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Basic Research

ABCA1 Agonist Mimetic Peptide CS-6253 Induces Microparticles Release From Different Cell Types by ABCA1-Efflux–Dependent Mechanism

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See editorial by Brunham, pages 705–706 of this issue.

ABSTRACT

Background: Small peptides based on the C-terminal domain of apo E have recently been proposed as ATP-binding cassette transporter A1 (ABCA1) agonist with therapeutic potential. Previous work has shown that a novel synthetic peptide, CS-6253, acts synergistically with apolipoprotein A-I or alone to generate high-density lipoprotein (HDL) particles; we have also shown that cells can release microparticles (50-350 nm in apparent diameter) in an ABCA1- and apolipoprotein A-I-dependent manner. The purpose of this study was to explore the ability of a novel synthetic peptide CS-6253 to induce microparticle release from various cell lines in the process of HDL biogenesis.

Methods: The effects of CS-6253 on microparticle formation through the ABCA1 transporter were examined *in vitro* using cell-based systems and pharmacologic manipulations.

Results: In cell-based systems combined with fast performance liquid

RÉSUMÉ

Contexte : Selon une hypothèse formulée récemment, certains petits peptides de même structure que le domaine C-terminal de l'apolipoprotéine E pourraient être des agonistes de la protéine de transport A1 à cassette de fixation à l'ATP (ABCA1) et présenter un potentiel thérapeutique. Des travaux antérieurs ont montré qu'un nouveau peptide synthétique, le CS-6253, agit seul ou en synergie avec l'apolipoprotéines A-I pour produire des particules de lipoprotéines de haute densité (HDL); nous avons également montré que les cellules peuvent libérer des microparticules (d'un diamètre apparent de 50 à 350 nm) de façon dépendante de la présence d'ABCA1 et d'apolipoprotéine A-I. L'objet de cette étude était d'explorer la capacité d'un nouveau peptide synthétique, le CS-6253, de provoquer la libération de microparticules dans différentes lignées cellulaires au cours du processus de biogenèse des HDL.

Small peptides based on the C-terminal domain of apo E have recently been proposed as ATP-binding cassette transporter A1 (ABCA1) agonist with therapeutic potential.[1] Previous work has shown that a novel synthetic peptide, CS-6253, acts synergistically with apolipoprotein A-I (apoA-I) or alone to generate high-density lipoprotein (HDL) particles enriched in cholesterol.[2] However, simply raising HDL cholesterol levels is not a successful strategy to prevent cardiovascular events.[3,4] HDL function is in large part mediated through the lipidation of apoA-I with cellular membrane lipids via ABCA1. Previous work from us and others has shown that apoA-I mediates the release of microparticles (MPs) from cholesterol-loaded cells through ABCA1.[5-8] ApoA-I contains amphipathic alpha-helical sequences essential for lipid transport and interaction with ABCA1. Here, we examined the effects of CS-6253, a 26-mer amphipathic alpha-helical peptide on MP release, using cell-based models and pharmacologic manipulation. The ABCA1 transporter is a phospholipid transporter with a large extracellular domain forming a tunnel for the transport of hydrophobic molecules that allows the transfer of free cholesterol to acceptor proteins such as apoA-I or apoE.[3,9,10] When expressed on the plasma membrane (PM), ABCA1 leads to the formation of microdomains for cholesterol efflux onto acceptor particles. ABCA1 creates extracellular vesicle domains in the process of releasing cholesterol in the presence of apoA-I.[5,8,9] Previous studies suggest a potential role for ABCA1 in facilitating outward bending or bulging of the PM.[11] Such membrane dynamics are a prerequisite for HDL biogenesis[12] and may also be required to release MPs to the medium.[13] It is interesting to note that MP release was

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chromatography and nano-sight-tracking analysis, we show that ABCA1 and CS-6253 mediate and increase the production of microparticles containing cholesterol. CS-6253 in baby hamster kidney cells not expressing ABCA1 (baby hamster kidney mock cells) did not alter cholesterol removal across the plasma membrane in the absence of ABCA1 expression even at high concentrations. We report that CS-6253 is not cytotoxic.

Conclusions: The present study shows that CS-6253 generates cholesterol containing microparticles with size heterogeneity (100-350 nm) in an ABCA1-dependent manner. We show that microparticles contribute to cell cholesterol efflux from monocyte-macrophage cells. At high doses, CS-6253 is not able to extract cholesterol from cells not expressing ABCA1, indicating that CS-6253 requires ABCA1 cooperation for cholesterol mobilization. We conclude that CS-6253 is an ABCA1 agonist peptide that promotes cellular cholesterol efflux through HDL biogenesis and microparticle formation.

Méthodologie : Les effets du CS-6253 sur la formation de microparticules par l'intermédiaire du transporteur ABCA1 ont été analysés *in vitro* à l'aide de systèmes cellulaires et de manipulations pharmacologiques.

Résultats : Dans des systèmes cellulaires, après analyse par chromatographie rapide en phase liquide et analyse du suivi des nanoparticules NanoSight, nous avons montré que les peptides ABCA1 et CS-6523 médient et stimulent la production de microparticules contenant du cholestérol. Dans une lignée de cellules rénales de bébé hamster n'exprimant pas l'ABCA1 (cellules rénales de bébé hamster témoins), la présence de CS-6253, même à concentration élevée, n'a eu aucun effet sur l'élimination du cholestérol à travers la membrane plasmique en l'absence d'expression du gène de l'ABCA1. Nous rapportons également que le peptide CS-6253 n'est pas cytotoxique.

Conclusions : Cette étude montre que le CS-6253 produit des microparticules de taille hétérogène (de 100 à 350 nm) contenant du cholestérol en fonction de la présence d'ABCA1. Nous montrons que les microparticules contribuent à l'évacuation du cholestérol cellulaire hors des monocytes/macrophages. À forte dose, le CS-6253 n'est pas capable d'extraire le cholestérol des cellules qui n'expriment pas l'ABCA1, ce qui indique qu'il a besoin de l'aide de l'ABCA1 pour mobiliser le cholestérol. Nous concluons que le CS-6253 est un peptide agoniste de l'ABCA1 qui favorise l'évacuation du cholestérol cellulaire par la biogenèse de HDL et la formation de microparticules.

reported in conditions of cellular cholesterol excess when incubating cells with apoA-I mimetics.[14,15] We have previously shown that apoA-I mediates the release of MPs;[8] in the present study, we sought to examine the effects of CS-6253 on MP release in the presence or absence of ABCA1. We show for the first time that CS-6253 induces MP release from various cells in the process of HDL biogenesis. The release of MPs from various cell lines follows that of nascent HDL (nHDL) formation, suggesting that these 2 processes are linked and ABCA1 dependent. The physiological relevance of MPs generated by CS-6253 is unknown. However, increasing cellular cholesterol efflux by both an ABCA1-mediated pathway and MP release may have therapeutic implication.

Materials and Methods

Cell culture

Baby hamster kidney (BHK) cells express ABCA1 and ABCG1 under the regulation of mifepristone and were a kind gift of the late Jack Oram, PhD (Seattle, Washington).[16] Human monocytes Tamm-Horsfall protein 1 (THP-1) cells were purchased from American Type Culture Collection (ATCC TIB-202; Cedarlane, Burlington, Ontario, Canada). Liver human hepatoma G2 cells (HepG2) were obtained from American Type Culture Collection (ATCC-HB8065, Camden, NJ) and used according to the manufacturer's recommendations. Briefly, BHK, HepG2, and THP-1 cells were respectively grown and maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI 1640) containing 10% fetal serum in a humidified 37°C, 5% CO_2 incubator until experimental treatment. Studies were carried out on standard 24-well tissue culture treated plates (Costar; Corning Inc, Corning, NY) (150,000 cells/well) unless stated otherwise. Cell culture media were purchased from Wisent (Montreal, QC), supplemented with 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen), and 10% phosphate buffered saline (FBS) (Wisent). Cells were used between passages 4 and 9. Phorbol 12-myristate-12 acetate, probucol (4,4′-(isopropylidenedithio) bis [2,6-di-tert-butylphenol]), methyl-β-cyclodextrin, and bovine serum albumin (BSA) essentially fatty acid free were obtained from Sigma-Aldrich (Canada). 3H-free cholesterol was purchased from Perkin Elmer (Norwalk, CT).

Cell viability

Cells were cultured in DMEM-CS-6253 at doses of 3, 10, 50, 100, and 500 μg/mL or in RPMI at increased time points (1, 2, 4, 24, and 48 hours) for a fixed dose of 3 μg/mL of CS-6253. The cellular viability was determined by using trypan blue staining, and the MTT assay (Thiazolyl Blue Tetrazolium Blue, Sigma).[17]

CS-6253 peptide

The CS-6253 peptide was synthesized (Biosynthesis Inc, Lewisville, TX) with all L-amino acids, capped with N-terminal acetyl and C-terminal amide groups. The lyophilized peptides were dissolved in 10 mM phosphate buffered-150 mM NaCl, pH = 7.4, saline (10 mM phosphate buffer pH = 7.4), sterilized by filtration (0.2 μm), and stored at 4°C. Peptide concentrations were determined by absorbance at 280 nm. Immunopurified polyclonal anti-CS-6253 antibody (Biosynthesis Inc) was used to detect HDL-CS-6253 particles and lipid-free CS-6253. The anti-CS-6253 is a purified rabbit IgG polyclonal antibody. Lack of cross-reactivity of this CS-6253 antibody to apoA-I and apo E was verified.[2]

Cholesterol depletion and loading

For acute cholesterol depletion, BHK-ABCA1 cells were incubated for 45 minutes at 37°C in DMEM containing 50

mM HEPES (pH 7.2) and 0.2% BSA (DMEM/BSA) and 10 mM methyl-β-cyclodextrin (CDX).[12] Control cells were incubated in the same medium lacking cyclodextrin. Methodology of loading THP-1 cells with cholesterol was performed as previously.[2,18] Briefly, THP-1 cells were grown in suspension in T175 culture flasks at 37°C in 5% CO_2 in bicarbonate-buffered RPMI containing 10% FBS (vol/vol), 50 μmol/L β-mercaptoethanol, and 50 μg/mL gentamicin at a cell density of 0.2 to 1.0 × 10^6/mL. Afterward, cells were plated at 0.5 × 10^6/mL in 100 mm petri dishes with 10% FBS, 50 μM β-mercaptoethanol, 50 μg/mL gentamicin, and 200 nM phorbol 12-myristate-12 acetate for 3 days to become differentiated macrophages. On day 3, increased radioactivities of 3H-free cholesterol (0.01, 0.1, 1, 2, and 4 μCi) respectively (0.15, 1.57, 15.79, 31.56, and 63.12 ng/mL) were used to label monolayers (24 hours) in RPMI with 1% FBS for 2-3 days. The medium was subsequently removed, and the macrophage monolayer washed 3 times with PBS and then incubated in RPMI in the absence or presence of CS-6253 (1 μM) for 24 hours. Concentrated medium from triplicate 100 mm dish THP-1 cells containing 3H-cholesterol was collected and centrifuged (4000 × *g* for 15 minutes and 10,000 × *g* for 30 minutes) to remove cell debris.[8,13] Next, the supernatant was collected and passed through a 0.2 μm filter and concentrated with an Amicon ultracentrifugal filter (10,000 molecular weight cutoff)[5,8] and analysed by fast performance liquid chromatography (FPLC). Radioactivity associated with each fraction was determined.

Cholesterol efflux

Cholesterol efflux was carried out by incubating cells in triplicate in a 24-well plate with 1 μM (3 μg/mL) CS-6253 for 24 hours at 37°C unless otherwise mentioned. At the end of the efflux period, medium was collected and cells were washed twice with cold PBS 1×. The medium was centrifuged at 3000 × *g* for 5 minutes to remove cell debris. Then, 0.2 mL of the supernatant was mixed with scintillation liquid and counted for radioactivity. The cells were lysed in 0.5 mL of 0.1 N NaOH overnight with constant shaking. Further, 0.2 mL of cell lysates from each well were mixed with scintillation liquid and counted for radioactivity.[19] The amount of cholesterol efflux from BHK cells expressing ABCA1 or THP-1 cells in the absence of CS-6253 was determined relatively to the total efflux when CS-6253 was present in the media as previously reported.[5]

FPLC analysis for MPs

Isolation of MPs was performed as previously described by Nandi et al.[5] Briefly, pooled medium from triplicate 100 mm dishes BHK (ABCA1) or THP-1 or HepG2 cells containing 3H-cholesterol was collected and centrifuged (4000 × *g* for 15

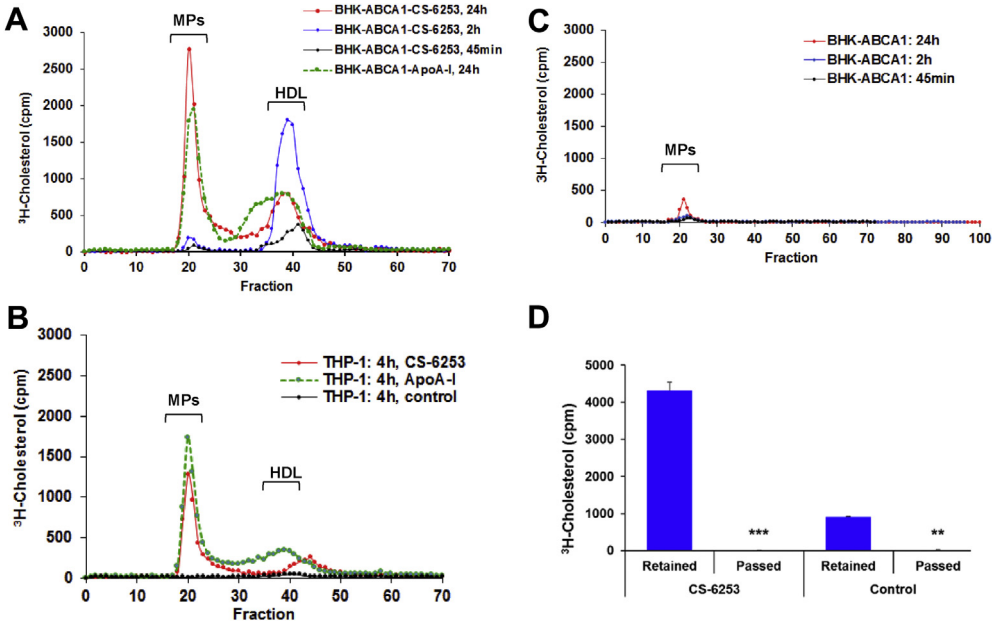

Figure 1. Time-dependent analysis of microparticles (MPs) released into the medium in the presence of CS-6253 from baby hamster kidney (BHK)-ATP-binding cassette transporter A1 (ABCA1) cells. (**A**) MP release in the presence or the absence of CS- 6253. 3H-cholesterol-labelled BHK cells expressing ABCA1 were incubated with CS-6253 (1 μM) or apoA-I (1 μM) at time points 45 minutes, 2 hours, and 24 hours. Media were collected, pooled, and concentrated by ultrafiltration. Concentrated medium from ABCA1 cells was analysed by fast performance liquid chromatography (FPLC) in triplicate. 3H-cholesterol-labelled BHK-mock cells were used as a control. Radioactivity associated with each fraction was determined. (**B**) CS-6253 induces MP release from human Tamm-Horsfall protein 1 (THP-1) macrophage. 3H-cholesterol-labelled THP-1 cells were incubated with CS-6253 (1 μM) or apoA-I (1 μM) for (4 hours). 3H-cholesterol-labelled THP-1 cells incubated alone were used as a control. (**C**) In the absence of ABCA1 expression, no high-density lipoprotein (HDL) nor MP is formed. All experiments were performed in triplicate 100-mm dishes from 2 independent experiments. (**D**) Analysis of the size of MPs derived from cells incubated with CS-6253. MPs were isolated by FPLC from the pooled concentrated efflux medium containing 3H-cholesterol-labelled BHK-ABCA1-expressing cells that were exposed or not to CS-6253 (1 μM). MPs were collected and filtered through 10 kDa molecular weight cutoff filter. The solute was retained on the cutoff membrane, and the filtrates were collected and counted for β-scintillation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by Student's *t*-test. Results represent the mean of triplicates ± standard deviation from 3 independent experiments.

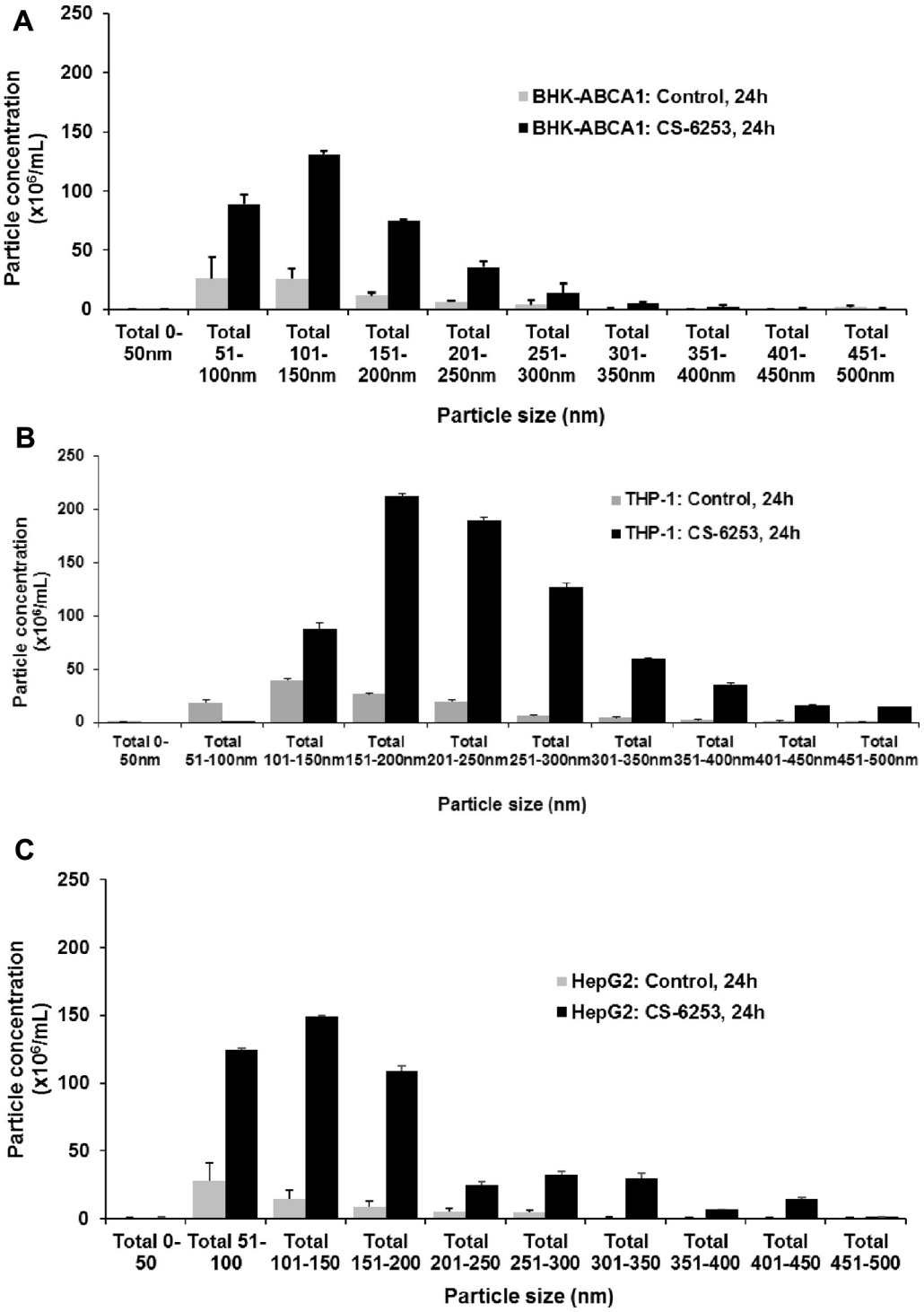

Figure 2. Size analysis of microparticles (MPs) by nanoparticle tracking analysis (NTA) (**A-C**). Analysis of MPs isolated by fast performance liquid chromatography (FPLC) with NTA. Media were first collected and concentrated by ultrafiltration before FPLC as indicated in "Materials and Methods." Concentrated medium from ATP-binding cassette transporter A1 (ABCA1) or mock cells, Tamm-Horsfall protein 1 (THP-1) cells, and HepG2 cells was analysed by FPLC. Next, NTA of MPs collected from FPLC peak after 24-hour incubation of CS-6253 (1 μM) with the indicated cell type (**A-C**). Results are shown as means ± standard deviation of triplicates, with measurements performed 5 times of the nano-sight analysis software from 3 independent experiments. BHK, baby hamster kidney.

minutes and 10,000 × *g* for 30 minutes) to remove cell debris.[8,13] Next, the supernatant was collected and passed through a 0.2 μm filter and concentrated with an Amicon ultracentrifugal filter (10,000 molecular weight cutoff).[5,8] MP profiles were obtained using FPLC separation with a Superose 6B column. A 200 μL aliquot of concentrated medium was loaded onto the column and eluted with 150 mM NaCl, pH 7.4, at a constant flow rate of 0.4 mL/min.

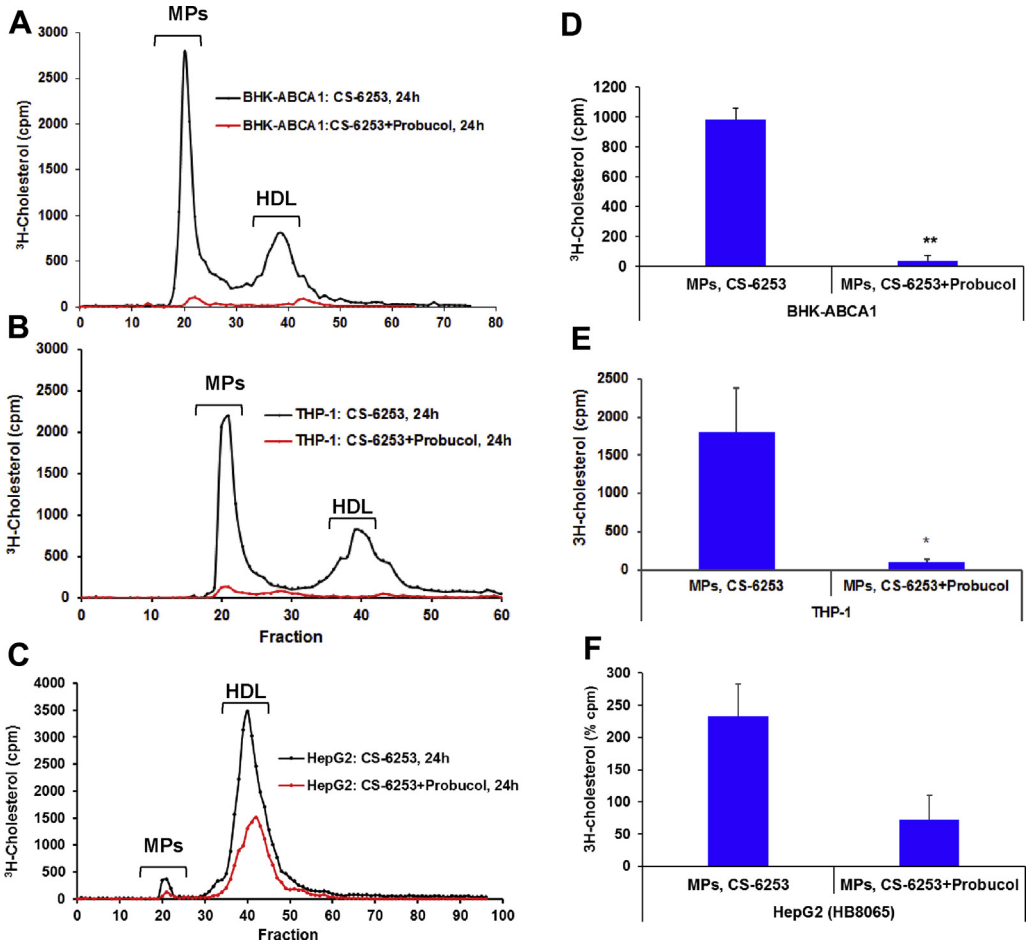

Figure 3. The effect of ATP-binding cassette transporter A1 (ABCA1) inactivation by probucol on the generation of microparticles (MPs) and high-density lipoprotein (HDL) by baby hamster kidney (BHK) cells expressing (**A**) ABCA1, (**B**) Tamm-Horsfall protein 1 (THP-1), and (**C**) HepG2 cells in the presence of CS-6253 (1 μM), 24 hours. (**A-C**) The conditioned medium of cells and probucol-loaded cells was tested by fast performance liquid chromatography (FPLC) after 24 hours of incubation. FPLC fractions corresponding to peak I were pooled and washed extensively by filtration to remove free 3H-cholesterol. 3H-cholesterol was counted and expressed as a cpm count (**D-F**): $*P < 0.05$; $**P < 0.01$ by Student's *t*-test. Results represent the mean of triplicates ± standard deviation from 2 independent experiments.

ABCA1 blocking

Efflux was promoted from various cell lines expressing ABCA1 after the incubation of cells with or without 10 μmol/L probucol. Probucol was prepared and incubated with cells as reported previously.[8,20] The effect of probucol ABCA1-mediated HDL biogenesis and on MP release was confirmed by ABCA1-mediated cellular lipid efflux to HDL-CS-6253 and medium alone without CS-6253, respectively.

Analysis of HDL species by gel electrophoresis

Two-dimensional-nondenaturing gradient gel electrophoresis (2D-PAGGE) 5% to 35% was performed as previously described[2,21] on media cell culture (80 μL) along with molecular weight protein standard mixture (GE Healthcare, UK). Molecular weight markers were revealed by Ponceau S sodium salt. CS-6253-containing particles were detected with an anti-human-CS-6253 antibody.

MP analysis

The number and size of MPs were analysed using NS500 nanoparticle tracking analysis (NTA; Nano Sight, Amesbury, UK) as reported previously with minor modifications.[8,22] Briefly, pooled culture medium from triplicate 100 mm dish BHK (ABCA1) or THP-1 or HepG2 cells containing 3H-cholesterol was collected and centrifuged (4000 × *g* for 15 minutes and 10,000 × *g* for 30 minutes).[8,13] Next, the supernatant was collected and passed through a 0.2 μm filter and concentrated with an Amicon ultracentrifugal filter (10,000 molecular weight cutoff). Pooled FPLC fractions corresponding to MPs are collected and concentrated with a 10 kDa filter, and 300 μL of each sample was loaded into the flow chamber.[22] The NTA technology has a dynamic range of 0.5-1000 nm for particles analysis, which allows determination of the size distribution of isolated particles based on the Brownian motion of vesicles in liquid suspension.[23] It is one of the proposed methods for the characterisation of the quality of the isolated vesicles.[24] NTA allows detection and tracking

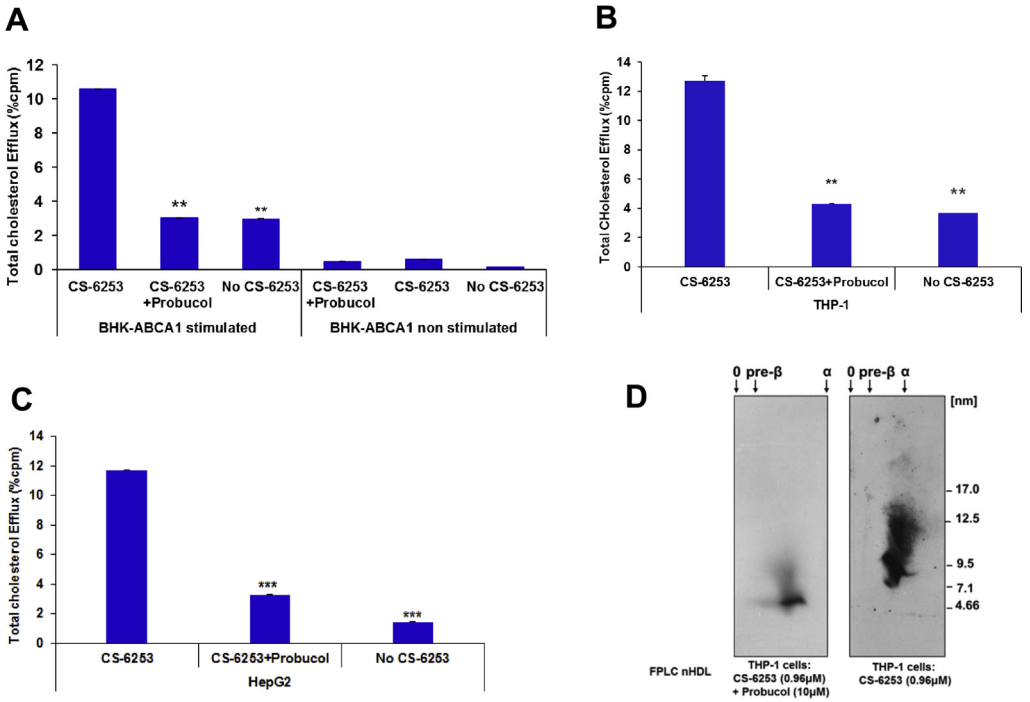

Figure 4. Effect of probucol on cellular ATP-binding cassette transporter A1 (ABCA1)-mediated lipid efflux and microparticle (MP) release, and evaluation of CS-6253-independent cholesterol efflux contribution to the total efflux. (**A**) Baby hamster kidney (BHK), (**B**) Tamm-Horsfall protein 1 (THP-1), and (**C**) HepG2 cells were labelled with 3H-cholesterol (24 hours). ABCA1 expression was induced by treating BHK cells with 10 nM mifepristone. Cells were then changed into fresh medium alone (no CS-6253). Medium was collected after 24 hours. Medium and cell-associated 3H radioactivity was counted from each well and presented as percentage of cholesterol in the medium relative to the total cholesterol (medium and cell-associated). Contribution of MPs released in the medium was calculated as radioactivity of medium in the absence of CS-6253 divided by radioactivity of medium containing high-density lipoprotein (HDL)-CS-6253 as reported previously.[5] Standard error bars smaller than symbols when not seen from 3 independent experiments. (**D**) Representative two-dimensional-nondenaturing gradient gel electrophoresis of HDL particles in the presence (**left**) and absence of probucol. $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test. FPLC, fast performance liquid chromatography; nHDL, nascent HDL.

of the Brownian motion of 0.5- to 1000-nm-sized vesicles, as indicated by the manufacturer. Briefly, isolated MP particles from FPLC fractions are collected and concentrated with a 10 kDa filter, and 300 µL were loaded into the flow chamber under automatic detection and batch processing settings.

Statistical analysis

Results were presented as means and standard deviations (SD) of triplicate determinations. All measurements using the nano-sight analysis were performed 5 times (30 seconds each) for each sample and repeated in 3 independent experiments. For FPLC analysis, cell culture experiments were performed in triplicates from pooled media and repeated at least twice. Cholesterol efflux data are representation of triplicate of 3 independent experiments. Data are presented as the average of independent experiments with the SD reflecting the difference between experiments. The SD bar is presented in all graphs. Student's *t*-tests were used to examine the difference between continuous variables; a *P* value of 0.05 (2-tailed) was considered significant. We examined the correlation between HDL biogenesis and MP formation by Spearman correlation. We used a commercially available statistical package for analysis (GraphPad Software Inc, La Jolla, CA).

Results

CS-6253 stimulates MP formation in an ABCA1-dependent fashion

We determined various physiological aspects of the novel synthetic(s) ABCA1 agonist peptide CS-6253 for its ability in interacting with the ABCA1 transporter for MP release.

We used BHK cells stably expressing human ABCA1 under mifepristone control. BHK cells not expressing ABCA1 (mock) were also used as controls. The elution profiles of 3H-cholesterol in lipid particles released in the presence of lipid-free CS-6253 (1 µM) show a time-dependent release of MPs (Fig. 1A). In the presence of CS-6253, ABCA1 expressing cells produced a second peak of nHDL; as we have shown previously, these particles contain CS-6253.[2] At 2 and 24 hours, we found that both the HDL-CS-6253 and MP increased. After 24-hour incubation, we found a shift in the HDL peak indicating an increase in 3H-cholesterol accumulation in HDL-CS-6253, whereas we did not observe a change in the MP peak, in keeping with apoA-I (Fig. 1A, in green).

A similar pattern was observed in THP-1 macrophages (Fig. 1B). BHK cells not expressing ABCA1 do not release MPs in the presence of CS-6253 (Fig. 1C). To further characterize the size of MPs released in the efflux medium from 3H-cholesterol-labelled, ABCA1 expressing cells were

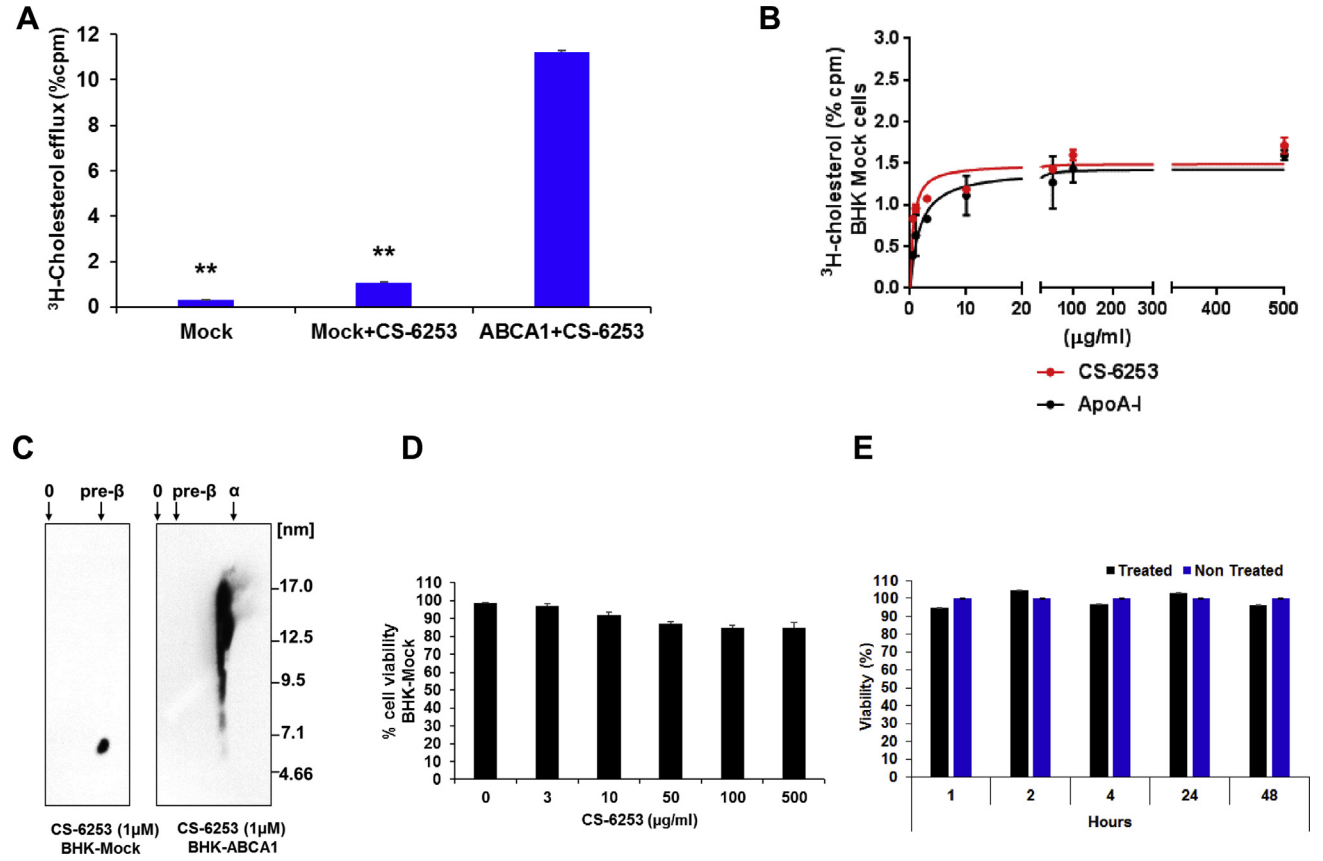

Figure 5. Effect of high doses of CS-6253 on cholesterol removal from baby hamster kidney (BHK)-mock cells. (**A**) ATP-binding cassette transporter A1 (ABCA1) activity is required for CS-6253-independent cholesterol efflux. 3H-cholesterol-labelled BHK-mock and ABCA1 cells were induced for 24 hours with mifepristone (10 nM) and incubated with Dulbecco's modified Eagle's medium with or without CS-6253 for 24 hours. (**B**) Cholesterol desorption from BHK-mock cells to increased doses of apoA-I (**black circle**) and CS-6253 (**red circle**) (3, 10, 50, 100, 500, 1000 µg/mL) was determined at 24-hour time point. Results represent the mean of triplicate wells ± standard deviation (SD) from 3 independent experiments. Kinetic parameters for cholesterol desorption from mock cells for CS-6253 peptide or apoA-I are as follows: CS-6253: $K_m = 0.67 \pm 0.15$ µg/mL, $V_{max} = 1.56\% \pm 0.05\%$ cpm/24 h; apoA-I: $K_m = 1.650 \pm 0.38$ µg/mL, $V_{max} = 1.42\% \pm 0.056\%$ cpm/24 h. (**C**) Ability of CS-6253 to interact with ABCA1 for generating nascent high-density lipoprotein particles. Particles in the medium (BHK-mock) and ABCA1 cells were analysed by two-dimensional-nondenaturing gradient gel electrophoresis after 24-hour incubation of lipid-free CS-6253 (1 µg/mL). CS-6253 proteins were detected by anti-CS-6253 and revealed by chemiluminescence. Molecular markers are indicated. (**D**) Cell viability (BHK-mock) was measured by the Trypan blue dye exclusion method, and the percentage of cell growth was calculated as a ratio of the number of treated cells at increased doses and control cell. Results were mean ± SD of the triplicate independent experiments. (**E**) Tamm-Horsfall protein 1 (THP-1) cell viability measured by the MTT assay, and the percentage of cell viability was calculated as a ratio of the optical density of CS-6253-treated cells and control cells. Results were mean ± SD of the triplicate experiments and are representative of 3 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

incubated with or without CS-6253 peptide (Fig. 1D). By using a 10 kDa molecular weight cutoff filter, we demonstrate that 3H-cholesterol was all retained, suggesting an MP diameter size of ≥20 nm, consistent with previous work.[5,25]

MP release exhibit size heterogeneity

The release of MPs was examined by nano-sight-tracking analysis where size (nm) and concentration (particles/mL) were measured. In BHK-ABCA1 cells, MP release increased significantly ($35.19 \times 10^6 \pm 46.77$, particles/mL) when compared with cells incubated alone (control) ($7.67 \times 10^6 \pm 10.40$, particles/mL) ($P = 0.004$) (Fig. 2A). The average of HDL particles released in this process was $16.56 \times 10^6 \pm 41.21$ ($P < 0.0001$). A similar observation was made for MP released in the presence of apoA-I ($8.15 \times 10^6 \pm 12.78$ vs $24.28 \times 10^6 \pm 59.22$, $P < 0.001$, respectively). We found a significant positive correlation between the amount of HDL and MPs released in the presence of apoA-I in a time-dependent fashion ($r = 0.83$, $P < 0.0001$). Similarly, the correlation between HDL and MP release in the presence of CS-6253 was high ($r = 0.85$, $P < 0.0001$). In THP-1 cells, CS-6253 significantly increased MPs in number and size ($40.73 \times 10^6 \pm 47.39$, particles/mL) when compared with cells incubated without CS-6253 (control) ($7.69 \times 10^6 \pm 9.37$, particles/mL) ($P = 0.001$) (Fig. 2B). In HepG2 cells, we found a similar pattern: ($49.25 \times 10^6 \pm 56$, particles/mL) when compared with cells incubated alone (control) ($6.30 \times 10^6 \pm 9$, particles/mL) ($P = 0.0015$) (Fig. 2C). The size of MPs was < 350 nm, with a peak in the 100-150 nm range (Fig. 2, A and C). Again, a similar pattern was observed for THP-1 and HepG2 cells but with a peak size between 100 and 350 nm (Fig. 2A).

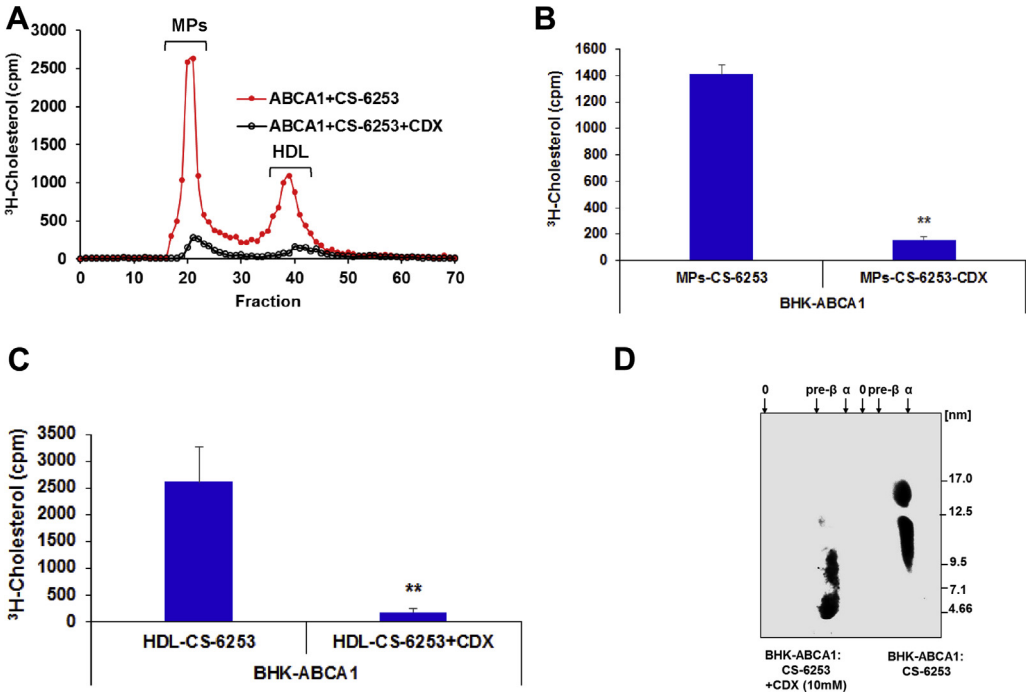

Figure 6. Effect of cellular cholesterol depletion on microparticles (MPs) and high-density lipoprotein (HDL) formation in the presence of CS-6253. (**A**) Radiolabelled (2 μCi) baby hamster kidney (BHK) cells expressing ATP-binding cassette transporter A1 (ABCA1) were incubated or not with methyl-β-cyclodextrin (CDX) (10 mM) for 45 minutes at 37°C. The cell medium of CDX-treated cells was separated by fast performance liquid chromatography (FPLC) after 24 hours. FPLC fractions corresponding to peak I (MPs) (**B**) and peak II (HDL) (**C**) were pooled and washed extensively by filtration to remove free 3H-cholesterol. 3H-cholesterol was expressed as cpm count. Results shown as means ± standard deviation of triplicate from duplicate independent experiments. $*P < 0.05$ and $**P < 0.01$ by Student's *t*-test. (**D**) Western blot analysis of 2-dimensional native gel electrophoresis of CS-6253 containing nascent HDL particles generated by incubation of BHK cells expressing ABCA1 with CS-6253 in the absence or presence of CDX (10 mM) showing the effect of CDX on HDL formation, 4 hours. CS-6253 proteins were detected by immunoblotting to a polyclonal anti-CS-6253 antibody.

Probucol inhibits MP release from cells

To further probe the importance of ABCA1 and CS-6253 in MP production we used probucol, an inhibitor of ABCA1-mediated cholesterol efflux.[20] Probucol was added to cells, and the culture medium was analysed by FPLC after 24-hour incubation of CS-6253. The peak representing MPs was collected and filtered to remove free 3H-cholesterol and counted. In all cell lines, an important reduction in MP cholesterol content was observed in the FPLC profiles after probucol treatment (Fig. 3, A-C). Quantification of cholesterol content in MPs isolated by FPLC showed a significant reduction (Fig. 3, D-F).

Probucol specifically inhibits ABCA1 cholesterol efflux and MP formation

The effects of probucol on ABCA1 were further probed by inhibiting cholesterol efflux in the presence of CS-6253 in various cell lines (Fig. 4, A-C). BHK nonexpressing ABCA1 cells were used as negative control (Fig. 4A, right panel). Not surprisingly, probucol did not completely inhibit HDL formation in HepG2 cells. Hepatic cells have multiple ABC transporters as well as the scavenger receptor, class B type I that contributes to the cholesterol flux between cells and acceptors. In addition, HepG2 cells secrete apolipoproteins, including apoA-I that may contribute to cholesterol removal.[26,27]

We next estimated MP release in relation to total cholesterol efflux when CS-6253 was incubated with cells. As indicated in Figure 4, the amount of cholesterol efflux from BHK cells expressing ABCA1 in the absence of CS-6253 was approximately 28% of the total efflux when CS-6253 was present (Fig. 4A). This was 30% in THP-1 cells (Fig. 4B) and 12% in HepG2 cells (Fig. 4C). Cholesterol removal by MP generation contributes substantially to the total cholesterol efflux from cells. The amount of cholesterol effluxed as MPs vs as HDL-CS-6253 is as follows: (2.96% ± 0.01% cpm vs 10.57% ± 0.79% cpm, $P < 0.01$, in ABCA1-BHK cells), (3.7% ± 0.02% cpm vs 12.71% ± 0.96% cpm, $P < 0.01$, in THP-1 cells), and (1.4% ± 0.9% cpm vs 11.67% ± 0.2% cpm, $P < 0.001$, in HepG2 cells), respectively. The observation that cholesterol efflux in ABCA1 expressing cells may occur in the absence of an acceptor may be attributed to passive efflux as we have previously reported.[2] Figure 4D shows a representative 2D-PAGGE showing a lack of formation of HDL particles in the presence of probucol in THP-1 cells.

CS-6253 is dependent on ABCA1 to generate MP release

We confirm that CS-6253 is unable to generate MP release in the absence of ABCA1. We tested CS-6253-independent efflux in BHK-mock cells. These cells were unable to efflux

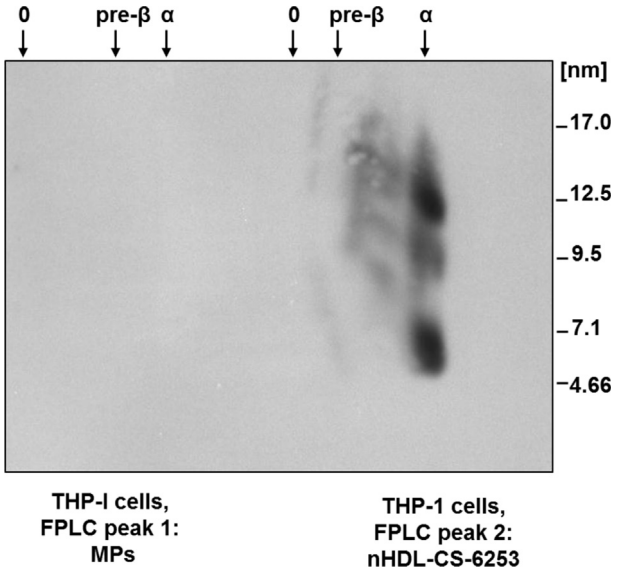

Figure 7. Microparticles (MPs) released by cells expressing ATP-binding cassette transporter A1 in the presence of CS-6253 do not contain CS-6253. 3H-cholesterol-labelled Tamm-Horsfall protein 1 (THP-1) cells were incubated with CS-6253 (1 μM) for 24 hours. Media were collected and concentrated by ultrafiltration. Concentrated medium from THP-1 cells was analysed by fast performance liquid chromatography (FPLC). FPLC fractions corresponding to elution peaks (MPs or high-density lipoprotein [HDL]) were concentrated by ultrafiltration and analysed by immunoblotting to detect CS-6253. Molecular markers are indicated. nHDL, nascent HDL.

cholesterol to apoA-I.[28] We found that BHK-mock cells were defective in CS-6253-independent efflux (Fig. 5A), which is significantly reduced when compared with BHK without ABCA1 ($P < 0.001$) (Fig. 5A).

To quantify these observations, we investigate 3H-cholesterol-labelled BHK-mock cell activity in the presence of CS-6253 at higher doses (Fig. 5B). In the presence of CS-6253 but not ABCA1, diffusion of cholesterol is approximately 3 times more efficient than for apoA-I (Fig. 5B). Passive efflux is involved in cholesterol efflux but less efficient than transporter-mediated efflux.[29] As shown for THP1 in Figure 4D, CS-6253 was not able to form HDL particles in BHK-mock cells lacking ABCA1 (Fig. 5C, left panel) and consequently to induce cholesterol efflux, in contrast to some other mimetic peptides.[14] CS-6253 mediates the formation of discrete HDL particles with apparent diameters of 9.5, 13, and 17 nm. There is no significant cytotoxicity at high concentrations of CS-6253 when incubated with BHK cells nonexpressing ABCA1 (BHK-mock), that is, was associated with a viability $\geq$ 90% (Fig. 5D). Furthermore, cellular viability showed no cytotoxicity with CS-6253 when incubated with THP-1 cells at variable time point as demonstrated by the MTT assay (Fig. 5E).

Cell membrane cholesterol depletion with cyclodextrin prevents MP release

We also examined the effects of PM cholesterol depletion in BHK-ABCA1 cells with CDX (10 mM) for 45 minutes, on MP formation (Fig. 6A). MP release was significantly reduced by CDX treatment (−87% ± 0.14%, $P < 0.01$) when compared with nontreated cells (Fig. 6B). After PM cholesterol CDX depletion, we also noticed impairment of HDL-CS-6253 formation (−94% ± 0.01%, $P < 0.01$) when compared with nontreated cells (Fig. 6C). As a control, we performed 2D-PAGGE analysis that confirmed the production of nHDL-CS-6253 with a size distribution ranging from 9 to 17 nm, but no HDL formation under CDX treatment conditions (Fig. 6D). It is noteworthy that in the presence of cyclodextrin, 2 small peaks of HDL-CS-6253 and MPs can be identified. It is likely that CDX does not completely deplete membrane cholesterol.

CS-6253 is not associated with MPs

MPs released by cells expressing ABCA1 in the presence of CS-6253 do not contain CS- 6253. The FPLC fractions corresponding to elution peaks (MPs or HDL) after CS-6253 treatment were analysed by immunoblotting to detect CS-6253. nHDL-CS-6253 particles were found only in the FPLC-HDL peak, and not in the MP peak (Fig. 7).

Cellular cholesterol loading increased MP release

MP production is ABCA1 and CS-6253 dependent. We investigated the release of MP under conditions of increased loading of 3H-free cholesterol (Fig. 8, A and B). The methodology of loading cells with cholesterol was performed as previously described.[8] Our results indicated that cellular cholesterol loading markedly increased 3H-cholesterol in MPs as well as HDL in the presence of CS-6253 (Fig. 8, A and B) without an apparent change in their sizes (nm). Given the changes in the membrane cholesterol content of cells when excess cholesterol is present, we investigated the effect of CS-6253 on MP release and cholesterol efflux in acetylated low-density lipoprotein loaded THP-1 cells, CS-6253 and apoA-I increased total cholesterol efflux (Fig. 8C).

Discussion

In this work, we report that the ABCA1 agonist CS-6253 increases MP generation *in vitro* in an ABCA1-dependent manner in a way similar to that of apoA-I, albeit with some differences.[5-8] CS-6253 is a modification of peptide ATI-5261[30,31] with a substitution of phenylalanine for leucine residues, and with substitution of arginine by citrulline.[1,2] CS-6253 was developed with the objective to optimize the structural features of the amphipathic class A α-helix motif to avoid *in vivo* muscle toxicity at high doses.[1,32] Here we report that CS-6253 increased MP release from cells. By using cell-based cholesterol efflux assays, we found that this interaction is ABCA1 dependent as demonstrated by contrasting experiments with cells not expressing ABCA1 and after inhibition with probucol. MP release is also dependent on PM cholesterol availability; MP generation drastically decreases after CDX-mediated cholesterol depletion. MP formation depends on ABCA1 and CS-6253 cooperation, and we hypothesize that there is stereoselective protein to protein interaction of lipid-free CS-6253 with ABCA1 facilitating lipid transport and MP generation.[14] Moreover, the level of MPs released depends on cholesterol load, illustrating the potential of amphipathic α-helices of CS-6253s to sequester cellular cholesterol by an

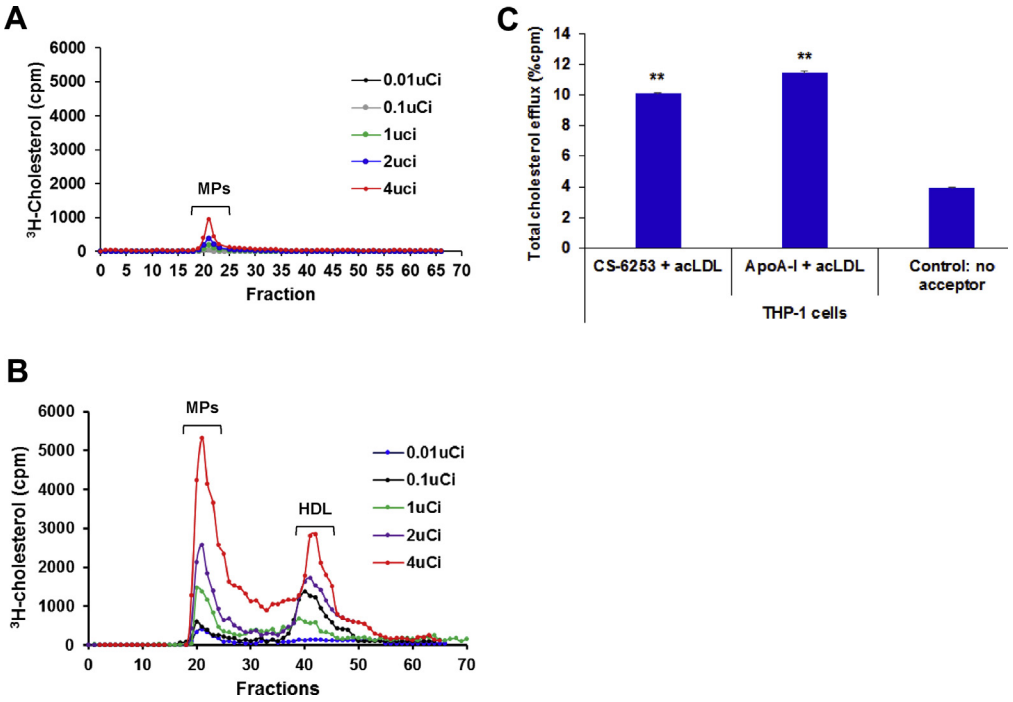

Figure 8. Analysis of lipid particles released to the medium in the absence (**A**) or presence (**B**) of CS-6253 with increased quantity (μCi) of 3H-cholesterol (0.01, 0.1, 1, 2, and 4 μCi). 3H-cholesterol-labelled Tamm-Horsfall protein 1 (THP-1) cells expressing ATP-binding cassette transporter A1 were incubated in the presence of (1 μM) CS-6253 for 24 hours. Media were collected, pooled, and concentrated by ultrafiltration as described in "Materials and Methods." Concentrated medium from THP-1 cells was analysed by fast performance liquid chromatography. Radioactivity associated with each fraction was determined from 2 independent experiments. (**C**) Effect of CS-6253 on microparticle (MP) release and cholesterol efflux in cells that contain excess cholesterol by loading with acetylated low-density lipoprotein (acLDL). THP-1 cells were incubated for 3 days in growth medium containing 2 μCi of 3H-free cholesterol with the presence of acLDL (100 μg/mL). Cholesterol efflux induced by CS-6253 and apoA-I were compared with control cells incubated alone. Data represent average from triplicate samples from 2 independent experiments. $^{**}P < 0.01$ by Student's t-test. HDL, high-density lipoprotein.

ABCA1-dependent process. This observation supports the concept that ABCA1 acts as a transporter and facilitates the efflux of cholesterol onto acceptor particles.[33] The mechanism of MP formation via ABCA1 remains to be elucidated. We postulate that the ABCA1-associated formation of exovesicular structures[12,13] may facilitate the release of membrane bound microdomains that are rich in cholesterol and form MPs and exosomes. This has been shown for apoA-I.[8] As we have previously reported, CS-6253 contributes efficiently to the removal of cholesterol from cells,[2] with some cell specificity, as shown here for THP-1, BHK, and HepG2 cells, suggesting that MP release is tissue specific.

We have previously reported that MPs contribute a significant proportion of total cellular cholesterol efflux, a measurement previously described as "passive diffusion."[8] Here, we extend these observations and confirm that the apolipoprotein mimetic CS-6253 modulated HDL biogenesis and MP release and these 2 events are highly correlated ($r = 0.85$; $P < 0.001$). Despite this close correlation, it is not clear whether the cholesterol pools from membrane microdomains that facilitate HDL biogenesis and MP release are similar. The physiological role of MP formation mediated by ABCA1 is poorly understood. Detailed analysis of these MPs from the proteomic, lipidomic, genomic (including micro-RNA) must now be performed in specific cells and tissues.

Another potentially interesting application of our finding is whether MP constitutes a biomarker for cellular cholesterol removal from macrophages and atherosclerotic plaques[34,35] and can be used as a surrogate measurement for the evaluation of therapeutic interventions. The effects of apoA-I and apo E mimetic peptides in the process of cellular cholesterol transport are a matter of intensive research.[36,37] In contrast to CS-6253, some other peptides have displayed detergent-like properties and showed ABCA1-independent cholesterol extraction from cells[14] potentially associated with cell lysis limiting their therapeutic application. We further notice that the doses of probucol (10 μM) and CDX (10 mM) have been selected based on our and other's studies. Probucol at a concentration of 10 μM is not toxic *in vitro*.[8,20] In addition, previous work from our lab[12] with 10 mM CDX treatment did not affect the levels of ABCA1 or induce cellular toxicity. At high pharmacologic concentrations, CS-6253 has not shown cell toxicity and our data suggest that CS-6253 does not influence cholesterol diffusion across the PM. This contrasts to the findings for native apoA-I where high concentrations cause substantial cholesterol diffusion. The structural design of CS-6253 is central to its mechanisms, its ability to remove cholesterol in an ABCA1-dependent manner, and is consistent with its favorable safety profile at high pharmacological doses *in vitro* and *in vivo*.[1,32]

In summary, we have shown that CS-6253 causes the release of MPs from ABCA1 expressing cells. MPs are released from various cell lines, including macrophages, and this process is dependent on the ABCA1 transporter; they exhibit size

heterogeneity, with an apparent diameter ranging from 50 to 350 nm (Fig. 2). The mechanism by which CS-6253 causes the release of MPs is incompletely understood. Native apoA-I and CS-6253 contain amphipathic type A α-helical peptide segments, and we hypothesize that these segments are involved in both HDL biogenesis and MP formation through the ABCA1 transporter.[2] The finding that a synthetic apolipoprotein mimetic peptide enhances cholesterol removal from cells may have a therapeutic potential.

Limitations

Our study has some limitations. We did not extensively characterize potential MP species that can be modulated by ABCA1/CS-6253 interactions. We believed that further studies may help to investigate the quality of MPs released in the process of HDL-CS-6253 biogenesis and their physiological impact on recipient cells in the context of cholesterol transport.

Acknowledgements

A. Hafiane and J. Genest conceived and designed the experiments. A. Hafiane performed the experiments. A. Hafiane and J. Genest analysed and interpreted the data. J. Genest and J.O. Johansson contributed reagents/materials/analysis tools. A. Hafiane and J. Genest wrote the paper. J.O. Johansson provided critical comments for the manuscript.

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Disclosures

J.O. Johansson holds patents for CS-6253 and is an employee of Artery Therapeutics, Inc. The rest of the authors have no relevant conflicts of interest to disclose.

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