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PCSK9-D374Y mediated LDL-R degradation can be functionally inhibited by EGF-A and truncated EGF-A peptides: An *in vitro* study

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HIGHLIGHTS

- PCSK9 acts by binding to the EGF-A domain of LDLR and promotes its degradation.
- Gain-of-function D374Y PCSK9 mutation causes severe hypercholesterolemia in humans.
- PCSK9-D374Y mediated LDLR degradation is inhibited by EGF-A analogs *in vitro*.

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ABSTRACT

Background and aims: Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to low density lipoprotein receptor (LDLR) through the LDLR epidermal growth factor-like repeat A (EGF-A) domain and induces receptor internalization and degradation. PCSK9 has emerged as a novel therapeutic target for hypercholesterolemia. Clinical studies with PCSK9 inhibiting antibodies have demonstrated strong LDL-c lowering effects, but other therapeutic approaches using small molecule inhibitors for targeting PCSK9 functions may offer supplementary therapeutic options. The aim of our study was to evaluate the effect of synthetic EGF-A analogs on mutated (D374Y) PCSK9-D374Y mediated LDLR degradation *in vitro*.

Methods: Huh7 human hepatoma cells were transiently transfected to overexpress the gain-of-function D374Y PCSK9 mutation, which has been associated with severe hypercholesterolemia in humans.

Results: Transient transfection of cells with PCSK9-D374Y expression vector very effectively enhanced degradation of mature LDLR in Huh7. Treatment with both EGF-A and EGF-A truncated peptides inhibited this effect and showed increased LDLR protein in Huh7 cells transfected with PCSK9-D374Y in a clear concentration dependent manner. Huh7 transfected cells treated with increasing concentration of EGF-A analogs also showed an increase internalization of labeled Dil-LDL.

Conclusions: The result of our study shows that EGF-A analogs are able to effectively hamper the enhanced degradation of LDLR in liver cells expressing PCSK9-D374Y.

1. Introduction

PCSK9 (proprotein convertase subtilisin/kexin type 9) regulates LDL cholesterol (LDL-C) levels by interacting with the LDL receptor (LDL-R) [1–6]. Secreted mature PCSK9 binds the LDL-R on the surface of hepatocytes and promotes their internalization and degradation in a post-ER complex. PCSK9 catalytic activity is not required for LDL-R degradation [1,4,7]. PCSK9 would function as a chaperone molecule that

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either prevents LDL-R recycling to plasma membrane from endosomes and/or targets LDL-R to lysosome for degradation. The secreted form of PCSK9 binds directly the extracellular domain (EGF-A domain) of LDL-R. PCSK9 binding to this site is absolutely required for LDL-R degradation [8]. Gain-of-function mutations (GOF) of *PCSK9* cause hypercholesterolemia and early-onset coronary heart disease [9–12], whereas loss-of-function mutations (LOF) result in low plasma cholesterol levels and protection against coronary heart disease without apparent negative consequences [13–15]. Therefore, inhibition of PCSK9 is being pursued as an approach to reduce plasma LDL cholesterol levels [16].

Although the molecular mechanism of action of PCSK9 is not yet completely clear, various approaches inhibiting the PCSK9/LDL-R binding interaction have been investigated and developed.

Amongst those, inhibition by fully human PCSK9 antibodies (evolocumab and alirocumab) has been studied in a wide range of patients and has shown to decrease LDL-C by ~50–70% [1]. Multiple phase 3 studies with these drugs have been already completed and the initial data show a reduction of cardiovascular endpoints [17–19].

Both alirocumab and evolocumab have been approved in 2015 for the treatment of hypercholesterolemia in the European Union and the United States.

Alternative therapeutic strategies are directed at reducing production of PCSK9 by antisense DNA [20] or RNA interference [21,22] technologies. However, these therapeutic approaches are not the most desirable for chronic asymptomatic conditions such as hyperlipidemia. Small molecule inhibitors for targeting PCSK9 functions [23–26], may offer potentially more convenient routes of administration and lower costs.

Peptides are now emerging as therapeutic candidates to bridge the gap between small-molecules (< 500 Da in mass) and large biologics, including antibodies (> 5000 Da) [27]. A small peptide would be a potential candidate to block the interaction between PCSK9 and the LDLR.

In this study, we investigated the possibility of disrupting the binding interface between LDL-R and the p.Asp374Tyr (D374Y) mutated PCSK9 variant, able to increase its activity (gain of function, GOF), by using *in vitro* two EGF-A analogs.

2. Materials and methods

2.1. Cloning and expression of PCSK9 cDNA

Huh7 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Italy) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin 100UI/ml, non essential amminoacids (NEAA) 1% at 37 °C in a humidified 5% CO_2 incubator.

Total RNA was isolated from Huh7 cells by using a RNA extraction commercial kit following the manufacturer's instructions (RNAwiz™, Ambion, Thermo Fisher Scientific, Italy).

2.2. Preparation of expression plasmids

Human full length wild type PCSK9 cDNA (GenBank NM_174936.2) was amplified from Huh7 cells using a tailed pair of primers (PCSK9–F -*gcaagctt*ATGGGCACCGTCAGCTCCAG -3′ HindIII tailed and PCSK9-R′-*cactcgag*CTGGAGCTCCTGGGAGGCCT -3′ XhoI tailed) in order to allow the oriented cloning of the 2076 bp product into a C-terminal FLAG tagging vector (pCMV-3Tag-8 Epitope Tagging Mammalian Expression vector - Stratagene, La Jolla, CA, USA). The PCSK9 wild type (PCSK9-WT) expression plasmid was purified by the Qiagen plasmid purification maxi kit (Qiagen, Milano, Italy), and the sequence of the cloned product was authenticated by direct sequencing in a 3500 Genetic Analyzer and the results were analyzed with the Seqscape software (both from Thermo Fisher, USA).

Biochemical and cellular analyses have revealed that the GOF D374Y mutation originally observed by Timms et al. [28] results from a 10–25-fold higher affinity of PCSK9 for the LDLR [28–30]. This is the most damaging GOF mutation of PCSK9, leading to severe hypercholesterolemia and early death from premature coronary heart disease [31].

The PCSK9-WT expression plasmid was used as template to prepare mutant PCSK9-D374Y using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenic primers used to introduce the D374Y (c.1120G > T) mutation were: PCSK9-D374Y_F 5′- TGGTGCCTCCAGC***T***AC**T**GCAGCACCTGCT - 3′ and PCSK9-D374Y_R 5′- AGCAGGTGCTGCA**G**T***A***GCTGGAGGCACCA -3' (the mutated nucleotide is indicated in boldface and codons specifying for amino acid change is underlined).

The mutant PCSK9-D374Y expression plasmid was purified and authenticated as described above.

2.3. Synthetic EGF-A domain preparation

A 40mer synthetic peptide of EGF-A domain of LDLR (TNECLDNNGGCSHVCNDLKIGYECLCPDGFQLVAQRRCED) was custom-synthesized and purchased from Genscript Corporation (USA). The guaranteed peptide purity was > 85% by HPLC. An additional peptide corresponding to the first 25aa of EGF-A peptide was custom synthesized (P1- TNECLDNNGGCSHVCNDLKIGYECL) (Genscript Corporation, USA). The guaranteed P1 peptide purity was 81.5% by HPLC.

2.4. Transient transfection of PCSK9-WT and PCSK9-D374Y in Huh7 cells

Huh7 cells were seeded at a density of 4.0×10^5 cells/well 24 h before transfection in a 24 wells plate (1.9 cm^2/well) in DMEM-FCS 10%, penicillin and streptomycin 100UI/ml, non essential amminoacids (NEAA) 1%.

At a cell confluence of 70%, plasmid vectors containing either PCSK9-WT or mutant PCSK9-D374Y cDNA were transfected into Huh7 cells with Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions.

Thirty hours after transfection, Huh7 cells were treated with serum free media or media containing EGF-A and P1 peptides at concentration of 12, 5 μM, 25 μM and 50 μM for 18 h.

In a subset of experiments, transfected cells with mutant PCSK9-D374Y cDNA were treated with alirocumab at a final concentration of 19.2 μg/mL as previously described [32].

After 18 h incubation cells were harvested and lysed in Ripa Buffer (NaCl 150 mM, 1% NP40, 0, 5% Na-deoxycholate, 0, 1% SDS, 50 mM TRIS ph 8).

Quantification of total proteins of whole-cell lysate from Huh7 cells was performed by BCA micro protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

2.5. Western blot analysis

Twenty μg of total proteins from cell lysates were suspended in Laemmli Sample Buffer (0.5 mM pH 6.8 Tris-HCl, 10% SDS, 20% glycerol and 0.3% bromophenol blue) (1:1) and DTT (0, 3 μl/10 μl), heated to 95 °C for 5 min and separated by linear 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a NuPage MES SDS Running Buffer 20x (Invitrogen, USA) at 180 mA. Cell lysates and media proteins were electro-transferred from the gel to a PVDF (polyvinylidene fluoride) membrane Hybond P (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The transfer was performed in a refrigerator at 35V (constant voltage) for 2 h. The membrane was incubated with 5% non-fat dry milk in PBS containing 0.05% of Tween-20 (blocking solution) at room temperature to block non-specific binding of the antibodies.

After 1 h incubation, the membrane was cut into two sections to be incubated with different antibody: the upper section (spanning from

100 kDa to 250 kDa) was incubated with anti-human LDL Receptor rabbit polyclonal antibody (Progen Biotechnik GmbH, Heidelberg, Germany) in PBS Buffer with 2% of non-fat dry milk and 0.05% of Tween-20, with a 1:500 ratio; the lower section (spanning from ~20 kDa to ~60 kDa) was incubated either with anti-human β-Actin mouse monoclonal antibody (Sigma, USA) or α-tubulin mouse monoclonal antibody (Santa Cruz, USA) in PBS Buffer with 2% of non-fat dry milk and 0.05% of Tween-20, with a 1:20,000 ratio for β-Actin and 1:250 ratio for α-tubulin.

After overnight incubation with primary antibodies at 4 °C, the two sections were washed twice with 0.1% Tween-20 in PBS Buffer. The upper section was incubated with Anti-Rabbit IgG (Sigma, USA) diluted in PBS Buffer with 5% non-fat dry milk and 0.05% Tween-20, with a 1:5000 ratio. The lower section was incubated with Anti-Mouse IgG (Sigma, USA) diluted in PBS Buffer with 5% non-fat dry milk and 0.05% Tween-20, with a 1:20.000 ratio. Immunocomplexes were detected by ECL plus Western Blotting Detection Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by autoradiography.

2.6. LDL internalization assay

Thirty hours after transfection with PCSK9-D374Y and empty vectors, Huh7 cells were treated with EGF-A and P1 peptides in increasing concentrations (12, 5 μM, 25 μM and 50 μM) in serum-free D-MEM.

After 14 h cells were then treated with, 40 μg/μl of Dil-LDL as described elsewhere [33].

Quantification of total protein content in each well was performed by BCA micro (Pierce Biotechnology, Rockford, IL, USA). Then whole cell extracts were analyzed at 520 nm and 570 nm by using the VICTOR™ X4 fluorimetric System (PerkinElmer Life Sciences) to measure fluorescent signal from internalized Dil-LDL. The results were normalized with respect to the total protein content. Results were expressed as percent of the Dil-LDL binding without competitors. All experiments were performed in triplicate.

2.7. Statistics

The internalization curve has been fitted using a Michaelis Menten first degree equation with a linear non specific component (see also supplementary methods); the values of dissociation constant (kd) and maximum amount (Bmax) of internalized LDL were evaluated together with the non specific internalization constant (klin) using a non-compartmental approach included in the SAAM II ver 1.0 software from RFKA (Seattle, USA).

3. Results

We evaluated whether a synthetic EGF-A domain of LDLR could inhibit LDLR degradation mediated by blocking its interaction with a D374Y PCSK9 mutant, so to restore LDL uptake using Huh7 liver cells as a model system.

Transient transfection of cells with PCSK9-WT and PCSK9-D374Y expression vectors enhanced degradation of mature LDLR in Huh7 compared to cells transfected with empty vector with a more marked effect in cells treated with the mutant PCSK9-D374Y vector (Fig. 1A and B; Fig. 2A and C).

The presence of EGF-A inhibited this effect and showed increased LDLR protein in Huh7 cells transfected with PCSK9-WT and PCSK9-D374Y. The inhibition was effective both in the Huh7 expressing PCSK9-WT and PCSK9-D374Y at 12.5 μM EGF-A, showing a clear concentration dependent increase at 25 and 50 μM EGF-A (Fig. 1A and B, Fig. 2A and C). In a parallel experiment, the treatment of PCSK9-D374Y transfected cells with the P1 synthetic peptide was also able to inhibit the mature LDLR degradation in a concentration dependent manner (12.5–50 μM). (Fig. 2B and C).

As control, we also tested the effect of treatment with alirocumab in cells transfected with PCSK9-D374Y. In this setting the inhibition of LDL-R degradation appeared less pronounced than EGFA treated cells (Fig. 3A and B).

EGF-A and P1 peptides inhibition of D374Y-PCSK9 function was further assayed by internalization of Dil-labeled LDL. Huh7 transfected cells treated with increasing concentration of both EGF-A and P1 peptides, showed an efficient inhibition of the LDL-R and D374Y binding affinity demonstrated by increase internalization of Dil-LDL (Fig. 4 and Supplemental Fig. 1).

4. Discussion

PCSK9 is a validated target for the treatment of hypercholesterolemia. Monoclonal antibodies (MAbs) that bind to PCSK9 and prevent PCSK9 interaction with LDL-R have been shown to reduce circulating LDL-C levels in pre-clinical animal models and in human clinical trials. The discovery of small molecules that can disrupt the interaction of PCSK9 with LDL-R has prompted to the identification of alternative approaches for antagonizing PCSK9 function.

Shan et al. were the first to show that, the induced degradation mediated by the PCSK9-LDL-R interaction is effectively inhibited by the presence in the cell media of a synthetic EGF-A domain peptide in PCSK9 treated HepG2 cells, and this effect resulted in increased LDL-R protein and LDL uptake [23].

Aspartic acid at position 374 in PCSK9 protein (D374) is crucial for binding the EGF-A domain of LDL-R; the GOF D374Y mutation confers higher affinity of PCSK9 for the LDL-R and this result in enhanced receptor degradation and severe hypercholesterolemia in humans [29–31].

Mutations in EGF-A have been also linked to familial hypercholesterolemia. In particular, the gain-of-function mutation p.His327Tyr (H327Y) in EGF-A is associated with increased pH-independent binding affinity for PCSK9 by forming a hydrogen bond with D374 in PCSK9 [25].

In our study, we investigated the possibility of inhibiting the interaction of PCSK9-D374Y GOF mutation with mature LDL-R, and promoting the internalization of labeled LDL-C by using both a synthetic full length EGF-A domain peptide and a 25-amino acid truncated EGF-A analog in a cell-based assay.

To pursue this objective we decided to transiently express PCSK9-D374Y in Huh7 and we next added in the medium a synthetic full length EGF-A domain peptide. A similar set of experiments were performed by adding a shorter 25-amino acid truncated EGF-A analog. Both peptides were able to inhibit the degradation of mature LDL-R mediated by PCSK9-D374Y and the inhibition of PCSK9-D374Y activity was reflected in a corresponding increase in Huh7 uptake of Dil-labeled LDL.

In our *in vitro* system the treatment with EGF-A analogs appeared to be more effective in the inhibition of the degradation of LDL-R compared to the treatment with monoclonal antibody against PCSK9 (alirocumab).

Schroeder et al. have previously shown that truncated EGF-A peptides restore LDL-R recycling in the presence of PCSK9 *in vitro* [34]. They elegantly showed that the smallest part of the EGF-A domain (EGF-A5) retains binding affinity similar to that of wild type EGF-A, but had a weaker potency to inhibit PCSK9-driven LDL-R as shown by functional assays [34]. A mutated peptide H306Y was able to promote increase binding affinity through a hydrogen bond with D374 in PCSK9 [34].

These studies are consistent with our data suggesting that the binding between PCSK9 and LDL-R can be modulated by peptides administration and that our therapeutic approach is suitable for patients affected by familial hypercholesterolemia due to GOF mutations in *PCSK9* gene.

Recently, the discovery of peptides competitive with EGF-A binding to PCSK9 has progressed and the identification of a lupin derived

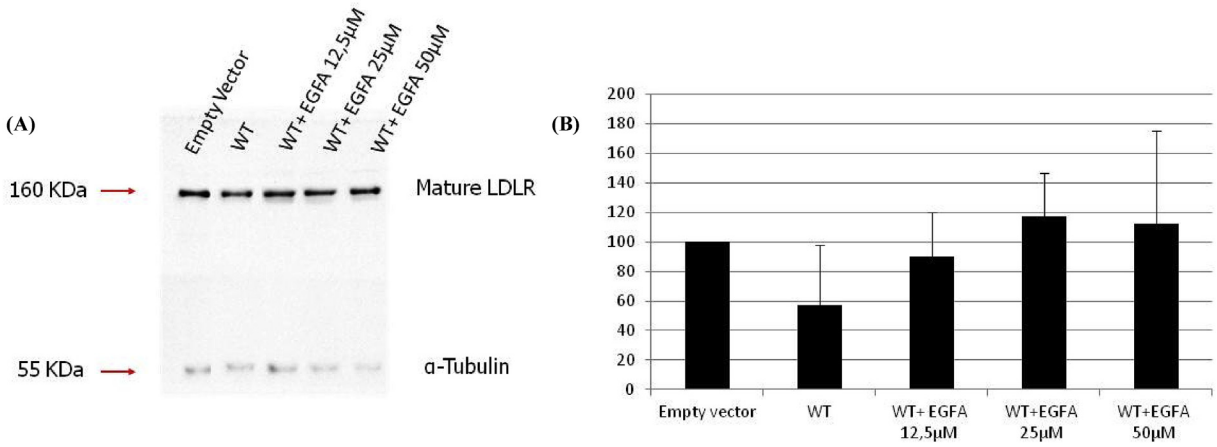

Fig. 1. EGF-A peptide inhibits WT PCSK9-mediated degradation of LDLR.
(A) Huh7 cells were transiently transfected with PCSK9-WT expression plasmid and empty vector (control) and treated with EGF-A at 12,5 µM, 25 µM and 50 µM as indicated in the Methods section. Whole cell protein lysates were prepared and duplicate samples analyzed for LDLR protein by Western blotting. The band corresponding to mature LDLR (160 kD) is indicated. (B) The amount of total LDLR protein relative to the amount of α-tubulin is shown. Results are expressed as percentage of LDLR protein relative to control cells transfected with empty vector. Error bars are standard deviation of duplicate experiments.

decapeptide [35] and an imidazole-based peptidomimetic [36] are some examples. Zhang et al. by screening linear and di-sulfide linked phage displayed peptides libraries identified potent PCSK9 inhibitors [37]. The most promising peptide was a 13 amino acid long peptide binding to PCSK9; this short peptide structurally overlap with the EGF-A binding site and is therefore competing with the EGF-A domain with a similar mode of action as the monoclonal antibodies [37]. More recently, the same research group reported on the discovery of a targetable cryptic N-terminal groove in PCSK9 very close to the EGF-A–PCSK9 interaction surface [38]. They identified an optimized peptide that was also able to restore LDL-R in HepG2 cells by combining phage display experiments and a structure guided design [38].

Additional functional domains in other parts of the PCSK9 protein have been identified and have not been reported yet. As an example, it

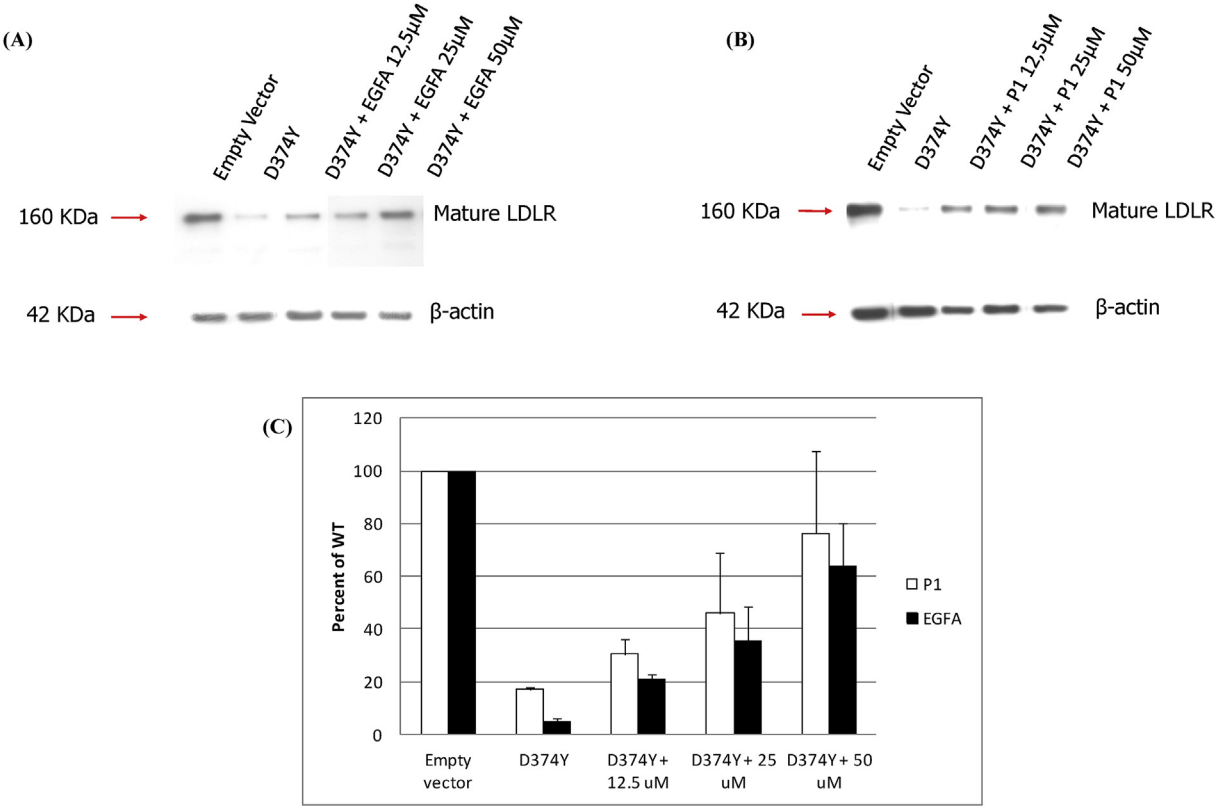

Fig. 2. EGF-A and truncated EGF-A peptides inhibit D374Y PCSK9-mediated degradation of LDLR.
(A and B) Huh7 cells were transiently transfected with mutant PCSK9-D374Y expression plasmid and empty vector (control) and treated with EGF-A at 12,5 µM, 25 µM and 50 µM (A) or truncated EGF-A peptide (P1) at 12,5 µM, 25 µM and 50 µM (B) as indicated in the Methods section. Whole cell protein lysates were prepared and duplicate samples analyzed for LDLR protein by Western blotting. The band corresponding to mature LDLR (160 kD) is indicated. (C) The amount of total LDLR protein relative to the amount of β-actin is shown. Results are expressed as percentage of LDLR protein relative to control cells transfected with empty vector. Error bars are standard deviation of duplicate experiments.

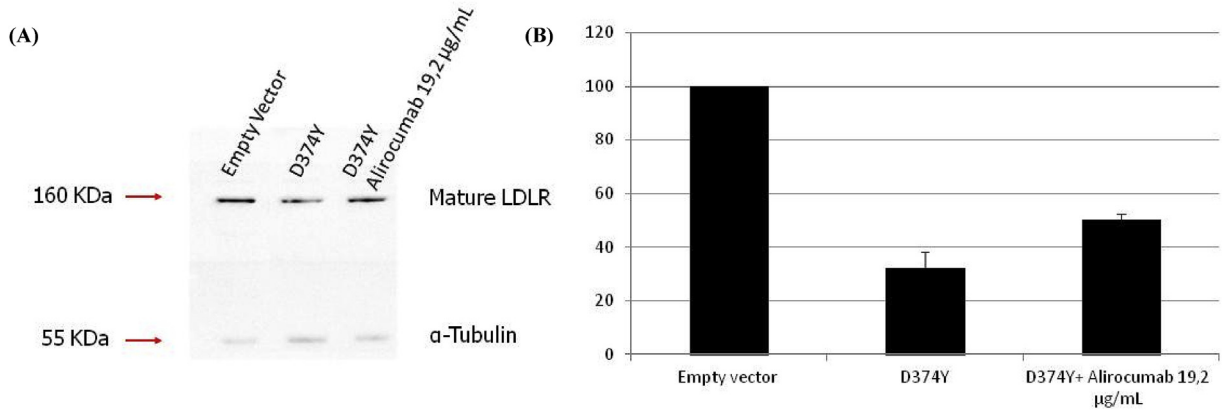

Fig. 3. Alirocumab inhibits D374Y PCSK9-mediated degradation of LDLR.
(A) Huh7 cells were transiently transfected with mutant PCSK9-D374Y expression plasmid and empty vector (control) and treated with alirocumab at 19.2 µg/mL as indicated in the Methods section. Whole cell protein lysates were prepared and duplicate samples analyzed for LDLR protein by Western blotting. The band corresponding to mature LDLR (160 kD) is indicated. (B) The amount of total LDLR protein relative to the amount of α-tubulin is shown. Results are expressed as percentage of LDLR protein relative to control cells transfected with empty vector. Error bars are standard deviation of duplicate experiments.

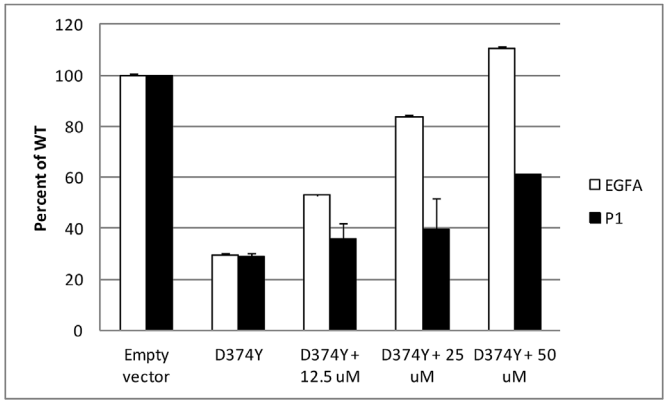

Fig. 4. Uptake of DiI-labeled LDL in Huh7 cells.
Forty µg/µl of Dil-LDL were added to medium of transiently transfected Huh7 cells with mutant PCSK9-D374Y expression plasmid treated with EGF-A at 12,5 µM, 25 µM and 50 µM (solid white columns) or truncated EGF-A peptide (solid black columns) at 12,5 µM, 25 µM and 50 µM. Results are expressed as percentage of Dil-LDL internalization relative to control cells transfected with empty vector. Error bars represent standard deviation of triplicate experiments.

has recently been reported that PCSK9 binding to proteoglycans is important for PCSK9-LDL-R binding, suggesting that the block of the PCSK9-proteoglycans interaction may represent an additional opportunity for small PCSK9 inhibitors [39].

Despite the progresses in understanding the PCSK9-LDL-R interaction at the molecular level and in identifying smaller peptides and peptidomimetics able to inhibit this interaction, needs to be said that the achievement of an oral drug with this mode of action remains a challenge.

In conclusion, the result of our study shows that EGF-A analogs are able to effectively hamper the enhanced degradation of LDL-R in liver cells expressing a gain of function mutation of PCSK9-D374Y responsible for FH in some patients. The identification of a small peptide of 25 aa able to work as the whole EGF-A might allow in the future to imagine an oral administration of the drug by using up-to-date nanotechnologies.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

ABC and MRA conceived the study; ABC, VV and AG and DN drafted the manuscript; VV, FF, RS, GIA, VI, AG, CS, FD set the experiments, acquired the analyzed data; ABC, MRA, DN, CB, GB supervised data analysis and interpreted the results; All authors provided critical comments on the manuscript.

All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.09.009.

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