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Review Article

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Evaluation of reconstituted high-density lipoprotein (rHDL) as a drug delivery platform – a detailed survey of rHDL particles ranging from biophysical properties to clinical implications

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Abstract

During the last decade, and with increasing intensity, the potential for using reconstituted high-density lipoprotein (rHDL) particles to deliver hydrophobic drugs to impaired cells and tissues has been explored. Here, we evaluate various parameters that should be considered when utilizing discoidal rHDL particles as a drug delivery platform. Key parameters such as preparation basics, pronounced statistical variation in drug incorporation across rHDL particles, effects of lipid composition on HDL/rHDL *in vivo* and *vitro* dynamics/particle stability, and pharmacokinetic/safety data from rHDL infusion studies in human subjects will be addressed including the innate receptors and native functions of HDL. The broad but detailed information presented in this work could also be deployed in other rHDL-related research. However, the major aim of this review is to point out factors that have the potential to advance rHDL research toward realizing the 'magic bullet' for lipophilic and hydrophilic drug delivery in various clinical contexts.
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Key words: Reconstituted high-density lipoproteins; rHDL; Nanodiscs; Nanodisks; Targeted drug delivery; Magic bullet

High-density lipoprotein (HDL) is a family of 7-12 nanometer sized particles comprised of apolipoproteins and lipids[1] that function, among other things, as a transport system for a variety of hydrophobic bio-molecules in vertebrates and insects. A notable key function of HDL is to transport cholesterol from the peripheral tissues to liver for catabolism or excretion as a part of the process of reverse cholesterol transportation (RCT). A great interest in this pathway has been fostered by epidemiologic evidence showing an inverse relationship between HDL-cholesterol levels and the risk of developing cardiovascular disease (CVD). Along these lines, much effort has been committed to utilizing either HDL-based or HDL-modulating therapies for prevention and management of CVD.[2-5] Nevertheless, these attempts have failed in passing all clinical phases thus far and recently an expert panel convened by the American National Lipid Association concluded that HDL-cholesterol is not a therapeutic target for the treatment of CVD at the present time.[4] However, they supported the need to continue to investigate the therapeutic effect of modulating HDL structure and function.

An attractive avenue for harnessing the biology of HDL in a therapeutic context would be to utilize reconstituted HDL (rHDL) as a drug delivery vehicle for hydrophobic cargo. HDL's native ability to transport various hydrophobic molecules and target specific tissues and organs combined with its inherent biocompatibility and biodegradability makes the HDL particle a strong candidate for safe and targeted delivery of hydrophobic drugs. In 2001, Rensen et al[6] presented the first examples of employing rHDL as a drug delivery vehicle for hydrophobic molecules. Since then, a steadily increasing amount of work has been produced on this subject, including a more detailed understanding of the diversity, dynamics and metabolic fate of reconstituted and the biological HDL particles. We therefore think it is timely to integrate and discuss these results following the 2001 review paper.[6]

Biological HDL exists in two different architectures, discoidal and spherical. The focus of this review will be on the discoidal rHDL (referred to as rHDL from here on if nothing else is specified) that mimics the most abundant endogenous nascent HDL particle, due largely to the ease of preparation and

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successful implementation of rHDL in completed phase 1 and 2 trials.[3] In addition, the bilayer configuration in discoidal HDL particles allows membrane proteins including extra- and intracellular domains to be incorporated, while the monolayer feature of spherical reconstituted particles is not ideally designed to host transmembrane proteins. For specific interest in reconstituted spherical HDL and HDL-mimicking particles, we recommend the following reviews.[7,8] It should be noted that the terms nanodisk and nanodisc are found in the literature and refer to the discoidal HDL including incorporated cargo or comprised of amino-terminal variants of human apolipoprotein A-I, respectively. A schematic illustration of the canonical discoidal 9.6 nm rHDL particle that mimics the biological nascent α_4 HDL is shown in Figure 1. This particular rHDL configuration is comprised of two amphipathic apoA-I proteins and a phospholipid bilayer and represents the primary focus of this review.

The rHDL particle has thus far been found to be useful in a wide variety of applications ranging from potential therapies for CVD, membrane-mimicking hosts for transmembrane proteins for elucidating the structure and function of biologically important but difficult-to-work-with proteins[14] and markers for medical imaging based on different imaging modalities such as MRI, optical, and CT[15,16] to drug and siRNA[11] delivery vehicles that target specific tissues/organs including cancer cells.[13] Figure 1 shows the rHDL-designs used in some of these various applications. For some of these exciting rHDL-based applications, it is important to continue to advance the effective targeting, stability, and drug delivery properties of these particles. We will discuss the bio- and chemophysical properties of rHDL and examine avenues for altering their targeting and delivery potential. In doing so, we provide a brief overview of the innate receptors and native functions of HDL, including the RCT pathway that it participates in. Next, we focus on the corresponding innate targeting features of rHDL and ways to change the rHDL targeting followed by useful examples that have utilized rHDL as a vehicle for incorporating various compounds including hydrophobic drugs. Finally, we address the various parameters that should be evaluated and considered regarding rHDL to assess the advantages and limitations of utilizing discoidal rHDL particles as a drug delivery vehicle. Key parameters and considerations include the preparation route, the pronounced statistical variation in drug incorporation into single rHDL particles, the effect of lipid composition on HDL/rHDL *in vivo* and *vitro* dynamics and particle stability, and pharmacokinetic and safety data from rHDL infusion studies in human subjects.

The major aim of this work is to examine and pinpoint refinements that can be made in terms of lipid, apolipoprotein, and drug cargo composition as well as presenting strategies that have the potential to push rHDL research toward realizing the 'magic bullet' for lipophilic and hydrophilic drug delivery in various clinical contexts.

Biological HDL

The more we learn about the biology of HDL particles and its native receptors, the more diverse and complex the HDL particle and its pathway becomes.

HDL - a heterogeneous lipoprotein family

From an early stage, it was known that HDL particles are heterogeneous in terms of density and size.[1] The heterogeneity of HDL particle size and shape is traditionally explained by the different stages that HDL undergoes during its maturation within the RCT pathway. Figure 2 depicts a schematic illustration of the RCT pathway including the well-known components that contribute to HDL subclass maturation: starting with the initial lipid loading of the most abundant HDL protein, apoA-I to form the lipid-poor apoA-I pre-β complex and α_4 HDL particles via ATP-binding cassette transporter (ABCA1)-mediated lipidation. The canonical 9.6 nm rHDL mimics the discoidal α_4 HDL particles that consist of a cholesterol-enriched phospholipid bilayer encircled by two edge-facing apoA-I proteins. The pre-β and α_4 HDL particles can then be transformed into spherical shaped $\alpha_{1\text{-}3}$ HDL particles, with α_3 being the smallest and α_1 the largest spherical HDL, through the recruitment and activation of lecithin-cholesterol acyltransferase (LCAT) that converts cholesterol into a more hydrophobic cholesteryl ester that further migrates to form the core of the HDL particle. The ATP-binding cassette transporter ABCG1 may be involved in maturation of the HDL by loading additional free cholesterol (FC) onto the HDL particle, while the cholesteryl ester transfer protein (CETP) is responsible for transfer and exchange of cholesteryl ester and triglycerides between the $\alpha_{1\text{-}3}$ HDL and any available apoB-containing very low-density (VLDL) and low-density lipoprotein (LDL) particles in blood. We have only shown the RCT pathway, since that is the most studied and presumably important pathway that HDL undergoes. (See Figure 2).

Intensive research into the biology of HDL has demonstrated a diverse portfolio of functions that HDL particles display. The RCT activity is probably the most studied and documented function of HDL. However, several other HDL-based biological mechanisms have been documented to play important roles within and beyond lipid transport and maintenance of cardiovascular homeostasis. HDL particles have been shown to possess anti-oxidative,[17] anti-inflammatory,[18] anti-apoptotic,[19] anti-platelet,[20,21] and immunogenic[22] properties. HDL has also been found to exhibit a positive influence on the maintenance of glucose homeostasis by inducing anti-apoptotic behavior of beta-cells[23] and stimulation of insulin secretion.[24] Some of these claims have been further supported by recent proteomic studies that independently identified more than 85 identical proteins associated with HDL and, intriguingly, suggest connections between these proteins and processes as diverse as acute phase response/inflammation, hemostasis, and immune responses.[25] Moreover, lipidomic studies have enabled the identification of more than 200 individual lipid species ranging from glycerophospholipids, sphingolipids, sterols, acylglycerols to fatty acids in the HDL of healthy subjects.[26] In addition to the diversity of HDL associated proteins and lipids, a broad range of other kinds of biomolecules have shown to be associated with HDL particles including carotenoids,[27] vitamins,[28-30] hormones,[31,32] and even small RNAs.[33,34]

All these observations call for further review and understanding of HDL particles as a family of HDL subspecies that each contain unique lipid, protein, and biomolecule composition as a

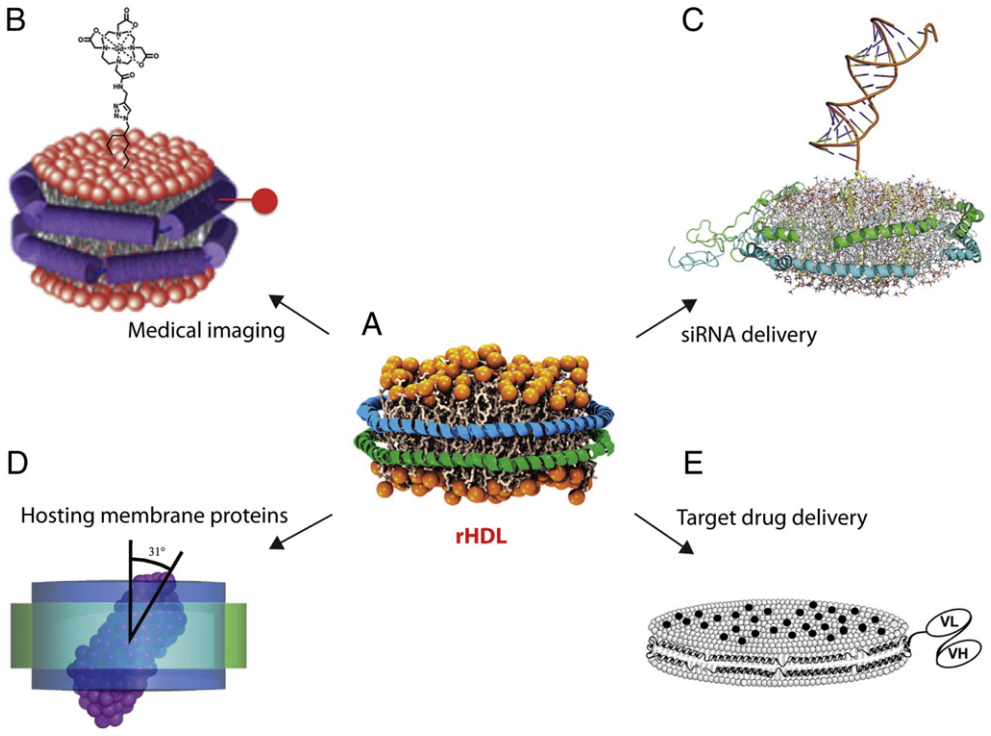

Figure 1. Schematic illustrations of rHDL particles comprised of phospholipids and two apoA-I proteins with and without incorporated compounds. **(A)** Molecular dynamic model of the canonical discoidal 9.6 nm rHDL that represents the most well established structural model of this particular rHDL species. The model is based on two amino-terminal, 43-residue truncation variants of human apoA-I (ΔN-apoA-I). Reprinted (adapted) with permission from.[9] Copyright (2007) American Chemical Society. **(B)** rHDL including synthetic lipids that coordinate gadolinium ions used for medical imaging. The red moiety corresponds to a red-fluorophore conjugated to ΔN-apoA-I. Reprinted (adapted) with permission from.[10] Copyright (2015) American Chemical Society. **(C)** Cholesterol-conjugated siRNA (chol-siRNA, yellow unit) embedded in rHDL used for posttranscriptional knockdown of target genes. Reprinted by permission from Macmillan Publishers Ltd: Molecular Therapy,[11] copyright 2012. **(D)** Bacteriorhodopsin (purple unit) incorporated into rHDL enabled novel structural studies of bacteriorhodopsin in a native-like phospholipid bilayer.[12] The green unit represents two ΔN-apoA-I proteins. This figure is reproduced with permission from the International Union of Crystallography. **(E)** A modified rHDL loaded with curcumin (solid black). A single chain variable fragment (comprised of the variable heavy (VH) and light (VL) chains from an antibody) fused to apoA-I to modify and target the rHDL towards B-cell lymphoma cells. Copyright 2015 Canadian Science Publishing or its licensors. Reproduced with permission from Ref.[13].

function of their size and shape that likely mediate functions well beyond cholesterol transport. Intriguingly, Kasey C. Vickers and Alan T. Remaley[5] suggested that HDL should possibly be rebranded as a general transporter of cargo between cells based on the diversity of molecules that are now known to be associated with HDL particles. It is highly likely that these advances in understanding the complexity and diversity of HDL particles and their biology can lead to refinements that lead to the creation of more efficient and targeted rHDL drug delivery solutions.

HDL's innate targets and receptors

An understanding of the receptor-ligands present in HDL and the corresponding cellular receptors of these ligands is important for understanding the function and eventual fate of endogenous HDL particles.

Various apolipoproteins have shown to be associated with HDL such as: apoA-I, apoA-II, apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, apoC-IV, apoD, apoE, apoF, apoH, apoJ, apoL-I, and apoM.[35] ApoA-I is by far the most abundant protein in HDL and accounts for almost *70%* of the protein content. Another relatively common, but far less abundant apolipoprotein component of HDL is apoE. Both of these apolipoproteins serve to determine and define the size and shape of HDL that then make these particle ligands for specific receptors that facilitate the efflux or influx of lipids to the recipient cell. These two proteins have so far been the predominant choice of apolipoproteins for the formulation of rHDL particles used for drug delivery purposes, and thus, we will focus on these apolipoprotein components with particular emphasis on apoA-I given its natural abundance and use in clinical HDL-based trials.[36-43]

The apoA-I containing HDL particle is known to bind various cellular receptors such as the scavenger receptor class B type 1 (SR-B1), ectopic F-ATPase, ABCG1, and cubilin. The cubilin protein is an endocytic receptor highly expressed in renal proximal tubules, where it, among other functions, mediates endocytotic uptake of apoA-I/HDL. Thus, it may control the reuse of apoA-I/HDL by transcytosis.[44] The ABCG1 and SR-B1 receptors mediate efflux of cholesterol to HDL particles from the peripheral cells including foam cells that have been shown to be a hallmark of atherosclerosis. However, the SR-B1 is generally thought to mediate lipid influx via selective uptake of cholesteryl ester molecules, where only the lipid load of HDL is transferred

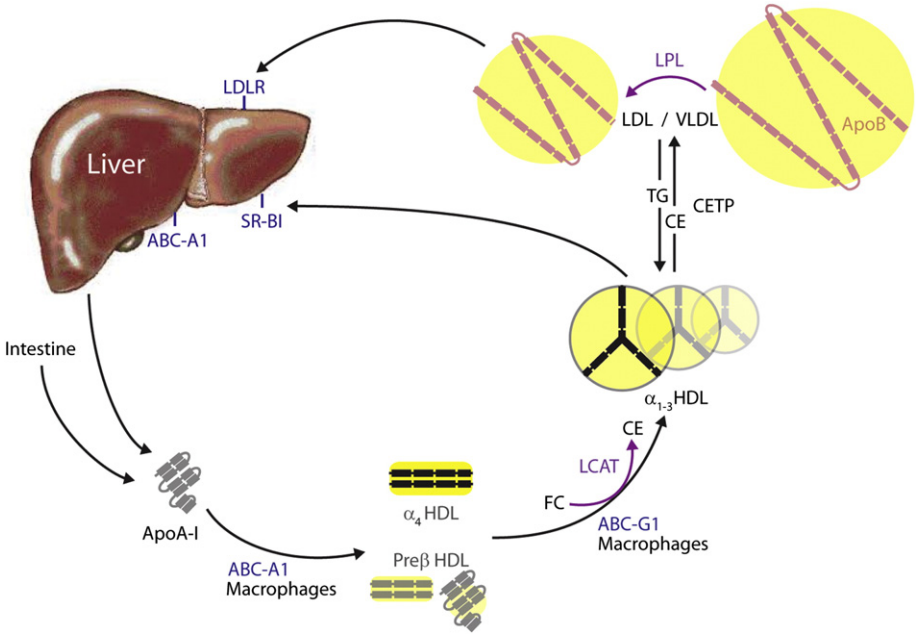

Figure 2. Diagram of the reverse cholesterol transport (RCT) pathway. The first step begins with the formation of nascent Preβ-HDL by the efflux of free cholesterol (FC) and phospholipids mediated by the ABCA1 transporter to apoA-I that is secreted from the liver and intestine. These poorly lipidated apoA-I particles are further lipidated by ABCA1 to generate larger discoidal HDL called α_4 HDL. These discoidal HDL particles acquire additional lipids by ABCA1 and ABCG1 transporters, and LCAT converts FC to cholesteryl esters (CE), which are then sequestered into the core of the HDL to reshape HDL into the spherical $\alpha_{1\text{-}3}$ forms of HDL. Cholesterol on HDL can be delivered directly to the liver following uptake by SR-BI or transferred in exchange for triglycerides (TG) to VLDL and LDL by CETP. Intact LDL is eventually delivered to the liver by the LDL receptor.

without concomitant catabolism of the HDL particle itself.[45] Thus, the SR-B1 is likely the most important receptor for delivery of rHDL-cargo to target cells. In addition, SR-B1 is a bona fide endocytic receptor as it mediates the internalization of liposaccharides and facilitates hepatitis C virus entry.[46,47] Therefore, it is conceivable that SR-B1 also mediates HDL endocytosis and the group of Tall has shown some evidence that this is the case in polarized hepatocyte cells.[48] However, the holo-particle uptake is not in agreement with recent structural work of the LIMP-2 protein[49] that is a member of the CD36 superfamily of scavenger receptor proteins including SR-BI. This work infers by homology modeling that the structure of SR-BI contains a lipid tunnel that serves to deliver cholesterol-ester from the bound HDL to the plasma membrane. Hence, the exact mechanism by which the SR-BI mediates lipid efflux and influx from HDL to cells requires further work to be fully elucidated.

The SR-B1 is abundant in hepatic, adrenal gland, ovarian and placental tissues,[50] with the highest abundance of SR-B1 protein localized in the liver[51] where it is thought to be the key receptor for cholesteryl ester uptake being delivered by HDL as a part of the RCT pathway. Interestingly, a study has demonstrated that the interaction between HDL and ectopic F-ATPase at the cell surface of hepatocytes stimulates endocytosis of the holo-HDL particle.[52] This non-traditional HDL-receptor for cholesteryl ester influx could challenge the traditional notion of the function of SR-B1.

ApoE is known to bind to members of the LDL receptor family that includes more than 10 different receptors that mediate endocytosis[53] and uptake of the holo-particle. The liver contains about 70% of the total LDL receptors in the body,[54] including the predominant apoE-dependent endocytic receptor, the low-density lipoprotein receptor (LDLR).

Based on the biodistribution of these cellular receptors for apoA-I and apoE as well as its function as the major storage and recycling organ for lipoprotein particles, the liver is the main target of both apoA-I and apoE-based HDL particles in healthy subjects as depicted in Figure 2.

rHDL targets

In addition to the targeting features of the apolipoproteins constituting HDL particles, C. Vickers and Alan T. Remaley recently proposed that HDL likely participates in the transport of iron from the gut and phagocytic cells to targeted recipient cells through the HDL-associated serotransferrin protein.[5] This finding illustrates that an example of redirecting HDL away from its classical cholesterol recipient organs can be found in a biologically relevant context. This finding also suggests that modifications made to HDL that anchor or incorporate cell receptor binding motifs to rHDL particles may serve to redirect rHDL particles away from canonical HDL apolipoprotein

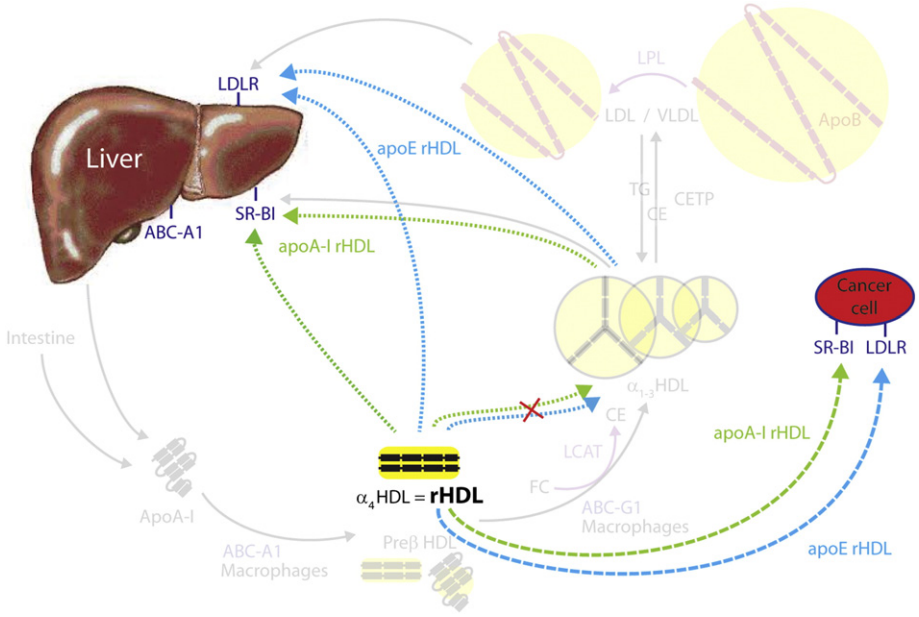

Figure 3. Diagram of the rHDL targets that include the liver and various cancer tumors. The target is likely to be independent of whether apoA-I or apoE are used in the preparation of rHDL, since both LDLR and SR-BI receptors are highly expressed in these tissues. The RCT pathway is shown behind (high transparency). A clearer diagram of this particular pathway is shown in Figure 2. The red cross indicates that efforts toward minimizing the LCAT mediated transformation of the discoidal rHDL into its spherical counterpart may improve the efficiency of the rHDL delivery platform as outlined in the *In vivo modeling of the rHDL* and *How to increase the circulation time of rHDL* sections.

binding receptors and towards other targeting tissues/organs that can be used in therapeutically relevant ways.

The inherent liver target of rHDL

The most predictable target of the rHDL drug delivery system reconstituted from either apoA-I or apoE is as mentioned above the liver. The liver coordinates many different functions, and thus, is prone to many diseases that could potentially be treated efficiently using the rHDL drug delivery in combination with an efficacious drug.

ApoA-I versus apoE rHDL

From a drug delivery point of view, the choice to use either apoA-I or apoE in rHDL formulation could, in part, be determined by the chemical stability of the drug cargo towards the acidic environment in lysosomes, which apoE-based rHDL will necessarily be exposed to as a part of the endocytic pathway mediated by the LDLR. Additionally, the endocytic influx of drugs mediated by the LDLR may turn out to be more efficient if the SR-B1 is unable to channel the hydrophobic drug through the cell membrane.

Targeting cancer

In addition to the predictable targeting of rHDL to liver, several studies emphasize that rHDL has great potential to target cancer cells based on upregulation of apolipoprotein receptors following tumorigenesis. It has been reported that SR-B1 is highly expressed in a variety of tumor cells of prostate,[55] breast,[56] colorectal,[57] ovarian cancers[58] as well as nasopharyngeal carcinoma.[59] It has also been shown that LDLR is over-expressed in numerous cancers.[60] The upregulation of SR-BI and LDLR in various cancer cells is most likely linked to the high demand for cholesterol and lipid to meet cell growth and proliferation demands[61] in cancer cells. The rapid growth and proliferation are known hallmarks of cancer cells.[62,63] The hypocholesteremia observed as a complication of acute lymphoblastic leukaemia[64] supports a high influx of cholesterol delivered by HDL and LDL to cancer cells. With this understanding in mind, the apoA-I or apoE based rHDL seems to be good candidates for targeting and delivering anti-cancer therapeutics to cancer cells via targeting of the upregulated SR-BI or LDLR receptors, respectively (See Figure 3). In addition, the small size of rHDL (typically ~10 nm) compared to liposomes (typically > 40 nm) can be considered advantageous as rHDL particles may become trapped in the leaky vasculature of tumors or able to find their way between the dense (less than 40 nm) extracellular collagen fibril matrix that are present in some cancer tumors[65] and thereby increase delivery efficiency to tumor sites.

Improving the targeting feature of rHDL

The incorporation of additional ligand motifs into rHDL could either increase the targeting efficiency of rHDL to liver and cancer cells or redirect the rHDL to target other desired tissues.

In the first published review on utilizing recombinant lipoproteins for drug targeting from 2001,[6] the asialoglycoprotein receptors (ASGPr), which are uniquely localized on hepatocytes and mediate the endocytosis of plasma glycoproteins, were suggested to be an attractive target for selective delivery of drugs. The review paper refers to work by Schouten et al[66] that demonstrated that spherical HDL-like particles

comprised of lactosylated apolipoproteins could target the ASGPr on liver cells. Moreover, it has been shown that a synthetic N-acetylgalactosamine–terminated glycolipid bound to endogenous HDL enhanced the cholesteryl ester uptake by the liver. Thus, applying ligands for the ASGPr to rHDL particles would most likely improve the efficiency of targeting the liver.

Folic acid has also been used in both liposome and rHDL formulations in order to target cancer cells. In one study,[67] folate was conjugated to apoA-I following rHDL formation. This approach showed a high selectivity towards targeting ovarian cancer cells in mice and only a minimal amount of the folate-labelled rHDL was found in the host liver when using an intraperitoneal administration. It should be noted that over 90% of nonmucinous ovarian cancers overexpress the folate receptor FR-α and the degree of FR-α expression correlates with the grade of malignancy,[68] which suggests that this approach of targeting the folate receptor with an rHDL drug delivery system shows strong therapeutic potential. Another study shows[69] that the uptake of the anti-cancer drug Paclitaxel (PTX) in ovarian cancer cells was improved two-fold by modifying the apoA-I with folate compared to rHDL without apoA-I modifications.

We have recently applied another strategy to successfully confer cell specificity of rHDL. Instead of incorporating a ligand moiety to the rHDL bilayer, we engineered a single-chain variable fragment (scFv) apoA-I chimera.[13] In this work, an scFv of the CD20 antibody was fused to apoA-I in order to target B-cell lymphoma cells that are known to express CD20 at all stages except the first and latter. Another similar example shows that a fusion protein comprised of apoA-I and an scFv moiety against the vimentin (VIM) antigen possesses the capacity to form rHDL and recognize the VIM antigen.[70] The extension of apoA-I with a small peptide rather than an scFv moiety of about 50-60 kDa has also proven successful.[71] In this work, rHDL-based genetic fusions of a truncated apoA-I and a transduction domain from HIV tat protein known as the TAT peptide loaded with the anti-cancer drug doxorubicin showed greater efficacy in suppressing tumor growth in mice than rHDL synthesized without the TAT moiety.

The scFv-apolipoprotein fusion protein or use of other ligands to either increase the targeting efficiency of rHDL to liver and cancer cells or target a different tissue is intriguing. This approach would increase the number of rHDL particles finding their way to desired target cells and could thereby potentially enhance the SR-BI or LDLR receptor-mediated cellular uptake of the rHDL cargo. However, it should be stressed that if the receptors of these ligands do not mediate cellular uptake of the cargo, the risk of a less efficient treatment exists, since the cargo may simply bind to the extracellular face of the receptor and never get internalized into the target tissue.

rHDL as a delivery vehicle for hydrophobic biomolecules and drugs

Here we present various examples that utilize rHDL as a vehicle for hosting and carrying various compounds including hydrophobic drugs. This summary is not intended to be complete and only addresses examples of cargo incorporated into rHDL, yet it helps to illustrate the variety of drugs and targets that have thus far been tested with rHDL as the delivery platform. We also suggest a few strategies for taking advantage of an rHDL drug delivery platform from a treatment perspective.

Biomolecules

Amphotericin B (AmpB) has been used in clinics for nearly half a century as an antifungal therapy. However, the therapeutic potential is limited by poor aqueous solubility. rHDL particle loaded with AmpB have been shown to strongly inhibit *Candida albicans* and *Aspergillus fumigatus* in mice when compared to previously available AmpB formulations.[72,73] It was shown that AmpB-rHDL particles exhibit a more favorable safety profile while maintaining uncompromised antifungal properties compared to both AmpB-deoxycholate and AmpB-liposomal formulations.[72] Furthermore, AmpB-rHDL has been shown to induce complete clearance of infection in *Leishmania major* infected mice[74] and the researched AmpB-rHDL formulation turned out to be more effective than its commercial liposomal counterpart (AmBisome®). It is notable that rHDL particles with incorporated AmpB exhibit a mean size of 40 to 80 nm depending on the additional incubation temperature. Thus, it appears, at least in this case, that the addition of drug does impact the outcome of the self-assembly in terms of the final rHDL size.

Retinoids, such as ATRA, are useful agents in cancer therapy as they exhibit a central role in cell growth, differentiation, and apoptosis.[75,76] Like AmpB, ATRA is insoluble in water and thus exhibits low bioavailability that, in combination with its toxicity at high doses, requires it to be formulated in a vehicle that is capable of delivering ATRA at the appropriate dose. ATRA-rHDL prepared by Redmond et al[77] and Singh et al[78] have shown that ATRA-ND are more effective at inducing apoptosis in mantle cell lymphoma cells compared to free ATRA.

Curcumin is a compound found in turmeric and known to exhibit several interesting therapeutic properties including anti-oxidant, anti-amyloid, anti-bacterial, anti-viral and anti-cancer effects among others. Several studies have shown that rHDL particles often referred to as nanodisks provide a solution to overcome the low absorption and poor bioavailability of bare curcumin using either apoA-I,[79,80] apoE or an N-terminal part of apoE[81] as a scaffold protein. Interestingly, one study[80] shows that rHDL loaded with curcumin induced apoptosis in mantle cell lymphoma.

Recently, work by Ryan's group[82] showed that rHDL particles comprised of cardiolipin (a lipid that contains four fatty chains and three glycerol moieties found almost exclusively in the cell's mitochondria) and apoA-I prevent TAZ-knockdown-induced apoptosis in myeloid progenitor cells. This study suggests a novel approach to treat diseases related to cardiolipin-associated disorders including Barth Syndrome that is characterized by loss of function of the mutated TAZ protein that functions in cardiolipin acyl chain remodeling.

Membrane proteins

It has been known for some time that rHDL particles provide a cell-membrane-like environment due to their lipid bilayer structure, and thus, are capable of hosting not only hydrophobic drugs but also membrane proteins. Various membrane proteins

such as bacteriorhodopsin[12,83] cytochrome P450,[84] G-protein coupled receptors and many more[85] have been incorporated into rHDL to obtain functional and structural information about the hard-to-crystalize membrane proteins. We have recently shown experimentally that the seven-transmembrane helix bundle in a membrane protein is tilted about 30% degrees perpendicular to the lipid bilayer in an rHDL-like particle[12] in agreement with theoretical calculations. We speculate that enzymatic or other integral membrane proteins could be used as 'drugs' by degrading or modifying harmful compounds in plasma or at the liver-plasma interface.

siRNA

Small interfering RNAs (siRNA) hold enormous therapeutic potential with their ability to specifically target and downregulate target mRNA levels post-transcriptionally. Specific gene silencing using siRNA can disrupt viral replication and turn off genes related to a number of detrimental processes (e.g. metastasis). One of the main obstacles in using RNA based 'drugs' has been the stabilization of RNA, but various improvements have been put into stabilizing the RNAs in vivo. Thus, the full potential of the siRNA technology is, in certain respects, linked to development of reliable and safe methods for systemic and targeted delivery of siRNA to the desired cell populations.[86-88] Various viral and non-viral delivery systems such as liposomes, aptamers, and dendrimers have been developed. However, for most of these systems, the safety and selectivity of the siRNA delivery still requires significant optimization. Interestingly, it has been shown that biological HDL transports endogenous microRNAs in plasma and is capable of delivering them to recipient cells in humans.[33,34] However, the large heterogeneity of these HDL particles in terms of size, protein and lipid composition has thus far made it difficult to figure out the exact composition that is required to effectively bind synthetic RNAs to rHDL particles. Nevertheless, these studies clearly show that using rHDL for transporting and delivering regulatory RNAs appears to be a promising approach that warrants further investigation. Several research groups have employed rHDL particles as a delivery system for siRNA due to the biocompatibility and inherent selectivity of these particles for liver. Wolfrum et al[89] showed that when cholesterol-conjugated siRNAs (chol-siRNA) targeting apoB were bound to endogenous HDL, the particles ended up predominantly in liver. They also showed that chol-siRNA associated with endogenous HDL particles was 8 to 15 times more effective at silencing apoB protein expression *in vivo* compared to naked chol-siRNA injected into the bloodstream. In a second similar study, Nakayama and coworkers[11] recently showed that rHDL containing chol-siRNA is also efficient at silencing apoB expression in the liver of mice. Mistuni et al[90] showed that rHDL containing the positively charged lipid 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP) can be used to bind naked siRNA through electrostatic interactions, and subsequently used to trigger a 60% knockdown of the house keeping gene GAPDH in cultured hepatoma cells. However, the work also showed a wide polydispersity of the rHDL particles with respect to size. The instability of the standard 9.6 nm rHDL when the lipid composition contains more than 10% by mole of DMTAP has been reported elsewhere.[91]

A few notable aspects regarding using chol-siRNA associated with rHDL have been observed. A single study shows that the cellular uptake of chol-siRNA is more efficient for rHDL loaded with one versus four chol-siRNAs per rHDL particle according to the fluorescence intensity from labelled-chol-siRNA taken up in hepatocytes.[11] The authors suggest that the presence of multiple chol-siRNA in single rHDL particles may impair the interaction between the SR-B1 or LDLR receptors and the apoA-I or apoE-based reconstituted lipoproteins, respectively. Interestingly, another study suggests that the mammalian homolog of Sid1 in *C. elegans* that is known to mediate cellular small RNAs uptake is proposed to take part in the cellular uptake of chol-siRNA.[89] To support this they showed, among other things, that the silencing of the Sid1 homolog in HepG2 cells or pretreatment of HepG2 cells with anti-Sid1 antibodies both reduced the uptake of chol-siRNA after incubation with chol-siRNA-rHDL.

The cholesterol fusion approach of delivering siRNAs via rHDL mediated uptake shows that the rHDL delivery platform is not limited to hosting and delivering hydrophobic-based drugs. As the ongoing developments of anti-sense technology improve alongside the expansion of flexible rHDL delivery systems, the conjugation of siRNA/anti-sense strands to cholesterol for promoting rHDL loading portends the future realization of an effective Trojan horse platform for delivering siRNA/DNA cargo more efficiently and effectively to target cells. The combination of modified-rHDL and siRNA/DNA has the potential to become a general therapeutic approach to target a wide range of diseases.

Anti-cancer drugs

Like the case of AmpB, ATRA and curcumin, solubility is a major limitation of anti-cancer drugs as an estimated 40% of new anti-cancer drugs are characterized as having poor water solubility.[92] The anti-cancer drug PTX is used in the clinic to treat various kinds of cancers but its inherent hydrophobicity impairs its bioavailability. PTX-loaded rHDL with and without succinyl modification of cholesterol and apoA-I[93] turned out to be more efficient than both commercial Taxol® (tradename of PTX) and liposomes loaded with PTX at targeting and accumulating in the tumor tissue as well as reducing tumor size in mice. A more efficient uptake of PTX using rHDL compared to free PTX has also been shown by Lacko et al.[69] In this work, they showed that the PTX uptake was 3-fold higher by SR-B1 overexpressing cells and that the uptake was reduced when spherical reconstituted (sr) HDL-loaded PTX was co-introduced to SR-B1-expressing prostate cancer cells with 10-fold plasma HDL or rHDL. These results clearly suggest that the SR-B1 receptor takes part in the cellular uptake of PTX presented via srHDL. Other anti-cancer agents such as valrubicin and doxorubicin hydrochloride (Dox) have also been incorporated into srHDL particles that have been shown to increase valrubicin solubility and functionality[94] and promote Dox-dependent reduction in tumor growth more effectively than liposomes.[95] The latter work claims that the hydrophilic

Dox compound is loaded into the core of a srHDL, yet, this is seemingly surprising given that the rHDL-like particles were prepared from egg phospholipids (PL) lacking cholesteryl esters that would normally constitute the hydrophobic core of the spherical HDL. Furthermore, a hydrophobic core is not considered conducive to hosting hydrophilic compounds.

How to improve anti-cancer drugs for rHDL delivery

In the case of targeting cancer cells/tissue, the incorporation of highly toxic anti-cancer drugs in the rHDL vehicle may end up harming the liver substantially due to the expected delivery of rHDL to liver. Thus, two strategies could be beneficially combined to reduce the impact on liver: (i) modify the rHDL particle with known cancer cell ligands to facilitate the efficient diversion and delivery of rHDL to cancer cells rather than the liver by either modifying the scaffold apolipoprotein (in a similar manner to the scFv(anti-CD20)-apoA-I and TAT-apoA-I fusion proteins presented in the *Improving the targeting feature of rHDL* section) or use lipid-anchored or folate-modified apoA-I hybrids[67] that are known to target cancer cells and/or (ii) use drugs/bioagents that may not significantly impair the liver, since a certain fraction of the incorporated rHDL drugs will eventually end up in the liver. It is also important to note that drugs and biomolecules that target the fast proliferating cancer cells are potential candidates, since the half-life of hepatocytes in rats is 440 days,[96] and thus, the half-life for human hepatocytes that constitute about 70% of the human liver is likely more than a year. One example of such an approach is to employ chol-siRNA to target cyclin D1 expression. A recent study has shown that siRNA targeting cyclin D1 expression both halts the proliferation and reduces the number of mantle cell lymphoma cells.[97] Cyclin D1 promotes cell growth and is highly expressed in mantle cell lymphoma cells, which demonstrates it promise for siRNA targeting with an appropriate delivery vehicle such as rHDL. Moreover, many of the commercially available chemotherapeutics that target cells undergoing frequent cell division are also highly hydrophobic (for example PTX), which makes these drugs potentially strong candidates for rHDL-based treatment assuming they can be stably associated with lipid and then successfully off-loaded to the desired target cells. The enhanced circulation time of protein- or lipid-pegylated rHDL could also increase successful delivery of rHDL to cancer cells by allowing the particles to be trapped within the leaky vasculature of tumors or between the extracellular collagen fibril matrix that are present in certain cancerous tumors.

A cocktail and a tandem approach

The flexibility of cargo integration allows for the incorporation of multiple kinds of drugs into the rHDL platform. The ability to deploy a complex arsenal of diverse and efficacious drugs within a single platform would presumably enhance the efficacy of cancer treatment. A cancerous tumor is a complex network that consists of different genetically modified cells and tissue types,[62] and thus, using a treatment approach based on a carefully chosen mixture of drugs would likely be more efficient in eradicating the tumor. The use of cocktails comprised of different anti-cancer therapies are already being applied in fighting certain cancers in the clinic. A similar approach could be taken with the rHDL design, whereby particle composition could be adjusted to facilitate efficient cargo delivery to the target cells and minimize the negative side effects that are known to be associated with these highly toxic anti-cancer drug cocktails.

Along with these considerations, a tandem approach of maximizing the beneficial effects of rHDL in certain readily applicable diseases such as CVD and diabetes as well as using rHDL to complement or enhance preexisting and well-known therapeutic options creates an interesting combination that could create synergistic effects with drug-loaded rHDL. A recent study[98] showed that statin-loaded rHDL inhibits atherosclerotic plaque formation in mice. In this work, the focus was on the anti-inflammatory properties of statin drugs and rHDL was used to harness HDL's natural ability to target macrophages and, thus, deliver statins to atherosclerotic lesions. Nevertheless, the plaque macrophage content tended to be lower in rHDL treated groups as compared to placebo treated groups.

Doxorubicin is an anti-cancer drug that is known for its cardiotoxic side-effects triggered by the generation of oxidative species. Interestingly, one study[99] showed that when the formulation included sphingosine-1-phosphate added to the doxorubicin-rHDL complex, these complexes protected cardiomyocytes from doxorubicin-induced apoptosis due most likely to the combined anti-inflammatory and anti-oxidative functions of sphingosine-1-phosphate-loaded rHDL.

The rHDL cocktail and/or tandem approach could also be applied to other indications than CVD and cancer. In diabetic patients, the documented increased gluconeogenesis in liver (along with the impaired insulin mediated cellular glucose uptake) contributes to critically high levels of glucose in the bloodstream. In addition, an increased level of the lipid intermediates diacylglycerol and ceramides in the liver and skeletal muscle cells have shown to lead to impaired insulin signaling and insulin resistance.[100] For these two reasons, incorporation of cargo that is capable of downregulating hepatic gluconeogenesis (like metformin®) and diacylglycerol and ceramide levels or inhibiting the action of these latter compounds (in the liver) into rHDL could be an interesting anti-diabetic approach to pursue. In addition, HDL has shown to induce anti-apoptotic behavior of beta-cells[23] and also stimulate insulin secretion.[24] Thus by combining the rHDL-associated positive effect on beta-cells with the delivery of therapeutics that can down-regulate hepatic gluconeogenesis and diacylglycerol/ceramide levels in the liver via rHDL may provide a novel and multi-functional approach to promoting insulin sensitivity and controlling the blood glucose level in diabetic and prediabetic patients (Table 1).

The examples above show that the rHDL can be successfully utilized as a drug delivery system. In the following sections, we discuss the chemophysical and pharmaceutical properties of rHDL in order to further optimize the rHDL-delivery platform.

The preparation and characterization of rHDL with and without drugs

The heterogeneity of biological HDL in terms of size and composition is also present in simplified rHDL systems, which

Table 1
Examples of various kinds of cargo incorporated into discoidal reconstituted HDL (rHDL) and their corresponding indications.

Cargo	Lipid composition	Apolipoprotein	Target - Disease	Model	Ref.
Amphotericin B	DMPC and DMPG	ApoA-I	Fungal	*C. albicans* (*CA*) and *A. fumigatus* (*AF*) strains and mice infected with *CA*	72
Amphotericin B	DMPC and DMPG	ApoA-I	Fungal	*S. cerevisiae, CA, AF, and C. neoformans*	73
Amphotericin B	DMPC and DMPG	ApoA-I	*Leishmania major*	*L. major-infected BALB/c mice*	74
ATRA	DMPC and DMPG	ApoA-I	Cancer (MCL*)	Granta, NCEB, and Jeko cells	77,78
Curcumin	DMPC	ApoA-I	Liver/cancer (MCL)	HepG2 and Jeko cells	79
Curcumin	DMPC	ApoA-I	Cancer (MCL)	Jeko and Granta cells	80
Curcumin	DMPC	ApoE-N-terminal	LDL-receptor/General	LDLr-IP† analysis	81
Curcumin	DMPC	scFv-ApoA-I‡	Cancer (BCL§)	Ramos and Granta cells	13
Cardiolipin	Cardiolipin	ApoA-I	Mitochondria (Barth syndrome)	HL60 cells	82
siRNA(ApoB)	POPC or DMPC	ApoA-I vs ApoE	Liver (CVD)	Mice	12
siRNA(GAPDH)	DMPC and DMTAP	ApoA-I	Liver/cancer (POP‖)	HepG2 cells	90
Statin	MHPC and DMPC	ApoA-I	Atherosclerotic lesions	ApoE$^{-/-}$ mice	98
Paclitaxal	Soy PC and MS¶	Succinated-apoA-I or bare apoA-I	Cancer	MCF-7 cells and tumor-bearing mice	93
Doxorubicin	Soy PC and MS	TAT-apoA-I#	Cancer	Cancer cells and cancer mice	71

* Mantle cell lymphoma.
† IP = Immunoprecipitation.
‡ A fusion protein comprised of a CD20 antigen single-chain variable fragment (scFv) and apoA-I.
§ B-cell lymphoma.
‖ Proof-of-principle.
¶ Monocholesteryl-succinate.
# A fusion protein comprised of apoA-I and a protein transduction domain (TAT).

calls for careful characterization of rHDL particles both with and without drug integration.

The preparation of rHDL

The rHDL particles are traditionally formed using either the detergent depletion method[101] or mixing liposomal lipids with apoA-I.[102] In the former method, lipids, detergents and apoA-I are mixed above the gel-liquid phase transition temperature of the lipid followed by detergent removal using either dialysis or hydrophobic biobeads. Using the appropriate conditions, the result is a formation of highly monodisperse rHDL particles with respect to size. The size of the formed rHDL is dependent on the molar ratio between the lipids and the apolipoprotein, and the choice of lipids and apolipoprotein. The preparation of 9.6 nm rHDL particles containing two apoA-I proteins and a lipid:protein ratio of roughly 100:1 (dependent of the mean molecular area of the lipid(s)) has historically been the most preferred choice. This particular configuration mimics the most abundant nascent HDL, the α_4 HDL. However, sizes of 9, 12, 14 and 17 nm have been reported based on a mixture of PL and FC or sphingomyelin lipids,[103] and rHDL particles as large as ~47 nm (still discoidal) containing an estimated 10 apoA-I apolipoproteins have been observed.[104]

The advantage of preparing rHDL by adding apoA-I to liposomes is that detergents that may exhibit toxicity in biological systems are avoided. The drawback is that the formation of rHDL using lipids with a phase transition below zero degrees, such as when using common unsaturated lipids such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), is difficult. It should be noted that sonication speeds up rHDL formation, however, sonication can also damage the lipids and/or protein. Defects in the artificial membrane are proposed to be highly abundant in small liposomes that are formed by sonication. The kinetics of rHDL formation from liposomal formulations are generally highly dependent on either (i) defects in lipid packing in the liposomes[105] that are highly abundant at the phase transition of the lipid due to the formation of phase boundary region, and in small liposomes due to the high curvature or (ii) the presence of negatively charged lipids. Interestingly, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which has a phase transition at 23.9 °C, is often used to prepare rHDL using the liposomal approach due to the convenient working conditions with respect to temperature. The accessibility of the apoA-I proteins to the negatively-charged oxygen on the phosphatic part of the lipid could be the driving mechanism for the initial interactions between apoA-I and the lipids. This accessibility is presumably high in the case of negatively-charged lipids and is most likely also present at the phase transition of zwitterionic PL. Thus, the author suggests that the mechanism by which apoA-I gets lipidated could be the same for both situations mentioned above (i and ii). Previous work suggests a two-step mechanism for the formation of rHDL wherein the C-terminus of apoA-I initiates interaction with the lipid membrane.[106] Interestingly, the calculated overall charge of the latter 18 amino acids in this C-terminal region is +1 at pH 7.4 based on the contribution of three lysines and two glutamic acids compared to a total charge of ~ −9 for the full length protein supports the hypothesis that the negatively charged moieties of the lipids are important for initiating the formation of rHDL particles. However, it should be mentioned that the degree of hydrophobicity of the C-terminus[107] and the glutamic acid at the

ninth to last position in the protein sequence[108] have also been shown to be important factors in the initial formation of rHDL particles.

Recently, a high-throughput method for preparing rHDL based on microfluidics was developed.[109] This method also allows for incorporation of the hydrophobic drug simvastatin into rHDL. Notable, the micro fluidic single-step reconstitution formed slightly smaller (<9 nm) or larger rHDL particles compared to the conventional liposome-apoA-I procedure that is known to form the canonical 9.6 nm rHDL particles. Interestingly, this new single-step microfluidic approach can result in continuous production of rHDL at a rate of 420 mg/h (total weight) and has the ability for further scale up by running multiple chips in parallel.[109]

Incorporation of drugs and other biomolecules into rHDL

Incorporation of drugs into rHDL can be obtained by various methods including incubating the drug with pre-assembled rHDL at a temperature above the phase transition temperature of the lipid if the drug compound is soluble in water (i.e. chol-siRNA).[11] If the compound is insoluble it can be reconstituted into the rHDL during the rHDL formation using the detergent depletion method. It has also been demonstrated that membrane proteins can be incorporated using this approach.[110] This particular work is based on using the ΔN-apoA-I variant. Lipophilic drugs and/or biomolecules can also be added to the liposomal lipid film when using the liposome/apoA-I method or added using an organic solvent carrier to the liposomes followed by addition of apoA-I and dialysis to remove the small amount of contaminating organic solvent.[79] It should be stressed that it is important to characterize and thoroughly understand the drug-rHDL assembly, since the drug may impact the overall particle structure as well as the final molecular composition of the assembled particles.

Interestingly, about 2/3 of the mass in the canonical 9.6 nm rHDL particle comes from the lipid mass of the bilayer, while a much smaller proportion is constituted by lipids in the bilayer for liposomes because of their appreciably larger size. Thus, it follows that the proportion of hydrophobic drugs that an rHDL particle can accumulate is significantly higher than for liposomes. Contrarily, the small size of rHDL particles compared to liposomes limits the absolute number of those incorporated into rHDL compared to liposomes, but this fact may not be critical since concentrations as high as 135 mg/kg of rHDL (with respect to the mass of apoA-I) have proven to be safe in human subjects.[40]

The inherent variation of drug and lipid composition in individual rHDL particles

The final composition of the drug loaded rHDL is most likely important in terms of both the therapeutic efficacy of the system and practical cost considerations. Thus, it should be noted that the variation of drug incorporation within a synthetic rHDL pool is suggested to be highly based on simple binomial distribution modeling that assumes that the binding of drug to rHDL is independent of whether the rHDL already contains one or more drug molecules. In the simple case where a bulk stoichiometry ratio of 1 drug molecule per rHDL is used, it is estimated that 37% of rHDL particles will lack any drug, 26% will contain 2 or more drug molecules, and only 37% of rHDL particles will be loaded with a single drug molecule. These values are based on an rHDL that contains 200 lipids and estimating that the drug occupies a volume equivalent to one lipid molecule in the rHDL bilayer. If the drug occupies the area of 2 lipids, an estimated 27% of rHDL particles will be loaded with a single drug molecule if a 1:1 bulk ratio between drug and rHDL particle is used. This kind of effect is also in play in the case of lipid composition variability where more than one type of lipid is present in the rHDL formulation. Along these lines, a recent study showed that the lipid variation in nanoscale liposomes is significantly larger than predicted by a simple binomial distribution.[111] Therefore, in the case that the cost value of the drug is much higher than the rHDL, it may be worth starting with a drug:rHDL ratio less than one to obtain a more cost effective therapeutic product. Moreover, in some cases the cellular uptake of the drug is reduced when more than one drug-molecule is incorporated in rHDL as previously shown in the case of chol-siRNA.[11]

Characterization of rHDL

A few standard techniques are often used to characterize the rHDL particles with respect to size. Size-exclusion chromatography (SEC) is a fairly simple and fast technique and is often used both to assess the overall size of the rHDL (based on the Stokes radius) and as a purification step. Native-polyacrylamide gel electrophoresis (native-PAGE) is also commonly used. More advanced methods such as transmission electron microscopy are commonly used to further evaluate the size and shape of the rHDL. A study using small-angle X-ray scattering to evaluate the structure of the rHDL (based on the ΔN-apoA-I) confirmed the discoidal shape of rHDL.[112] In addition to basic structural analysis, the lipid and apoA-I composition are commonly addressed using chemical phosphorus analysis, enzymatic assays or mass spectrometry to determine the lipid concentration. Absorbance measurements at 280-nm, bicinchoninic acid assay (BCA) or Bradford assays are also often used for determining concentration of the apolipoprotein. The method applied to determine the mean drug to rHDL ratio depends on the chemophysical properties of the drug.

The various aspects of the formation of drug loaded rHDL stresses the importance of a thorough characterization of the rHDL-based end product for determining the consistency and predictability of the final rHDL product.

The author would like to recommend using SEC as a gold standard for assessing the overall size and size-distribution of rHDL particles, since it is an easy and highly reliable method. Compared to Native-PAGE, SEC does not depend on the overall charge of the particles.

The role of lipids in native and reconstituted HDL

The destiny of the HDL particle in circulation is not only limited to the apolipoprotein component; the lipids also play an important role in the fate of the HDL and stability of rHDL.

Lipid composition alters HDL stability and metabolism

Various studies support the idea that the lipid composition of the HDL and rHDL inextricably determines the biophysical and biological properties of these particles along with the apolipoprotein component. For example, the impact of sphingomyelin (SM) on rHDL properties has been investigated in various studies and the main findings are summarized in the review paper by Martinez-Beamonte et al.[113] Increased SM compared to PL enrichment of human HDL enhances stability of HDL (using spherical HDL) in terms of antioxidant effect and increases apoA-I affinity compared to PL alone. SM enrichment has been shown to induce an ordered and rigid liquid lipid bilayer environment in HDL particles[113] Along these lines, it has been suggested that the lipid composition of nascent HDL closely resembles that of lipid rafts,[114] which are known membrane microdomains containing greater cholesterol and SM content compared to the rest of the lipid bilayer. We suggest that the high affinity of SM for apoA-I protein together with SM's ability to form a more rigid lipid environment may explain why enrichment of high density lipoproteins with SM inhibits LCAT-mediated cholesterol esterification by 50%[115] and causes ~ 90% inhibition of lipoprotein lipase (LPL) activity in triolein-SM emulsions.[116]

Some of the above observations could also be explained by cholesterol's preferential affinity for SM over PL. The most striking difference between SM and PL is that SM possesses both hydrogen bond-accepting and donating groups at the polar/non-polar interface, while PL only has hydrogen bond-accepting capacity. This molecular feature enables efficient hydrogen bonding between SM and the hydroxyl group in cholesterol. Moreover, the saturated fatty acids are more abundant in SM compared to PL, a feature that is known to promote stronger binding to cholesterol.[117] A differential scanning calorimetry study conducted by McMullen and McElhaney[118] suggests that the optimal hydrophobic match between cholesterol and PL is obtained for PL comprised of 17 carbon (C_{17}) saturated acyl chains. In addition, it has been shown that DMPC lipid comprised of saturated C_{14} acyl chains binds stronger to cholesterol than POPC that contains a saturated C_{16} and mono-unsaturated C_{18} acyl chain.[117] Thus, the degree of saturation of the acyl chains has a greater impact on the interaction with cholesterol as compared to the length of the acyl chains. These aforementioned properties of SM in HDL particles are all attractive in terms of forming stable rHDL formulations for drug delivery. However, it is important to recognize a study that has shown that SM enriched HDL particles exhibit reduced cholesterol-ester influx into COS-7 cells through the SR-B1 receptor compared to their PL-containing counterpart.[119] Contrarily, the efflux of cholesterol from COS-7 cells to the enriched HDL particles was significantly higher for PL- compared to SM-enriched HDL,[119] while another study showed that SM-enriched rHDL compared to PL-enriched rHDL promoted the greatest cholesterol efflux from macrophage cells.[120] A slightly different pattern was observed when LDL particles were employed as PL donors.[121] In this case, SM-loaded HDL was more efficiently transferred to cells by SR-BI as compared with PL and PE. Thus, the effect of SM on HDL functionality is not completely well understood and further studies are needed to confirm whether SM is more advantageous than phospholipids to prepare rHDL for drug delivery.

Like SM, charged lipids are also known to alter the properties of HDL particles. We have shown that the presence of negatively charged lipids in rHDL enhances the *in vitro* stability of rHDL particles due to interparticle electrostatic repulsion.[91] Furthermore, we[91] and others suggest that the local positively charged polar/non-polar interface between apoA-I and the head groups of the peripheral lipids in the lipid bilayer is stabilized by negatively charged lipids. In addition to the increased stability, Rigotti et al have interestingly demonstrated that anionic PL-based liposomes bind to the SR-BI receptor.[122] Thus, forming rHDL based on anionic lipids may enhance binding to the SR-BI receptor. It should be noted that the macrophage engulfment of negatively charged particles such as liposomes[123] and apoptotic cells takes place *in vivo*. On the other hand, the small size of the rHDL compared to liposomes and cells may allow rHDL to escape macrophage engulfment.

The exact trade-off of applying SM and/or anionic lipid components in the rHDL drug delivery formulation has to be tested more rigorously. For example, utilizing cholesterol-anchored drugs in conjunction with SM lipids to associate the drug more tightly to the rHDL vehicle may be an interesting avenue to explore. A study by Wolfrum et al[89] showed that the activity of an siRNA 'drug' tethered to a hydrophobic moiety correlated positively with the affinity of the drug (hydrophobic part) to the rHDL. This work also showed that the chol-siRNAs exhibit the highest affinity for HDL compared to other lipid candidates such as lauroyl (C_{12}), myristoyl (C_{14}), palmitoyl (C_{16}), steroyl (C_{18}), oleoyl (C_{18},mono-saturated), linoleoyl (C_{18}, di-saturated), docosanyl (C_{22}) etc., and, importantly, that the chol-siRNA had a higher affinity for HDL compared to highly abundant plasma serum albumin. As mentioned above, the drug's affinity to rHDL may also impair the cellular uptake of the drug as mediated by the SR-B1 receptor. This latter effect was observed by Yancey and coworkers.[119] What these studies collectively demonstrate is that the exact optimal conditions for binding specific drug payloads to rHDL particles requires further testing and refinement on a case by case basis, yet the studies and data suggest that there is great possibility for promoting strong association between the lipid and apolipoprotein components of rHDL and the desired drug or siRNA payload required for the specific therapeutic application.

The heterogeneous lipid environment and dynamics of rHDL lipids

The presence of amphipathic apolipoproteins in the lipid-protein HDL complex changes the properties of the HDL lipids in a heterogeneous fashion. The lipids in close proximity to the apolipoproteins, known as the boundary lipids, behave differently from the lipids expanding the core of the rHDL particle. The effect of such a heterogeneous environment has been illustrated by the example of the proposed preferential arrangement of cholesterol within and among the core lipids compared to boundary PL.[103,124] These well-designed studies examined either the relationship between rHDL size and lipid composition[103] or used differential scanning calorimetry and fluorescent techniques[124] to show that cholesterol prefers to be associated with the core rather than the boundary lipids. In

addition, negatively charged lipids are suggested to pack more favorably within the boundary layer compared to PL due to strong electrostatic interactions between the highly positively charged apoA-I at the polar/non-polar interface of the apolipoprotein and the associated negatively charged lipids.[91,103] Thus, the degree of incorporation of drugs, the stability of the drug-rHDL complex, and the influx properties of a drug incorporated into an rHDL particle could depend on the preferential affinities of the drug toward the lipid core environment or the apolipoprotein and/or the boundary lipid species. It is notable that approximately 40% of the lipids in the canonical 9.6 nm rHDL particle are in direct contact with the apolipoproteins based on geometrical calculations. The dynamics of HDL lipids are also different from its bulk properties. It has been shown that the PL transfer between DMPC apoA-I-based rHDL particles is faster than the DMPC exchange between liposomes.[125] According to the authors, the accelerated lipid desorption from rHDL particles is triggered by the unfavorable and stressed lipid close packing induced by apoA-I and the hydrophobic hydration at the apoA-I to lipid interface. In addition, it has been demonstrated that the gel-liquid phase transition temperature of DMPC in rHDL particles is about 3 °C above the phase transition temperature of DMPC-liposomes,[102] which supports an increase in the lateral pressure of the acyl-chains in the rHDL particles, that then leads to tighter packing of lipids. A recent molecular dynamic study[126] also confirmed that the overall acyl order is higher for lipids in HDL-like particles compared to liposomes and that the lipids at the center of the particle are highly ordered compared to the lipids in contact with the perimeter scaffold protein. Another study[127] suggests that the spontaneous transfer of PL from rHDL to LDL particles (half-life of 6-7 h) have considerably higher rates than those observed between liposomes (half-life of 63 to 83 h).[128] Furthermore, the exchange rate between those lipoproteins increases four-fold by adding PL transfer protein,[127] which may further exacerbate the differences between the HDL lipid composition in a biological setting.

These biophysical and biochemical studies emphasize that the lipids and lipid environment in rHDL due to the presence of apolipoprotein are significantly different from the protein-free environment of liposomes. The binary composition in terms of protein and lipid components introduces a heterogeneous lipid environment in the rHDL particle that provides both challenges regarding the dynamics of the system as mentioned above, but may also provide flexibility for diverse drug incorporation and precision for controlling the rate of drug release and subsequent targeted uptake. These properties could be adjusted by changing the hydrophobic match/mismatch between apoA-I and the lipids and/or drugs to tailor the formulation for the specific *in vivo* application. The additional modulation of lipid content and overall rHDL structure by *in vivo* actors such as LCAT and CETP will be addressed in the following part.

Pharmacological issues regarding the rHDL

It should be emphasized that the mechanisms responsible for the turnover of plasma HDL are still under intensely active investigation and thus are still largely undefined.

Pharmacokinetics of rHDL in rodents and human subjects

The HDL hypothesis that "high-density lipoproteins protect from atherosclerosis" has been rigorously studied over the years and has led to various clinical trials[3] investigating drugs that promote increased endogenous HDL synthesis as well as direct administration of rHDL. Five different rHDL formulations have entered phase 1 trials.[3] Another of these phase 1 studies employed a recombinant version of a naturally-occurring apoA-I mutant known as apoA-I-Milano, which contains an arginine-to-cysteine substitution at position 173,[36] while two others based on the non-mutated human apoA-I proceeded to phase 2 trials[41,42] that were completed in 2013 and 2014, respectively. Unfortunately, these latter trials showed no significant positive outcome of rHDL treatment. Nevertheless, clinical rHDL infusion studies have provided relevant insights into the pharmacological aspects of rHDL exposure to human plasma. A recent clinical trial evaluated the pharmacokinetics of multiple intravenous infusions of rHDL comprised of human apoA-I and PL from soybean in a 1:55 ratio (CSL-112).[38] The main findings were that the plasma half-life of the apoA-I protein ranged from 19 to 93 h, while the corresponding half-life of the PL ranged from 3 to 82 h. In addition to the full-range values, the 6.8 g dose group gave rise to half-life mean values of 39.7 h (last infusion) to 60.8 h (first infusion). Although the range of half-life values is rather wide, the significant differences between the half-life values of apoA-I and PL show that the clearance of apoA-I in circulation cannot be explained by the clearance of intact exogenous rHDL particles alone. It should also be noted that the CSL-112 formulation is based on a low lipid to apoA-I ratio (55:1) which gave rise to smaller rHDL-like particles. These particles were designed to optimize cholesterol efflux by ABCA1.[39] Thus, this formulation may undergo remodeling and clearance faster than the highly lipidated 9.6 nm rHDL. A small rHDL infusion study on 7 healthy human subjects showed a similar trend regarding faster clearance of the lipid component compared to apoA-I protein.[129] In addition, the authors state that the fractional catabolic rate of apoA-I is comparable to the endogenous HDL apoA-I which is around 0.3,[130] meaning that the half-life is above 24 h. Another clinical trial based on the infusion of rHDL (CER-001)[43] showed that the apoA-I level returned to baseline by 24 h after dosing for doses up to 10 mg/kg but remained in circulation longer than 72 h for doses of 10 mg/kg and above (Table 2).

Studies based on LDLr$^{-/-}$ mice treated with CER-001 and apoE$^{-/-}$ mice infused with CLS-111 exhibit a half-life of 4[131] and 8 h,[132] respectively, when using recombinant human apoA-I. When considering the consequences of these studies, particularly for their application to humans and human clinical trials, it should be stressed that mouse metabolism is significantly faster than seen in humans.

The data on rHDL half-life and dwell time vary quite a lot, but generally indicate that the half-life of rHDL is comparable to another commonly used drug delivery system, pegylated liposomes. Comprehensive pharmacokinetic studies of doxorubicin encapsulated in pegylated liposomes (Doxil®) have been conducted at patients with a variety of solid tumors.[133] An initial study with pilot Doxil®[134] showed that doxorubicin clearance

Table 2
Key formulation and corresponding pharmacokinetic and safety data of rHDL from clinical trials and a few additional mice studies.

Apolipoprotein	Lipid(s)	L:P* ratio	Trivial name	Subject	Dose mg/kg†	Dose frequency Weekly	Safety	ApoA-I half-life Hours	Ref.
r-human apoA-I Milano	POPC	~35‡	ETC-216	Patients with ACS	15 and 45	5	N/A§	N/A	36
Human apoA-I	Soybean PC	150	CLS-111	Patients with ACS	40 and 80	4	Low conc. well tolerated‖	N/A	37
Human apoA-I	Soybean PC	55	CLS-112	Healthy H.¶ subjects	3.4 g or 6.8 g	4#	Well tolerated	19–93 & (3–82)**	38,39
Human apoA-I	Soybean PC	55	CLS-112	Healthy H. subjects	5-135	Single	Well tolerated	~24††	39,40
Human apoA-I	Soybean PC	55	CLS-112	Patients with ACS	1.7 g–6.8 g	Single	N/A	N/A	41
r-human apoA-I	SM and DPPG‡‡	~110§§	CER-001	Patients with ACS	3–12	6	Generally well tolerated	N/A	42
r-human apoA-I	SM and DPPG‡‡	~110§§	CER-001	Healthy H. subjects	0.25–45	Single	Well tolerated	N/A‖‖	43
Human apoA-I	Soybean PC	150	CLS-111	ApoE$^{-/-}$ mice	40	Single	N/A	8	132
Pegylated human apoA-I	Soybean PC	150	CLS-111¶¶	ApoE$^{-/-}$ mice	40	Single	N/A	49	132
r-human apoA-I	SM and DPPG‡‡	~110§§	CER-001	LDLr$^{-/-}$ mice	10	Single/Multiple	N/A	4	131

* Lipid:Protein.
† Doses are defined as the apoA-I concentration present in the dosing solution.
‡ Calculated from a given mass ratio of 1:1.
§ N/A = not available.
‖ A dose of 80 mg/kg was associated with a high incidence of liver function test abnormalities. These changes returned to normal after stopping the treatment.
¶ H = human.
# Once or twice weekly.
** Based on the PC clearance.
†† Estimated by eye from apoA-I circulation data in Ref.[37].
‡‡ In a ratio 97:3 by weight.
§§ Calculated from a mass ratio of 1:2.7.
‖‖ Doses up to 10 mg/kg returned to apoA-I baseline by 24 hours post-dose, while doses ≥10 mg/kg remained in circulation longer than 72 h post-dose.
¶¶ Pegylated-modified CLS-111.

exhibits a biphasic clearance pattern. The initial half-life is 1-3 h (~30% of the injected dose was cleared), while the second phase which includes more than 95% of the AUC in humans, has a half-life of about 45 h. These values are proposed to represent the pharmacokinetics of pegylated liposomes, since the corresponding half-lives of free doxorubicin are significantly shorter (0.06 and 10.4 h). Other studies show both single and biphasic elimination patterns where the half-lifes are ranging from 33.3-89.8 h (for the single half-life values) depending on dose and tumor type.[133] A comprehensive study of the dwell-time of rHDL that tracks both the lipid and apoA-I components independently along with tissue biodistribution as a function of lipid composition and size would provide useful information that could be applied to engineer more efficient rHDL drug delivery formulations.

In vivo remodeling of the rHDL

The half-life of apoA-I in circulation is one hallmark of the pharmacokinetics of rHDL. However, it does not appear to accurately reflect the lifespan of the intact rHDL particle. At most, it gives a theoretical upper limit of the half-life of the intact rHDL particle. The primary biological HDL target that mediates lipid influx in the case of apoA-I-based HDL is, as previously mentioned, the SR-B1 receptor. The SR-B1 can mediate influx of the lipid payload in the rHDL or it can transfer lipids (including FC) to the rHDL particles and thereby activate LCAT and CETP enzymes to transform rHDL into the spherical HDL forms (see Figure 2). This remodeling of infused rHDL has been observed in rabbits.[135] In the rabbit model, two hours after the infusion of FC-free rHDL (CSL-112), the majority of human apoA-I was seen in particles larger than the parent rHDL, suggesting an *in vivo* remodeling and maturation of the rHDL particles into spherical particles within the circulation. Smaller species of human apoA-I were also seen during the remodeling process, presumably due to a SR-BI-mediated influx of rHDL lipids.

A key question in terms of using the rHDL as a drug delivery vehicle is whether the remodeling of the rHDL particle as acted on by various biological agents in the HDL pathways may bias the targeting of the intact particle, the cellular uptake of the drug, and/or introduce drug leakage. A study based on PTX-loaded rHDL particles that contain FC and PL,[93] which are the known substrates of LCAT, shows that PTX leakage is a noted consequence of the remodeling process. Interestingly, the authors showed that a chemical succinate modification of cholesterol and cholesteryl-succinate modification of apoA-I served to decrease the overall remodeling process and, thus, lower drug leakage from the particles during their time in circulation. However, the effect on the remodeling of PTX-loaded rHDL particles lacking FC was not

investigated, which leaves an open question regarding the relative importance of dual LCAT substrates (FC and PL) as a part of the remodeling process.

Any infused apoA-I rHDL is most likely encountered first by SR-B1 expressing cells. The SR-BI is a bidirectional lipid receptor. Thus, if the molecular gradient controls the directional flux of the lipids between the cell membrane and the rHDL, it is likely that any hydrophobic drug payload has a preference for cellular influx. By contrast, FC is more likely to efflux to the rHDL (if the rHDL is free of FC), a feature that has motivated the use of rHDL to treat CVD patients. The rHDL is thought to remove the high content of FC in foam cells of the arterial wall that are cholesterol and triglyceride-laden macrophage cells that are a known contributor to and hallmark of atherosclerotic plaque-build up. One way to promote cellular SR-BI-mediated drug uptake could be to use rHDL containing cholesterol. The drawback of this approach is that the LCAT-mediated remodeling of rHDL-containing FC and PL is activated, but it is possible that this could be mitigated by using functionalized cholesterol or SM that would diminish *in vivo* LCAT remodeling activity. If drug-loaded rHDL is not taken up by the SR-B1 expressing cells through either selective lipid influx or holo-particle uptake, the destiny of the drug cargo depends on the chemical properties of the drug as to whether it can withstand leakage from the rHDL during remodeling. If the drug is capable of staying on the rHDL during LCAT- and CETP-mediated remodeling, the vast majority of it will eventually end up associating with the SR-B1 receptor (see Figure 2). Thus, no matter which pathway the rHDL takes within the RCT framework shown in Figure 2, the rHDL vehicle has a high preference for finding its way to SR-B1 expressing cells. Thus, the drug cargo may be taken up during the first pass encounter with SR-BI or if the drug is not removed at first, the drug-rHDL will likely undergo LCAT- and CETP-mediated remodeling where the chemical properties of the drug may be important to avoid drug leakage. If significant drug leakage is avoided, the mature spherical rHDL will then deliver its content to cells following the second pass encounter with SR-BI-expressing tissues. However, depending on the molecular selectivity of the SR-B1 receptor, it may or may not take up the drug at all. In the case that SR-B1-mediated uptake results in endocytosis, the molecular structure of the drug may be irrelevant.

In the case of apoE-based rHDL delivery the pathways are almost identical. However, instead of interacting with a bidirectional lipid transporter like the SR-BI receptor, apoE is taken up primary by liver-expressed LDLR that mediates the endocytosis of the holo-particle. Thus, the transport of apoE-dependent rHDL cargo is meant to go unidirectional towards LDLR-expressing cells followed by eventual release of the payload into the target cells by lysosomal degradation (presuming, of course, that the drug payload can survive the acidification of the lysosomal compartment). Thus, the drug used in the apoE-rHDL is most likely more efficient if it can withstand the acidic environment of the lysosomes.

How to increase the circulation time of rHDL

Macrophages are thought to play an important role in the clearance of liposomes. Thus, by incorporating pegylated lipids into liposomes, the half-life can be increased because pegylation avoids rapid liposomal recognition by macrophages. These pegylated liposomes have thus come to be known as stealth liposomes and show decreased clearance rates. Recently, Tall and coworkers showed that pegylation of apoA-I could also be successfully employed to improve the circulation dwell time of rHDL apoA-I.[132] By incorporating mono-pegylated apoA-I (via the N-terminus) into intact rHDL (CLS-111), the half-life of human apoA-I increased from 8 to 49 h in hypercholesterolemic $ApoE^{-/-}$ mice. As mentioned before, and importantly, the pegylated lipids may also provide a handle for altering the kinetics of rHDL clearance to increase the opportunity for drug payload uptake. A chemical succinate modification of cholesterol and apoA-I have also shown to improve the *in vivo* stability of rHDL.[93] Moreover, by engineering the apoA-I in such a way that it no longer interacts with plasma remodeling factors like LCAT, an additional method could be employed to efficiently prolong the circulation time of rHDL in plasma (See Figure 3). Importantly, the central helices 5, 6, and 7 of apoA-I are known to activate LCAT.[136] As pointed out in section *Lipid composition alters HDL stability and metabolism*, SM lipids alter the LCAT activity and we suggest in section *In vivo remodeling of the rHDL* that the incorporation of modified cholesterol that are unable to interact with LCAT may also reduce the potential leakage of drug cargo during LCAT-mediated maturation of rHDL.

It should be noted that the pharmacokinetics of the bare rHDL is one consideration, yet the incorporated cargo may alter the pharmacokinetic properties in additional and even unexpected ways depending on the particular properties of the payload.

The safety of rHDL infusion in human subjects

A single ascending-dose study of CSL-112 used in healthy subjects found that a dose ranging from 5 to 135 mg/kg with respect to the mass of apoA-I was well tolerated.[40] A multi dose study of the same formulation (CSL-112) confirmed the safety of rHDL treatments and noted that a multiple dose approach (1 to 2 infusions over four weeks) was also well tolerated in healthy subjects. No rHDL or human apoA-I-specific binding antibodies were observed for any subject in the former study. Notable, a dose of 135 mg/kg corresponds to about two times the amount of endogenous apoA-I in healthy subjects.

Contrary to CLS-112, the CLS-111 formulation showed that a dose of 80 mg/kg was associated with a high incidence of liver functional test abnormalities.[37] These changes returned to a normal range after stopping the treatment, and there was no evidence of liver failure or permanent damage. It has been proposed that residual bile acid used in the preparation of the rHDL (CLS-111) for the clinical trial mentioned above could have caused adverse effects and may therefore limit the quantity of rHDL that can be administered.[132] Another dialysis procedure was applied to CLS-112 versus the CLS-111 study to (most likely) remove more of the excess bile acid and avoid, or at least, reduce safety issues due to residual amount of bile acid. The study based on the CER-001 rHDL formulation evaluated dose levels from 0.25 to 45 mg/kg and all doses of CER-001 were safe and well tolerated.[43]

Table 3
Summary of factors and strategies that are proposed to increase the efficacy of rHDL-loaded therapies.

Category				Section
Stability				
Lipid	In vitro	Reduce LCAT remodeling	in vivo	
Sphingomyelin		+*		Lipid composition alters…
Anionic lipids	+			Lipid composition alters…
Pegylated-lipids			(+)†	How to increase…
Modified-cholesterol‡		+	+	*In vivo* remodeling...
Apolipoprotein-modifications				
Succination		+	x	*In vivo* remodeling...
Pegylation			+	How to increase…
Mutate alfa-helixes 5-7		(+)	(+)	How to increase…
Target cell/tissues				
Apolipoprotein	Target			
ApoA-I, apoE	Liver			The inherent liver target…
ApoA-I, apoE	Various cancers			Targeting cancer
Lactosylate-apoA-I	Liver			Improving the targeting…
ScFv/peptide-apoA-I	Depends on the scFv/peptide fragment			Improving the targeting…
Folate-apoA-I	Various cancers			Improving the targeting…
Drugs/Biomolecules				
Cargo incorporated into rHDL	See Table 1			Biomolecules to Anti-cancer…
Trojan horse approach	Cholesteryl-drug conjugates			siRNA
Avoid drugs that impairs the liver	Use drugs that targets proliferation			How to improve anti-cancer…
Combinational approaches				
Cocktail approach	Use multiple drugs			A cocktail and a tandem…
Tandem approach	Combine the therapeutic properties of rHDL and the incorporated compound			A cocktail and a tandem…
Preparation route				
Incorporation method	Depends on the chemical properties of the cargo			Incorporation of drug…
Composition variation				
A bulk 1:1 drug:rHDL ratio implies that 27% of the rHDL particles are loaded with 1 drug§				The inherent variation…
Characterization of rHDL				
Size-exclusion chromatography should (according to the author) be the golden standard for characterizing the overall size/distribution of rHDL particles				Characterization of rHDL
Heterogenous lipid environment in rHDL				
The lipid environment in rHDL is different from liposomes				The heterogeneous lipid...
Cargo may exhibit preferential affinity for the boundary lipids, the core lipids or the apolipoprotein				
Half-life in humans				
Bare rHDL	3-93 hours‖			Pharmacokinetics of rHDL…
Safety dose in humans				
Bare rHDL	At least 135 mg/Kg¶			The safety of rHDL infusion…

* + refers to a documented property.
† (+) corresponds to a property proposed by the author (JBS).
‡ Could also improve the cellular uptake due to a higher cholesterol gradient across the membrane.
§ Based on a binomial distribution. See the detailed assumptions in section *The inherent variation of the drug and lipid composition…*
‖ The data varies a lot between rHDL formulations and studies. The range is defined by the highest and lowest value of apoA-I and the lipids.
¶ Defined by the concentration of apoA-I.

It should be stressed that adding additional functional groups, protein modifications and drugs to the rHDL particles could trigger immune responses that should be tested. The clinical data above only point out that the bare rHDL drug delivery vehicle seems to be non-immunogenic.

In conclusion, the half-life time of rHDL is supposed to be comparable to pegylated liposomes and by applying the pegylated approach to rHDL the half-life time is increased substantially. Moreover, according to various clinical trials, the administration of bare rHDL is regarded as safe in a wide range of concentrations assuming proper preparation conditions are observed.

Summary and outlook

A goal of this work was to organize and present various considerations that anyone interested in using rHDL in general and in particular as a drug delivery system should be aware of and consider. We have summarized key molecular factors driving HDL biology and pertinent strategies for manipulating rHDL systems and optimizing drug efficacy discussed herein (Table 3). It should be emphasized that even hydrophilic cargo molecules can be applied to the rHDL drug delivery platforms, as illustrated by several studies that use rHDL to deliver chol-siRNA to liver. A strategy that is based on cholesterol

conjugation to promote incorporation of particular cargo molecules into rHDL combines the idea of a Trojan horse delivery system with the targeting feature of rHDL in one potent drug delivery platform.

The main concerns when using rHDL as a drug delivery platform focus on the overall dynamics of rHDL, including the lipids and lipoprotein components that may contribute to cargo leakage. To address these issues, we call for more detailed *in vivo* studies where both the apoA-I, lipids and ideally, the chosen cargo are differentially labeled in order to more completely probe the integrity of the rHDL complex on an individual component basis and hopefully track the cargo from initial administration to delivery to the target cells/tissue. With that being said, we have shown in the *rHDL as a delivery vehicle for hydrophobic biomolecules and drugs* part that rHDL is an efficient platform for delivering various kinds of cargo into target cells and tissues. This provides concrete evidence for the promise of rHDL-based drug delivery systems for addressing a variety of future therapeutic needs.

We have also emphasized the complexity of the rHDL particles with regard to their structure, composition, dynamics and function. Despite this complexity, the advantages of rHDL lie in the high degree of tuneability of such a system following sufficient investigation and testing of the relevant therapeutic molecule's compatibility with the rHDL platform. Along these lines, we think it could be interesting to explore several of the factors listed in Table 3 across a combination of categories to further optimize and refine the utility of rHDL for a range of therapeutic applications. Hopefully this approach can push the potential of the rHDL delivery platform towards realizing the 'magic bullet' of multiple, highly-efficient and efficacious treatment options in a range of therapeutic areas where the current need is high. We also welcome further studies that attempt to increase the reach of the rHDL drug delivery approach into other diseases beyond what has been tested thus far. The combination of targeted-modified-rHDL loaded with cholesterol conjugates such as chol-siRNA provides a general formulation that could potentially be deployed in the treatment of a wide range of diseases.

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References

1.. Jonas A, Phillips MC. Lipoprotein structure. In: Vance DE, Vance JE, editors. *Biochemistry of Lipids. Lipoproteins and Membranes*. 5th ed. Amsterdam: Elsevier; 2008. p. 485-506.
2. Kingwell BA, Chapman MJ. Future of high-density lipoprotein infusion therapies: potential for clinical management of vascular disease. *Circulation* 2013;**128**(10):1112-21.
3. Kingwell BA, Chapman MJ, Kontush A, Miller NE. HDL-targeted therapies: progress, failures and future. *Nat Rev Drug Discov* 2014; **13**(6):445-64.
4. Toth PP, Barter PJ, Rosenson RS, Boden WE, Chapman MJ, Cuchel M, et al. High-density lipoproteins: a consensus statement from the National Lipid Association. *J Clin Lipidol* 2013;**7**(5): 484-525.
5. Vickers KC, Remaley AT. Thematic review series: high density lipoprotein structure, function, and metabolism HDL and cholesterol: life after the divorce? *J Lipid Res* 2014;**55**(1):4-12.
6. Rensen PCN, de Vrueh RLA, Kuiper J, Bijsterbosch MK, Biessen EAL, van Berkel TJC. Recombinant lipoproteins: lipoprotein-like lipid particles for drug targeting. *Adv Drug Deliv Rev* 2001;**47**(2–3): 251-76.
7. Lacko AG, Sabnis NA, Nagarajan B, McConathy WJ. HDL as a drug and nucleic acid delivery vehicle. *Front Pharmacol* 2015;**6**.
8. Damiano MG, Mutharasan RK, Tripathy S, McMahon KM, Thaxton CS. Templated high density lipoprotein nanoparticles as potential therapies and for molecular delivery. *Adv Drug Deliv Rev* 2013;**65**(5): 649-62.
9. Shih AY, Freddolino PL, Sligar SG, Schulten K. Disassembly of nanodiscs with cholate. *Nano Lett* 2007;**7**(6):1692-6.
10. Carney CE, Lenov IL, Baker CJ, MacRenaris KW, Eckermann AL, Sligar SG, et al. Nanodiscs as a modular platform for multimodal MR-optical imaging. *Bioconjug Chem* 2015;**26**(5):899-905.
11. Nakayama T, Butler JS, Sehgal A, Severgnini M, Racie T, Sharman J, et al. Harnessing a physiologic mechanism for siRNA delivery with mimetic lipoprotein particles. *Mol Ther* 2012;**20**(8):1582-9.
12. Kynde SAR, Skar-Gislinge N, Pedersen MC, Midtgaard SR, Simonsen JB, Schweins R, et al. Small-angle scattering gives direct structural information about a membrane protein inside a lipid environment. *Biol Crystallogr* 2014;**70**:371-83.
13. Crosby NM, Ghosh M, Su B, Beckstead JA, Kamei A, Simonsen JB, et al. Anti-CD20 single chain variable antibody fragment-apolipoprotein A-I chimera containing nanodisks promote targeted bioactive agent delivery to CD20-positive lymphomas. *Biochem Cell Biol* 2015;**93**(4): 343-50.
14. Borch J, Hamann T. The nanodisc: a novel tool for membrane protein studies. *Biol Chem* 2009;**390**(8):805-14.
15. Skajaa T, Cormode DP, Falk E, Mulder WJ, Fisher EA, Fayad ZA. High-density lipoprotein-based contrast agents for multimodal imaging of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2010; **30**(2):169-76.
16. Huda P, Binderup T, Pedersen MC, Midtgaard SR, Elema DR, Kjaer A, et al. PET/CT based in vivo evaluation of Cu-64 labelled nanodiscs in tumor bearing mice. *Plos One* 2015;**10**(7).
17. Kontush A, Therond P, Zerrad A, Couturier M, Negre-Salvayre A, de Souza JA, et al. Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL3 particles - relevance to antiapoptotic and antioxidative activities. *Arterioscler Thromb Vasc Biol* 2007;**27**(8):1843-9.
18. Patel S, Drew BG, Nakhla S, Duffy SJ, Murphy AJ, Barter PJ, et al. Reconstituted high-density lipoprotein increases plasma high-density lipoprotein anti-inflammatory properties and cholesterol efflux capacity in patients with type 2 diabetes. *J Am Coll Cardiol* 2009;**53**(11): 962-71.
19. de Souza JA, Vindis C, Negre-Salvayre A, Rye KA, Couturier M, Therond P, et al. Small, dense HDL 3 particles attenuate apoptosis in endothelial cells: pivotal role of apolipoprotein A-I. *J Cell Mol Med* 2010;**14**(3):608-20.
20. Nofer JR, Brodde MF, Kehrel BE. High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. *Clin Exp Pharmacol Physiol* 2010;**37**(7):726-35.
21. Calkin AC, Drew BG, Ono A, Duffy SJ, Gordon MV, Schoenwaelder SM, et al. Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux. *Circulation* 2009;**120**(21):2095-104.
22. Feingold KR, Grunfeld C. The role of HDL in innate immunity. *J Lipid Res* 2011;**52**(1):1-3.
23. von Eckardstein A, Widmann C. High-density lipoprotein, beta cells, and diabetes. *Cardiovasc Res* 2014;**103**(3):384-94.

24. Drew BG, Rye KA, Duffy SJ, Barter P, Kingwell BA. The emerging role of HDL in glucose metabolism. *Nat Rev Endocrinol* 2012;**8**(4): 237-45.
25. Shah AS, Tan L, Long JL, Davidson WS. Thematic review series: high density lipoprotein structure, function, and metabolism proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond. *J Lipid Res* 2013;**54**(10): 2575-85.
26. Kontush A, Lhomme M, Chapman MJ. Thematic review series: high density lipoprotein structure, function, and metabolism unraveling the complexities of the HDL lipidome. *J Lipid Res* 2013;**54**(11):2950-63.
27. Traber MG, Diamond SR, Lane JC, Brody RI, Kayden HJ. Beta-carotene transport in human lipoproteins - comparisons with alpha-tocopherol. *Lipids* 1994;**29**(10):665-9.
28. Alwaili K, Bailey D, Awan Z, Bailey SD, Ruel I, Hafiane A, et al. The HDL proteome in acute coronary syndromes shifts to an inflammatory profile. *Biochim Biophys Acta* 2012;**1821**(3):405-15.
29. Balazs Z, Panzenboeck U, Hammer A, Sovic A, Quehenberger O, Malle E, et al. Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an in vitro blood–brain barrier model. *J Neurochem* 2004;**89**(4):939-50.
30. Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Investig* 2007;**117**(3):746-56.
31. Tikkanen MJ, Vihma V, Jauhiainen M, Hockerstedt A, Helisten H, Kaamanen M. Lipoprotein-associated estrogens. *Cardiovasc Res* 2002; **56**(2):184-8.
32. Benvenga S, Cahnmann HJ, Gregg RE, Robbins J. Characterization of the binding of thyroxine to high-density lipoproteins and Apolipoproteins A-I. *J Clin Endocrinol Metab* 1989;**68**(6):1067-72.
33. Regazzi R, Widmann C. Genetics and molecular biology: miRNAs take the HDL ride. *Curr Opin Lipidol* 2012;**23**(2):165-6.
34. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol* 2011;**13**(4):423-33.
35. Annema W, von Eckardstein A. High-density lipoproteins - multifunctional but vulnerable protections from atherosclerosis. *Circ J* 2013; **77**(10):2432-48.
36. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, et al. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* 2003;**290**(17):2292-300.
37. Tardif JC, Gregoire J, L'Allier PL, Ibrahim R, Lesperance J, Heinonen TM, et al. Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA* 2007; **297**(15):1675-82.
38. Easton R, Gille A, D'Andrea D, Davis R, Wright SD, Shear C. A multiple ascending dose study of CSL112, an infused formulation of ApoA-I. *J Clin Pharmacol* 2014;**54**(3):301-10.
39. Gille A, Easton R, D'Andrea D, Wright SD, Shear CL. CSL112 enhances biomarkers of reverse cholesterol transport after single and multiple infusions in healthy subjects. *Arterioscler Thromb Vasc Biol* 2014;**34**(9):2106-14.
40. Gille A, Easton R, Wright S, Shear C. CSL112, a novel formulation of human apolipoprotein A-I, dramatically increases cholesterol efflux capacity in healthy subjects: a, placebo-controlled, double-blinded, randomized single ascending dose study. *Circulation* 2012;**126**(21).
41. Gille A, D'Andrea D, Easton R, Tricoci P, Alexander JH, Wright SD, et al. CSL112, a novel formulation of human apolipoprotein A-I, dramatically increases cholesterol efflux capacity in patients with stable atherothrombotic disease: a multicenter, randomized, double-blind, placebo-controlled, ascending-dose study. *Circulation* 2013; **128**(22).
42. Tardif JC, Ballantyne CM, Barter P, Dasseux JL, Fayad ZA, Guertin MC, et al. Effects of the high-density lipoprotein mimetic agent CER-001 on coronary atherosclerosis in patients with acute coronary syndromes: a randomized trial. *Eur Heart J* 2014;**35**(46):3277-86.
43. Keyserling CH, Hunt TL, Klepp HM, Scott RA, Barbaras R, Schwendeman A, et al. CER-001, a synthetic HDL-mimetic, safely mobilizes cholesterol in healthy dyslipidemic volunteers. *Circulation* 2011;**124**(21).
44. Aseem O, Smith BT, Cooley MA, Wilkerson BA, Argraves KM, Remaley AT, et al. Cubilin maintains blood levels of HDL and albumin. *J Am Soc Nephrol* 2014;**25**(5):1028-36.
45. Acton S, Rigotti A, Landschulz KT, Xu SZ, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 1996;**271**(5248):518-20.
46. Voisset C, Callens N, Blanchard E, Dubuisson J, Vu-Dac N. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem* 2005;**280**(9):7793-9.
47. Vishnyakova TG, Bocharov AV, Baranova IN, Chen ZG, Remaley AT, Csako G, et al. Binding and internalization of lipopolysaccharide by Cla-1, a human orthologue of rodent scavenger receptor B1. *J Biol Chem* 2003;**278**(25):22771-80.
48. Silver DL, Wang N, Xiao X, Tall AR. High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. *J Biol Chem* 2001;**276**(27):25287-93.
49. Neculai D, Schwake M, Ravichandran M, Zunke F, Collins RF, Peters J, et al. Structure of LIMP-2 provides functional insights with implications for SR-BI and CD36. *Nature* 2013;**504**(7478):172-6.
50. Cao GP, Garcia CK, Wyne KL, Schultz RA, Parker KL, Hobbs HH. Structure and localization of the human gene encoding SR-BI/CLA-1 - evidence for transcriptional control by steroidogenic factor 1. *J Biol Chem* 1997;**272**(52):33068-76.
51. Krieger M. Charting the fate of the "good cholesterol": Identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu Rev Biochem* 1999;**68**:523-58.
52. Martinez LO, Jacquet S, Esteve JP, Rolland C, Cabezon E, Champagne E, et al. Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. *Nature* 2003;**421**(6918):75-9.
53. Goldstein JL, Brown MS, Anderson RGW, Russell DW, Schneider WJ. Receptor-mediated endocytosis - concepts emerging from the Ldl receptor system. *Annu Rev Cell Biol* 1985;**1**:1-39.
54. Bilheimer DW, Goldstein JL, Grundy SM, Starzl TE, Brown MS. Liver-transplantation to provide low-density-lipoprotein receptors and lower plasma-cholesterol in a child with homozygous familial hypercholesterolemia. *Med* 1984;**311**(26):1658-64.
55. Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, et al. Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. *Prostate* 2010; **70**(4):390-400.
56. Cao WM, Murao K, Imachi H, Yu X, Abe H, Yamauchi A, et al. A mutant high-density lipoprotein receptor inhibits proliferation of human breast cancer cells. *Cancer Res* 2004;**64**(4):1515-21.
57. Lobo MV, Huerta L, Ruiz-Velasco N, Teixeiro E, de la Cueva P, Celdran A, et al. Localization of the lipid receptors CD36 and CLA-1/SR-BI in the human gastrointestinal tract: towards the identification of receptors mediating the intestinal absorption of dietary lipids. *J Histochem Cytochem* 2001;**49**(10):1253-60.
58. Su F, Kozak KR, Imaizumi S, Gao F, Amneus MW, Grijalva V, et al. Apolipoprotein A-I (apoA-I) and apoA-I mimetic peptides inhibit tumor development in a mouse model of ovarian cancer. *S A* 2010; **107**(46):19997-20002.
59. Zheng Y, Liu YY, Jin HL, Pan ST, Qian Y, Huang C, et al. Scavenger receptor B1 is a potential biomarker of human nasopharyngeal carcinoma and its growth is inhibited by HDL-mimetic nanoparticles. *Theranostics* 2013;**3**(7):477-86.
60. Vitols S. Uptake of low-density lipoprotein by malignant cells–possible therapeutic applications. *Cancer Cells* 1991;**3**(12):488-95.

61. Siperstein MD. Role of cholesterogenesis and isoprenoid synthesis in DNA-replication and cell-growth. *J Lipid Res* 1984;**25**(13): 1462-8.
62. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**(5):646-74.
63. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**(1): 57-70.
64. Pugliese L, Bernardini I, Pacifico N, Peverini M, Damaskopoulou E, Cataldi S, et al. Severe hypocholesterolaemia is often neglected in haematological malignancies. *Cancer* 2010;**46**(9):1735-43.
65. Pluen A, Boucher Y, Ramanujan S, McKee TD, Gohongi T, di Tomaso E, et al. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. *S A* 2001;**98**(8):4628-33.
66. Schouten D, Vanderkooij M, Muller J, Pieters MN, Bijsterbosch MK, Vanberkel TJC. Development of lipoprotein-like lipid particles for drug targeting - neo-high density lipoproteins. *Mol Pharmacol* 1993;**44**(2): 486-92.
67. Corbin IR, Ng KK, Ding L, Jurisicova A, Zheng G. Near-infrared fluorescent imaging of metastatic ovarian cancer using folate receptor-targeted high-density lipoprotein nanocarriers. *Nanomedicine* 2013; **8**(6):875-90.
68. Kalli KR, Oberg AL, Keeney GL, Christianson TJ, Low PS, Knutson KL, et al. Folate receptor alpha as a tumor target in epithelial ovarian cancer. *Gynecol Oncol* 2008;**108**(3):619-26.
69. Mooberry LK, Nair M, Paranjape S, McConathy WJ, Lacko AG. Receptor mediated uptake of paclitaxel from a synthetic high density lipoprotein nanocarrier. *J Drug Target* 2010;**18**(1):53-8.
70. Iovannisci DM, Beckstead JA, Ryan RO. Targeting nanodisks via a single chain variable antibody–apolipoprotein chimera. *Biochem Biophys Res Commun* 2009;**379**(2):466-9.
71. Murakami T, Wijagkanalan W, Hashida M, Tsuchida K. Intracellular drug delivery by genetically engineered high-density lipoprotein nanoparticles. *Nanomedicine* 2010;**5**(6):867-79.
72. Burgess BL, He Y, Baker MM, Luo B, Carroll SF, Forte TM, et al. NanoDisk containing super aggregated amphotericin B: a high therapeutic index antifungal formulation with enhanced potency. *Nanomedicine* 2013;**8**:4733-43.
73. Oda MN, Hargreaves PL, Beckstead JA, Redmond KA, van Antwerpen R, Ryan RO. Reconstituted high density lipoprotein enriched with the polyene antibiotic amphotericin B. *J Lipid Res* 2006;**47**(2):260-7.
74. Nelson KG, Bishop JV, Ryan RO, Titus R. Nanodisk-associated amphotericin B clears Leishmania major cutaneous infection in susceptible BALB/c mice. *Antimicrob Agents Chemother* 2006;**50**(4):1238-44.
75. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. *Nat Rev Cancer* 2001;**1**(3):181-93.
76. Soprano DR, Qin P, Soprano KJ. Retinoic acid receptors and cancers. *Annu Rev Nutr* 2004;**24**:201-21.
77. Redmond KA, Nguyen TS, Ryan RO. All-trans-retinoic acid nanodisks. *Pharm* 2007;**339**(1–2):246-50.
78. Singh AT, Evens AM, Anderson RJ, Beckstead JA, Sankar N, Sassano A, et al. All trans retinoic acid nanodisks enhance retinoic acid receptor mediated apoptosis and cell cycle arrest in mantle cell lymphoma. *Haematol* 2010;**150**(2):158-69.
79. Ghosh M, Singh ATK, Xu WW, Sulchek T, Gordon LI, Ryan RO. Curcumin nanodisks: formulation and characterization. *Nanomedicine* 2011;**7**(2):162-7.
80. Singh AT, Ghosh M, Forte TM, Ryan RO, Gordon LI. Curcumin nanodisk-induced apoptosis in mantle cell lymphoma. *Leuk Lymphoma* 2011;**52**(8):1537-43.
81. Khumsupan P, Ramirez R, Khumsupan D, Narayanaswami V. Apolipoprotein E LDL receptor-binding domain-containing high-density lipoprotein: a nanovehicle to transport curcumin, an antioxidant and anti-amyloid bioflavonoid. *Biochim Biophys Acta* 2011;**1808**(1): 352-9.
82. Ikon N, Su B, Hsu FF, Forte TM, Ryan RO. Exogenous cardiolipin localizes to mitochondria and prevents TAZ knockdown-induced apoptosis in myeloid progenitor cells. *Biochem Biophys Res Commun* 2015;**464**(2):580-5.
83. Bayburt TH, Sligar SG. Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers. *Protein Sci* 2003;**12**(11):2476-81.
84. Baas BJ, Denisov IG, Sligar SG. Homotropic cooperativity of monomeric cytochrome P450 3A4 in a nanoscale native bilayer environment. *Arch Biochem Biophys* 2004;**430**(2):218-28.
85. Bayburt TH, Sligar SG. Membrane protein assembly into nanodiscs. *FEBS Lett* 2010;**584**(9):1721-7.
86. Tokatlian T, Segura T. siRNA applications in nanomedicine. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2010;**2**(3):305-15.
87. Leng Q, Woodle MC, Lu PY, Mixson AJ. Advances in systemic siRNA delivery. *Drugs Future* 2009;**34**(9):721.
88. Davidson BL, McCray Jr PB. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 2011;**12**(5):329-40.
89. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, et al. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotechnol* 2007;**25**(10):1149-57.
90. Ghosh M, Ren G, Simonsen JB, Ryan RO. Cationic lipid nanodisks as an siRNA delivery vehicle. *Biochem Cell Biol* 2014;**92**(3):200-5.
91. Wadsater M, Maric S, Simonsen JB, Mortensen K, Cardenas M. The effect of using binary mixtures of zwitterionic and charged lipids on nanodisc formation and stability. *Soft Matter* 2013;**9**(7):2329-37.
92. Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. *ACS Nano* 2009;**3**(1):16-20.
93. Wang J, Jia JT, Liu JP, He HL, Zhang WL, Li ZH. Tumor targeting effects of a novel modified paclitaxel-loaded discoidal mimic high density lipoproteins. *Drug Deliv* 2013;**20**(8):356-63.
94. Sabnis N, Nair M, Israel M, McConathy WJ, Lacko AG. Enhanced solubility and functionality of valrubicin (AD-32) against cancer cells upon encapsulation into biocompatible nanoparticles. *Nanomedicine* 2012;**7**:975-83.
95. Yuan Y, Wang W, Wang B, Zhu H, Zhang B, Feng M. Delivery of hydrophilic drug doxorubicin hydrochloride-targeted liver using apoAI as carrier. *J Drug Target* 2013;**21**(4):367-74.
96. Chandar N, Amenta J, Kandala JC, Lombardi B. Liver cell turnover in rats fed a choline-devoid diet. *Carcinogenesis* 1987;**8**(5):669-73.
97. Weinstein S, Emmanuel R, Jacobi AM, Abraham A, Behlke MA, Sprague AG, et al. RNA inhibition highlights Cyclin D1 as a potential therapeutic target for mantle cell lymphoma. *Plos One* 2012;**7**(8).
98. Duivenvoorden R, Tang J, Cormode DP, Mieszawska AJ, Izquierdo-Garcia D, Ozcan C, et al. A statin-loaded reconstituted high-density lipoprotein nanoparticle inhibits atherosclerotic plaque inflammation. *Nat Commun* 2014;**5**:3065.
99. Frias MA, Lang U, Gerber-Wicht C, James RW. Native and reconstituted HDL protect cardiomyocytes from doxorubicin-induced apoptosis. *Cardiovasc Res* 2010;**85**(1):118-26.
100. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell* 2012;**148**(5):852-71.
101. Jonas A. Reconstitution of high-density lipoproteins. *Methods Enzymol* 1986;**128**:553-82.
102. Tall AR, Small DM, Deckelbaum RJ, Shipley GG. Structure and thermodynamic properties of high density lipoprotein recombinants. *J Biol Chem* 1977;**252**(13):4701-11.
103. Lund-Katz S, Lyssenko NN, Nickel M, Nguyen D, Chetty PS, Weibel G, et al. Mechanisms responsible for the compositional heterogeneity of nascent high density lipoprotein. *J Biol Chem* 2013;**288**(32): 23150-60.
104. Massey JB, Pownall HJ. Cholesterol is a determinant of the structures of discoidal high density lipoproteins formed by the solubilization of phospholipid membranes by apolipoprotein A-I. *Biochim Biophys Acta* 2008;**1781**(5):245-53.
105. Pownall HJ, Pao Q, Hickson D, Sparrow JT, Kusserow SK, Massey JB. Kinetics and mechanism of association of human-plasma apolipoproteins with dimyristoylphosphatidylcholine - effect of

protein-structure and lipid clusters on reaction-rates. *Biochemistry* 1981;**20**(23):6630-5.
106. Saito H, Dhanasekaran P, Nguyen D, Holvoet P, Lund-Katz S, Phillips MC. Domain structure and lipid interaction in human apolipoproteins A-I and E, a general model. *J Biol Chem* 2003;**278**(26):23227-32.
107. Lyssenko NN, Hata M, Dhanasekaran P, Nickel M, Nguyen D, Chetty PS, et al. Influence of C-terminal alpha-helix hydrophobicity and aromatic amino acid content on apolipoprotein A-I functionality. *Biochim Biophys Acta* 2012;**1821**(3):456-63.
108. Kono M, Tanaka T, Tanaka M, Vedhachalam C, Chetty PS, Nguyen D, et al. Disruption of the C-terminal helix by single amino acid deletion is directly responsible for impaired cholesterol efflux ability of apolipoprotein A-I Nichinan. *J Lipid Res* 2010;**51**(4):809-18.
109. Kim Y, Fay F, Cormode DP, Sanchez-Gaytan BL, Tang J, Hennessy EJ, et al. Single step reconstitution of multifunctional high-density lipoprotein-derived nanomaterials using microfluidics. *ACS Nano* 2013;**7**(11):9975-83.
110. Ritchie TK, Grinkova YV, Bayburt TH, Denisov IG, Zolnerciks JK, Atkins WM, et al. Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Method Enzymol* 2009;**464**:211-31.
111. Larsen J, Hatzakis NS, Stamou D. Observation of inhomogeneity in the lipid composition of individual nanoscale liposomes. *J Am Chem Soc* 2011;**133**(28):10685-7.
112. Skar-Gislinge N, Simonsen JB, Mortensen K, Feidenhans'l R, Sligar SG, Moller BL, et al. Elliptical structure of phospholipid bilayer nanodiscs encapsulated by scaffold proteins: casting the roles of the lipids and the protein. *J Am Chem Soc* 2010;**132**(39):13713-22.
113. Martinez-Beamonte R, Lou-Bonafonte JM, Martinez-Gracia MV, Osada J. Sphingomyelin in high-density lipoproteins: structural role and biological function. *Mol Sci* 2013;**14**(4):7716-41.
114. Sorci-Thomas MG, Owen JS, Fulp B, Bhat S, Zhu X, Parks JS, et al. Nascent high density lipoproteins formed by ABCA1 resemble lipid rafts and are structurally organized by three apoA-I monomers. *J Lipid Res* 2012;**53**(9):1890-909.
115. Subbaiah PV, Liu M. Role of sphingomyelin in the regulation of cholesterol esterification in the plasma-lipoproteins - inhibition of lecithin-cholesterol acyltransferase reaction. *J Biol Chem* 1993;**268**(27):20156-63.
116. Arimoto I, Saito H, Kawashima Y, Miyajima K, Handa T. Effects of sphingomyelin and cholesterol on lipoprotein lipase-mediated lipolysis in lipid emulsions. *J Lipid Res* 1998;**39**(1):143-51.
117. Kucerka N, Marquardt D, Harroun TA, Nieh MP, Wassall SR, de Jong DH, et al. Cholesterol in bilayers with PUFA chains: doping with DMPC or POPC results in sterol reorientation and membrane-domain formation. *Biochemistry* 2010;**49**(35):7485-93.
118. McMullen TP, Lewis RN, McElhaney RN. Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. *Biochemistry* 1993;**32**(2):516-22.
119. Yancey PG, de la Llera-Moya M, Swarnakar S, Monzo P, Klein SM, Connelly MA, et al. High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J Biol Chem* 2000;**275**(47):36596-604.
120. Ma CIJ, Beckstead JA, Thompson A, Hafiane A, Wang RHL, Ryan RO, et al. Tweaking the cholesterol efflux capacity of reconstituted HDL. *Biochem Cell Biol* 2012;**90**(5):636-45.
121. Urban S, Zieseniss S, Werder M, Hauser H, Budzinski R, Engelmann B. Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. *J Biol Chem* 2000;**275**(43):33409-15.
122. Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem* 1995; **270**(27):16221-4.
123. Ahsan F, Rivas IP, Khan MA, Torres Suarez AI. Targeting to macrophages: role of physicochemical properties of particulate carriers–liposomes and microspheres–on the phagocytosis by macrophages. *J Control Release* 2002;**79**(1–3):29-40.
124. Massey JB, She HS, Gotto Jr AM, Pownall HJ. Lateral distribution of phospholipid and cholesterol in apolipoprotein A-I recombinants. *Biochemistry* 1985;**24**(25):7110-6.
125. Nakano M, Fukuda M, Kudo T, Miyazaki M, Wada Y, Matsuzaki N, et al. Static and dynamic properties of phospholipid bilayer nanodiscs. *J Am Chem Soc* 2009;**131**(23):8308-12.
126. Debnath A, Schafer LV. Structure and dynamics of phospholipid nanodiscs from all-atom and coarse-grained simulations. *J Phys Chem B* 2015;**119**(23):6991-7002.
127. Jonas A, Kezdy KE, Williams MI, Rye KA. Lipid transfers between reconstituted high-density lipoprotein complexes and low-density lipoproteins - effects of plasma-protein factors. *J Lipid Res* 1988; **29**(10):1349-57.
128. Phillips MC, Johnson WJ, Rothblat GH. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim Biophys Acta* 1987;**906**(2):223-76.
129. Nanjee MN, Doran JE, Lerch PG, Miller NE. Acute effects of intravenous infusion of ApoA1/phosphatidylcholine discs on plasma lipoproteins in humans. *Arterioscler Thromb Vasc Biol* 1999;**19**(4): 979-89.
130. Brinton EA, Eisenberg S, Breslow JL. Human HDL cholesterol levels are determined by apoA-I fractional catabolic rate, which correlates inversely with estimates of HDL particle size. Effects of gender, hepatic and lipoprotein lipases, triglyceride and insulin levels, and body fat distribution. *Arterioscler Thromb* 1994;**14**(5):707-20.
131. Tardy C, Goffinet M, Boubekeur N, Ackermann R, Sy G, Bluteau A, et al. CER-001, a HDL-mimetic, stimulates the reverse lipid transport and atherosclerosis regression in high cholesterol diet-fed LDL-receptor deficient mice. *Atherosclerosis* 2014;**232**(1):110-8.
132. Murphy AJ, Funt S, Gorman D, Tall AR, Wang N. Pegylation of high-density lipoprotein decreases plasma clearance and enhances antiatherogenic activity. *Circ Res* 2013;**113**(1):e1-9.
133. Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin - review of animal and human studies. *Clin Pharmacokinet* 2003;**42**(5):419-36.
134. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res* 1994;**54**(4):987-92.
135. Diditchenko S, Gille A, Pragst I, Stadler D, Waelchli M, Hamilton R, et al. Novel formulation of a reconstituted high-density lipoprotein (CSL112) dramatically enhances ABCA1-dependent cholesterol efflux. *Arterioscler Thromb Vasc Biol* 2013;**33**(9):2202-11.
136. Sorci-Thomas MG, Bhat S, Thomas MJ. Activation of lecithin: cholesterol acyltransferase by HDL ApoA-I central helices. *Clin Lipidol* 2009;**4**(1):113-24.