Inhibition of ABCA1 protein degradation promotes HDL cholesterol efflux capacity and RCT and reduces atherosclerosis in mice

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Abstract ABCA1 plays a key role in the initial lipidation of apoA-I, which generates circulating HDL cholesterol. Whereas it is known that the transcriptional upregulation of ABCA1 promotes HDL formation and reverse cholesterol transport (RCT), it is not known how the inhibition of ABCA1 protein degradation impacts HDL function. Employing the small molecule triacetyl-3-hydroxyphenyladenosine (IMM-H007), we determined how the attenuation of ABCA1 protein degradation affects HDL cholesterol efflux capacity, RCT, and atherosclerotic lesion formation. Pulse-chase analysis revealed that IMM-H007 inhibits ABCA1 degradation and facilitates its cell-surface localization in macrophages, and additional studies in macrophages showed that IMM-H007 thereby promotes cholesterol efflux. IMM-H007 treatment of Paigen diet-fed mice caused an increase in circulating HDL level, it increased the cholesterol efflux capacity of HDL, and it enhanced in vivo RCT from macrophages to the plasma, liver, and feces. Furthermore, ABCA1 degradation suppression by IMM-H007 reduced atherosclerotic plaque formation in apoE−/− mice. Thus, via effects on both ABCA1-expressing cells and circulating HDL function, the inhibition of ABCA1 protein degradation by IMM-H007 promotes HDL cholesterol efflux capacity and RCT and attenuates atherogenesis. IMM-H007 potentially represents a lead compound for the development of agents to augment HDL function.

Clinical and epidemiological studies have shown an inverse relationship between plasma levels of HDL cholesterol or its major apolipoprotein, apoA-I, and cardiovascular disease risk. For example, in the Framingham Heart Study, for every 1% increase in circulating HDL, there was a 2% decrease in the global risk of developing coronary heart disease (1). The direct infusion of reconstituted apoA-I particles reduces atherosclerotic plaque size in humans (2), and the overexpression of apoA-I in mice results in elevated HDL levels and the prevention of plaque progression (3, 4). Therefore, raising HDL cholesterol appears to be an attractive therapeutic strategy for atherosclerosis risk reduction.

However, it has been observed that therapies such as CETP inhibitors and niacin (5, 6) that increase HDL level are not sufficient to elucidate HDL’s atheroprotective properties. Alternatively, the cardioprotective potential of HDL may be primarily related to its function, namely to promote reverse cholesterol transport (RCT) from peripheral tissues and cells to the liver. Thus, the synergistic enhancement of HDL function, as well as quantity, may represent a more effective means to harness HDL biology for therapeutic gain (7). The ABCA1 has been identified as the key and rate-limiting transporter facilitating the...
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initial steps of HDL formation and of RCT, with hepatic, intestine, or adipose ABCA1 activity being closely associated with plasma HDL level (8). Increased ABCA1 expression in macrophages would be anticipated to enhance RCT and reduce atherosclerosis risk. Thus, ABCA1 has been identified as an emerging target for new pharmacological agents designed to leverage the cardiovascular protective potential of HDL. It is well-established that liver X receptor (LXR)-induced transcriptional upregulation of ABCA1 promotes HDL formation and RCT (9), and a previous study demonstrated that two oxidized products of probe col inhibit ABCA1 degradation, increase HDL level, and reduce atherosclerosis (10). However, how altering ABCA1 protein degradation impacts HDL function is unknown.

In the present study, we determined whether an increase in endogenous ABCA1 expression due to altered posttranslational regulation leads to enhanced HDL function and RCT, and consequently an alleviation of atherosclerosis formation. We did so using a novel agent, triacetyl-3-hydroxyphenalenosine (IMM-H007), which we formerly observed impacts lipid levels in hyperlipidemic hamsters (11). We discovered that IMM-H007 inhibits intracellular ABCA1 degradation, and that this is related to the suppression of calcium-activated calpain activity. The actions of IMM-H007 on ABCA1 result in greater cell-surface ABCA1 content, increased circulating HDL, enhanced HDL cholesterol efflux capacity, the promotion of ABCA1-mediated RCT, and a reduction in atherosclerotic plaque size in hypercholesterolemic mice. As such, IMM-H007 is a promising lead agent candidate for raising HDL cholesterol efflux capacity, the promotion of ABCA1-mediated RCT, and a reduction in atherosclerotic plaque size in hypercholesterolemic mice. As such, IMM-H007 is a promising lead agent candidate for raising HDL, and likely more importantly, for enhancing HDL function.

MATERIALS AND METHODS

Cell culture

THP-1 and J774 cells were obtained from the Cell Culture Center of Peking Union Medical College (China), and maintained as described (12–14). THP-1 cells were grown in suspension in RPMI 1640 supplemented with 10% FBS, and they were differentiated to macrophages by incubating in the presence of 10 ng/ml of PMA (Sigma) for 48 h. J774 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO₂ incubator.

Animals

Wild-type C57BL/6 mice, apoE⁻/⁻ mice, and hamsters were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in a temperature-controlled environment with 12 h light/12 h dark cycles. Age-matched animals had free access to water and were fed standard rodent chow (16% protein, 4% fat, 6% fiber) or a Paigen diet (7.5% hard, 1.25% cholesterol, 0.5% sodium cholate, 0.125% choline chloride) (15). Probucol or IMM-H007 (99.86% purity by HPLC) was dissolved in a 0.3% sodium carboxymethylcellulose solution that served as control vehicle, and they were administered by oral gavage at the indicated doses once a day. At the end of study, the animals were given the last gavage dose, fasted for 2 h, and euthanized. For plasma lipid analyses, animals were fasted for 4 h and then bled from the retro-orbital plexus. All procedures were conducted in conformity with the Public Health Service policy, and they were approved by the Animal Care and Use Committee of the Institute of Material Medica, Chinese Academy of Medical Sciences, and Peking Union Medical College (Beijing, China).

ABCA1 degradation analysis

To determine the degradation rate of ABCA1, THP-1 macrophages were incubated with either DMSO (vehicle) or 10 μmol/l IMM-H007 for 1 h. Cells were washed with PBS and then maintained in 0.2% BSA medium with 20 μg/ml cycloheximide (CHX) for the indicated time periods. To examine calpain-mediated degradation, THP-1 macrophages were incubated with EGTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetate-AM (BAPTA), or calpeptin (calpain inhibitor) plus CHX for 60 min. In other studies, after treatment with EGTA/BAPTA (1 μM/100 μM) for 1 h, the cells were treated with CHX plus various extracellular Ca²⁺ concentrations with/without IMM-H007 for 30 min (16). ABCA1 protein abundance was analyzed by immunoblot analysis. Intracellular calpain activity was measured using a commercially available kit (Millipore).

ABCA1 labeling and tracing

To investigate ABCA1 abundance on the plasma membrane and the degradation of the cell surface-resident protein, biotinylation was carried out on THP-1 macrophages as previously described (13, 17, 18). Briefly, cells were incubated at 4°C for 60 min with PBS containing EZ-Link sulfo-NHS-SS-biotin (Pierce). After quenching, cells were washed and lysed in RIPA buffer, and biotinylated proteins were isolated by incubating with streptavidin-agarose resin (Pierce) overnight at 4°C. The bound proteins were eluted with loading buffer, and then subjected to immunoblot analysis.

To trace ABCA1 internalization, the biotinylation of the cell surface protein was cleaved with 50 mmol/l reduced glutathione (Sigma) at pH 7.8 twice for 20 min, and the remaining intracellular biotinylated ABCA1 was determined as the internalized portion. To examine ABCA1 recycling, intracellular ABCA1 was prelabeled as above, cells were treated with vehicle or IMM-H007 for the indicated periods, and cell surface biotin was again removed. Resurfaced ABCA1 was estimated by comparing biotinylated ABCA1 with and without the second cleavage (19).

ABCA1 flow cytometry and confocal microscopy

After treatment with vehicle or IMM-H007 (10 μmol/l), differentiated THP-1 macrophages were harvested and suspended in PBS. Cells were blocked with normal goat serum on ice for 30 min and stained with an anti-ABCA1 antibody (Abcam) at 1:50 dilution for 1 h on ice. Following washing, cells were stained with green fluorescent Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:100 dilution), cells were washed and re-suspended in 1% paraformaldehyde, and FACSScan analysis was performed with CellQuest software.

Standard protocols for immunofluorescence microscopy were employed as described previously (20). THP-1 cells plated on cover glass were treated with or without IMM-H007 overnight, washed with PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, blocked with normal goat serum, and stained with anti-ABCA1 antibody (1:50) overnight at 4°C. Cells were re-washed twice and incubated with AlexaFluor 488 anti-mouse IgG secondary antibody for 30 min. Images were captured with an Olympus confocal microscope and analyzed with Olympus confocal software.
Plasma lipid profiles

Plasma HDL levels were measured by cholesterol enzymatic kit (BioSino Corporation) after precipitation of apoB-containing lipoproteins by adding 100 μl plasma to 40 μl 20% PEG 8000 (P-2139 in 200 mmol/l glycine, pH 10; Sigma-Aldrich) solution (21, 22). Plasma lipoprotein levels were analyzed by fast protein liquid chromatography (FPLC) using Superose HR6 columns, followed by fluorescent enzymatic assays for cholesterol.

Fecal neutral sterol determinations

Mice were individually housed and fed a chow diet or Paigen diet with/without IMM-H007 treatment for 14 days. Feces were collected over the last 3 days and were dried, weighed, and ground to a powder. An aliquot of 500 mg of feces was extracted, and the neutral sterol content (cholesterol, stigmasterol, coprostanol, and β-sitosterol) was quantitated by gas chromatography/mass spectrometry as described (23). 5-Cholestene was used as an internal control.

In vitro cholesterol efflux

Cholesterol efflux experiments were performed as previously described (24–26). J774 cells or differentiated THP-1 macrophages grown in 24 multi-well plates were labeled with 3H-cholesterol (2 μCi/ml) and loaded with 25 μg/ml acetylated (ac)LDL in DMEM or RPMI 1640 plus 1% FBS for 24 h. Cells were washed and equilibrated overnight in either the presence or absence of IMM-H007 (25, 50, and 100 μM) in medium with 0.2% BSA. Cholesterol efflux was assessed over 4 h in medium plus 0.2% BSA, with various lipid acceptors added, as detailed in the figure legends. Radioactivity was measured in the medium and cell lysate, and efflux was calculated as the percent radioactivity in the medium relative to the total radioactivity in the cells and medium (27).

In vivo macrophage RCT

Experiments were carried out as described previously (24, 25, 28). apoE−/− mice were fed a Paigen diet and treated with vehicle or IMM-H007 by oral gavage for 2 weeks. J774 macrophages were loaded with 50 μg/ml acLDL and 5 μCi/ml [3H]cholesterol for 24 h, equilibrated in DMEM plus 0.2% BSA overnight, scraped into DMEM/0.2% BSA, centrifuged (1,200 g, 5 min), and resuspended in DMEM. The labeled J774 cells (4.5 × 106 cells/mouse, 3 × 106 cpm in 0.25 ml DMEM, n = 5/group) were injected intraperitoneally into individually housed mice. Plasma samples were obtained at 6, 24, and 48 h after injection, and 10 μl aliquots were counted in a scintillation counter. Feces were collected continuously from 0 to 48 h and soaked in distilled water overnight at 4°C, homogenized in an equal volume of ethanol, and 20 μl aliquots were counted. Mice continued to receive vehicle or IMM-H007 during the 48 h RCT study. At study termination, mice were euthanized, 100 mg liver was removed for lipid extraction, and 10 μl aliquots were counted. Gall bladders were also isolated and 20 μl of bile was counted. Results were expressed as percentage of counts per minute injected.

Quantitative real-time RT-PCR

To evaluate gene expression in liver or cells, total RNA was extracted using TRIzol reagent and subjected to reverse transcription using a high-capacity cDNA reverse transcription kit (Takara). Quantitative RT-PCR analyses were performed by SYBR green methodologies and relative mRNA levels were calculated using the comparative Ct method (20). Each sample was tested in triplicate, and transcript abundance was normalized to β-actin mRNA level. Relative transcript expression was determined using a control sample as a calibrator and the ΔΔCT method. The following specific primers were used: mouse ABCA1 forward (5′GCCTGGATCTACTCTGTGCCG3′) and reverse (5′GCCATTGTCGACACCCCATGA3′), human ABCA1 forward (5′CTCGGGTGCAGGCGAATCTAT3′) and reverse (5′CAGCTACTTCGCTCGCAAT3′).

Immunoblot analysis

Proteins were extracted in RIPA buffer containing a cocktail of protease inhibitors, separated by SDS-PAGE in 5–10% gel, transferred to polyvinylidene fluoride membranes, and incubated with antibodies to ABCA1 (1:1,000), ABCG1 (1:2,000), scavenger receptor class B type I (SR-BI) (1:2,000), LCAT (1:400), apoA-I (1:2,500), apoE (1:100), LXRα (1:300), or β-actin (1:10,000) at 4°C overnight, followed by incubation with appropriate HRP-conjugated secondary antibodies (1:2,000). The protein blots were visualized and quantified using chemiluminescence (ECL Plus Western blotting detection system; GE Healthcare UK Ltd.) and PhotoShop analysis software.

Atherosclerosis analysis

To induce atherosclerosis in apoE−/− mice, animals were fed with the Paigen diet (containing 1.25% cholesterol) ad libitum for 10 weeks. Proabul (200 mg/kg) was employed as a positive control intervention. Mouse aorta preparation and atherosclerosis quantification was conducted as previously described (29). The entire aorta was stained with Oil Red O for en face analysis and photographed with a Nikon D600 digital camera. Aortic roots were frozen in OCT, and 7 μm-thick sections were stained with Oil Red O and hematoxylin to evaluate lipid content. Lesion areas were quantified with ImageJ software.

Statistical analyses

Results are presented as mean ± SEM. Differences between means of two groups were evaluated by Student’s t-test (two tailed). ANOVA was used to compare findings over time or between three or more groups, with Tukey’s or Sidak’s post hoc testing. Analyses were performed using GraphPad Prism 5, and statistical significance was assumed for P ≤ 0.05.

RESULTS

IMM-H007 increases ABCA1 protein but not mRNA level in vitro and in vivo

Previous studies have demonstrated that posttranslational processes play a pivotal role in the regulation of ABCA1 expression (30). Whether IMM-H007, whose structure is shown in Fig. 1A, affects ABCA1 protein abundance was first tested in THP-1 cells. We found that treatment of THP-1 cells with IMM-H007 increased the protein but not mRNA expression of ABCA1 in a time- and concentration-dependent manner (Fig. 1B–E). Identical results were obtained in J774 cells (supplementary Fig. 1A–C), and in mouse peritoneal macrophages (supplementary Fig. 1D, E). IMM-H007 did not alter the expression of LXR, which is a key upstream promoter of ABCA1 expression (supplementary Fig. 2A, B). Consistent with the in vitro findings, hepatic ABCA1 expression was increased in 200 mg/kg IMM-H007-treated mice fed either a Paigen diet (Fig. 1F) or standard chow (supplementary Fig. 1F), and there were no changes in hepatic ABCA1 mRNA levels in either the treated mice on Paigen diet (Fig. 1G) or the treated mice on standard chow (supplementary Fig. 1G). These data indicate
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that IMM-H007 promotes ABCA1 protein but not mRNA expression in vitro and in vivo.

**IMM-H007 retards ABCA1 protein degradation and facilitates its surface localization**

In combination with reports that ABCA1 protein and mRNA expression are discordant (31), these findings led us to hypothesize that IMM-H007 increases ABCA1 expression by altering the stability of the ABCA1 protein. Using protein synthesis inhibition with CHX, we observed that treatment of THP-1 macrophages with IMM-H007 delays ABCA1 protein degradation (Fig. 2A). Next, we determined whether the inhibition of ABCA1 degradation leads to its accumulation on the cell surface, because the activation of ABCA1-mediated cholesterol efflux and HDL biogenesis take place at the cell surface. Using biotinylation, we observed that cell surface ABCA1 in THP-1 cells increases after IMM-H007 stimulation (Fig. 2B), paralleling the preservation of total cellular ABCA1.

To further investigate the mechanism for the elevation of cell surface ABCA1, the internalization and recycling of ABCA1 were examined. In the presence of the calpain inhibitor, calpeptin, there was decreased ABCA1 internalization (supplementary Fig. 2C), and it was found that IMM-H007 also inhibits ABCA1 internalization (Fig. 2C). Furthermore, we observed that internalized ABCA1 gets degraded rapidly in the absence of IMM-H007 and less rapidly in the presence of IMM-H007 (supplementary Fig. 2D), and there is a resulting greater recycling of ABCA1 to the cell surface in the presence of the agent (Fig. 2D, E). Calpeptin similarly caused increased abundance of ABCA1 recycled to the plasma membrane (supplementary Fig. 2E). The enhancement of cell surface ABCA1 by IMM-H007 was further verified by flow cytometry and confocal imaging (Fig. 2F, G).
Fig. 2. IMM-H007 retards ABCA1 protein degradation and facilitates its surface localization. A: IMM-H007 delays ABCA1 protein degradation. THP-1 cells were incubated with CHX (20 μg/ml) for the indicated times after 1 h of vehicle or IMM-H007 treatment. Cell lysates were processed for immunoblotting. B: IMM-H007 increases cell surface ABCA1. THP-1 cells were treated with vehicle or IMM-H007 (10 μM) for the indicated times, labeled with sulfo-SS-biotin at 4°C, and biotinylated protein was selectively absorbed by streptavidin-agarose and analyzed by immunoblotting. C: IMM-H007 retards surface ABCA1 internalization. After biotinylation, surface ABCA1 was internalized in the presence or absence of IMM-H007 at 37°C for the indicated times. Surface biotinylation was cleaved and the remaining intracellular biotinylated ABCA1 was quantified to evaluate the internalized fraction. D, E: IMM-H007 facilitates the recycling of internalized ABCA1 to the plasma membrane. After internalization at 37°C for 1 h, biotinylated protein in the vehicle- or IMM-H007-treated cells was recycled at 37°C for the indicated times. Remaining intracellular biotinylated ABCA1 (ic) and total biotinylated ABCA1, which includes intracellular biotinylated ABCA1 (ic) plus surface biotinylated ABCA1 (s), after recycling were detected by immunoblotting. The difference between ic
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Fig. 3. IMM-H007 inhibits ABCA1 protein degradation by suppressing calpain activity, and it promotes ABCA1 cell surface trafficking by palmitoylation. A: Calcium chelating-agents and the calpain inhibitor, calpeptin, reduce ABCA1 degradation. Cells were incubated with CHX (20 μg/ml) and vehicle (1); EGTA, 100 μM (2); BAPTA, 100 μM (3); EGTA+BAPTA, 1 μM+100 μM (4); calpeptin, 100 μM (5); or IMM-H007, 10 μM (6) for 1 h, and ABCA1 protein abundance was determined. B: IMM-H007 suppresses Ca<sup>2+</sup>-dependent ABCA1 protein degradation. Cells were exposed to various Ca<sup>2+</sup> concentrations in the presence or absence of 10 μM IMM-H007 at 37°C for 30 min, and analyzed for ABCA1 abundance. C: Intracellular calpain activity is suppressed by IMM-H007. Cells were treated as described in (A), and calpain activity was determined. D: IMM-H007 does not affect ABCA1 stability in calpain-deficient cells. Cells were incubated in the presence of CHX for 1 h in the absence or presence of IMM-H007 (10 μM) following transfection with control siRNA or siRNA targeting calpain. ABCA1 abundance was then evaluated. In (A–C), data are mean ± SEM of three independent assays. *P < 0.05 and **P < 0.01 versus control or vehicle.

Thus, IMM-H007 increases cell surface ABCA1 by suppressing ABCA1 degradation and internalization, and by facilitating ABCA1 recycling to the cell surface.

IMM-H007 restraints calpain activity to inhibit ABCA1 protein degradation

Calpain-mediated proteolysis plays a major role in regulating the activity of ABCA1 (32). We therefore evaluated the impact of IMM-H007 on calpain-mediated ABCA1 degradation in THP-1 cells. As expected, the inhibition of calpain-mediated degradation with calpeptin or by chelating of calcium ion increased ABCA1 protein level (Fig. 3A), and increasing medium Ca<sup>2+</sup> concentrations accelerated ABCA1 degradation (Fig. 3B). IMM-H007 treatment suppressed Ca<sup>2+</sup>-dependent ABCA1 degradation (Fig. 3B). Moreover, mirroring the actions of calpeptin, IMM-H007 decreased intracellular calpain activity (Fig. 3C), although calpain abundance was invariant (data not shown). The depletion of intracellular calpain expression by siRNA increased ABCA1 protein stability, and IMM-H007 had no further effect on ABCA1 abundance in the setting of calpain silencing (Fig. 3D). These findings indicate that IMM-H007 increases ABCA1 abundance by restraining calpain-mediated ABCA1 protein degradation.

IMM-H007 increases HDL cholesterol and facilitates cholesterol excretion

Next we measured plasma HDL in vehicle-, probucol-, or IMM-H007-treated mice and hamsters. Six weeks of treatment with probucol in Paigen diet-fed apoE<sup>−/−</sup> mice caused a modest 13.2% decrease in circulating HDL, whereas IMM-H007 (100 and 200 mg/kg) resulted in 20.2% and 51.3% increases in HDL (Fig. 4A), although plasma apoA-I was not affected (supplementary Fig. 3A). In WT mice fed a Paigen diet, the plasma HDL was elevated 21.3% by IMM-H007 (200 mg/kg) administration for two weeks.
HDL isolated from IMM-H007-treated chow-fed mice enhanced cholesterol efflux from J774 and THP-1 macrophages, promoting efflux up to 1.4 ± 0.75-fold (P < 0.01) and 1.6 ± 0.53-fold (P < 0.01), respectively, at the highest dose of agent administered in comparison with HDL from vehicle-treated mice. Regarding ABCA1-mediated cholesterol efflux (Fig. 5A, B), HDL from mice treated with 200 mg/kg IMM-H007 raised efflux up to 3.18-fold and 6.89-fold in J774 and THP-1 cells, respectively. The treatment of J774 or THP-1 cells with IMM-H007 raised cholesterol efflux to apoA-I (Fig. 5C, D, respectively). Parallel findings were obtained when primary macrophages were employed (supplementary Fig. 4). In contrast, such treatment of J774 cells did not affect SR-BI-mediated efflux, which was determined as the component of efflux to PEG-HDL from vehicle-treated mice that was attenuated by block lipid transport-1 (BLT-1), which is a specific inhibitor of SR-BI-mediated lipid transfer (33) (Fig. 5E). The treatment of J774 cells with IMM-H007 also did not affect efflux mediated by aqueous diffusion, which was evaluated by measuring efflux to methyl-β-cyclodextrin (12) (Fig. 5F).

Thus, the increased efficiency of HDL isolated from IMM-H007-treated mice to remove cholesterol from macrophages, and the enhanced efflux observed following the direct treatment of macrophages with IMM-H007 are both attributable to more efficient cholesterol efflux by ABCA1.
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IMM-H007 suppresses atherosclerotic lesion formation in apoE−/− mice

We next tested the hypothesis that the enhanced RCT resulting from the suppression of ABCA1 protein degradation by IMM-H007 decreases atherosclerotic plaque formation in hypercholesterolemic mice. As expected, positive control treatment with probucol caused a decrease in lesion abundance (Fig. 7A–E). Compared with vehicle-treated apoE−/− mice with HDL cholesterol levels of 4.50 ± 0.56 mmol/l, IMM-H007 reduced the number and size of plaques in the aortic arch region (Fig. 7A, B). There were no adverse effects of IMM-H007 on liver enzymes (supplementary Fig. 5). The effect was maximal at 200 mg/kg IMM-H007 treatment, which yielded HDL levels of 7.22 ± 1.33 mmol/l (supplementary Table 3). In the entire en face aorta, the amount of lipid-laden plaque area was reduced 3- to 4-fold in mice treated with 200 mg/kg IMM-H007 (Fig. 7B, D). Oil Red O staining of cross-sections of the aortic root also revealed that...
IMM-H007 suppresses plaque formation in a dose-dependent manner (Fig. 7C, E), paralleling what was observed in the aortic arch region. In addition, IMM-H007 treatment resulted in reduced macrophage accumulation in the atherosclerotic plaque (supplementary Fig. 6). Together, these outcomes indicate that the blunting of degradation of the ABCA1 protein by IMM-H007 treatment increases both HDL quantity and function, resulting in reduced atherosclerotic plaque generation.

DISCUSSION

In the current work, we show that the small molecule IMM-H007 inhibits intracellular ABCA1 protein degradation by suppression of calpain activity and ABCA1 internalization, leading to an elevation of cell surface-resident ABCA1 in macrophages, increased circulating HDL that has enhanced capacity to efflux cholesterol, the promotion of ABCA1-mediated RCT, and the alleviation of the formation of atherosclerosis in apoE<sup>−/−</sup> mice. Thus, via effects on both ABCA1-expressing cells and circulating HDL function, the attenuation of ABCA1 degradation by IMM-H007 results in greater capacity for HDL to mediate RCT, and this likely underlies the atheroprotection that the intervention affords.

The ABCA1 protein undergoes degradation via both calpain-mediated and ubiquitin-mediated processes (18, 32). The former is dependent on the protease calpain to hydrolyze the ABCA1 protein, and the PEST-enriched motif is required (30), and ubiquitin mediates ABCA1 protein proteolysis by both lysosomal and nonlysosomal degradation pathways (18). We determined that IMM-H007 decreases intracellular calpain activity and it also decreases Ca<sup>2+</sup>-dependent ABCA1 turnover, indicating that the agent inhibits calpain-mediated ABCA1 degradation. Following calpain silencing, IMM-H007 had no impact on ABCA1 turnover, indicating that the primary effect of the small molecule is on calpain-mediated ABCA1 degradation.

The small molecule IMM-H007 was derived from cordycepin, which is an adenosine analog isolated from the fungus Cordyceps militaris, and synthesized as a purine analog. We previously found that cordycepin decreases LDL cholesterol levels in vivo (34), but its efficacy is limited due to poor bioavailability. IMM-H007 was synthesized and observed to stimulate the phosphorylation of AMPK and to decrease lipid biosynthesis (35). AMPK activation by IMM-H007 may partially explain the observed declines in LDL cholesterol and triglycerides in plasma and liver because AMPK activation attenuates cholesterol synthesis, lipogenesis, and triglyceride synthesis (36). However, the observed increases in ABCA1 protein stability, in HDL cholesterol efflux capacity, and in RCT with IMM-H007 are not likely related to AMPK activation because previous studies in macrophage-derived foam cells have shown that although AMPK stimulation increases ABCG1 expression, it has no effect on ABCA1 (37). Whether IMM-H007 binds directly to ABCA1 to impact its fate, and/or whether other processes participate now warrant investigation.

It is known that the ABCA1 inhibitor probucol reduces HDL cholesterol levels yet decreases atherosclerosis. This is thought to be due to hepatic ABCA1 antagonism and the diversion of HDL cholesterol from efflux back into the plasma to excretion in the bile (25). The two oxidized products of probucol, spiroquinone and diphenooquinone, have been found to reduce ABCA1 protein degradation. Studies in cholesterol-fed rabbits have further shown that spiroquinone and diphenooquinone decrease lipid deposition in atherosclerotic lesions, and this was associated with increases in plasma HDL (10). However, how the inhibition...
Fig. 7. IMM-H007 alleviates atherosclerotic plaque development in apoE<sup>−/−</sup> mice. apoE<sup>−/−</sup> mice (n = 10–12) fed a Paigen diet were treated with vehicle, probucol (200 mg/kg), or the indicated doses of IMM-H007 for 10 weeks. A: Plaques (arrows) in aortic arches and thoracic aortas of representative apoE<sup>−/−</sup> mice are shown. En face Oil Red O stained aortas (B) and cryosections of mouse aortic roots (C) from vehicle and treated apoE<sup>−/−</sup> mice are shown. Mean atherosclerotic lesion areas of aorta (D) and aortic root (E) were determined using ImageJ software; each data point represents an individual animal. The horizontal lines denote the mean of each group. Scale bars: 1 cm (B) and 20 μm (C).
of ABCA1 protein degradation affects HDL function has been unknown. Enhanced macrophage RCT is generally believed to be the crucial mechanism by which HDL has atheroprotective potential, with the most important step being the initial efflux of cholesterol from the macrophage to lipid-poor apoAI via the ABCA1 pathway (7). Our findings in the current study reveal that ABCA1 protein stabilization by IMM-H007 increases macrophage RCT, both by effects on macrophage ABCA1 and on HDL cholesterol efflux capacity. In vivo this results in greater cholesterol transfer to the liver, to bile, and to feces, and this likely underlies the capacity of IMM-H007 to reduce atherosclerotic plaque formation. In contrast to LXR agonists which promote fatty liver (38), we found that hepatic cholesterol and triglyceride are decreased by IMM-H007. Thus, the small molecule enhances ABCA1 expression without causing the adverse effects on the liver that complicate LXR-targeted treatments.

In conclusion, our study shows that the small molecule IMM-H007 inhibits ABCA1 degradation by suppressing calcium-activated calpain activity, resulting in increases in circulating HDL that has enhanced capacity to efflux cholesterol, the promotion of ABCA1-mediated RCT, and a reduction in atherosclerotic plaque formation in hypercholesterolemic mice. These findings indicate that the attenuation of ABCA1 protein degradation may primarily have cardiovascular benefit not as a result of elevating circulating HDL levels, but instead by increasing the capacity of HDL to perform RCT. Furthermore, they reveal that IMM-H007 may be a promising lead agent candidate for enhancing HDL function.

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