

## Activation of naturally occurring LCAT mutants by a novel activator compound

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### **List of non-common abbreviations**

apoB-D, apolipoprotein B-depleted

CE, cholesteryl esters

CER, cholesterol esterification rate

DS, Daiichi Sankyo

FLD, familial LCAT deficiency

LCAT, lecithin:cholesterol acyltransferase

LOF, loss of function

rHDL, reconstituted HDL

rhLCAT, recombinant human LCAT

MD, molecular dynamics

UC, unesterified cholesterol

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## ABSTRACT

Lecithin:cholesterol acyltransferase (LCAT) is the unique plasma enzyme able to esterify cholesterol and plays an important role in HDL maturation and promotion of reverse cholesterol transport. Familial LCAT deficiency (FLD, OMIM #245900) is a rare recessive disease due to loss of function mutations in the *LCAT* gene, for which there is no cure. In this study, we assessed the *in vitro* efficacy of a novel small molecule LCAT activator. Cholesterol esterification rate (CER) and LCAT activity were tested in plasma from 6 controls and 5 FLD homozygous carriers of various LCAT mutations at different doses of the compound (0.1, 1 and 10 µg/mL). In control plasma, the compound significantly increased both CER ( $P < 0.001$ ) and LCAT activity ( $P = 0.007$ ) in a dose dependent manner. Both CER and LCAT activity increased by 4-5 fold, reaching maximum activation at the dose of 1 µg/mL. Interestingly, DS compound produced an increase in CER in 2 of the 5 tested LCAT mutants (Leu372---Arg and Val309---Met), while LCAT activity increased in 3 LCAT mutants (Arg147---Trp, Thr274---Ile and Leu372---Arg); mutant Pro254---Ser was not activated at any of the tested doses. The present findings pose the basis for personalized therapeutic interventions in FLD carriers and support the potential LCAT activation in secondary LCAT defects.

## SIGNIFICANCE STATEMENT

We characterized the pharmacology of a novel small-molecule LCAT activator *in vitro* on a subset of naturally occurring LCAT mutants. Our findings pose the basis for personalized therapeutic interventions in FLD carriers, who can face severe complications and for whom no cure exists.

## Introduction

Familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD, OMIM #245900) is a rare autosomal recessive disorder due to loss of function (LOF) mutations in the *LCAT* gene. In homozygous and compound heterozygous carriers, the lack of LCAT catalytic activity is complete, thereby affecting the ability of the enzyme to esterify free cholesterol on all plasma lipoproteins (Calabresi et al. 2012). As a consequence, cholesteryl esters (CE) are virtually absent in plasma. Clinical manifestations of FLD include corneal opacity, haemolytic anaemia, proteinuria and renal disease (Calabresi et al. 2012). Of note, glomerulosclerosis is the major cause of morbidity and mortality in FLD cases and may ultimately lead to renal failure in early life (from 30 years of age onwards) (Santamarina-Fojo et al. 2001). Renal transplantation represents an option in severe cases with end-stage renal disease, but the disease can rapidly reoccur in the transplanted kidney within only a few years (Strom et al. 2011). Hence, there is no cure for FLD, and the present treatment consists on current available therapies mainly to delay the evolution of chronic nephropathy. Enzyme replacement therapy with recombinant human LCAT (rhLCAT) is the most clinically advanced therapy for FLD (Shamburek et al. 2016a, Shamburek et al. 2016b), but it is expensive, it requires intravenous infusion and can cause immunogenicity in treated patients, which is a relevant issue in chronic treatments. Small-molecule activators able to partially restore enzyme activity in LCAT deficient patients represents a possible alternative to rhLCAT. Compound A is a previously described small-molecule, which proved to activate LCAT *in vitro* (Chen et al. 2012, Freeman et al. 2017) and to increase plasma CE and HDL-cholesterol levels in mice and hamsters (Chen et al. 2012). A novel class of reversible piperidinylpyrazolopyridine and piperidinylimidazopyridine activators were reported to activate human LCAT (Patent n. US9796709B2), and to increase HDL-cholesterol when administered to cynomolgus monkeys (WO2015/087996). Activators from this series have been shown to bind membrane binding domain in LCAT, without altering HDL binding capacity (Manthei et al. 2018). The purpose of the current study was to characterize the pharmacology of a novel LCAT activator (DS compound, Daiichi Sankyo) *in vitro* on a subset of naturally occurring LCAT mutants.

## Materials and methods

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

**Subjects.** Five homozygous carriers (2 males and 3 females, age  $43 \pm 6$  y) of different *LCAT* mutations, belonging to the Italian cohort (Calabresi et al. 2005, Calabresi et al. 2012), and 6 healthy donors (control subjects; 3M/3F, age  $41 \pm 17$  y) were asked to participate in the study. All the subjects were fully informed of the modalities and end-points of the study and

signed an informed consent. The study was approved by the Ethical Committee of the University of Milan (Approval no. 43/17) and conformed to the guidelines set out in the Declaration of Helsinki.

**Activator.** DS compound (International publication number: WO2015/087994) (Figure 1) was synthesized and stored at  $-20^{\circ}\text{C}$ , protected from light. The compound was solubilized in DMSO prior to use.

**Biochemical analyses.** Blood samples were collected in EDTA-tubes after an overnight fast and plasma separated by low-speed centrifugation at  $4^{\circ}\text{C}$ . Plasma total cholesterol, HDL-cholesterol, triglyceride and apolipoprotein levels were determined with certified methods using a Roche Integra c311 autoanalyzer (Roche Diagnostics). LDL-cholesterol was calculated using Friedewald's formula. When triglycerides  $> 400$  mg/dL, LDL-cholesterol were assessed using direct measurement. Unesterified cholesterol (UC) was determined by using a previously described enzymatic technique (Ossoli et al. 2019). Plasma LCAT concentration was measured by a specific competitive enzyme-linked immunoassay (Murakami et al. 1995). Plasma cholesterol esterification rate (CER) and LCAT activity, that reflect the ability of endogenous LCAT to esterify cholesterol within endogenous lipoproteins and exogenous HDL, respectively, were assessed by using previously described methods (Calabresi et al. 2005). LCAT activity and CER in plasma of controls and FLD subjects were assessed in presence or absence of the DS compound at 3 different doses (0.1, 1 and 10  $\mu\text{g}/\text{mL}$ ). The apolipoprotein B - depleted (apoB-D) plasma was obtained from the whole plasma by precipitating the apoB-containing lipoproteins with a polyethylene glycol (PEG) solution as already described (Arnaboldi et al. 2020). Shortly, whole plasma was incubated for 20 min with a 20% polyethylene glycol 6000 solution. After sample centrifugation at 10,000 rpm, the supernatant was collected and stored at  $4^{\circ}\text{C}$ .

LCAT activity on apoB-D plasma from controls and FLD subjects was tested only at the dose of 10  $\mu\text{g}/\text{mL}$  of activator, which was confirmed to be the enzyme activating dose in LCAT deficient plasma. All substrates were assayed in triplicate, with the average used for subsequent analysis.

**Molecular modelling and simulation analyses.** The initial LCAT::DS-compound complex was generated using the crystallographic structure published by Manthei et al (2018) (PDB ID: 6MVD), in which LCAT is co-crystalized with a drug analogue of DS compound. DS compound was then superposed on the crystalized ligand and the complex was refined with an all-atom minimization to convergence. DS compound binding site was also validated by probing the entire LCAT surface with molecular docking (see supplementary material). Molecular dynamics (MD) simulations were run to assess the impact of DS compound on LCAT dynamic and structural features, comparing two 500 ns long simulations run in the presence or absence of the ligand of interest. LCAT modelling,

simulations and analyses were performed using the Schrödinger Small Molecule Drug Discovery Suite 2020-2 with the OPLS3e force field (Schrödinger, LLC, New York, NY, 2020).

**Statistical analyses.** Data are presented as mean $\pm$ SD, unless otherwise stated. Changes vs. baseline are presented as absolute difference in activity. Comparisons between different activator doses in control subjects were performed by one-way ANOVA for repeated measures followed by Bonferroni correction. Tests were two-sided and *P* values < 0.05 were considered as statistically significant. Statistical analysis was performed using SPSS version 24.0 software (SPSS Inc., Chicago, USA).

## Results

Plasma lipid levels in the examined subjects are summarized in Table 1. FLD carriers enrolled in the present study displayed the peculiar lipid profile described in homozygous FLD carriers (Calabresi et al. 2012). HDL-cholesterol, apolipoprotein A-I, and apolipoprotein B levels were dramatically reduced in all carriers, while triglyceride levels were increased. Plasma UC levels were increased and on average 3 times higher in carriers compared to controls. As expected, CER and LCAT activity were null in all carriers. LCAT mass was reduced but measurable in all FLD carriers (Table 1).

**DS compound increases LCAT activity in plasma of control subjects.** DS compound significantly increased both CER and LCAT activity in all tested control plasma samples, already at the lower dose. Indeed, the mean basal CER of 44.1 $\pm$ 16.3 nmol/mL/h doubled to 86.1 $\pm$ 24.9 nmol/mL/h (*P*<0.001) at the dose of 0.1  $\mu$ g/mL. The activator leads to three times increase in CER at the dose of 1  $\mu$ g/mL, up to 121.5 $\pm$ 43.0 nmol/mL/h (+166.2 $\pm$ 77.0%, *P*<0.001). No further activation was observed at the dose of 10  $\mu$ g/mL, with CER remaining stable at 122.1 $\pm$ 42.8 nmol/mL/h (+199.8 $\pm$ 67.0% nmol/mL/h, *P*<0.001) (Figure 2a). In the same way, the mean basal LCAT activity in control plasma increased from 35.6 $\pm$ 16.3 nmol/ml/h to 92.5 $\pm$ 50.6 nmol/mL/h (+159.2 $\pm$ 70.9%, *P*=0.035) at the dose of 0.1  $\mu$ g/mL, and to 140.1 $\pm$ 72.3 nmol/mL/h (+295.2 $\pm$ 106.4%, *P*=0.007) at the dose of 1  $\mu$ g/mL. Again, no further activation was observed at the dose of 10  $\mu$ g/mL (+245.7 $\pm$ 110.7%, *P*=0.012) (Figure 2b). Specific LCAT activity, calculated dividing LCAT activity by plasma LCAT concentration, increased from 5.4 mmol/ $\mu$ g/h to 18.6 mmol/ $\mu$ g/h at the maximum tested dose. These results clearly show that the DS compound is a potent activator of human LCAT in a dose dependent manner, reaching a plateau at the dose of 1  $\mu$ g/mL (Figure 2).

In addition, to verify the effect of DS activator on endogenous HDL, we tested apoB-D plasma as substrate instead that synthetic HDL. DS compound at the tested dose of 10

$\mu\text{g/mL}$  increased LCAT activity from  $31.5 \pm 3.5$  nmol/mL/h to  $55.4 \pm 1.3$  nmol/mL/h (+76.8%, Supplemental Figure 1).

**DS compound stabilizes the LCAT lid-loop.** The analysis of protein::ligand interactions during the MD simulation highlighted key residues involved in the molecular recognition mechanism of DS compound by LCAT. In particular, persistent hydrogen bonds (> 90% of the simulated time) form between DS compound and Asp63, Asn78 and Try51, including also a  $\pi$ - $\pi$  stacking with the DS condensed rings, while Leu68 and Cys74 contribute with hydrophobic interactions. The dynamic behavior of residues of the apo and the complexed (with DS compound) forms of LCAT show that the mobility of DS compound binding pocket remains unaltered, while residues 45-47 of the membrane binding domain gain increased mobility; moreover, the lid loop, responsible of regulating the accessibility of substrates to LCAT binding site, is much more stable in an open conformation when DS compound is bound to LCAT (Figure 3; Supplemental Figures 2 and 3).

**DS compound increases LCAT activity for some naturally occurring LCAT mutants.** The ability of DS compound to activate LCAT mutants, all showing complete enzymatic defect, was tested in plasma from 5 FLD subjects carrying different LCAT mutations (Table 2, Figure 4). Mutant Arg147---Trp was activated by DS compound at the dose of 10  $\mu\text{g/mL}$ , with LCAT activity increasing from 0 to 26.6 nmol/mL/h; on the contrary CER remained null at all tested doses. Specific LCAT activity of the Arg147---Trp mutant reached a value of 14.7 mmol/ $\mu\text{g/h}$ , similar to that reached by wild-type LCAT. Mutant Thr274---Ile was also activated by the DS compound; at the dose of 0.1  $\mu\text{g/mL}$  LCAT activity increased to 31.2 nmol/mL/h, and the activation was maintained at the higher doses (27.5 and 34.4 nmol/mL/h, respectively). Specific LCAT activity of the Thr274---Ile mutant reached a value of 47.1 mmol/ $\mu\text{g/h}$  at the highest dose of the activator, thus showing that activation was independent of LCAT mass. Also for this mutant, CER remained null at all tested doses. Trying to clarify these apparently conflicting results, we tested the ability of the DS compound to activate the two LCAT mutants measuring LCAT activity on endogenous HDL (apoB-D plasma). As shown in Supplemental Figure 1, no activation was observed, thus suggesting that the DS compound is able to activate the mutant enzyme leading to cholesterol esterification on exogenous (synthetic) HDL, having no effect on endogenous lipoproteins (both apoB- and apoA-I-containing). Mutant Leu372---Arg was activated by the DS compound at the highest tested dose, leading to cholesterol esterification on all tested substrates (endogenous and exogenous lipoproteins). Specific LCAT activity of the Leu372--Arg mutant reached a value of 5.7 mmol/ $\mu\text{g/h}$  at the highest dose of the activator. In addition, when apoB-D plasma was tested, LCAT activity increased to 9.2 nmol/mL/h (Supplemental Figure 4). The Val309---Met mutant was also activated at the highest tested dose of the DS compound. While CER increased up to 20.3 nmol/mL/h, LCAT activity did not

change (Table 2, Figure 4), thus suggesting that only the beta-activity of LCAT is increased. Unfortunately, no more sample was available from this subject to test the apoB-D plasma. Mutant Pro254---Ser was not activated by DS compound at any of the tested doses, despite a plasma concentration similar to that of other tested mutants (Table 2, Figure 4).

## Discussion

FLD is a rare hereditary disease with high morbidity and no treatment. Phenotypic manifestations could be highly heterogeneous depending on the underlying mutation, and metabolic and environmental factors. The use of rhLCAT has been previously investigated as potential therapy and it has been demonstrated to be safe and well tolerated (Shamburek et al. 2016a); however, a recombinant protein is expensive and requires intravenous infusions, which makes demanding the chronic use. In this context, small-molecule LCAT activators could represent a better treatment option, proved that they are able to activate LCAT mutants causing FLD. The therapeutic use of small molecules offers several advantages to patients, including the oral administration route and no need for inpatient treatment, leading to an improved quality of life and lower social costs. Previously tested Compound A, the first developed LCAT activator, has already been shown to increase LCAT activity and raise HDL-cholesterol levels in the plasma of wild-type mice and hamsters (Chen et al. 2012). When tested *in vitro* using plasma of FLD carriers it was able to activate 1 out of 4 naturally occurring LCAT mutants.

Here we have tested a novel compound, taking advantage of the availability of plasma samples from a large cohort of carriers of *LCAT* mutations (Calabresi et al. 2005, Calabresi et al, 2012). To better characterize the effect of the compound on the esterification process, we measured both CER and LCAT activity. While the first reflects the ability of LCAT to esterify cholesterol on endogenous lipoproteins (HDL and apoB-containing particles), the latter measures the esterification on synthetic HDL (Calabresi et al. 2005). DS compound enhanced activity of wild-type LCAT up to 5.6-fold when measured as CER and up to 4.2-fold in LCAT activity, thus demonstrating efficacy in augmenting LCAT ability to esterify cholesterol in all substrates, already at the dose of 1  $\mu\text{g}/\text{mL}$ . According to our molecular modeling data, DS compound activity is related to its ability to stabilize the LCAT lid-loop in an open conformation, and to enhance the hydrophobicity of the LCAT membrane-binding domain, thus facilitating its interaction with lipids. These data allow us to hypothesize a potential increase of the LCAT  $V_{\text{max}}$  without significantly affecting the  $K_m$ , as described also for compound A (Freeman et al. 2017).

The efficacy of the compound to activate naturally occurring LCAT mutants was then tested selecting FLD homozygous carriers belonging to the large Italian cohort. DS compound produced an increase in CER in 2 of the 5 tested FLD mutations, while LCAT

activity increased in 3 FLD mutations. Only one of the tested mutants, the Pro254---Ser, which introduces a hydrophilic residue within a pack of hydrophobic residues close to the lid loop region likely disrupting LCAT fold, was not activated by DS compound in any experimental conditions, thus likely excluding carriers of this variant from benefit from this activator. DS compound activated similarly Arg147---Trp and Thr274---Ile mutants, by increasing LCAT activity and not CER, thus suggesting that the DS compound activates the ability of the mutant LCAT to esterify cholesterol only on synthetic HDL, having no effect on endogenous lipoproteins. This was confirmed by the absence of esterification activity on apoB-D plasma (endogenous HDL) of the same carriers. Circulating HDL particles are largely heterogenous, containing different phospholipids and proteins. Although synthetic HDL resemble nascent HDL particles, they are not representative of the complex diversity of circulating HDL and contain only apoA-I and a single PL species. Mutation Arg147---Trp is in a region involved in phospholipid binding in active site. Thr274---Ile, despite being far from LCAT active site, has been instead predicted to associate with a steric bulk which causes a local rearrangement decreasing lipoprotein binding area (Sensi et al. 2014). Among tested LCAT mutants, Leu372---Arg, which falls in a region likely involved in apoA-I recognition (Manthei et al. 2018), was the only mutant to be activated in all tested conditions (CER, LCAT activity on synthetic HDL, and LCAT activity on apoB-D plasma). DS compound at the dose of 1 µg/mL was already able to boost LCAT activity on a simple form of HDL, as synthetic HDL, while higher doses were required to activate the more complex plasma lipoproteins (apoB-D plasma). The Val309---Met mutant, a mutation far from the active site and from lipoprotein binding site, was activated only on endogenous substrates, already at the lowest dose of the DS compound, while no effect of the activator was observed on synthetic HDL. This suggest that only the activity on apoB-containing lipoproteins (VLDL/LDL), but not on HDL, is increased. Whether this may be beneficial or not needs to be further addressed by *in vivo* studies. Indeed, evidences from LCAT deficient carriers support the observation that increasing cholesteryl esters in VLDL/LDL is not beneficial, since it would possibly increase their atherogenic properties (Oldoni et al. 2018). On the other hand, it will remove unesterified cholesterol from the circulation and possibly reverse renal disease in FLD subjects, by reducing the formation of a nephrotoxic lipoprotein, called lipoprotein X. LpX is enriched in free cholesterol and was found to accumulate in the kidney, where it induces the peculiar glomerular damage (Ossoli et al. 2016, O et al. 1997).

In conclusion, the DS compound is a novel LCAT activator able to increase in a dose dependent manner LCAT ability to esterify cholesterol within all substrates. The use of an LCAT activator could be beneficial in conditions characterized by secondary LCAT defects, such as acute coronary syndrome (Ossoli et al. 2019), and chronic kidney disease (Calabresi et al. 2015, Baragetti et al. 2020). Interestingly, the compound can also activate

some naturally occurring LCAT mutants, rendering them able to esterify cholesterol in some or all lipoprotein substrates. Animal studies are required to prove that the activation of LCAT mutants by the DS compound will lead to a reduction in circulating unesterified cholesterol, which is the goal of therapy in FLD patients. The present results thus pose the basis for a personalized therapeutic intervention in FLD carriers, who can face severe clinical complications and for whom no cure exists.

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### Authorship Contribution

*Participated in research design:* Calabresi, Eberini, Ossoli, Pavanello, Kono, Yamada, Kiyosawa

*Conducted experiments:* Laurenzi, Turri, Simonelli, Strazzella

*Performed data analysis:* Pavanello, Simonelli

*Wrote or contributed to the writing of the manuscript:* Eberini, Pavanello, Ossoli, Turri, Calabresi, Kiyosawa

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## Figure legends

**Fig. 1. Chemical structure of DS compound.**

**Fig. 2. Dose-response for CER (a) and LCAT activity (b) in control plasma.** CER and LCAT activity were measured in plasma from n=6 control subjects before (baseline) and after incubation with DS compound at different doses. The differences in the mean values among the treatment doses were analyzed by one-way ANOVA for repeated measures, followed by Bonferroni correction for multiple comparisons. Data are expressed as mean±SD. \*\**P* <0.01, \**P* <0.05

**Fig. 3. Comparison between LCAT apo and DS-compound bound forms.** The root mean squared fluctuation (RMSF) values of LCAT residues during MD simulations are reported. RMSF is a measure of residues overall mobility. Residues interacting with DS compound and the lid-loop are highlighted.

**Fig. 4. Activation of CER (a) and LCAT activity (b) in FLD plasma.** CER and LCAT activity were measured in plasma from n=5 FLD patients (baseline CER and LCAT activity = 0 nmol/mL/h for all) after incubation with DS compound at the dose of 10 µg/mL. Data are expressed as absolute increase.

TABLE 1

Characteristics of controls and FLD carriers.

| Laboratory parameters            | Controls<br>(n=6) | Arg147--Trp | Thr274--Ile | Leu372--Arg | Pro254--Ser | Val309--Met |
|----------------------------------|-------------------|-------------|-------------|-------------|-------------|-------------|
| Total Cholesterol (mg/dL)        | 160.8±33.2        | 74          | 216         | 62          | 243         | 140         |
| Unesterified Cholesterol (mg/dL) | 46.2±9.5          | 74          | 193         | 62          | 191         | 124         |
| LDL Cholesterol (mg/dL)          | 80.0±23.0         | 44          | 156         | 26          | 104         | 56          |
| HDL Cholesterol (mg/dL)          | 65.2±14.6         | 13          | 4           | 16          | 7           | 7           |
| Triglycerides (mg/dL)            | 77.0±24.9         | 85          | 278         | 98          | 658         | 337         |
| Apolipoprotein A-I (mg/dL)       | NA                | 37          | 51          | 20          | 38          | 30          |
| Apolipoprotein B (mg/dL)         | NA                | 47          | 58          | 25          | 108         | 56          |
| CER (nmol/mL/h)                  | 44.1±16.3         | 0           | 0           | 0           | 0           | 0           |
| LCAT activity (nmol/mL/h)        | 35.6±16.3         | 0           | 0           | 0           | 0           | 0           |
| LCAT concentration (µg/mL)       | 5.4±0.8           | 1.7         | 0.8         | 2.7         | 1.0         | 0.9         |

Values are expressed as mean±SD. NA, not available

TABLE 2  
CER and LCAT activity in FLD plasma before and after incubation with DS compound.

| Mutation  | CER<br>(nmol/mL/h) |             |        |         | LCAT activity<br>(nmol/mL/h) |             |        |         |
|-----------|--------------------|-------------|--------|---------|------------------------------|-------------|--------|---------|
|           | Baseline           | DS compound |        |         | Baseline                     | DS compound |        |         |
|           |                    | 0.1µg/mL    | 1µg/mL | 10µg/mL |                              | 0.1µg/mL    | 1µg/mL | 10µg/mL |
| Arg147Trp | 0.0                | 0.0         | 0.0    | 0.0     | 0.0                          | 0.0         | 0.0    | 25.2    |
| Thr274Ile | 0.0                | 0.0         | 0.0    | 0.0     | 0.0                          | 25.8        | 27.6   | 37.7    |
| Leu372Arg | 0.0                | 0.0         | 0.0    | 24.1    | 0.0                          | 0.0         | 15.5   | 15.5    |
| Pro254Ser | 0.0                | 0.0         | 0.0    | 0.0     | 0.0                          | 0.0         | 0.0    | 0.0     |
| Val309Met | 0.0                | 21.7        | 10.6   | 20.3    | 0.0                          | 0.0         | 0.0    | 0.0     |

Figure 1

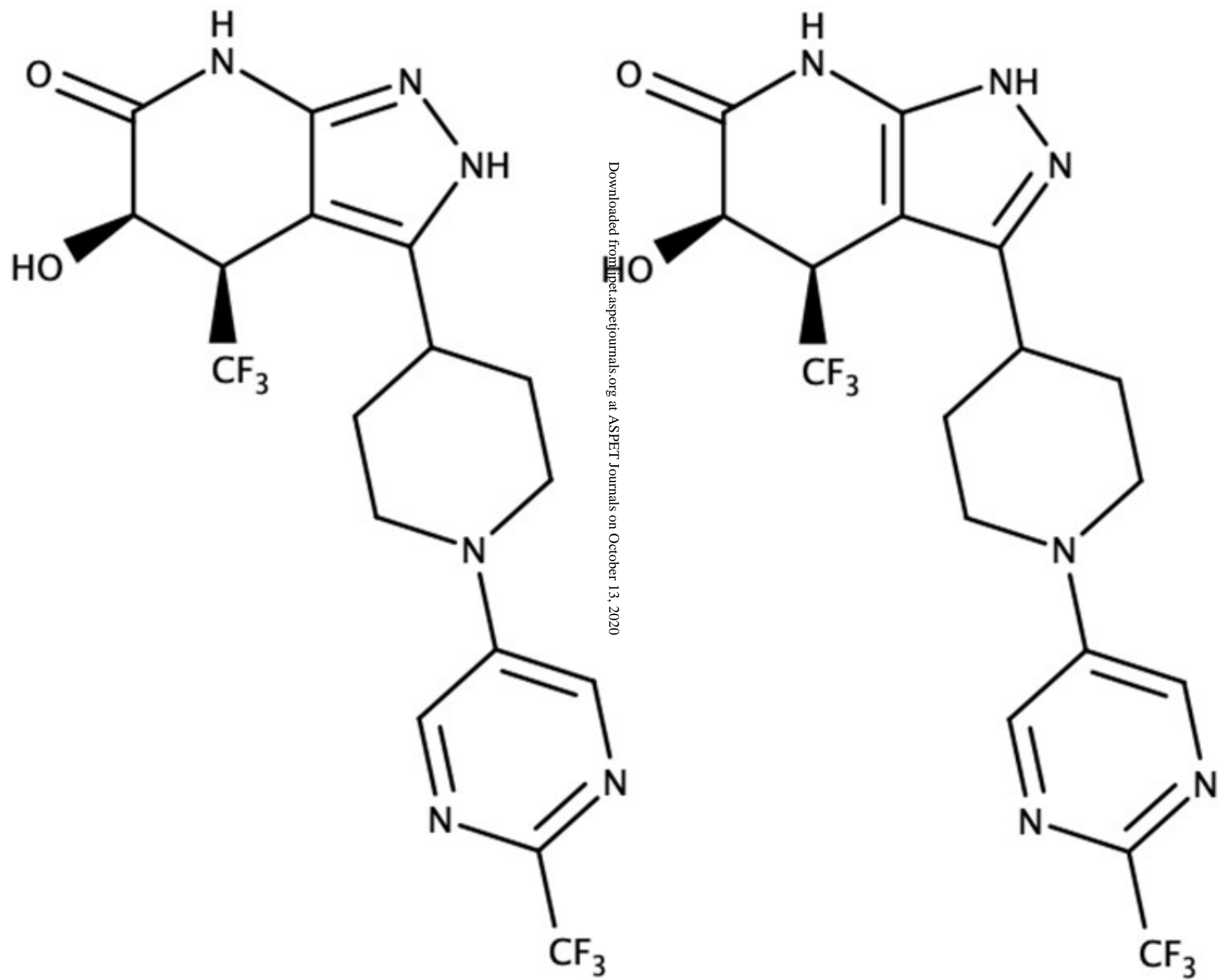
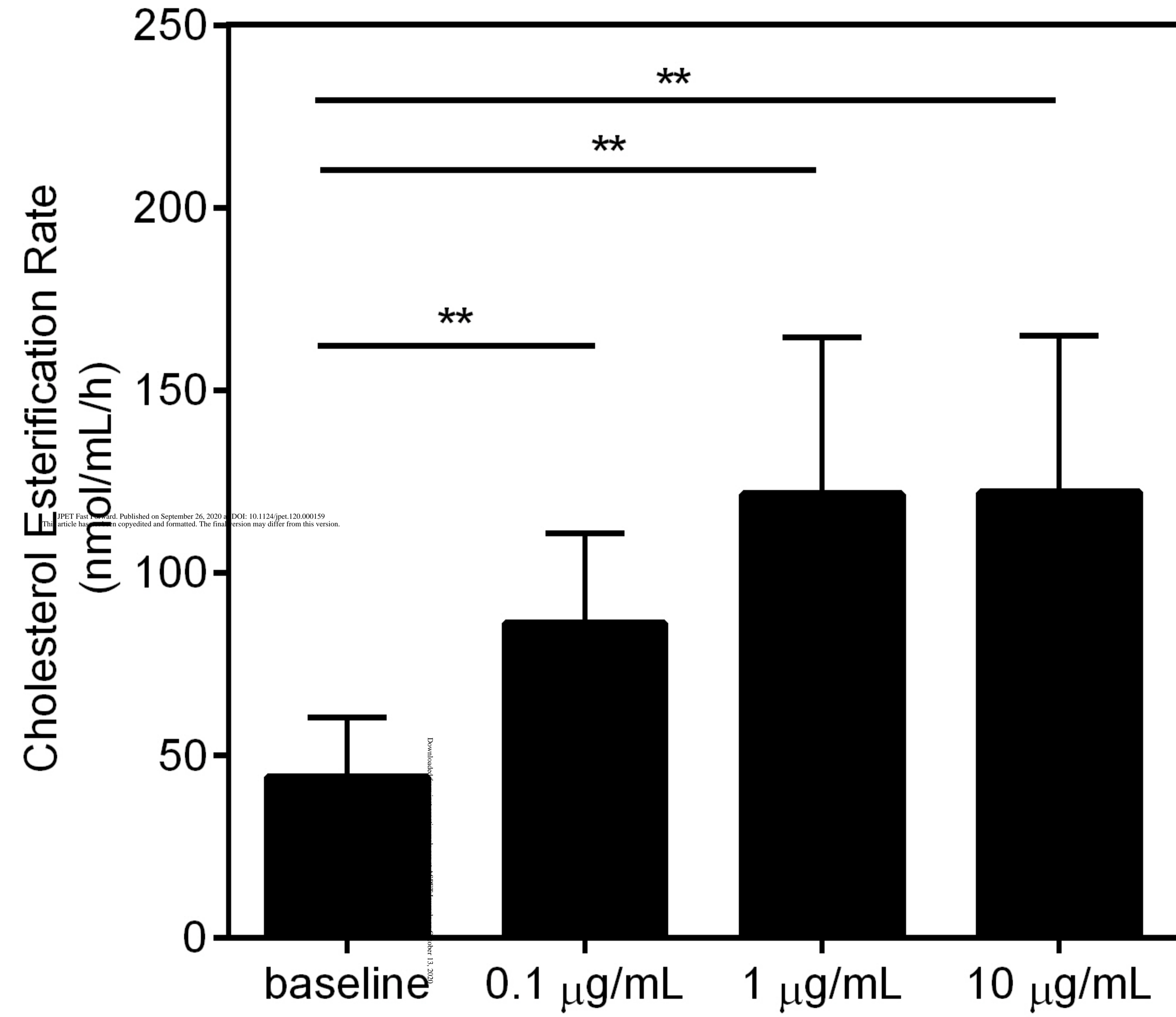


Figure 2

(a)



(b)

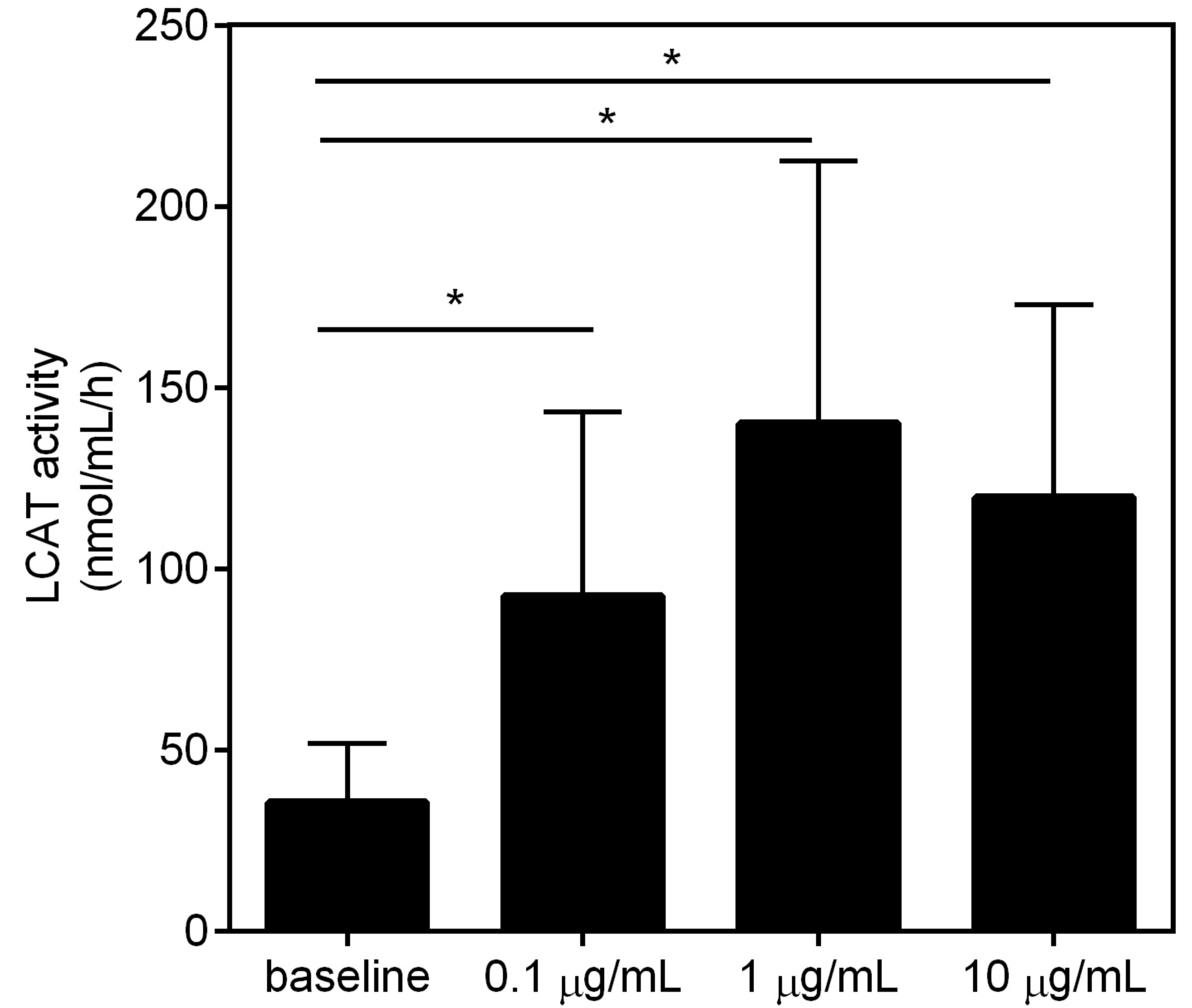
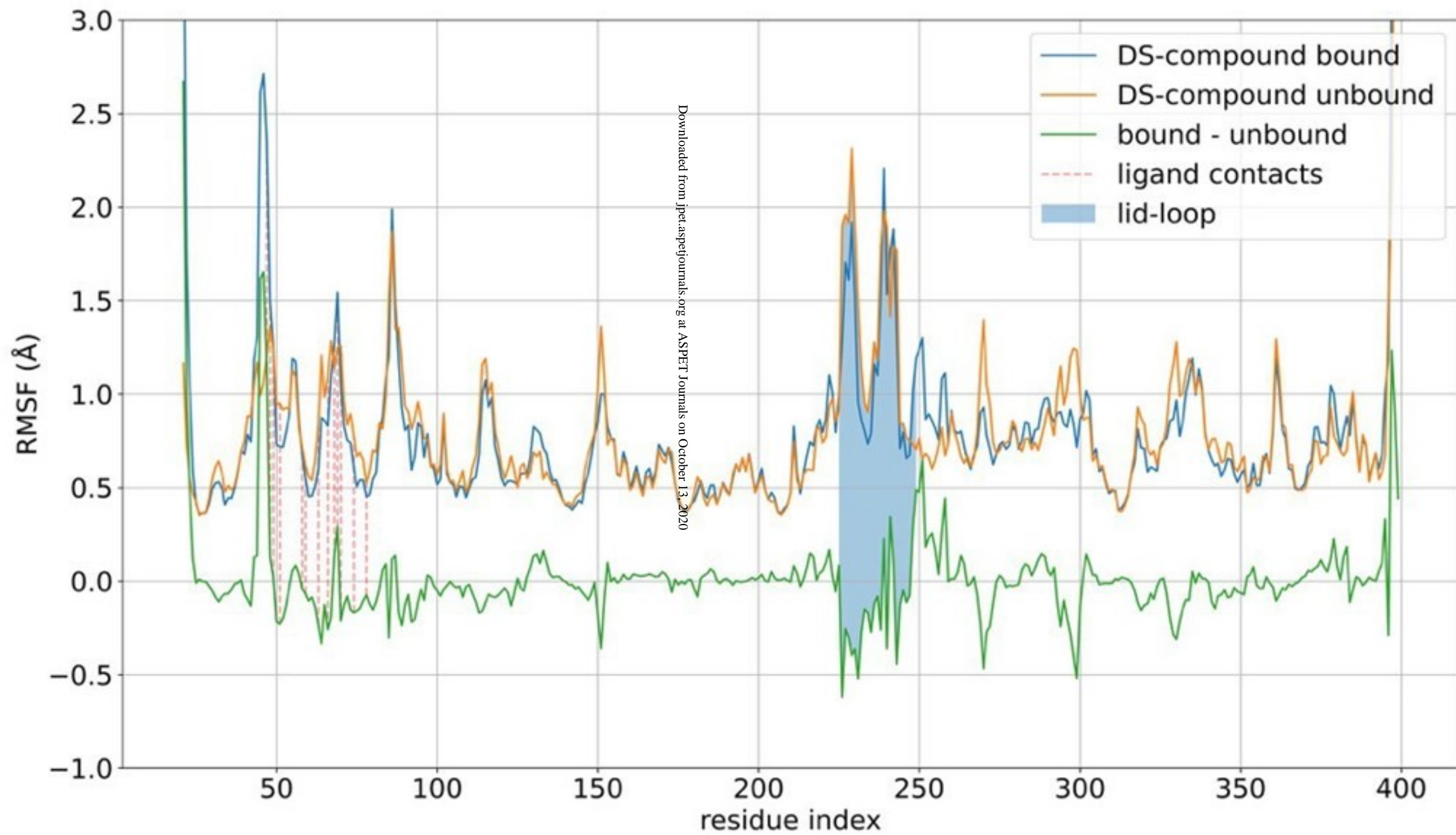
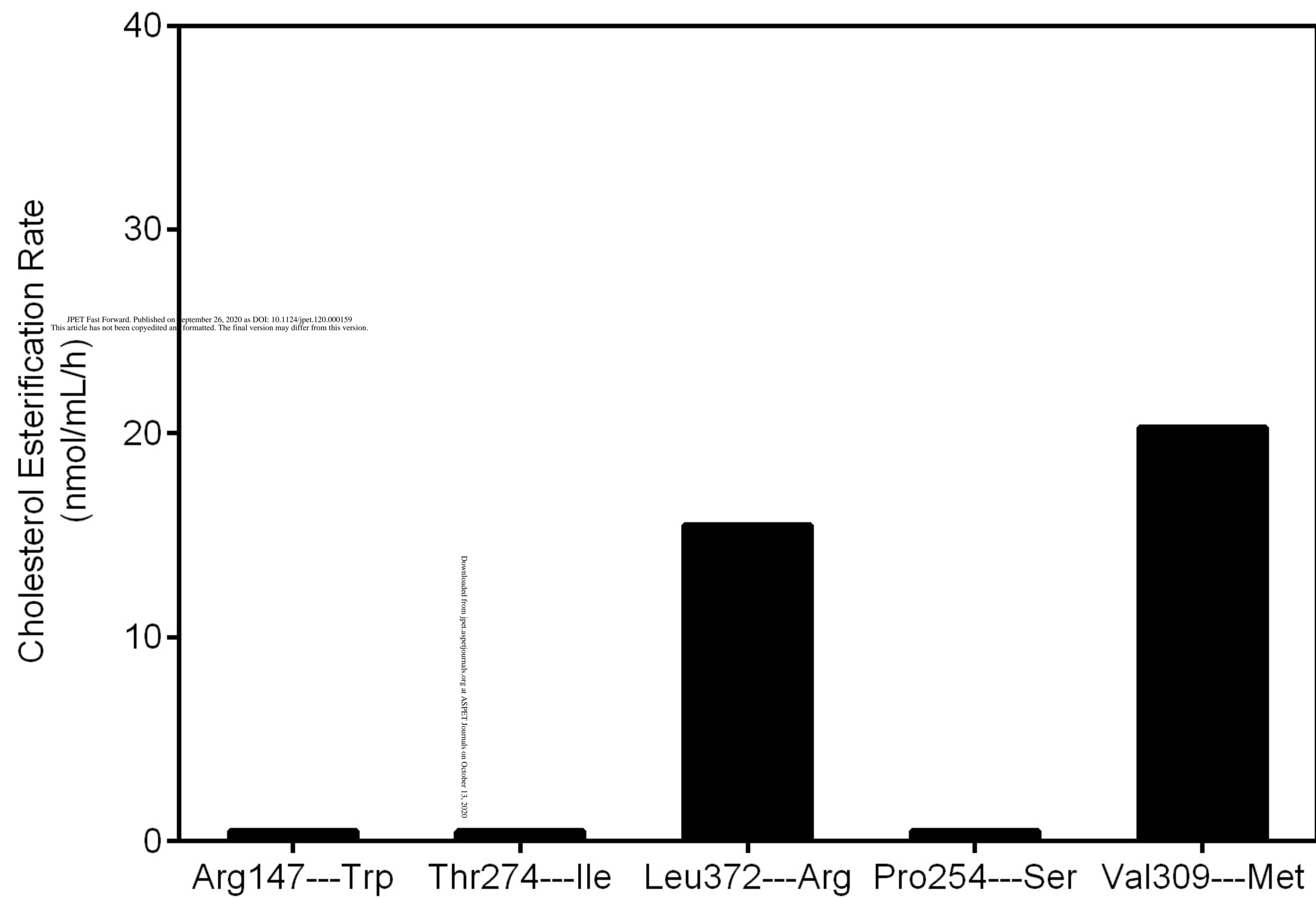


Figure 3



# Figure 4

(a)



(b)

