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# Zaragozic Acid A Enhances the Internalization of VLDL in Human Hepatocyte-Like C3A Cells

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## Abstract

High Low Density Lipoprotein (LDL) cholesterol levels are well-known to cause atherosclerotic diseases. The hepatic LDL receptor is the major determinant of plasma LDL-cholesterol levels, and as a result, a greater understanding of the regulatory mechanisms that control the expression and function of this gene is essential. Herein, we decided to examine whether the function of the LDL receptor increased in response to the cholesterol biosynthesis inhibitor, Zaragozic Acid A (ZA), which has been shown to significantly decrease serum cholesterol levels in rats. The results demonstrated that ZA increased the binding and internalization of Very Low Density Lipoprotein (VLDL), but not of LDL. This ZA-dependent increase in the internalization rate of VLDL was unrelated to an increase in the overall expression levels of the LDL receptor at the cell surface. These results suggest that ZA could reduce plasma cholesterol levels by preventing the synthesis of LDL from VLDL.

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## Introduction

Statins are considered the preferred treatment option to improve survival of individuals at risk of atherosclerotic related diseases [1-5]. Statins protect against atherosclerosis by decreasing serum low density lipoprotein (LDL)-cholesterol levels through up regulation of the LDL receptor pathway [5, 6]. It has been previously shown that treating rats with statins or other cholesterol biosynthesis inhibitors, such as Zaragozic Acid A (ZA), significantly increases hepatic LDL receptor mRNA levels and synthesis rate of LDL receptor protein [7, 8]. However, the steady-state levels of receptor protein remain unchanged [7, 8]. This apparent contradiction is explained by a corresponding increase in degradation rate of hepatic LDL receptor protein [8].

The LDL receptor removes LDL from the circulation through the pathway known as cycling of the LDL receptor [9]. This cycle leads to degradation of receptors and replacement of degraded LDL receptor molecules with newly synthesized receptors [9]. Thus, an induction in LDL receptor protein synthesis and degradation without altering the total number of receptor molecules present at the plasma membrane could be a

consequence of increased cycling (function) of the LDL receptor.

Since ZA has been shown to significantly reduce serum cholesterol levels in rats [8], suggesting an increase in receptor function, lipoprotein binding and internalization studies, in the presence and absence of ZA, were carried out to confirm this possibility. The internalization studies showed that ZA increased the internalization of VLDL but not LDL. These results suggest that ZA could reduce plasma cholesterol levels by preventing the synthesis of LDL from VLDL.

## Materials and Methods

### Materials

The cell lines, human hepatocyte-like C3A and mouse hybridoma secreting mouse C7 anti-LDL receptor antibody, were obtained from the American Type Culture Collection (Manassas, VA). Low Glucose (5.55 mM) Dulbecco's Modified Eagle's Medium (LG-DMEM), Fetal Bovine Serum (FBS), precast 4-20% SDS-PAGEs, and BODIPY®-LDL were purchased from Invitrogen (Carlsbad, CA). BD™ MITO + serum extender was from BD Biosciences (Sparks, MD). ZA from Merck and the rabbit anti-LDL receptor specific antibody were kindly provided by Dr. Gene C. Ness (Department of Molecular Medicine, University of South Florida, Tampa, FL). TRI Reagent was purchased from Molecular Research Center (Cincinnati, OH). The reverse transcriptase system and the SYBR green PCR Master Mix were obtained from Applied Bio systems (Foster City, CA). RIPA buffer, sulfo-NHS-SS-biotin, protein G/A magnetic beads, the BCA protein assay kit, Lane Marker Reducing Sample Buffer, and the Super Signal West Pico chemiluminescence substrate were purchased from Pierce (Rockford, IL). The mouse anti-proprotein convertase subtilisin kexin 9 (PCSK9) antibodies was from Cayman Chemicals (Ann Arbor, MI). Goat anti-actin and all the HRP-labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dil (3, 3'-dioctadecylindocarbocyanine)-VLDL was from Kalen Biomedical (Montgomery Village, MD). All other chemicals were purchased from Fisher Scientific or Sigma–Aldrich (St. Louis, MO).

### C3A cell culture

Cells were maintained at a density of $7 \times 10^8$ cells per 75-$cm^3$ flask in LG-DMEM supplemented with 10% FBS and antibiotics at 37°C under humidified atmosphere and 5% carbon dioxide. For experiments, cells were set in maintaining medium and incubated for 24 hours. Then, the medium was changed to a medium where FBS was replaced with 1 ml/L of BD™ MITO + serum extender (MITO+ medium). As previously reported, supplementing the medium with BD™ MITO + serum extender does not cause detectable morphological and/or growth changes in the C3A cells [10]. The MITO+ medium was used in these studies as a serum-free, lipid/cholesterol deficient medium [10]. Incubation in MITO+ medium was carried out for 24 hours. ZA (2.6 µM) was added at that point followed by an additional 24 hours incubation. The confluency of the cells at the time of harvesting was maintained between 70-85%. For cycloheximide experiments, in addition to treating with and without ZA, cells were incubated with 100 µM cycloheximide for 5 hours before harvesting. The dose and incubation time for cycloheximide were determined in optimization studies (data not shown). In some experiments, medium samples were collected prior to harvesting cells to determine secreted proprotein PCSK9 levels using ELISA as previously described [10].

### RNA preparation and quantitation using real-time PCR

Total RNA was prepared using TRI Reagent. RNA samples were DNase I treated and reverse transcribed using standard methods. Real-time PCR reactions were performed using 100 ng of ssDNA, the Applied Bio systems SYBR green PCR Master Mix, and the AB real-time PCR system (Applied Bio systems; Foster City, CA). Human LDL receptor, PCSK9, and 18s rRNA specific primers were obtained from SA Biosciences (Frederick, MD). The parameters for PCR were: denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 30 seconds. Quantitation was performed using the Comparative CT method [11].

### Preparation of RIPA proteins and Western Blotting Analysis

Lysates were prepared using ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphate inhibitors) according to the manufacturer's recommendations. Protein concentrations were determined using the BCA protein assay. Equivalent amounts of proteins were denatured in reducing sample buffer at 70°C for 5 minutes and subjected to electrophoresis on precast 4-20% SDS-PAGEs. Electro blotting onto nitrocellulose membranes, staining with 0.1% Ponceau S (in 5% acetic acid) to verify protein loading, and Western blotting analysis were performed at that point. Primary antibodies used herein were rabbit anti-LDL receptor (diluted 1:2000), mouse anti-PCSK9 (diluted 1:1000), and goat anti-actin (diluted 1:250; internal control). Primary antibodies bound to membranes were detected using HRP-labeled secondary antibodies and the Super Signal West Pico chemiluminescence substrate. Multiple exposures ranging from 0.5 s to 20 minutes and quantitation of the exposures were made using a Kodak Image Station 4000R Pro Imaging System and the Kodak Molecular Imaging software (New Haven, CT).

### Cell surface Biotinylation studies

After treating with ZA for 24 hours, cells were cooled on ice for 30 minutes to stop receptor cycling and washed three times with ice-cold PBS. Biotinylation of plasma membrane proteins was carried by incubating cells for 2 hours at 4°C with 0.8 mM of the membrane impermeable sulfo-NHS-SS-biotin (Sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3'-dithiopropionate) dissolved in PBS. At that point, cells were washed once with 100 mM Tris-HCl, pH 7.7, followed by incubation in a fresh aliquot of 100 mM Tris-HCl, pH 7.7, for 30 minutes at 4°C, to quench any residual biotin reagent. After washing twice in ice-cold PBS, cell extracts were prepared in immuno precipitation buffer (0.25 M sucrose, 0.05% SDS, 1% triton X-100, 0.5% sodium deoxycholate, 0.32 M NaCl, 2 mM $CaCl_2$, and protease inhibitors). Protein concentrations were measured using the BCA assay. Four hundred µg of cell lysates were incubated overnight at 4°C with 5 µg of mouse C7 anti-LDL receptor antibody in dilution buffer (1 mg/mL BSA, 0.1% triton X-100, 1 mM Tris-HCl, pH 8.0, 140 mM NaCl, and protease inhibitors). Next, 50 µl of protein G/A magnetic beads equilibrated in dilution buffer were added, and the samples were incubated for an additional 2 hours at room temperature. Beads were pulled to the side of the tube using a magnetic stand. After discarding solution, the beads were washed two times with dilution buffer and re suspended in 100 µl of 0.1 M Glycine pH 2.0. Elution of the protein/antibody complexes was completed by incubating at room temperature for 10 minutes. After applying magnetic field to pull beads, the solution was transferred to a fresh tube containing 15 µl of 1 M Tris-HCl pH 8.5. Western blotting analysis was carried out using avidin-HRP to determine biotinylated (plasma membrane) LDL receptor levels. Western blotting analysis using the rabbit anti-LDL receptor antibody was carried to correct for total LDL receptor proteins loaded into each well.

### BODIPY®-LDL and DiI-VLDL internalization studies

For this experiment, cells were plated in black opaque/clear bottom 96 well plate for fluorescent plate reading or standard 6 well plates for flow cytometry, both coated with poly-D-lysine as previously described [12]. After treating with ZA, cells were incubated on ice for 30 minutes to stop receptor cycling. At that point, 0.5 µg/ml of BODIPY®-LDL or 1.0 µg/ml of DiI-VLDL prepared in ice-cold MITO+ medium, supplemented with and without ZA depending on the original treatment, was added to some wells. Lipoprotein binding was allowed by incubating for 1 hour at 4°C. Binding data were collected at that point. For this, cells were washed twice with ice-cold PBS and either analyzed directly on the plate using a BMG Labtech PHERAstar™ fluorescent plate reader (ex/em: 485/520 nm for BODIPY®; ex/em: 554/571 nm for DiI) or suspended by gently scraping into PBS/2 mM EDTA followed by analysis in an Accuri C6 Flow Cytometer. This flow cytometer is equipped with 2 lasers, exciting probes at 488 and 640nm, and has fluorescence detection capabilities allowing simultaneous collection of 4-channel fluorescence data at wavelengths ranging from 510 to

780 nm. BODIPY®-fluorescence was detected using 488nm excitation and the FITC emission channel, FL1 (505 LP, 530/10 BP). DiI fluorescence was detected using 488nm excitation and the emission channel, FL2 (550 LP, 585/40 BP). The efficiency of excitation of DiI – labeled lipids using these cytometric conditions has been previously documented [13]. To determine internalization rates, after allowing lipoprotein binding for 1 hour at 4°C, the medium was replaced with warm (37°C) medium supplemented with fluorescent-labeled lipoproteins in the presence or absence of ZA depending on the original treatment. Incubation at 37°C to allow internalization was carried out for 15, 30, and 45 minutes. Washing with ice-cold PBS and analysis with either the fluorescent plate reader or the flow cytometer were carried out as described above. The percentage fluorescent cells were defined as the percentage of cells within each subpopulation with fluorescence intensity exceeding that of the maximum level of autofluorescence of unlabeled cells (background subtraction) in each experiment. For flow cytometry, mean fluorescence intensities were recorded for 10,000 events for each experiment.

### Statistical Analysis

Data from the individual parameters were compared by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test or t-test, when applicable, using the Graph Pad Prism 5 software (Graph Pad Software, Inc., La Jolla, CA). A $p<0.05$ was considered significant for all tests.

## Results

We started by examining whether the human hepatocyte-like C3A cell line was a good experimental model for the internalization studies. To accomplish this, cells were treated with and without ZA (2.6 μM) for 24 hours in MITO+ medium. After incubation, cells were employed in the preparation of RNA and RIPA protein samples to be analyzed by quantitative real-time PCR and Western blotting, respectively. PCSK9 was used herein as the positive control for these studies since up regulation in PCSK9 mRNA and protein expression in response to ZA treatment has been previously reported [14]. As shown in Figure 1A, ZA was able to enhance the mRNA expression of the LDL receptor and PCSK9 by 5.34- ($p<0.001$) and 5.05-fold ($p<0.01$), respectively. Figure 1B illustrates representative Western blots for LDL receptor, PCSK9, and the internal control actin. As shown in Figure 1C, no significant differences ($p=0.279$) in protein expression levels for the LDL receptor were observed in the presence or absence of ZA. As expected, intracellular expression of PCSK9 protein was increased 3.26-fold ($p<0.001$) as a result of ZA treatment (Figure 1C). ELISA analysis revealed that the secreted (medium) levels of PCSK9 were also increased 2.9-fold ($p<0.05$; $n=5$) due to ZA treatment (data not shown).

**Figure 1 - Ivaturi *et al.***

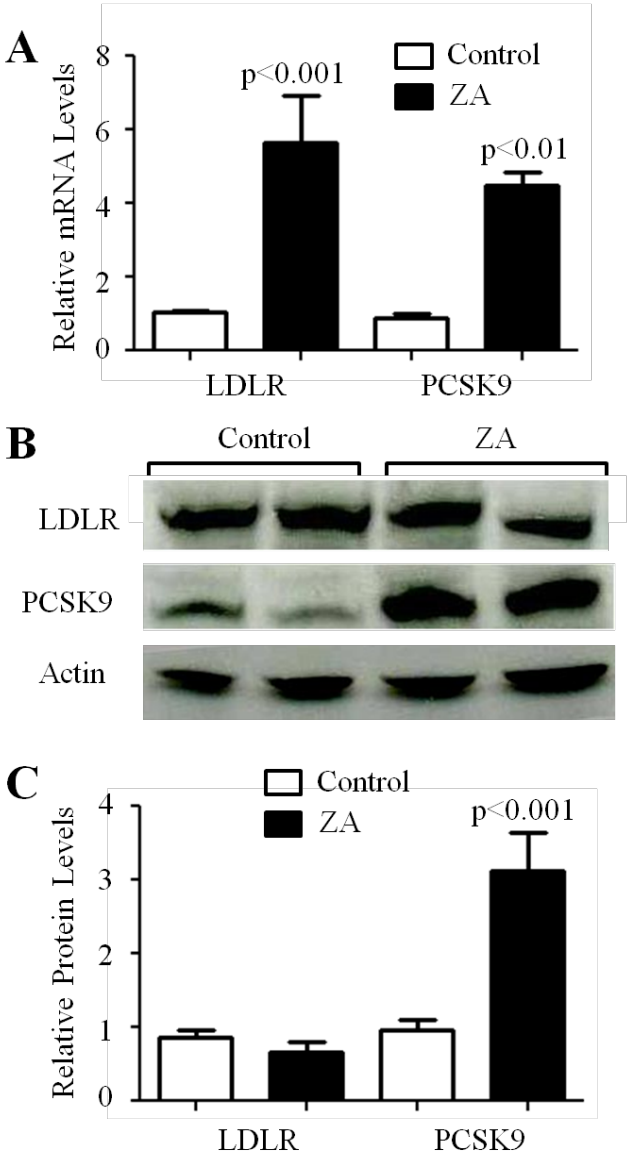


**Figure 1**: Validation of the human hepatocyte-like C3A cells for the internalization experiments. Cells were treated with 2.6 μM of Zaragozic Acid A (ZA) for 24 hours. RNA and protein samples were prepared and analyzed by real-time PCR (A) and Western blotting (B & C), respectively. (A) Data are presented as mean ± standard error of the mean (SEM; n=7). (B) Typical Western blots for LDL receptor, PCSK9, and actin (internal control) are shown. Twenty μg of total protein was loaded per lane. (C) Several blots were quantitated, and LDL receptor and PCSK9 signals were corrected using actin. Data represented as corrected mean ± SEM are from n=5. "p" values were obtained by comparing control and ZA groups for the same gene.

The findings about the LDL receptor expression shown in Figure. 1 coincides with previous reports showing that ZA is able to increase LDL receptor mRNA levels but not receptor protein expression [7, 8]. In animal studies, this is explained by a corresponding increase in the degradation rate of LDL receptor protein [7, 8]. Thus, the next step was to carry out cycloheximide studies to confirm the effects of ZA on human LDL receptor protein degradation. ZA treatment significantly reduced (23.5%; p=0.0364; n=3) the amount of human LDL receptor protein remaining at 5 hours in the presence of 100 μM cycloheximide as compared to control, indicating that the degradation rate of the LDL receptor protein was increased in the ZA treated cells (data not shown). These results suggested that a similar regulatory mechanism of the LDL receptor by ZA appears to be happening in the human C3A cells confirming that this cell line was a good in vitro model for studying the function of this receptor. Confirmation that the ZA treatment did not result in an increase in the number of LDL receptor molecules found at the cell surface (plasma membrane) was done using Biotinylation studies. We found no significant differences in the amount of LDL receptor protein present at the plasma membrane (p=0.9979; n=4) when comparing control and ZA samples (data not shown). These results suggested that if ZA increased the receptor function, the increase was not related to increased expression of the LDL receptor at the surface of cells.

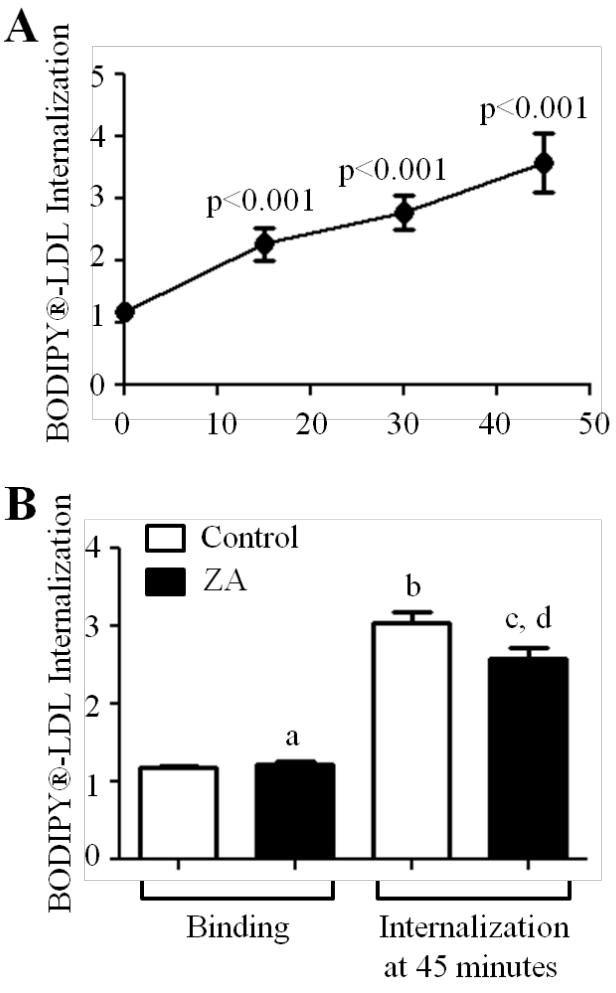
To determine whether the receptor function could be enhanced in the presence of ZA, binding and internalization studies using BODIPY®-LDL were performed. For this, C3A cells were treated with and without ZA (2.6 μM) in MITO+ medium for 24 hours as described above. BODIPY®-LDL binding at 4°C (also considered the 0 time point for the internalization) was done for 1 hour, and internalization at 37°C was performed for 15, 30 and 45 minutes. Based on previous studies, these time points will ensure at least 1 (1.25 exactly), 2 (2.5 exactly), and 3 (3.75 exactly) complete cycles, respectively, of the LDL receptor cycling [9]. Cells labeled with BODIPY®-LDL were detected as described under Materials and methods. The fluorescent values were first background subtracted using cells incubated with unlabeled LDL, and then represented as relative to the "0" time (set to 1).

Figure 2A shows a typical time course for BODIPY®-LDL internalization in control cells as measured using a fluorescent plate reader. As shown, the internalization of BODIPY®-LDL was linear during the time analyzed. The binding levels between control and ZA treated cells were similar (p=0.4092, n=8; Figure 2B), which correlated with the lack of induction in LDL receptor protein levels at the plasma membrane. Surprisingly, although there was internalization at 45 minutes in the presence of ZA, these levels were slightly lower (14.5%, p<0.01) than the levels seen in the control group at 45 minutes. No statistical significance differences were observed using flow cytometry (1.387 ± 0.262 for control vs. 1.453 ±0.174 for ZA; p=0.837) (data no shown). These data demonstrated that ZA treatment did not enhance the internalization of BODIPY®-LDL in C3A cells.

**Figure 2**: Effects of ZA on binding and internalization of BODIPY®-LDL in C3A cells. Treatment with ZA was carried out for 24 hours. Labeling with BODIPY®-LDL and analysis using a fluorescent plate reader. Data were expressed as relative BODIPY®-LDL internalization levels (mean ± SEM), where the value for the "0" time point (BODIPY®-LDL binding) was set to 1.0. (A) Time course for BODIPY®-LDL internalization (mean ± SEM; n=8 per time point) in control (untreated) cells.

"p" values obtained by comparing to the "0" time point are shown. (B) BODIPY®-LDL binding at 4°C and internalization levels after 45 minutes for control and ZA samples (mean ± SEM) for n=8 for all treatments. “a” refers to p=0.4092, when comparing binding levels for control and ZA samples. “b” refers to p<0.001, when comparing binding and internalization levels for control samples. “c” refers to p<0.001, when comparing binding and internalization levels for ZA samples. "d" refers to p<0.01, when comparing internalization levels for control and ZA samples.

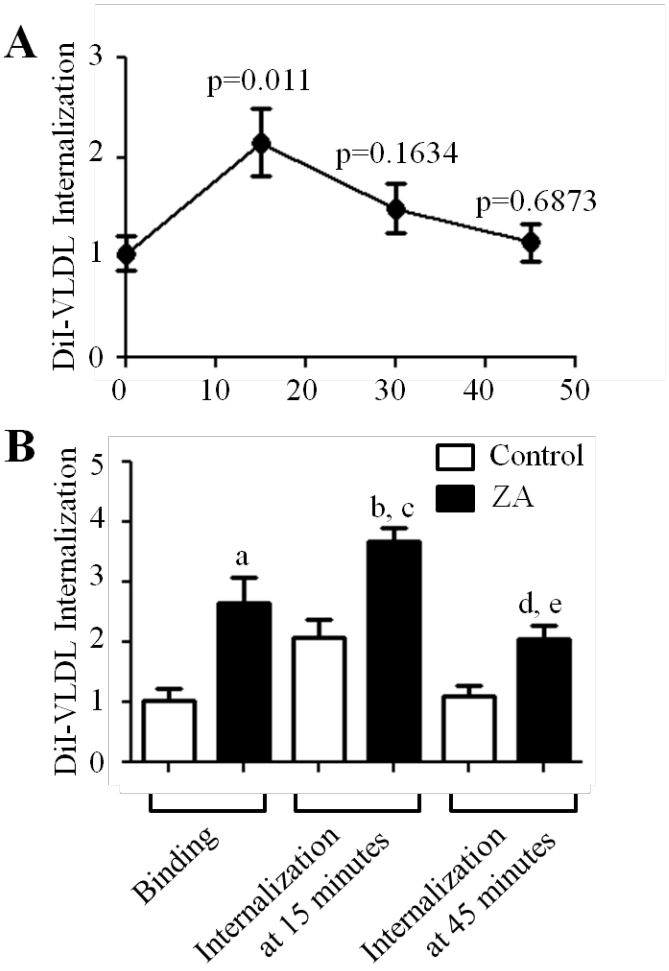
**Figure 2 - Ivaturi *et al.***




Although the results from the Bodipy®-LDL internalization studies agreed with those from the Western blotting analysis and the Biotinylation assay reported herein, as well as with published findings in animal studies related to the steady-state levels of the LDL receptor [7, 8], they did not correlate with the observations in rats demonstrating that ZA is able to decrease serum cholesterol levels without affecting receptor protein expression [7]. It is well-known that LDL is not the only lipoprotein removed by the LDL receptor. Other lipoproteins removed by this receptor are chylomicron remnant, VLDL, and βVLDL [15, 16]. In addition, it has been demonstrated that LDL arises from the metabolism of VLDL in the serum [16]. Accordingly, binding and internalization studies in the presence of DiI-VLDL were also carried out. Once again, cells were incubated with and without ZA (2.6 μM) for 24 hours before the addition of 1.0 μg/ml DiI-VLDL. As for BODIPY®-LDL, the rates of binding at 4°C for 1 hour and then internalization at 37°C for 15, 30, and 45 minutes were measured. The results are shown in Figure 3. Unlike for Bodipy®-LDL, in control (untreated) samples, the internalization of DiI-VLDL significantly increased at 15 minutes (2.06-fold; p=0.011) but then declined progressively (Figure 3A). Although a similar pattern was seen for ZA, the binding and internalization levels of DiI-VLDL in the ZA treated samples were significantly higher than the levels seen in the control samples, at every time point. As illustrated in Figure 3B, at 15 minutes, cells treated with ZA internalized 1.79-fold more DiI-VLDL (p=0.001; n=8) than control cells. At 45 minutes, once again, ZA treated cells internalized 1.87-fold more DiI-VLDL (p=0.0033; n=8) than control cells. Similar results were obtained using flow cytometry (data not shown). These results clearly demonstrated that treating with ZA resulted in increased internalization of DiI-VLDL in C3A cells.

**Figure 3**: Effects of ZA on binding and internalization of DiI-VLDL in C3A cells. Once again, treatment with ZA was carried out for 24 hours. Labeling with DiI-VLDL and analysis using a fluorescent plate reader. Data were expressed as relative DiI-VLDL internalization levels (mean ± SEM), where the value for the "0" time point (DiI-VLDL binding) was set to 1.0. (A) Time course for DiI-VLDL internalization (mean ± SEM; n=8 per time point) in control cells. "p" values obtained by comparing to

the "0" time point are shown. (B) DiI-VLDL binding at 4°C and internalization levels after 15 and 45 minutes for control and ZA samples (mean ± SEM; n=8 for all treatments). “a” refers to p=0.0033, when comparing binding levels for control and ZA samples. “b” refers to p=0.001, when comparing internalization levels at 15 minutes for control and ZA samples. “c” refers to p=0.0463, when comparing binding and internalization levels at 15 minutes for ZA samples. "d" refers to p=0.0033, when comparing internalization levels at 45 minutes for control and ZA samples. "e" refers to p=0.2187, when comparing when comparing binding and internalization levels at 45 minutes for ZA samples.




## Discussion

Herein, we demonstrated for the first time that treating human hepatic C3A cells with the cholesterol biosynthesis inhibitor ZA resulted in an increased internalization of VLDL, but not of LDL. These data suggest that ZA acts mainly by preventing LDL synthesis. The reduction in LDL synthesis could explain the ZA-dependent decrease in serum cholesterol levels seen in the animal studies [7, 8]. It will be interesting to examine whether other anti-cholesterolemic drugs, such as statins, act in a similar fashion.

Since the steady-state levels of LDL receptor protein were unchanged in response to ZA treatment, it could be assumed that the receptor cycling rate (function) was increased in order to internalize more VLDL. However, there is one critical question that needs to be answered, which is how could the cell discriminate between the two lipoprotein particles? Once possible answer to this question is that the LDL receptor was located within a plasma membrane micro domain that favors the binding and internalization of VLDL over LDL in response to ZA. Interestingly, despite that the levels of cell surface LDL receptors were the same in both treatment groups, ZA treatment led to higher VLDL binding than the binding seen in untreated cells. LDL binding was comparable in both treatment groups.

According to several studies, the LDL receptor could internalize different lipoprotein particles via diverse pathways [17-19]. The efficient internalization of LDL particles, for example, has been shown to require the acid-dependent mechanism of lipoprotein release in the endosome and the presence of an intact LDL receptor adaptor protein-1 (LDLRAP1), factors that are not required for the internalization of VLDL and/or β-VLDL [17, 18]. This suggests that the removal of LDL by the receptor needs an intact clathrin-dependent internalization pathway, whereas internalization of VLDL can occur through an alternative pathway like caveolae. In fact, it has been shown that mice lacking caveolin-1 have elevated levels of VLDL but not of LDL supporting this hypothesis [19]. We have previously demonstrated that C3A

cells grown in MITO+ medium have more LDL receptors in the clathrin-coated pits than in caveolae and that ZA treatment in rats increases the distribution of the LDL receptor towards the clathrin-coated pits [20]. This clearly explains the increased efficiency of the LDL receptor in removing lipoprotein particles but not the ability of the cells to internalize more VLDL than LDL.

Another possibility could be that the cells secreted a protein/factor (known or unknown) that modulates the binding of specific lipoproteins to the receptor. As shown herein, PCSK9 levels were significantly elevated in cells treated with ZA as compared to control cells. Thus, it might possible that PCSK9 is this factor that leads to higher binding and internalization of VLDL in cells treated with ZA. In fact, it has been reported that PCSK9 is able to interact with LDL but not with VLDL, both in vitro and in vivo [21]. Additional studies are required to determine the exact mechanism involved in the differential internalization of lipoprotein particles in hepatic cells treated with ZA.

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