

An *In vitro* Cell-Based LDL Uptake Model for Screening PCSK9 Modulators

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Abstract

PCSK9 plays a critical role in cholesterol metabolism by controlling the level of low-density lipoprotein receptor (LDLR). Recent population genetic studies have shown that PCSK9 is a genetic validated target for the reduction of LDLc. Several anti-PCSK9 antibodies are currently undergoing Phase II/III trials in patients with hypercholesterolemia with promising results. However, developing novel compounds that inhibit PCSK9 function is pharmaceutically preferred over both antibody and siRNA routes. We have recently developed a cell-based, functional assay incorporating recombinant PCSK9 protein for high-throughput screening of human liver cell HepG2. A pilot screen of the NINDS compound library has been successful in identifying several potential compounds in duplicate screening. One of the lead compounds, colchicine, has been further validated with the Western blot assay for PCSK9-mediated LDLR degradation in dose-responsive assay. Colchicine is a prescribing drug for the treatment of acute gout flares and familial Mediterranean fever. Identification of colchicine as a modulator of PCSK9 function highlights the potential of the novel use of this drug for cardiovascular disease treatment.

Keywords: PCSK9; LDL; High-throughput screen assay; Colchicine; Cholesterol; Modulator; Cardiovascular disease

Abbreviations: PCSK9: Proprotein convertase subtilisin/kexin type 9; NINDS: The National Institute of Neurological Disorders and Stroke compound library; HTS: High-Throughput Assay; CVD: Cardiovascular Disease

Introduction

Cardiovascular Disease (CVD) is among the main causes of premature death in the world. Plasma LDL cholesterol is a major determinant of CVD. Recent genetic studies have shown, one of the key genes, PCSK9, plays a critical role in cholesterol metabolism by controlling the level of low-density lipoprotein receptor (LDLR) [1-3]. Recent population genetic studies have shown that the nonsense mutations of PCSK9 were associated with a 28 percent reduction in mean LDLc, and an 88 percent reduction in the risk of a CVD in African-American, indicating PCSK9 is a genetic validated target for the reduction of LDLc in human population [4].

PCSK9 inhibition is currently a “hot drug target” for developing new cholesterol lowering therapy. Therapeutic RNAi targeting PCSK9 gene has been shown to have profound LDLc lowering effect in rodents and nonhuman primates [5]. Several pharmaceutical companies have developed therapeutic antibodies against PCSK9 for treatment of CVD. At least five phase II clinical trials of patients with AMG 145 antibodies (Amgen) or SAR236553/REG727 antibody (Regeneron Pharmaceutical) indicated that anti-PCSK9 antibodies reduced LDL in patients with hypercholesterolemia [6].

However, developing novel compounds that inhibit PCSK9 function is pharmaceutically preferred over both antibody and siRNA routes. We have recently developed a cell-based, functional assay incorporating recombinant PCSK9 protein for high-throughput screening of human liver cell HepG2. A pilot screen of the NINDS compound library has been carried out and we have identified several potential compounds in duplicate screening and one of the compounds, colchicine, has been validated with a western blot assay for PCSK9-mediated LDLR

degradation in dose-responsive assay. This is the first successful assay of its kind developed for screening for PCSK9 inhibitor.

Materials and Methods

Cell culture, protein purification and western blot assays

HepG2 cells were obtained from European collection of cell culture (Wiltshire, UK). Cells were grown in the DMEM medium containing 25 mM glucose and 10% foetal calf serum, as described [7]. A stable expressed FLAG-tagged wild-type PCSK9 cell line and a ‘gain-of-function’ mutant PCSK9-D374Y cell line were developed as previously described [8]. The FLAG-tagged PCSK9 protein was isolated using the FLAG-immunoprecipitation kit (Sigma) following by elution with the 3xFLAG peptide (final concentration 150 ng/ml of 3xFLAG peptide) [8]. The protein purity was determined by SDS-PAGE and visualized by Coomassie Blue stain with over 90% purity. Protein concentration was measured using Bio-Rad Protein Assay kit (Cat: 500-0006, Bio-Rad, UK). The anti-human LDLR antibody was a rabbit antibody directed against amino acids 184-196 of human LDLR (Research Diagnostics Inc. Flanders, NJ, USA). Anti-human PCSK9 antibody was from the Cayman Chemical (Cat: 10007185, Cayman, USA). Cell lysis and western blot analysis were carried out as previously described [7]. Western blot densitometry was carried out using the Vision works LS software (UVP, Cambridge, UK). All data were analysed by GB-Stat V5.4.4 program using student t-Test (two-tailed). The cell viability was determined by the trypan blue staining using the Trypan blue exclusion solution (Gibco, Cat 15250-061, Life Technologies Ltd, Paisley, UK).

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NINDS library

The National Institute of Neurological Disorders and Stroke (NINDS) compound library is a collection of known drugs and pharmacologically active compounds originally collated by the National Institute of Neurological Disorders and Stroke (US) [9,10]. The library contains 1041 compounds (http://iccb.med.harvard.edu/screening/compound_libraries/ninds.htm), a collection of known drugs and pharmacologically active compounds, consisting mainly of FDA-approved drugs. The NINDS library was kindly supplied to us by Dr Nicole Mathon and Dr Catherine Kettleborough from the Medical Research Council Technology, UK(MRCT). All the compounds in the NINDS were dissolved in DMSO at 1mM concentration, therefore, the sensitivity of the cell-based assay were determined in different dilutions of DMSO on the standard cell culture condition.

High-throughput screening of the cell- based LDL uptake assay

High-throughput screening was carried out in high throughput robotics facility in the university of Sheffield [11]. Human liver HepG2 cells were seeded in 96 well tissue plates at concentration 2×10^5 cells/ml. After 24 h, the medium was replaced with DMEM medium without FBS and purified PCSK9-D374Y (1 $\mu\text{g/ml}$ PCSK9=13.4 nM) plus pooling 5 compounds were added, resulting each compound concentration at 200 nM. In the positive control wells, only PCSK9-D374Y protein was added. The negative control was vehicle (DMSO) plus medium only. Vehicle (0.2% DMSO) was used in all assays. After 16h incubation, the medium was replaced with fresh medium containing 10 $\mu\text{g/ml}$ Bodipy FL LDL (Cat: L-3483, Invitrogen, UK) without FBS. The cells were incubated for 4 h at 37°C. The cells were washed with PBS and LDL uptake was quantified on the Fusion fluorescence plate reader (Packard BioScience, Beaconsfield, UK) with the excitation filter 485 nm and emission filter 530 nm.

Statistical analysis

The high-throughput analysis was calculated as Z' factor. The Z factor was determined according to Zhang et al. [12] using the following equation:

$$Z' = 1 - 3(SD_{\text{neg}} + SD_{\text{pos}}) / |(\text{Mean}_{\text{neg}} - \text{Mean}_{\text{pos}})|.$$

Z' values >0.5 have acceptable characteristics for high-throughput screening (HTS).

Secondary screening analysis of PCSK9-mediated LDLR degradation by western blot

HepG2 cells were seeded in 6 well tissue plates at a concentration of 2×10^5 cells/ml. After 24 h, the medium was replaced with DMEM medium without FBS and added the purified D374Y-PCSK9 protein (1 $\mu\text{g/ml}$). In the control experiments, without PCSK9 protein was added. In compound function assay, the test compounds were added together with the purified D374Y-PCSK9 protein. After 16h, cells were washed twice in PBS and harvested for western blot analysis as previous described [8].

Results and Discussion

Establishment of a cell-based assay

We have set up a cell-based screening method using fluorescent-labelled LDL uptake in HepG2 cells as an end point assay. We treated human liver HepG2 cells with a 'gain-of-function' mutant D374Y-

PCSK9 protein at 1 $\mu\text{g/ml}$ concentration (13.4 nM). As shown in Figure 1A, the fluorescence -labelled LDL uptake was significantly decreased in the PCSK9-D374Y-treated cells than that of untreated cells. This fluorescence intensity difference can be easily detected by the fluorescence plate reader (Figure 1B, 1st two columns). Because all compounds in our screening assay were dissolved in DMSO, we have further tested the DMSO effects on the PCSK9-mediated LDL uptake. We found that 200-fold dilution of DMSO (0.5 μl DMSO into 100 μl medium) (Figure 1B, 2nd two columns) worked well in our cell-base assay ($P < 0.05$) (Figure 1B). Therefore, we have carried out the screening program at this dilution. The Z' factor of the assay was determined to be 0.52, indicating this fluorescence-cell based assay would perform well in the large-scale screening process (Figure 1C).

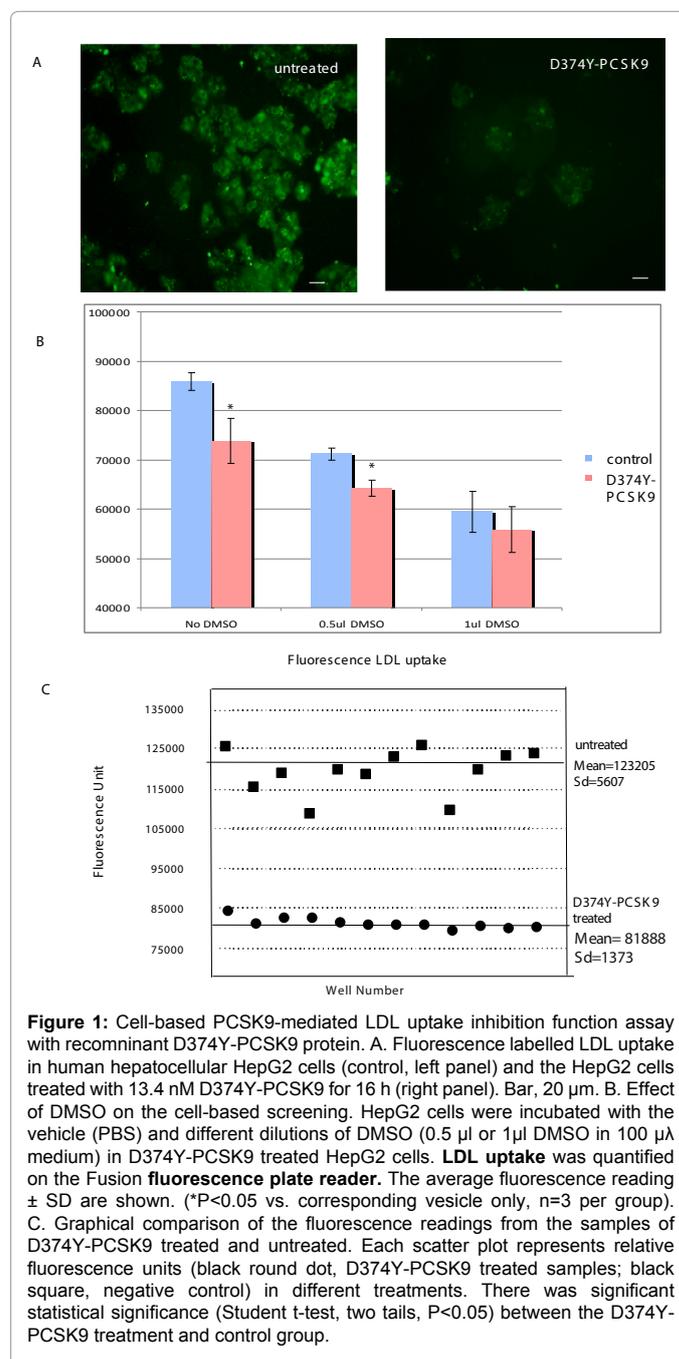


Figure 1: Cell-based PCSK9-mediated LDL uptake inhibition function assay with recombinant D374Y-PCSK9 protein. A. Fluorescence labelled LDL uptake in human hepatocellular HepG2 cells (control, left panel) and the HepG2 cells treated with 13.4 nM D374Y-PCSK9 for 16 h (right panel). Bar, 20 μm . B. Effect of DMSO on the cell-based screening. HepG2 cells were incubated with the vehicle (PBS) and different dilutions of DMSO (0.5 μl or 1 μl DMSO in 100 μl medium) in D374Y-PCSK9 treated HepG2 cells. LDL uptake was quantified on the Fusion fluorescence plate reader. The average fluorescence reading \pm SD are shown. (* $P < 0.05$ vs. corresponding vehicle only, $n = 3$ per group). C. Graphical comparison of the fluorescence readings from the samples of D374Y-PCSK9 treated and untreated. Each scatter plot represents relative fluorescence units (black round dot, D374Y-PCSK9 treated samples; black square, negative control) in different treatments. There was significant statistical significance (Student t-test, two tails, $P < 0.05$) between the D374Y-PCSK9 treatment and control group.

Screen the NINDS compound library

Initially, a pool of 5 compounds was added into 100 μ l of medium in each well in 96-well plate format. In the positive control wells, PCSK9-D374Y protein (13.4 nM final concentration) was added. The negative control was culture medium with vehicle (0.5% DMSO) only. After 16 h, the medium was replaced with DMEM without serum. After 24 h, the medium was replaced with fresh medium containing 10 μ g/ml Bodipy FL LDL without serum. The cells were incubated for 4 h at 37°C. The cells were washed with PBS and LDL uptake was quantified on the Fusion fluorescence plate reader. We have successfully screening the NINDS compound library by pooling 5 compounds into each well (200 nM final conc.), screening by duplication (Figure 2). Hit molecule was defined as a molecule that gave significantly increased LDL fluorescence in the compound treated cells in comparison with untreated cells. Each screen was carried out in duplication. Only the hit polls on both duplicates were selected and broken down into each individual compound (Figure 2).

As shown on Figure 3, the wells that contain D374Y-PCSK9 protein had significant lower fluorescence-labeled LDL uptake in comparison with the control wells (untreated) (Figure 3, 1-2 column). Initially, we have identified 9 positive pools in total 209 pools, which contain all 1041 compounds. These 9 positive pools were then broken down to each individual compound and 6 positive hits have been detected in a duplication assay (Figure 3, column A-F, Table 1).

Western blot analysis

We next used the western blot assay to access the 6 potential hits identified by the HTS assay. HepG2 cells were treated with 1 μ l/ml PCSK9-D374Y protein (13.4 nM) together with each of the 6 compounds with 200 nM final concentration. After 16 h in serum-free DMEM medium incubation, the whole cell extracts were isolated and subjected to western blot analysis with the anti-human LDLR antibody. As shown on Figure 4A, there was a significant increase of

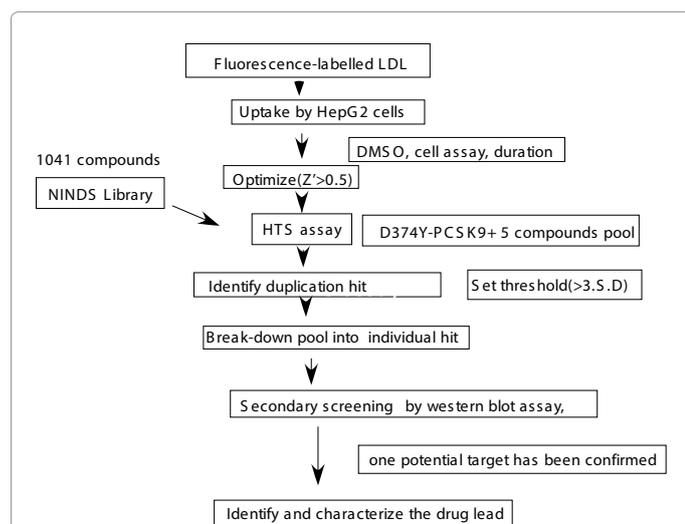


Figure 2: Experimental design of a cell-based high-throughput assay for identifying PCSK9 inhibitors. HepG2 cells were seeded in 2×10^5 /ml in 96-wells plate. Vehicle (PBS) or D374Y-PCSK9 protein (13.4 nM) was added in the culture medium with pooling 5 compounds into each well (200 nM final conc.) from the NINDS library. The positive pools in duplication were broken down to each individual compound. Each individual positive compound was then subject to the secondary screen assay by western blot.

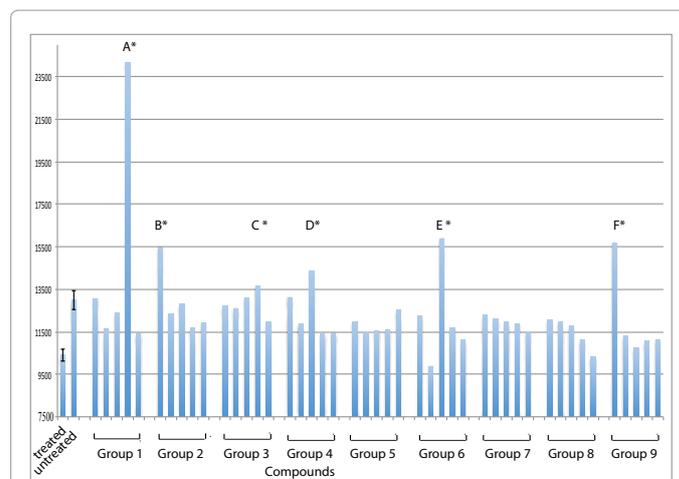


Figure 3: Representing readings of the fluorescence LDL uptake in the high-throughput assay. The positive pools (total 9) were broken down to each individual compound and 6 positive hits have been detected in a duplication assay to have significant higher LDL uptake than control wells ($*p < 0.05$ vs. corresponding vesicle only, $n = 6$).

Compounds	Molecule Name	fmla MolStructure	mol weight	therapeutic
A	Acriflavinium Hydrochloride	C14H14ClN3	259.7408	antiinfective, intercalating agent
B	Chochicine	C22H25NO6	399.4477	antimitotic, antigout agent
C	Sulpiride	C15H23N3O4S	341.4323	dopamine receptor antagonist, antipsychotic
D	Emodic acid	C15H8O7	300.2268	cathartic, purgative agonist
E	Almotriptan	C17H25N3O2S	335.4717	5HT 1B/2D receptor agonist
F	Creatinine	C4H7N3O	113.1199	metabolic enhancer

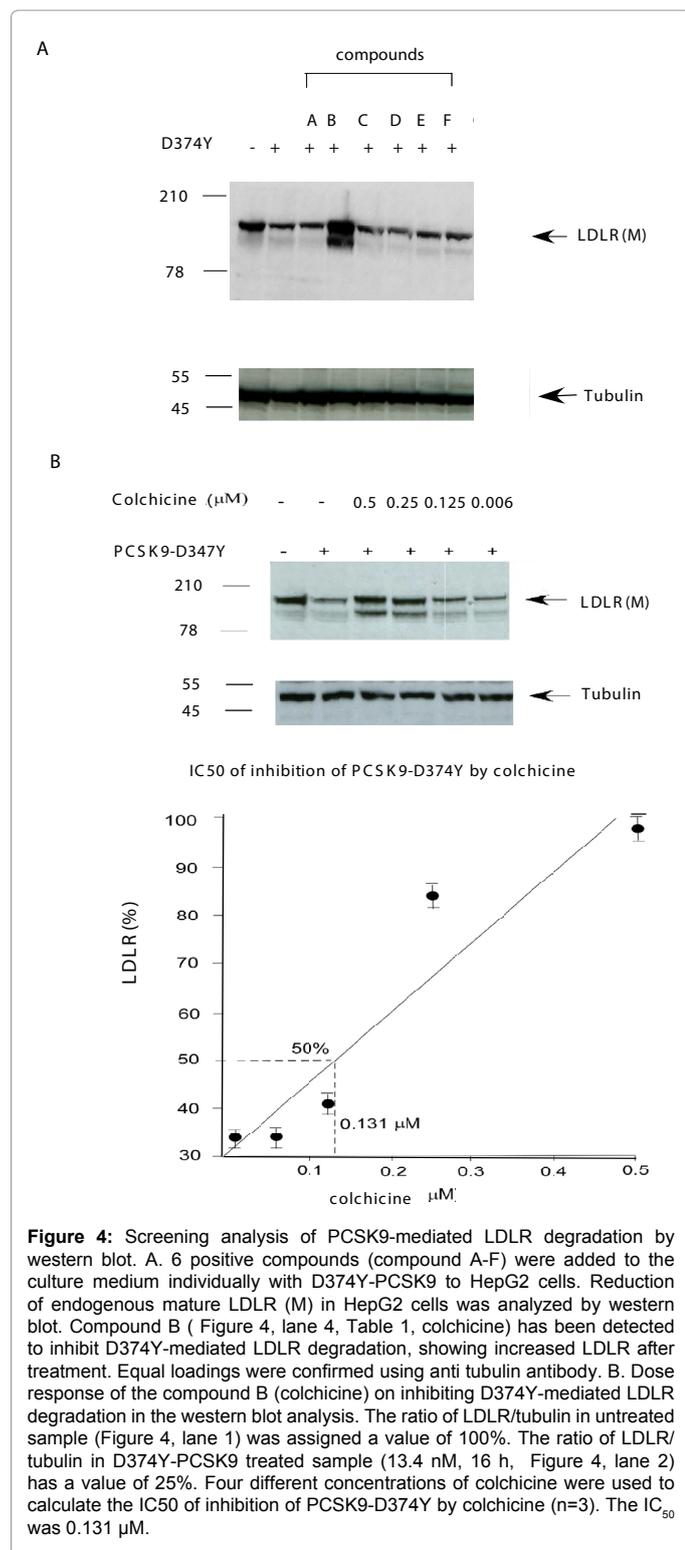
Table 1: List of the potential positive hits in the high-throughput assay.

LDLR protein in one of the compounds, compound B (colchicine) treatment (Figure 4A, lane 4). In fact, the level of LDLR in colchicine treated cells was similar to that of the untreated group (Figure 4A, lane 1). We further observed dose-dependent increase of the LDLR protein content (Figure 4B). The half maximum inhibition of colchicine to PCSK9-D374Y degradation of LDLR protein was calculated as 0.131 μ M (Figure 4B).

Because colchicine is cytotoxic, we have examined the cell viability of HepG2 after colchicine treatment using the trypan blue exclusion method. We found that treatment of HepG2 cells at current low concentrations of colchicines (0.05 μ M to 0.2 μ M) had no observed toxicity in 16 h-24 h period (data not shown).

Discussion

Compound Colchicine discovered in our screen of PCSK9 inhibitor is a well known ancient remedy that has been used to treat gouty arthritis over 1,400 years (Alexander of Tralles during the 6th century) [13]. More recently, FDA proved colchicine as a prescribing drug for



treatment of acute gout flares and familial Mediterranean fever [14]. Despite its historical role in gout, the metabolic effects of colchicine have not been explored. Therefore, our finding on colchicine inhibition of PCSK9 degradation of LDLR is a novel finding and giving an ancient remedy a new usage for cardiovascular disease treatment.

In fact, looking for past literatures we found there are two previous reports showing cholesterol lowering effects of colchicine. In 1967, Faloon et al reported that in six obese patients receiving constant low-calories diet, being treated with colchicine, serum cholesterol fell by 40 to 80 mg/100 ml in 6 days in four of the five patients [15]. Again, in the 1970, Rubulis et al reported the two hypercholesterolemia patients have been shown significant fall in serum cholesterol in 5 days treatment of colchicine 2.5 mg daily [16]. Despite these reports, the metabolic effects of colchicine on cholesterol regulation have not been explored. Our high-throughput result has pointed for the first time that colchicine interaction with PCSK9/LDLR pathway could be its key function in mediation of cholesterol regulation.

Colchicine is known as a potent binder to tubulin in the microtubules network [17]. Interestingly, a recent study has shown that the endocytic recycling of the LDL receptor returning to the plasma membrane depends on microtubule-dependent motility [18]. In a separate *in vitro* cell culture model of the Alzheimer's disease, increased APP expression and A β exposure alters microtubule function, leading to reduced transport of LDLR to the plasma membrane, moving it toward the Golgi apparatus and lysosomes [19]. PCSK9 is known forming a complex with LDLR in the early endosomes and lysosomes [8], that makes the PCSK9/LDLR complex is likely moving on the microtubules network, which is vulnerable to the disruption by colchicine. The detail mechanism of colchicine/PCSK9 action remains to be further explored.

Conclusion

We set up a cell-based HTS screen assay for PCSK9 inhibitors and screened a NINDS compound library with 1041 compounds. We have identified a chemical hit-Colchicine in this assay and used western blot assay as a secondary assay to further confirm that colchicine inhibited PCSK9 mediated LDLR degradation, highlighting the potential of the novel use of this drug for cardiovascular disease treatment.

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