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(71) Demandeur/Applicant:
HANSSON, GORAN K., SE

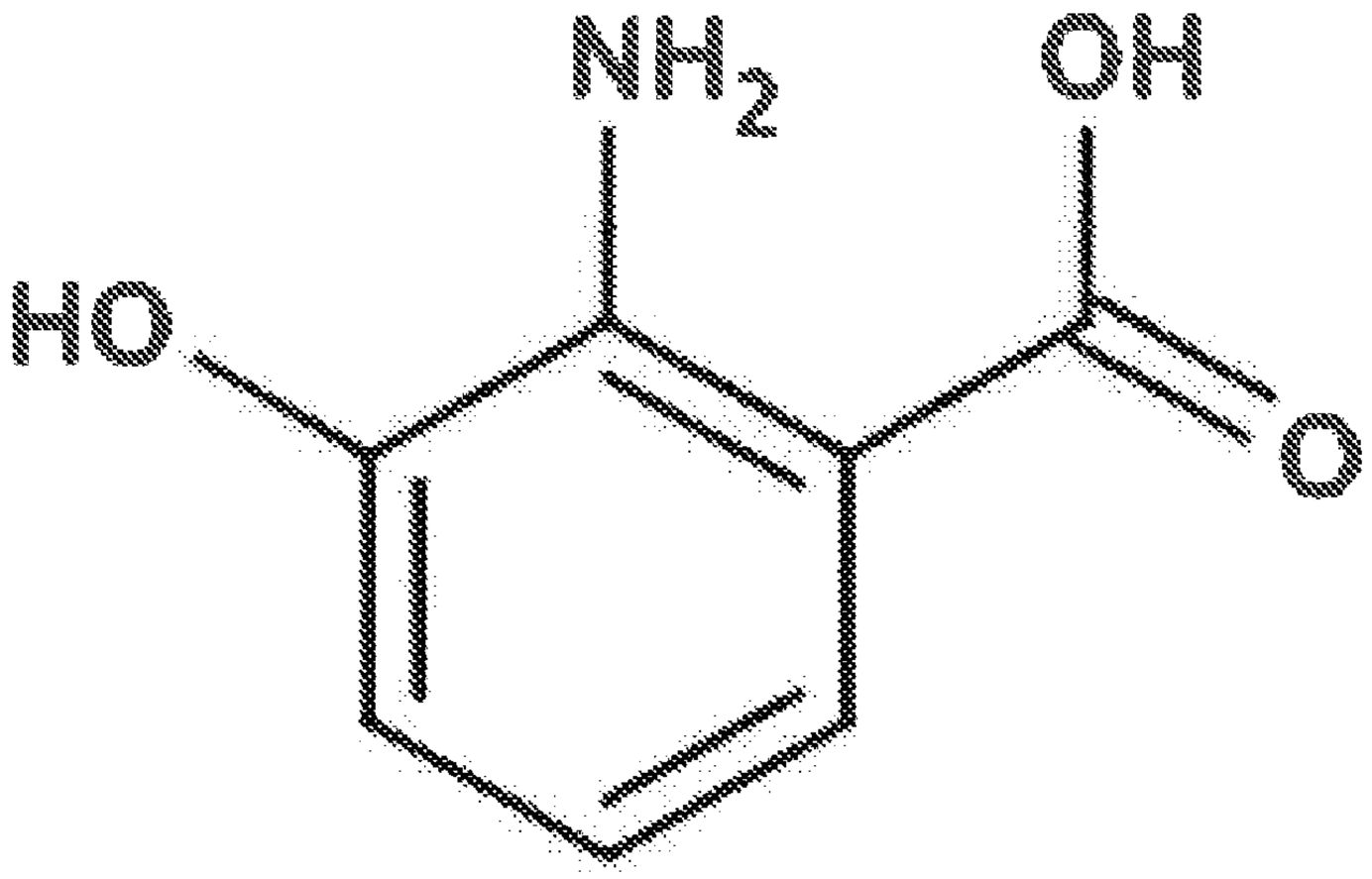
(72) Inventeurs/Inventors:
HANSSON, GORAN K., SE;
KETELHUTH, DANIEL, SE

(74) Agent: FETHERSTONHAUGH & CO.

(54) Titre : THERAPIE PAR ACIDE 3-HYDROXYANTHRANILIQUE (3-HAA) POUR PREVENTION ET TRAITEMENT DE L'HYPERLIPIDEMIE ET SES COMPLICATIONS CARDIOVASCULAIRES

(54) Title: 3-HYDROXYANTHRANILIC ACID (3-HAA) THERAPY FOR PREVENTION AND TREATMENT OF HYPERLIPIDEMIA AND ITS CARDIOVASCULAR COMPLICATIONS

Fig1.



(57) Abrégé/Abstract:
The present invention relates to the use of a tryptophan metabolite, 3- hydroxyanthranilic acid (3-HAA)or a functional analogue thereof, for prophylactic and/or therapeutic treatment of mammals, in special humans, against hyperlipidemia and its cardiovascular complications, i.e atheroma formation, myocardial infarction and heart failure, ischemic stroke and transient ischemic attacks, renal impairment, aortic aneurysms and critical limb ischemia caused by atherosclerosis.

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(72) Inventor; and
(71) Applicant : **HANSSON, Göran K** [SE/SE]; Sankt Eriksgatan 14, S-112 39 Stockholm (SE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **KETELHUTH, Daniel** [SE/SE]; Cembalogatan 2, S-126 28 Stockholm (SE).

(74) Agent: **AWAPATENT AB**; Box 5117, S-200 71 Malmö (SE).

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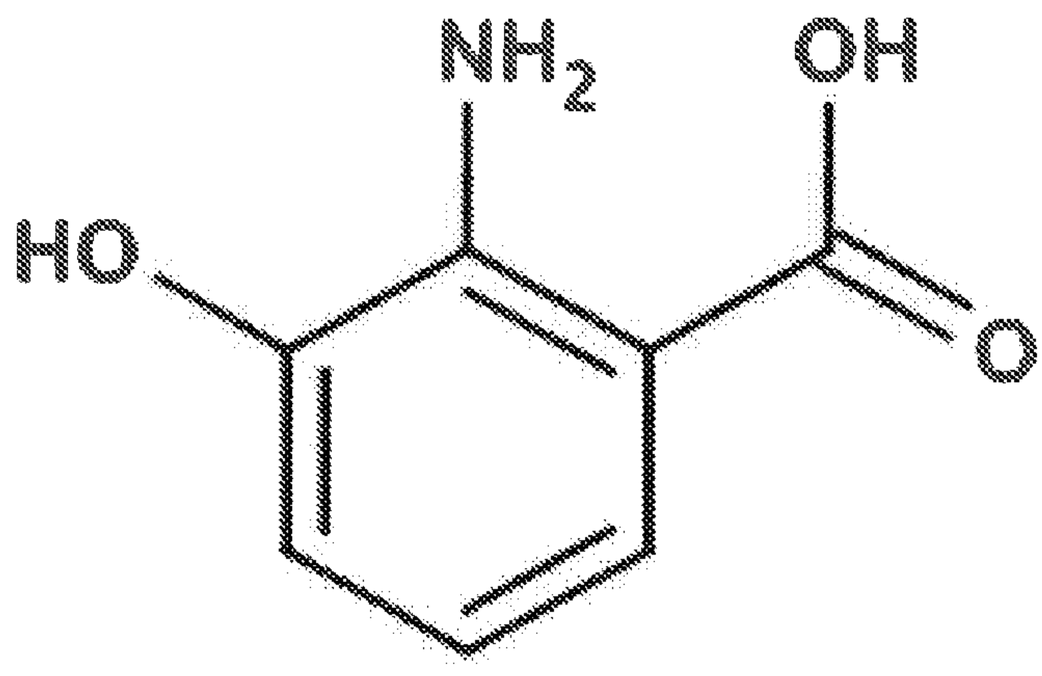

(57) Abstract: The present invention relates to the use of a tryptophan metabolite, 3- hydroxyanthranilic acid (3-HAA)or a functional analogue thereof, for prophylactic and/or therapeutic treatment of mammals, in special humans, against hyperlipidemia and its cardiovascular complications, i.e atheroma formation, myocardial infarction and heart failure, ischemic stroke and transient ischemic attacks, renal impairment, aortic aneurysms and critical limb ischemia caused by atherosclerosis.

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3-HYDROXYANTHRANILIC ACID (3-HAA) THERAPY FOR PREVENTION AND TREATMENT OF HYPERLIPIDEMIA AND ITS CARDIOVASCULAR COMPLICATIONS

FIELD OF THE INVENTION

The present invention relates to the use of the tryptophan metabolite 3-hydroxyanthranilic acid (3-HAA) or a functional analogue thereof for treatment of hyperlipidemia. In particular the invention includes: the use of 3-HAA or a functional analogue thereof, as such or together with a suitable vehicle, as a lipid-lowering therapy for prevention and/or treatment of hyperlipidemia and the cardiovascular complications associated with hyperlipidemia, specifically atheroma formation, myocardial infarction, ischemic stroke and transitory ischemic attacks, renal impairment, aortic aneurysms and critical limb ischemia caused by atherosclerosis.

BACKGROUND OF THE INVENTION

Cardiovascular diseases (CVD), largely caused by formation of atherosclerotic atheroma in the arteries, are the main cause of death in the Western world and also increasingly in developing countries [1]. However, progress in prevention of hyperlipidemia using lipid-lowering drugs, e.g. statins, has a proven survival benefit for both primary prevention (i.e. in individuals without clinical evidence of CVD) and secondary prevention (i.e. in patients with established CVD) [2-4]. Additionally, other classes of drugs, e.g. fibrates, which raise HDL levels, have been shown to reduce the progression of coronary disease in clinical trials [5, 6] and to reduce the incidence of cardiovascular events in outcome studies [7, 8].

Atherosclerosis is a chronic inflammatory condition initiated by retention and accumulation of apolipoprotein B100 (ApoB100)-containing lipoproteins, in particular Low density lipoprotein (LDL), in the artery wall leading to a maladaptive set of responses of macrophages and T cells and the formation

of atheroma in the arteries [9, 10]. Therefore, lowering circulating ApoB100 lipoproteins may have other beneficial effects in addition to decreasing the probability of lipids to accumulate in the arterial wall.

High levels of LDL and its precursor particle, very-low-density lipoprotein (VLDL), are associated with an increased risk for myocardial infarction, stroke, and other complications of atheroma. Elevated LDL and VLDL levels are reflected in high cholesterol and triglyceride levels in samples of blood serum or plasma. Such elevated blood lipid levels constitute the condition of hyperlipidemia which, as mentioned, is associated with increased risk for myocardial infarction, stroke and other atherosclerotic complications.

Hyperlipidemic levels are defined in the SCORE criteria of the European Society of Cardiology, which also identify target levels that should be reached by lipid-lowering therapy [11]. The cardiovascular risk impacted by high LDL cholesterol or total cholesterol is lowered when HDL cholesterol levels are high, as outlined in the same publication. Vice versa, low HDL levels increase the cardiovascular risk of elevated total cholesterol or LDL cholesterol. Current levels for hyperlipidemia as defined by the Swedish Medical Products Agency (Läkemedelsverket) are: Total cholesterol > 5 mmol/L, LDL-cholesterol >3 mmol/L, and HDL-cholesterol <1 mmol/L [12].

HDL particles exert their protective effect on arteries by mediating the removal of cholesterol from cells. After incorporation into HDL, cholesterol molecules are transported to the liver, converted into bile acids, and eliminated through the intestines.

Current therapy for hyperlipidemia is dominated by the group of drugs called statins. These compounds have good effects on LDL cholesterol in most individuals but only minor effects on HDL[13]. Furthermore, some individuals do not tolerate statins or other lipid-lowering drugs. For these reasons, there is a need for development of new lipid-lowering agents.

Modified phospholipids of LDL, such as lysophosphatidylcholine and 1-palmitoyl-2-(5′-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), can stimulate endothelial cells, smooth muscle cells, and macrophages [14, 15]. It is now known that such atherogenic lipids initiate innate immune response

through activation of Toll-like receptors [16-18]. Furthermore, cholesterol accumulating in macrophages that have internalized LDL particles can form microcrystals that directly activate the inflammasome, leading to production of the proinflammatory cytokine, interleukin-1beta [18]. Collectively, these data imply that modification of LDL generates endogenous ligands that trigger activation of innate immune responses that can promote atheroma formation.

Adaptive immunity also plays a key role in the development of atherosclerotic atheroma. Antigen-presenting cells encounter and internalize antigens in the intima, including LDL particles that are internalized through scavenger receptors [19]. After proteolysis, fragments of the LDL protein ApoB100 associate intracellularly with MHC class II proteins, traffic to the cell surface and are presented to T cells. The latter event leads to T cell activation and the production of cytokines which will trigger and maintain local inflammation [20].

In addition to their effects on the local pathological process, immune cells and mediators modulate lipid metabolism on the systemic level. Thus, the proinflammatory cytokine tumor necrosis factor (TNF) inhibits lipoprotein lipase, a key enzyme in triglyceride metabolism, leading to hypertriglyceridemia [21]. Another TNF-like protein, LIGHT, inhibits another lipase, hepatic lipase, by acting in the liver [22, 23]. This leads to reduced triglyceride catabolism and accumulation in the blood of large lipoproteins that contain triglycerides and cholesterol. As a third example, the cholesterol-lowering drugs of the statin class exert significant immunomodulatory effects by reducing T cell activation and inhibiting several autoimmune diseases [24, 25].

All these data demonstrate an intricate crosstalk between the immune and metabolic systems and suggest that it might be possible to treat hyperlipidemia and prevent atheroma formation by using immune cell-dependent pathways.

Recent data shows that IDO (Indoleamine 2,3-Dioxygenase) and IDO-catalyzed tryptophan metabolism play critical roles in the induction of immune suppression and tolerance (reviewed in [26]). IDO is coded by the IDO1 gene (encoded by 10 exons in the chromosome 8, 8p12-p11 in humans). The enzyme metabolizes L-Tryptophan (L-Trp) into N-formylkynurenine, a product

that is rapidly converted by formamidase to L-kynurenine (KYN), which in turn can either enter the bloodstream or be further metabolized to downstream Kynurenines (Kyns). These Kyns include 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), and quinolinic acid (QUIN) [27].

It has been shown that downstream tryptophan metabolites such as 3-hydroxyanthranillic acid (3-HAA, Fig1) can inhibit the function of Th1 and Th2 cells and increase the percentage of regulatory T cells (Tregs). Further, administration of 3-HAA has been shown to decrease inflammation induced by Th17 cells and protect mice against autoimmune encephalitis [28, 29]. In fact, data suggests that 3-HAA could control autoimmunity by direct effects on T cells or by indirect effects through altered antigen presentation on dendritic cells and macrophages [30].

SUMMARY OF THE INVENTION

Hyperlipidemia is the condition of abnormally elevated levels of blood lipids, in particular cholesterol and triglycerides transported in the plasma lipoproteins LDL and VLDL. High lipid levels are followed by sub-endothelial retention and accumulation of LDL in the artery wall; this leads to a chronic maladaptive inflammatory response of macrophages and T cells and to atheroma formation. In this context, prevention of hyperlipidemia using lipid-lowering drugs, e.g. statins and fibrates has proven to decrease the number of cardiovascular events and increase the survival of patients at risk or with established CVD [2-4, 7, 8].

The present inventors found that the immunomodulatory compound 3-HAA demonstrated an unexpected potent effect against hyperlipidemia and significantly reduced total plasma cholesterol and triglyceride levels in a series of experiments. Besides, 3-HAA significantly raised HDL levels and decreased VLDL/HDL and LDL/HDL ratios. Hence, the beneficial changes in plasma lipids were accompanied by a significant reduction in atherosclerotic plaque development. Therefore, 3-HAA and functional analogues thereof can be recognized as a new class of lipid-lowering agent for the prevention and treatment of hyperlipidemia and its cardiovascular complications, i.e. atheroma formation, myocardial infarction, ischemic stroke and transient

ischemic attacks, renal impairment, aortic aneurysms and critical limb ischemia caused by atherosclerosis.

Thus, the present invention relates to use of 3-HAA or functional analogues thereof in the treatment of hyperlipidemia or in the prevention of a cardiovascular complication of hyperlipidemia.

With functional analogues of 3-HAA is contemplated oxidation and reduction products of 3-HAA and substituted variants of 3-HAA, which retain the same or essentially the same effect on the lipid metabolism as 3-HAA.

According to one embodiment, the hyperlipidemia is selected from the group consisting of hypercholesterolemia, hypertriglyceridemia and combined (forms of) hyperlipidemia. In a preferred embodiment the hyperlipidemia is associated with low levels of high-density lipoprotein (HDL) in plasma.

In a preferred embodiment the cardiovascular complication of hyperlipidemia is atheroma formation.

In a further embodiment of the present invention, the cardiovascular complication of hyperlipidemia is a clinical manifestation of atheroma formation.

According to one embodiment the 3-HAA or a functional analogue thereof is used for prevention of myocardial infarction and/or heart failure.

In one embodiment the 3-HAA or functional analogue thereof is for use for prevention of angina pectoris.

In a preferred embodiment the 3-HAA is for use for prevention of ischemic stroke and/or transient ischemic attacks.

In another embodiment the 3-HAA or functional analogue thereof is for use for prevention of peripheral ischemia, gangrene, renal impairment, aortic aneurysms, and/or critical limb ischemia.

According to one embodiment of the present invention, the complication of hyperlipidemia is a dermatological complication of hyperlipidemia.

According to another embodiment, the dermatological complication of hyperlipidemia is xanthomas.

Brief description of the drawings

Fig1. Molecular representation of 3-Hydroxy anthranilic acid (3-HAA)

Molecular formula: $C_7H_7NO_3$. Molar mass 153.14 g/mol. IIUPAC name: 2-Amino-3-hydroxybenzoic acid.

Fig2. Uptake of FITC-oxLDL by peritoneal macrophages

Peritoneal macrophages from *Ldlr-/-* mice injected with 3-HAA (200 mg/Kg) or PBS (8 weeks treatment) were incubated with FITC-oxLDL at a concentration of 20 µg protein/ml for 2h at 37 °C. The uptake was quantified by flow cytometry. (A) Values given are MFI of FITC-oxLDL, means ± SE of triplicate wells for each mouse (n= 5 and 6, for 3-HAA or PBS respectively). (B) The graph shows representative histograms from peritoneal macrophages from 3-HAA or PBS treated mice. **) P<0.01.

Fig3. Plasma lipid analysis

Levels of (A) total cholesterol and (B) triglycerides were evaluated in plasma from 3-HAA or PBS treated mice (8 weeks treatment). (C) Size analysis of lipoprotein profiles from plasma of 3-HAA or PBS treated mice. The cholesterol concentration in each fraction (y axis) is plotted against retention fraction number (x axis); curves show mean ± SEM for 3-HAA and PBS treated mice. Values are expressed as mean ± SEM (n= 5 and 6, for 3-HAA or PBS respectively). **) P<0.01.

Fig4. 3-HAA reduces the development of atheroma

Twelve weeks old *Ldlr-/-* mice were treated with 3-HAA or PBS as control. The mice were sacrificed after 8 weeks on a Western diet. Dissected arches were stained with Sudan IV *en face* and % lesion area of total vessel area was calculated using Image J image analysis software (NIH, Bethesda, MD). The additive area of all the plaques in a given aortic arch was calculated as a percent of the total surface area of the arch. (n= 7 and 10, for 3-HAA or PBS respectively). ***) P<0.001.

EXPERIMENTAL

Procedure

It was hypothesized that induction of protective immunity, with effects on metabolism and inflammation, could be achieved by injecting 3-HAA (200

mg/kg) intraperitoneally, 3 times per week, for 8 weeks. Twelve weeks old *Ldlr-/-* mice were treated with 3-HAA, or PBS as control. The mice were fed a high-fat diet starting 2 days after the first injection until sacrifice, 8 weeks later with CO_2.

Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits according to the manufacturer's protocol. Dissected aortic arches were stained with Sudan IV *en face* and % lesion area of total vessel area was calculated. Additionally, the effect of 3-HAA on the uptake oxLDL was evaluated in cultures of peritoneal macrophages from 3-HAA or PBS treated mice.

MATERIAL AND METHODS

Animals and animal treatments

Ldlr-/- mice were bred and housed in the Animal Experimentation Unit at the Center for Molecular Medicine, Karolinska University Hospital. The mice (n= 7-10 per group) received 3 intra-peritoneal injections of PBS (200 µl) or 3-HAA (200 mg/Kg in PBS) per week for 6 weeks and 1 injection per week in the following two weeks (total 24 injections) and were sacrificed with CO_2. The mice were fed a high-fat diet (corn starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals and vitamins; 17.2% protein, 21% fat (62.9% saturated, 33.9 unsaturated and 3.4% polyunsaturated), 0.15% cholesterol, 43% carbohydrates, 10% H_2O and 3.9% cellulose fibers; R638 Lantmännen, Sweden) starting 2 days after the start of treatment.

Low-density lipoprotein (LDL) isolation

LDL (d = 1.019 - 1.063 g/mL) was isolated from pooled plasma of healthy donors by ultracentrifugation, as described [31]. 2mM Benzamidine, 0.5mM PMSF and 0.1U/ml Aprotinin were added immediately after the plasma was prepared and LDL was dialyzed extensively against PBS after isolation. One millimolar EDTA was added to an aliquot of LDL to prevent modification of LDL particles.

Preparation of FITC (fluorescein isothiocyanate) labeled oxidized LDL (FITC-oxLDL)

LDL was labeled using a modification of a previously described method [32]. Briefly, LDL (1.5 - 2 mg/ml) was dialyzed overnight against 500 mM $NaHC0_3$ pH 9.5. Next, 50 µg of FITC (Sigma-Aldrich, St.Louis, MO, USA), dissolved in DMSO (1 mg/ml), was added for each mg of protein in LDL, and incubated at room temperature for 2 hours. After incubation conjugates were separated from the free fluorochrome by gel filtration using a PD10 column (GE Healthcare, Uppsala, Sweden) and PBS for elution. FITC conjugation was evaluated by absorption spectroscopy against a FITC standard curve at 495 nm. Protein concentration was determined by Bradford assay (Biorad, CA, USA). Oxidized FITC-LDL (FITC-oxLDL) was obtained by incubating 1 ml of FITC-LDL (1 mg/ml) in the presence of 20 µM $CuSO_4$ for 18 h at 37°C. The extent of oxidation was evaluated by the TBARS assay, as described [33].

OxLDL uptake by peritoneal macrophages

Twenty four hours after the last injection of 3-HAA or PBS, peritoneal macrophages were isolated from *Ldlr-/-* mice by peritoneal lavage with PBS. After 2 to 3 hours in PBS, the macrophages were plated in 96-well plates at a density of $1x10^5$ cells per well with 20 µg/ml FITC-oxLDL in RPMI 1640 medium containing 1% FCS. After 2 h incubation at 37°C cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS. The cells were analyzed on a CyAn™ ADP flow cytometer (Dako, Glostrup, Denmark).

Plasma lipid analysis

Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits (Randox Lab. Ltd. Crumin, UK) according to the manufacturer's protocol.

Lipoprotein profiles

Plasma cholesterol lipoprotein profiles were determined using a modification of the method of Okazaki et al [34]. Briefly, plasma samples (50 µl) from mice treated with 3-HAA or PBS were fractionated using an HR10/30

Superose 6 column (GE Healthcare, Uppsala, Sweden) and a Discovery BIO GFC-500 as pre-column (5 cm x 7.8 i.d.; Supelco®,Sigma-Aldrich, PA, USA) coupled to Prominence UFLC system (Shimadzu, Kyoto, Japan) and equilibrated with Tris-buffered saline, pH 7.4. Fractions of 200 µl were collected using Foxy Jr® fraction collector (Teledyne Isco Inc, NE, USA) and total cholesterol was determined in each fraction using enzymatic colorimetric kit (Randox Lab. Ltd. Crumin, UK).

Lesion analysis

En face lipid accumulation was determined in the aortic arch from 3-HAA and PBS treated mice using Sudan IV staining. Briefly, dissected arches were fixed in 4% neutral buffered formalin. Samples were then cut longitudinally, splayed, pinned and subjected to Sudan IV staining (red color). Images were captured using a Leica DC480 camera connected to a Leica MZ6 stereo microscope (Leica, Wetzlar, Germany). The additive area of all the plaques in a given aortic arch was calculated as a percent of the total surface area of the arch (not including branching vessels). Quantitation of plaques was performed using Image J software (NIH, Bethesda, USA).

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. The nonparametric Mann-Whitney U test was used for comparisons between groups. Differences were considered significant at P values below 0.05.

RESULTS

3-hydroxyanthranilic acid inhibits oxLDL uptake

The uptake of fluorescent FITC-oxLDL was quantified by flow cytometry as described in the Methods section. As shown in Fig.2, peritoneal macrophages from mice treated with 3-HAA displayed approx 79% reduced uptake of FITC-oxLDL. Therefore, 3-HAA inhibits uptake of modified LDL particles, the key event in foam cell formation.

3-hydroxyanthranilic acid has a potent lipid-lowering effect

Unexpected for an immunomodulatory compound, administration of 3-HAA to *Ldlr-/-* mice was associated with a marked, statistically significant reduction in plasma total cholesterol (approx 50%) and triglycerides (approx 72%) (Fig.3). Additionally, 3-HAA effectively raised HDL levels and reduced VLDL/HDL (4.0 ± 0.55 and 1.4 ± 0.31, mean ± SEM of PBS and 3-HAA treated mice respectively; P< 0.01) and LDL/HDL ratios (2.3 ± 0.24 and 1.4 ± 0.16, mean ± SEM of PBS and 3-HAA treated mice respectively; P<0.05). These data identify 3-HAA as a new type of lipid-lowering agent. 3-HAA administration had no effect on body weight (data not shown).

Reduction of plasma lipids induced by 3-hydroxyanthranilic acid leads to reduced atheroma formation

Finally, we examined whether 3-HAA could affect atheroma formation. *Ldlr-/-* mice, which develop atheromatous lesions when treated with high fat diet, were treated with 3-HAA for 8 weeks. The dramatic reduction in total plasma cholesterol and triglyceride levels led to an approximately 90 % reduction in atherosclerotic lesion area in *en face* preparations of the aortic arch (Fig.4).

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27 -05- 2013

CLAIMS

1. 3-HAA for use in the treatment of hyperlipidemia or in the prevention of a cardiovascular complication of hyperlipidemia.

2. 3-HAA for use according to claim 1, wherein said hyperlipidemia is selected from the group consisting of hypercholesterolemia, hypertriglyceridemia and combined (forms of) hyperlipidemia.

3. 3-HAA for use according to claim 1 or 2, wherein said hyperlipidemia is associated with low levels of high-density lipoprotein (HDL) in plasma.

4. 3-HAA for use according to anyone of the previous claims, wherein said cardiovascular complication of hyperlipidemia is atheroma formation.

5. 3-HAA for use according to anyone of claims 1 to 3, wherein said cardiovascular complication of hyperlipidemia is a clinical manifestation of atheroma formation, which clinical manifestation is chosen from myocardial infarction and/or heart failure.

6. 5. 3-HAA for use according to any one of claims 4 to 5, for prevention of angina pectoris.

7. 3-HAA for use according to any one of claims 4 to 5, for prevention of ischemic stroke and/or transient ischemic attacks.

8. 3-HAA for use according to any one of claims 4 to 5, for prevention of peripheral ischemia, gangrene, renal impairment, aortic aneurysms, and/or critical limb ischemia caused by atherosclerosis.

9. 3-HAA for use according to claim 1 or 2, wherein the complication of hyperlipidemia is xanthomas.

Fig1.

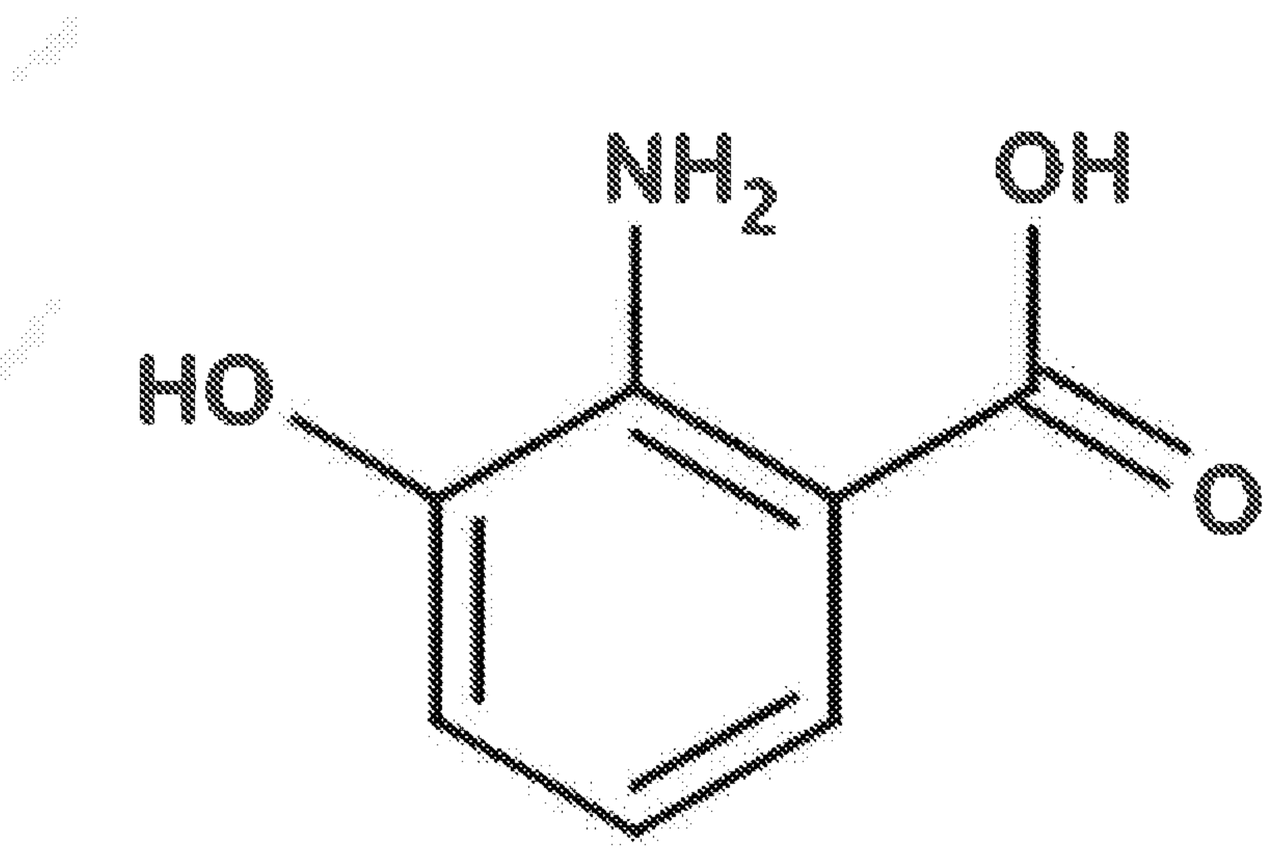


Fig2.

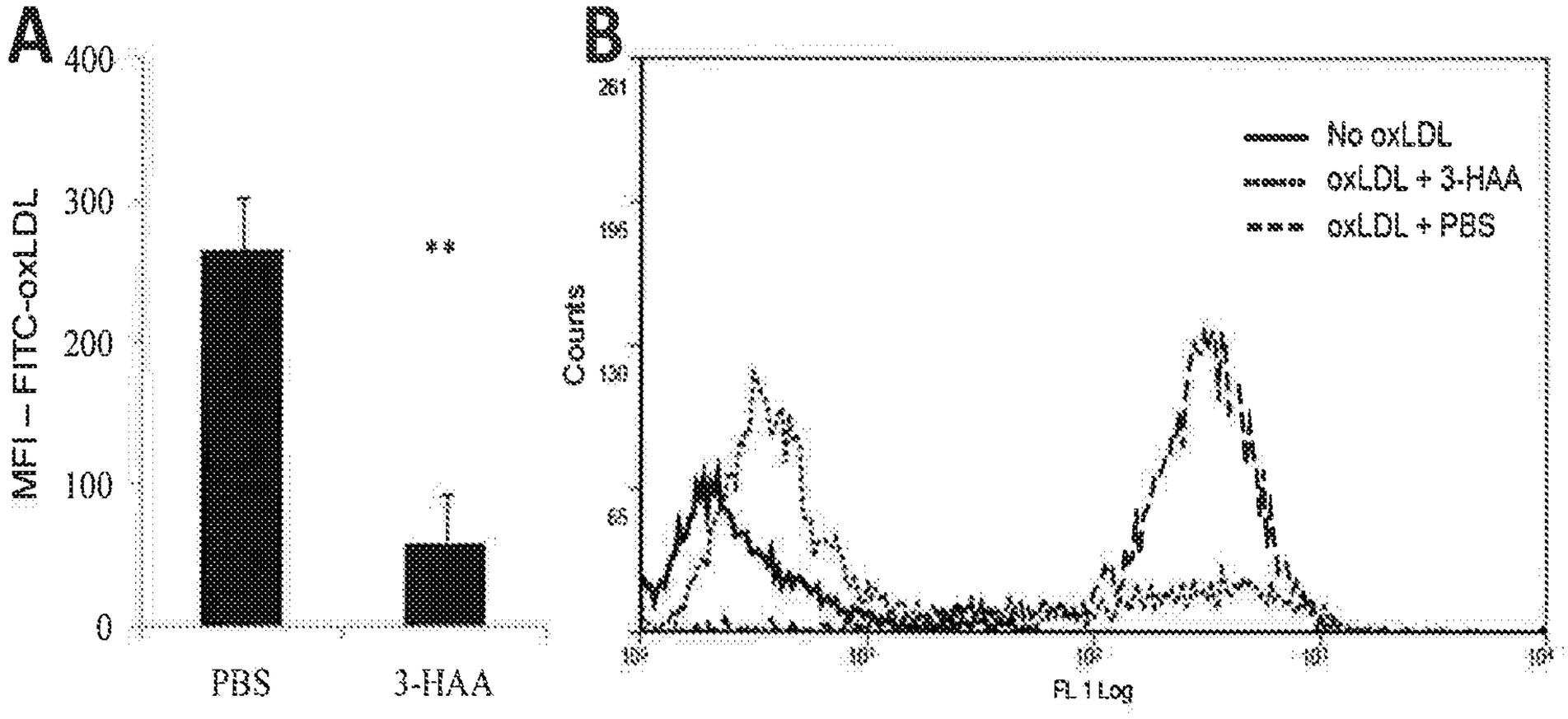
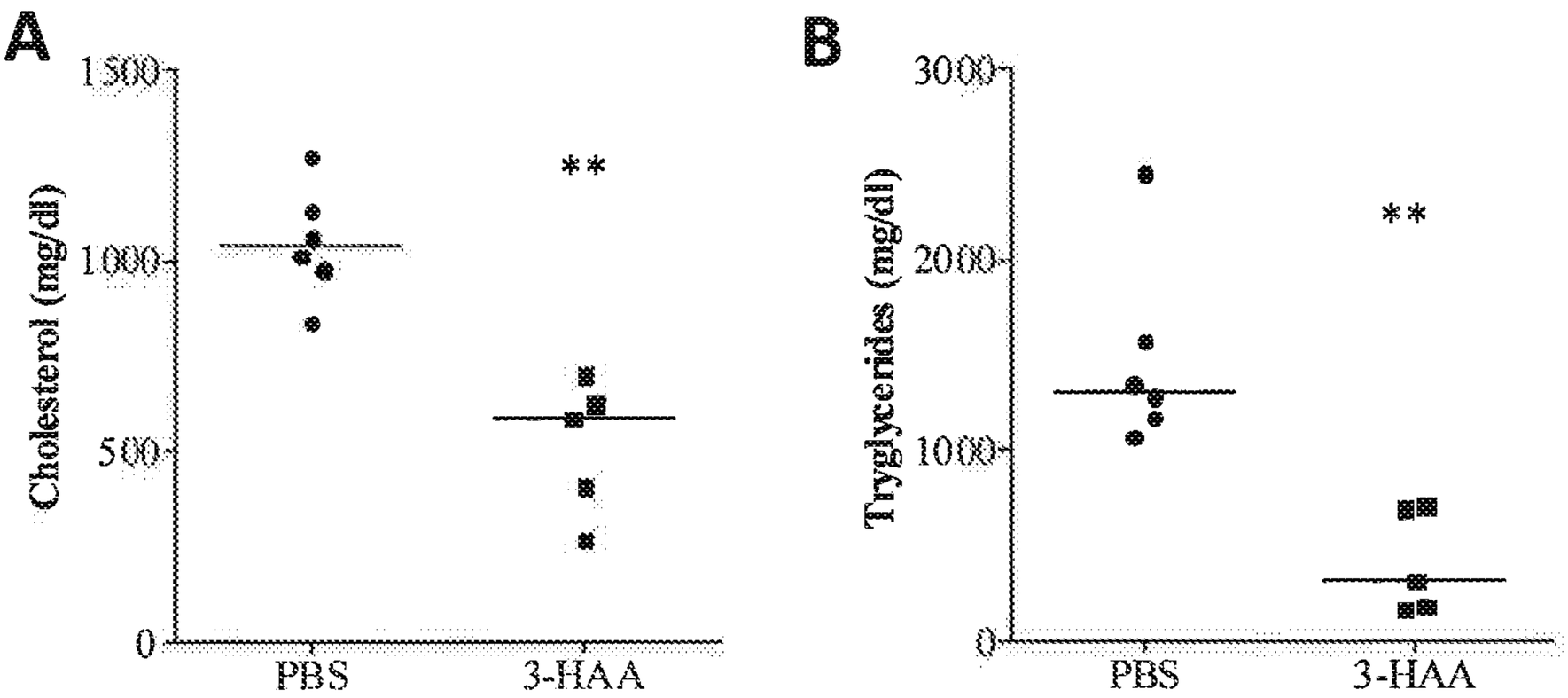

A
MFI – FITC-oxLDL
400
300
200
100
0
**
PBS
3-HAA
B
Counts
261
195
130
65
No oxLDL
oxLDL + 3-HAA
oxLDL + PBS
FL1 Log

Fig3.


A
1500
1000
500
0
Cholesterol (mg/dl)
**
PBS
3-HAA
B
3000
2000
1000
0
Tryglycerides (mg/dl)
**
PBS
3-HAA

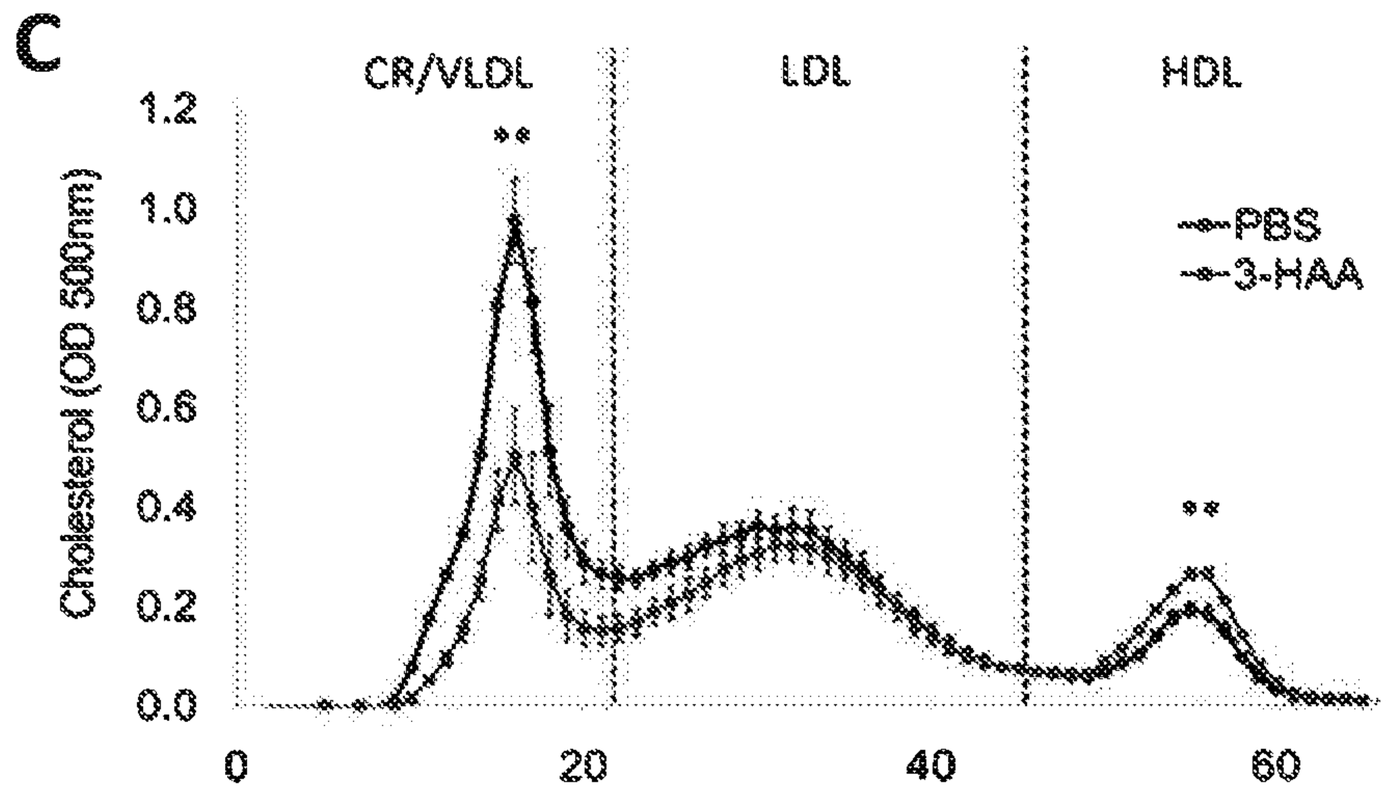

C
CR/VLDL
LDL
HDL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cholesterol (OD 500nm)
**
PBS
3-HAA
0
20
40
60

Fig4.

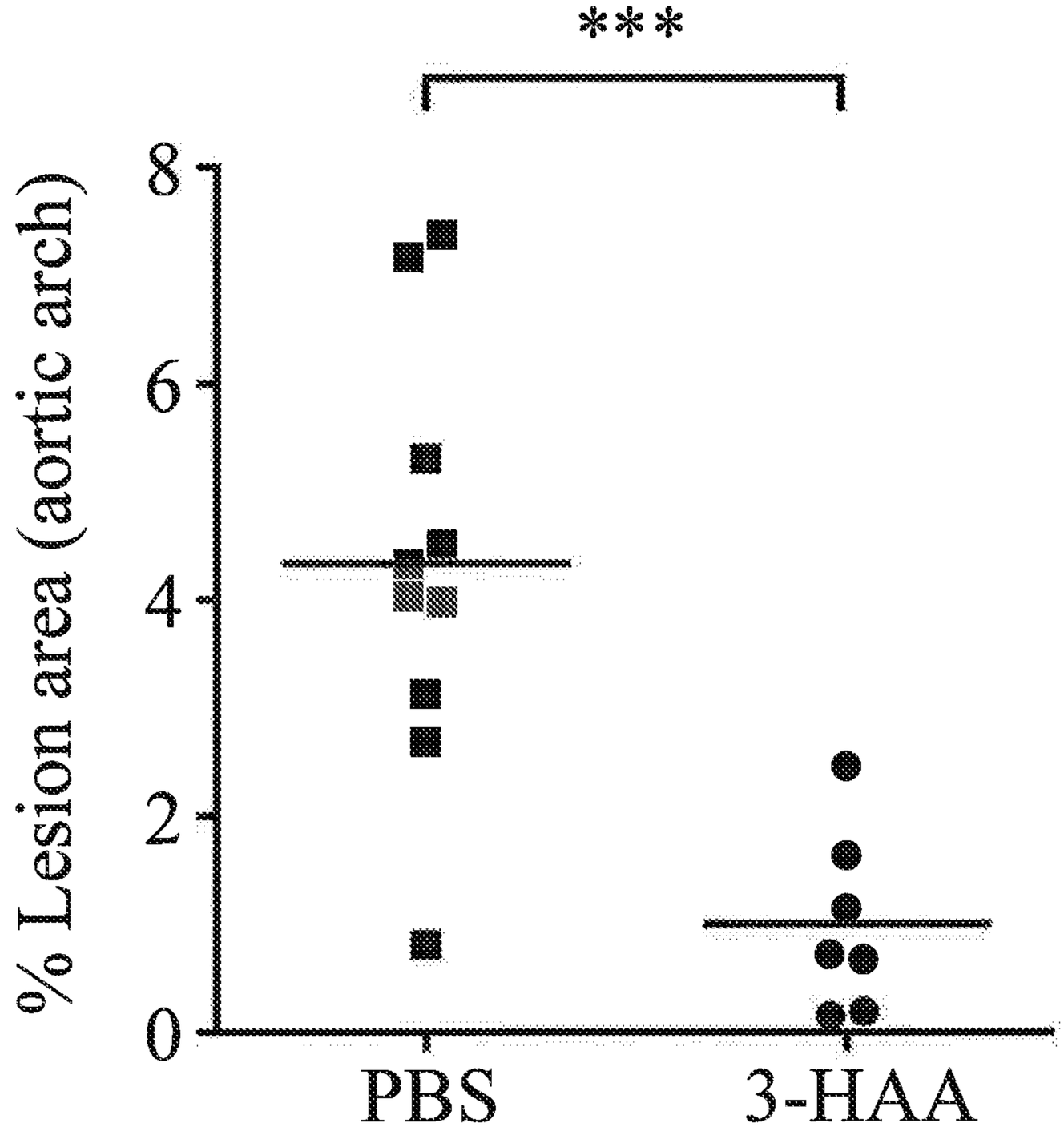
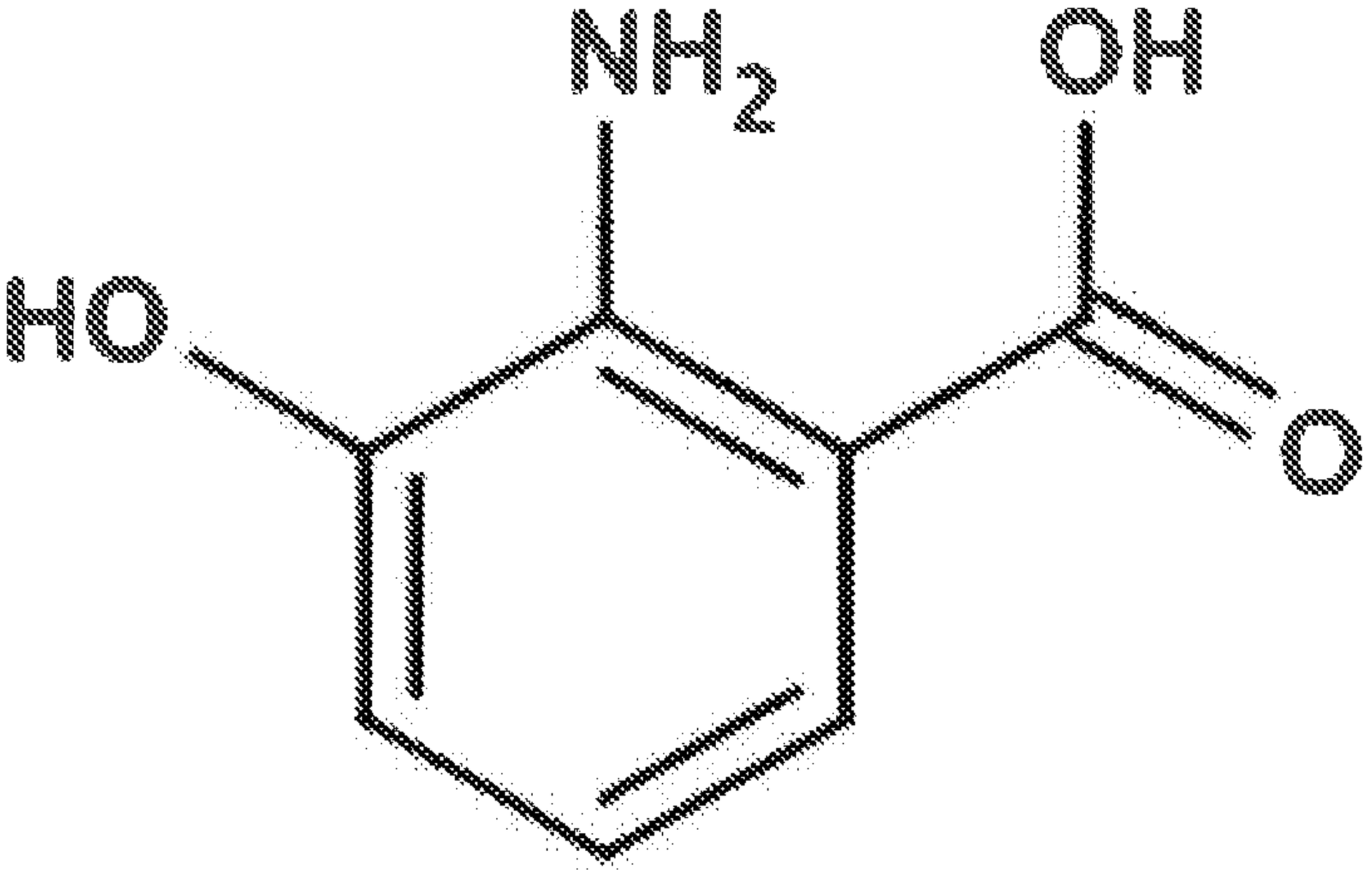



Fig1.