Erdei A. Complement receptor type 1 (CD35) mediates inhibitory signals in human B lymphocytes. J Immunol. 2002 Mar 15;168(6):2782-8.

18. Wu J, Wu YQ, Ricklin D, Janssen BJ, Lambris JD, Gros P. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. Nat. Immunol. 2009;10(7):728-733.

19. Józsi M, Oppermann M, Lambris JD, Zipfel PF. The C-terminus of complement factor H is essential for host cell protection. Mol. Immunol. 2007;44(10):2697-2706.

20. Kajander T, Lehtinen MJ, Hyvärinen S, Bhattacharjee A, Leung E, Isenman DE, et al. Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. Proc. Natl. Acad. Sci. USA. 2011;108(7):2897-2902.

21. Morgan HP, Schmidt CQ, Guariento M, Blaum BS, Gillespie D, Herbert AP, et al. Structural basis for engagement by complement factor H of C3b on a self surface. Nat. Struct. Mol. Biol. 2011;18(4):463-470.

22. Heinen S, Hartmann A, Lauer N, Wiehl U, Dahse HM, Schirmer S, et al. Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. Blood. 2009;114(12):2439-2447.

23. Timmann C, Leippe M, Horstmann RD. Two major serum components antigenically related to complement factor H are different glycosylation forms of a single protein with no factor H-like complement regulatory functions. J. Immunol. 1991;146(4):1265-1270.

24. Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, et al. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. Circulation. 1999;99(17):2302-2309.

25. Smith GP, Smith RA. Membrane-targeted complement inhibitors. Mol. Immunol. 2001;38(2-3):249–255.

26. Smith RA. Targeting anticomplement agents. Biochem. Soc. Trans. 2002;30(Pt 6):1037-1041.

27. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders:

therapeutic interventions. J Immunol. 2013 Apr 15;190(8):3839-47.

28. Kishore U, Sim RB. Factor H as a regulator of the classical pathway activation. Immunobiology. 2012;217(2):162-168.

29. Kuznetsova NR, Vodovozova EL. Differential binding of plasma proteins by liposomes loaded with lipophilic prodrugs of methotrexate and melphalan in the bilayer. Biochemistry (Mosc). 2014;79(8):797-804.

30. Schmidt CQ, Bai H, Lin Z, Risitano AM, Barlow PN, Ricklin D, Lambris JD. Rational engineering of a minimized immune inhibitor with unique triple-targeting properties. J. Immunol. 2013;190(11):5712-5721.

31. Nichols EM, Barbour TD, Pappworth IY, Wong EK, Palmer JM, Sheerin NS, et al. An extended mini-complement factor H molecule ameliorates experimental C3 glomerulopathy. Kidney Int. 2015 Jul 29. doi: 10.1038/ki.2015.233. [Epub ahead of print]

32. Strobel S, Abarrategui-Garrido C, Fariza-Requejo E, Seeberger H, Sánchez-Corral P, Józsi M. Factor H-related protein 1 neutralizes anti-factor H autoantibodies in autoimmune hemolytic uremic syndrome. Kidney Int. 2011;80(4):397-404.

33. Goicoechea de Jorge E, Caesar JJ, Malik TH, Patel M, Colledge M, Johnson S, et al. Dimerization of complement factor H-related proteins modulates complement activation in vivo. Proc. Natl. Acad. Sci. USA. 2013;110(12):4685-4690.

34. Tortajada A, Yébenes H, Abarrategui-Garrido C, Anter J, García-Fernández JM, Martínez-Barricarte R, et al. C3 glomerulopathy-associated CFHR1 mutation alters FHR oligomerization and complement regulation. J. Clin. Invest. 2013;123(6):2434-2446.

35. Józsi M, Tortajada A, Uzonyi B, Goicoechea de Jorge E, Rodríguez de Córdoba S. Factor H-related proteins determine complement-activating surfaces. Trends Immunol.

2015;36(6):374-384.

36. Baldo BA. Adverse events to monoclonal antibodies used for cancer therapy: Focus on hypersensitivity responses. Oncoimmunology. 2013;2(10):e26333.

37. Kimby E. Tolerability and safety of rituximab (MabThera). Cancer Treat Rev. 2005;31(6):456-473.

38. Boross P, Leusen JH. Mechanisms of action of CD20 antibodies. Am J Cancer Res. 2012;2(6):676-690.

39. Hörl S, Bánki Z, Huber G, Ejaz A, Windisch D, Muellauer B, et al. Reduction of complement factor H binding to CLL cells improves the induction of rituximab-mediated complement-dependent cytotoxicity. Leukemia. 2013;27(11):2200-2208.

40. Hakobyan S, Tortajada A, Harris CL, de Córdoba SR, Morgan BP. Variant-specific quantification of factor H in plasma identifies null alleles associated with atypical hemolytic uremic syndrome. Kidney Int. 2010;78(8):782-788.

41. Harris CL, Heurich M, Rodriguez de Cordoba S, Morgan BP. The complotype: dictating risk for inflammation and infection. Trends Immunol. 2012;33(10):513-521.

42. Kozma GT, Mészáros T, Weiszhár Zs, Schneider T, Rosta A, Urbanics R, et al. Variable association of complement activation by rituximab and paclitaxel in cancer patients in vivo and in their screening serum in vitro with clinical manifestations of hypersensitivity: a pilot study. Eur. J. Nanomed. 2015;7(4): 289–301.

43. Fülöp TG, Józsi M, Metselaar J, Storm G, Rosivall L, Szebeni J. The possible role of factor H in complement activation-related pseudoallergy (CARPA): a failed attempt to correlate blood levels of FH with liposome-induced hypersensitivity reactions in patients with autoimmune disease. Eur. J. Nanomed. 2015;7(1):7–14.

Figure legends

Figure 1. Figure showing the point of action of FH in the complement system and schematic drawing of FH and the used recombinant proteins.

(A) Simplified overview of the complement cascade. Complement activation can be initiated via three pathways, the classical, lectin and alternative pathway. These merge at the central component C3, which is cleaved into C3a and C3b. The latter forms the basis of the amplification loop of the alternative pathway and also initiates the terminal pathway. The serum complement inhibitor FH helps the inactivation of C3b and the dissociation of the enzyme necessary for C3b generation.

(B) FH consists of 20 complement control protein (CCP) domains. Mini-FH, a recombinant construct contains only the main functional domains of FH (CCPs 1-4 and 19-20).[15] FH constructs used in the experiments were FH1-4, which contains the CCPs 1-4, and FH15-20 consisting of CCPs 15-20. Factor H-related protein 1 (CFHR1) is composed of five domains that are shown aligned with the homologous FH domains. The numbers above the CFHR1 domains show the amino acid sequence identity in % to the corresponding FH domains. GAG, glycosaminoglycan.
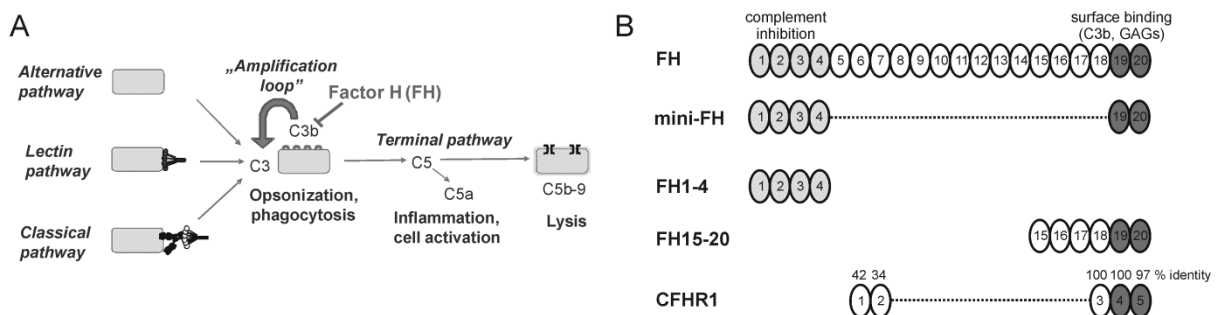
Mészáros et al. 2015
Figure 1

Figure 2. Factor H (FH) inhibits AmBisome-induced complement activation.

(A) Complement activation was measured in normal human sera by assessing the formation of SC5b-9 with ELISA. As positive control, we used zymosan A, which strongly activates complement. AmBisome (AmBi) caused significant, ~8-fold rise of SC5b-9 over baseline, although this rise was less than that caused by zymosan A. Exogenous FH, added at 200 μg/mL (1.3 μM) concentration, led to >50% inhibition of liposome-induced complement activation. FH slightly but non-significantly reduced zymosan-induced complement activation.

(B) Liposomes of various compositions were prepared as described in Methods and Table 1, and caused various degrees of complement activation when added to normal human sera as in (A). FH was added at 1.3 μM concentration. Chol-2P: liposomes containing cholesterol-anchored PEG2000.[16]

Data are mean + SEM of at least three measurements. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$, Student's *t*-test. ns, not significant.

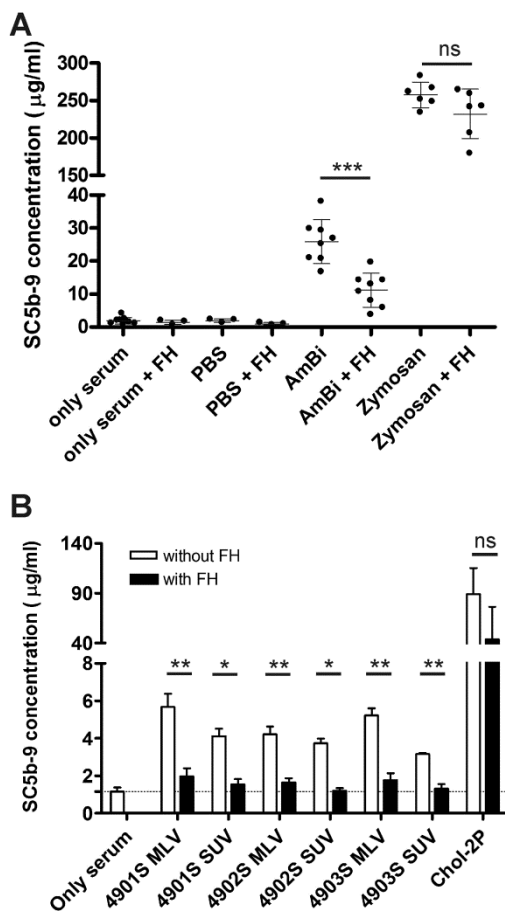Mészáros et al., 2015
Figure 2

Figure 3. Mini-factor H (mini-FH) more strongly inhibits drug-induced complement activation compared with full-length factor H (FH).

Complement activation was measured in five (A) and six (B) normal human sera by assessing the formation of SC5b-9 with ELISA. Both AmBisome (A) and Cremophor EL (B) caused significant increase of SC5b-9. Exogenous FH, added at 1.3 µM concentration, significantly reduced SC5b-9 formation. Mini-FH,[15] a recombinant compact form of factor H consisting only the main complement inhibitory domains (CCPs 1-4) and surface recognition domains (CCPs 19-20), added also at 1.3 µM concentration, more potently inhibited SC5b-9 generation compared with FH. Serum only: no Ambisome/CrEL added. Data are mean + SEM, *, $p < 0.05$, and ***, $p < 0.001$ (ANOVA).

(C) Ambisome was added to serum alone (PBS) or together with increasing concentrations (0.32-1.3 µM) of mini-FH, as indicated. Serum only: no Ambisome added. SC5b-9 was measured by ELISA. Data are mean + SEM from experiments with five human sera. *, $p < 0.05$, and ***, $p < 0.001$ (ANOVA).
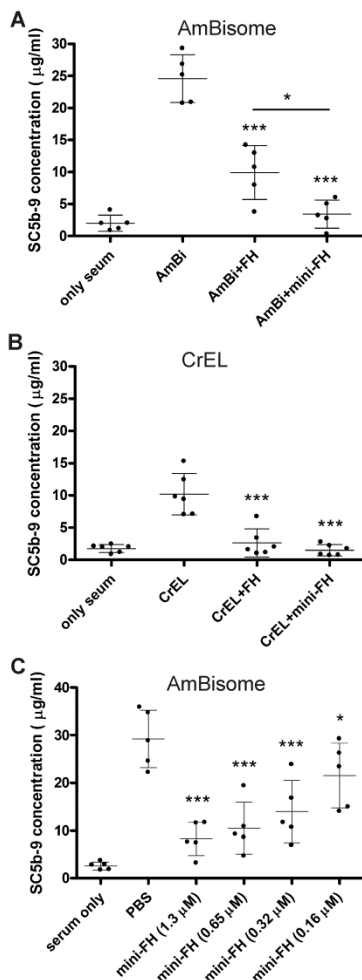

Mészáros et al., 2015
Figure 3

Figure 4. Evaluation of factor H fragments FH1-4 and FH15-20, and the factor H-related protein CFHR1.

Complement activation was measured in 3 normal human sera by assessing the formation of SC5b-9 with ELISA. Recombinant FH1-4, FH15-20, and the FH-related protein CFHR1 were tested for their capacity to inhibit complement activation induced by AmBisome (A) and Cremophor EL (B) in 3 normal human sera in the presence of the various proteins at 1.3 µM concentration by measuring the formation of SC5b-9 with ELISA. For comparison and as a positive control, the inhibitory effect of exogenous FH was also tested at 1.3 µM concentration. Serum only: no Ambisome/CrEL added. Data are mean + SEM, *, $p < 0.05$, and **, $p < 0.01$ (ANOVA).
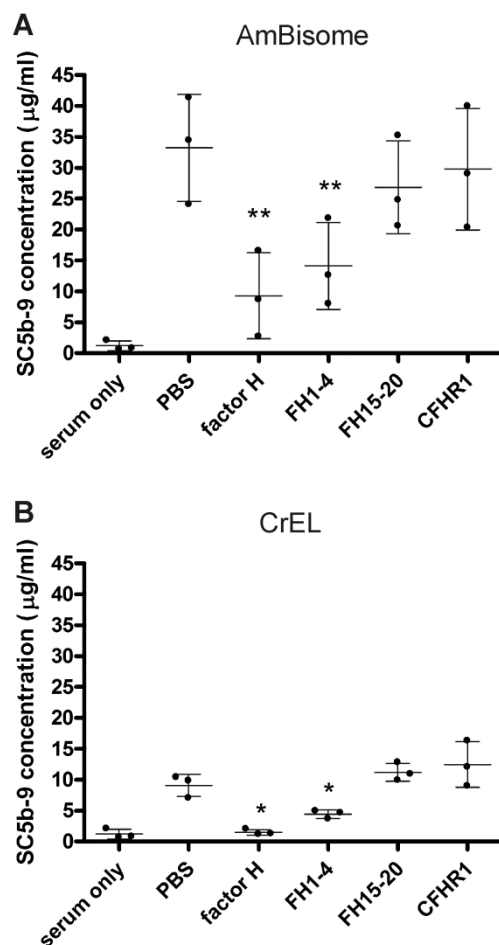

Mészáros et al., 2015
Figure 4

Figure 5. Factor H (FH) and mini-FH inhibit rituximab-induced complement activation.

(A) Rituximab induces complement activation in anti-coagulated whole blood. Whole blood assay was performed by addition of 300 µg/ml rituximab for the indicated time. In samples with lepirudin, an anticoagulant not interfering with complement activation, a rise in SC5b-9 can be observed compared with samples with heparin. A representative experiment is shown perfomed with three blood samples. Significantly higher concentration of SC5b-9 can be detected upon addition of rituximab in samples with lepirudin compared with those with heparin ($p = 0.0002$, Student's t-test).

(B) Rituximab was added in the indicated concentrations to whole blood containing lepirudin. FH and mini-FH were added at 1.3 µM concentration, and SC5b-9 was measured by ELISA. Data are mean + SEM of experiments with three blood donors. *, $p < 0.05$, and ns, not significant (ANOVA).
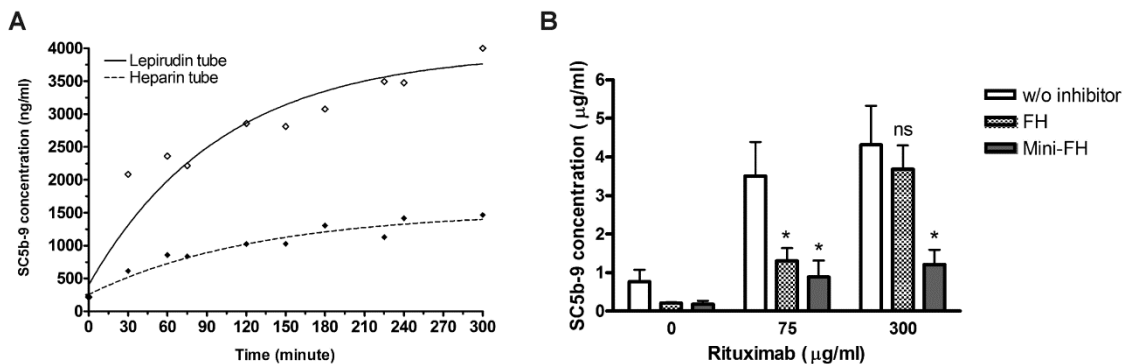

Mészáros et al., 2015
Figure 5

Figure 6. Effect of FH and FH1-4 on the survival of rituximab-treated B cells in human serum. Tonsil B cells (A) or BL-41 lymphoma cells (B) were incubated in 50% NHS. Rituximab was added at 10 µg/ml and the complement inhibitors were added in 1.3 µM concentration. Percentage of living cells after treatment is indicated. Dead cells are identified as propidium iodide (PI)-positive, and living cells as PI-negative cells. VBS, veronal buffered saline; RTX, rituximab; NHS, pooled normal human serum; FH, factor H; FH1-4, recombinant FH domains CCP1-4.
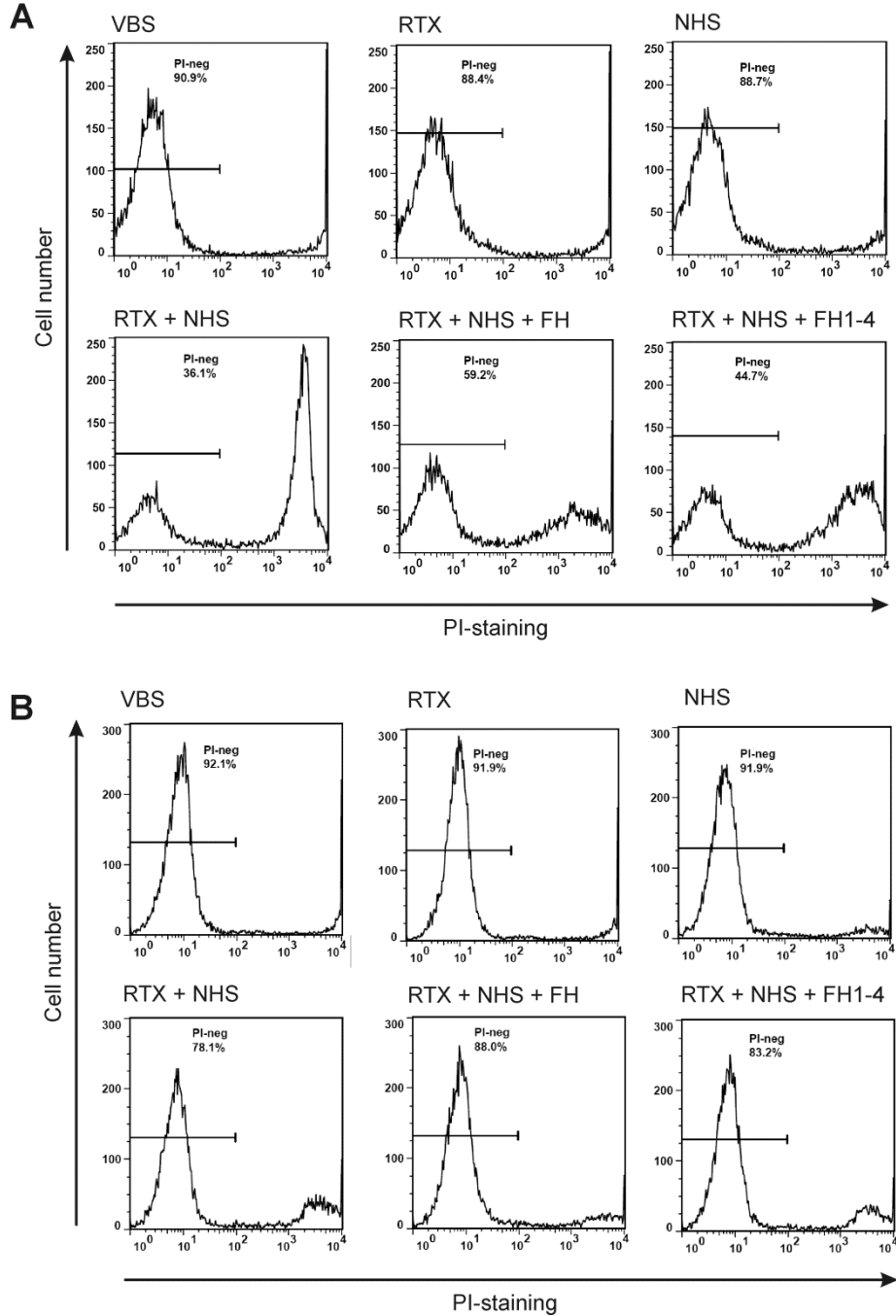
Mészáros et al., 2015
Figure 6

Table 1. Composition of the various liposomes used in this study.

Liposome ID	Compositon (Chol:PEG:HSPC)	Extrusion	Size (d.nm)
4901S MLV	39:0:61	no	269.5
4901S SUV	39:0:61	yes	102.4
4902S MLV	39:5:56	no	530.1
4902S SUV	39:5:56	yes	82.8
4903S MLV	39:10:51	no	323.4
4903S SUV	39:10:51	yes	69.3

MLV, multilamellar vesicle; Chol, cholesterol; PEG, polyethyleneglycol; HSPC, hydrogenated soy phosphatidyl choline