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Olivier Bocquet, Amandine Wahart, Thomas Sarazin, Elise Vincent, Christophe Schneider, et al.. Adverse Effects of Oseltamivir Phosphate Therapy on the Liver of LDLR 2/2 Mice Without Any Benefit on Atherosclerosis and Thrombosis. *Journal of Cardiovascular Pharmacology*, 2021, 77 (5), pp.660-672. 10.1097/FJC.0000000000001002 . hal-03358077

HAL Id: hal-03358077

<https://hal.science/hal-03358077>

Submitted on 1 Oct 2021

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Adverse effects of oseltamivir phosphate therapy on the liver of LDLR^{-/-} mice without any benefit on atherosclerosis and thrombosis

Olivier Bocquet¹, Dr. Amandine Wahart¹, Thomas Sarazin¹, Elise Vincent¹, Dr. Christophe Schneider¹, Dr. Anne Fougerat², Dr. Stéphanie Gayral², Aubéri Henry¹, Dr. Sébastien Blaise¹, Dr. Béatrice Romier-Crouzet¹, Dr. Stéphane Jaisson^{1,3}, Pr. Philippe Gillery^{1,3}, Dr. Amar Bennisroune¹, Dr. Hervé Sartelet¹, Dr. Muriel Lafargue², Pr. Laurent Martiny¹, Pr. Laurent Duca¹, Dr. Pascal Maurice¹.

¹ UMR CNRS 7369 Matrice Extracellulaire et Dynamique Cellulaire (MEDyC), Université de Reims Champagne Ardenne (URCA), UFR Sciences Exactes et Naturelles, Reims, France.

² INSERM UMR1048 I2MC, Université Paul Sabatier, Toulouse, France.

³ University Hospital of Reims, Department of Biochemistry-Pharmacology-Toxicology, Reims, France.

* Corresponding author:

Dr Pascal Maurice, UMR CNRS/URCA 7369 MEDyC, Team 2 "Matrix aging and Vascular remodelling", UFR Sciences Exactes et Naturelles, Moulin de la Housse, BP1039, 51687 Reims cedex 2, France.

Phone: (+33) 326 91 32 75 / e-mail: pascal.maurice@univ-reims.fr

Short running title: Oseltamivir phosphate and vascular diseases

Sources of funding: This work was supported by funding from CNRS, URCA, and the Fondation de France

Keywords: oseltamivir phosphate; sialidase; atherosclerosis; thrombosis; liver; inflammation

Abstract

Desialylation, governed by sialidases or neuraminidases, is strongly implicated in a wide range of human disorders, and accumulative data show that inhibition of neuraminidases, such as NEU1 sialidase, may be useful for managing atherosclerosis. Several studies have reported promising effects of oseltamivir phosphate, a widely used anti-influenza sialidase inhibitor, on human cancer cells, inflammation and insulin resistance. In this study, we evaluated the effects of oseltamivir phosphate on atherosclerosis and thrombosis and potential liver toxicity in LDLR^{-/-} mice fed with high fat diet. Our results showed that oseltamivir phosphate significantly decreased plasma levels of LDL-cholesterol and elastin fragmentation in aorta. However, no effect was observed on both atherosclerotic plaque size in aortic roots and chemically-induced thrombosis in carotid arteries. Importantly, oseltamivir phosphate administration had adverse effects on the liver of mice and significantly increased mRNA expression levels of F4/80, IL-1 β , TGF- β 1, MMP-12 and collagen. Taken together, our findings suggest that oseltamivir phosphate has limited benefits on atherosclerosis and carotid thrombosis and may lead to side effects in the liver with increased inflammation and fibrosis.

Introduction

Human neuraminidases, or sialidases, are glycoside hydrolases which release terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides (1). Four types of mammalian neuraminidases (NEU), encoded by different genes, have been described with distinct substrate specificity and subcellular localization: NEU1 (2), NEU2 (3), NEU3 (4) and NEU4 (5). Sialidases have been implicated in a wide range of human disorders, including cancers, metabolic and cardiovascular diseases (6). Accumulative data highlighted sialidases as promising therapeutic targets, and modulating the activity of these enzymes may have beneficial effects in several pathologies.

Due to the lack of selective inhibitors, the vast majority of studies reported so far have used DANA (2,3- dehydro-2-deoxy-N-acetylneuraminic acid), a non-selective inhibitor of the four NEU isoenzymes, or oseltamivir phosphate (OP), an anti-influenza drug (7). OP is a prodrug which is converted by carboxyl esterase enzymes into the active oseltamivir carboxylate. OP is a sialic acid analogue which interacts with and blocks sialidase enzymes of the influenza virus, blocking their ability to release virions in the infected cells (8). Although OP demonstrates almost no appreciable inhibition of human sialidases (9, 10), contradictory results were obtained with this drug when tested on human cancer cells using both *in vitro* and *in vivo* models (11-15). It was also reported that OP reverses the epithelial to mesenchymal transition process and increases drug sensitivity of chemoresistant human cancer cells (16, 17). Moreover, in human monocytic cells, OP has been shown to decrease pro-inflammatory cytokines production in response to LPS (18, 19) or thymoquinone, derived from the nutraceutical black cumin oil (20). Both agonists activate toll-like receptors and trigger NF κ B activation through NEU1 and NEU4, respectively. Finally, reported effects of OP on NEU1-mediated regulation of insulin receptor (IR), phosphorylation of IR β and insulin receptor substrate-1 (IRS-1) in response to insulin (21) and on IR transactivation by G-protein coupled

receptors (22), suggest potential application of OP treatment in insulin resistance and type 2 diabetes.

Accumulative evidences also showed that neuraminidases inhibition, and more precisely inhibition of NEU1 sialidase, may be useful for managing atherosclerosis development (23-25). Atherosclerosis induces the progressive luminal narrowing of arteries, which is the underlying cause of myocardial infarction and ischemic stroke. Formation of atherosclerotic plaques involve the accumulation of cholesterol and inflammatory cells in the sub-endothelial space of the vessel intima and is closely linked to extracellular matrix remodeling and elastic fibers degradation (26). However, evaluation of OP efficacy in animal models of atherosclerosis has never been reported so far. In addition, the use of OP in patients has been associated with adverse effects such as liver toxicity (9, 27-29), and the benefits versus harms of OP remains to be further evaluated before potential application in human for treatment of cancer, metabolic and cardiovascular diseases.

In the present paper, we evaluated the effects of OP administration on atherosclerosis and chemically-induced arterial thrombosis in LDLR^{-/-} mice fed with a high fat diet (HFD). We assessed the effects of OP on plasma lipid parameters, elastin fragmentation in aorta, and liver inflammation and fibrosis. Whereas OP was shown to significantly reduce plasma LDL-cholesterol levels and elastic fibers fragmentation in aorta, no benefit was observed on atherosclerosis development after 12 weeks of HFD, and on the time for complete carotid artery occlusion following FeCl₃ injury. Importantly, adverse effects were observed in the liver of mice with increased inflammation and fibrosis. In conclusion, this study suggests that OP has limited effects on atherosclerosis and carotid thrombosis and that caution must be taken regarding its **potential** adverse effects on the liver.

Materials and methods

Animals

All mouse experiments were approved by the Ethics Committee for Animal Welfare of the University of Reims Champagne-Ardenne (CEEA-RCA no 56) and the French Ministry of Research (APAFIS # 5464-2016052416307263v4) being compliant with the European Directive 2010/63/UE. LDLR^{-/-} (B6.129S7-Ldlr^{tm1Her}/J) female mice were purchased from Charles River Laboratories (L'Arbresle, France). LDLR^{-/-} mice (12 weeks-old) were fed for 12 weeks with HFD + 0.15% cholesterol or normal diet (ND) (Special Diet Services, DIETEX France Sarl, Argenteuil, France). The HFD was constituted by crude protein 22.6% (w/w), crude fat 23.0% (w/w), crude fibers 4.6% (w/w), Ash 4.2% (w/w), carbohydrate 39.8% (w/w).

Oseltamivir phosphate

OP capsules (75 mg, Roche) were reconstituted in sterile phosphate-buffered saline (PBS), and centrifuged at 1,000 rpm for 10 minutes to obtain OP in the supernatant as previously reported (13). The stock-extracted OP solution had a concentration of 15 mg/mL. OP was used at a concentration (50 mg/kg) that completely ablates tumor vascularization, tumor growth and spread to the lungs in a mouse model of heterotopic xenografts of MDA-MB-231 tumors (13) and twice lower than the one used by Gilmour et al in a mouse model of heterotopic xenografts of human pancreatic cancer (12). OP or vehicle were intraperitoneally injected each week, starting at the onset of HFD, and for 12 weeks.

Intraperitoneal glucose tolerance test

Glucose tolerance tests were performed according to Heikkinen *et al* (30). Briefly, 20% aqueous glucose solution was administrated by intraperitoneal injection (2 mg glucose/g body

weight) in mice fasted for 4 h. Glycemia was then measured from a blood drop taken from the tail of mice at baseline and 15, 30, 45, 60 and 90 min after glucose administration.

Blood measurements

At diet completion, mice were fasted for 6 h and blood was collected from the retro-orbital sinus into heparin tubes, centrifuged and plasma aliquots were kept at -80°C. Plasma glucose, insulin, total-, LDL-, and HDL-cholesterol, triglyceride and free fatty acids levels were measured by the Institut Clinique de la Souris (Ilkirch, France). Homeostatic model assessment (HOMA) for insulin resistance (HOMA-IR) was calculated according to (30): $HOMA-IR = (FPI \times FPG) / 22.5$ where FPI was the fasting plasma insulin concentration ($\mu U/mL$) and FPG was the fasting plasma glucose concentration (mmol/L). Glycated haemoglobin (HbA_{1c}) was quantified by immunoassay using the DCA Vantage Analyzer (Siemens Healthcare, Erlangen, Germany).

Quantification of elastin fragmentation in the aorta

Mice were sacrificed by cervical dislocation, and aorta were collected rapidly and embedded within optimal cutting temperature (OCT) (Tissue-Tek) compound before placing them at -80°C until analysis. Cryosections (10 μm) from aorta was visualized under an epifluorescence microscope (Olympus BX51WIF, Olympus France S.A.S., Rungis, France) connected to a digital camera (Olympus CAM-UC90). Elastin has autofluorescent properties and emits fluorescence at 500- 560 nm when excited at 488 nm (31).

Aortic pulse wave velocity

Doppler ultrasound (Indus Mouse Doppler System, Webster, TX) was performed under isoflurane anesthesia. Mice were placed supine on a heating board with legs secured to

electrocardiographic electrodes. Doppler probes (10 and 20 MHz) were placed on the transverse aortic arch (10 MHz) and abdominal aorta (20 MHz) and the distance between the probes was determined with a precision caliper. Pre-ejection time, the time between the R-wave of the electrocardiogram and the foot of the Doppler signal, was determined for each site. Aortic PWV (aPWV) was calculated by dividing the distance (cm) between the probes by the difference in pre-ejection times (milliseconds) of the thoracic and abdominal regions.

Quantification of atherosclerotic lesions

The hearts were prepared as described previously (32) and the atherosclerotic lesions were estimated according to Paigen et al. (33). Briefly, the hearts were washed in PBS and incubated in PBS at 4°C for 12 h. Each heart was embedded in OCT compound and stored at -80°C. One hundred sections of 10 µm thickness were prepared from the top of the left ventricle, where the aortic valves were first visible, up to a position in the aorta where the valves were just disappearing from the field. After drying for 2 h, the sections were stained with Oil Red O (ORO) and counterstained with Mayer's haematoxylin. After ORO staining, surface lesion areas were measured by computer assisted image quantification using the Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Sony (Tokyo, Japan) 3CCD video camera.

Mouse model of chemically-induced arterial thrombosis

The carotid artery of anesthetized mice was isolated from the surrounding tissue, and placed forward of a small piece of non-transparent black plastic film to prevent fluorescence interferences by underlying structures during the visualization of blood flow. Rhodamine 6G (1 mg/mL) was then retro-orbitally injected. Redox-induced vascular endothelial injury was achieved by applying a piece of filter paper (2 × 1 mm) saturated with a 7.5% ferric chloride

(FeCl₃) solution directly on the carotid artery for 1 minute. The filter paper was then removed, and thrombus formation was observed in real time using an epifluorescence microscope (Olympus BX51WIF, Olympus France S.A.S., Rungis, France) connected to a digital camera (Olympus CAM-UC90). Time for complete thrombotic occlusion was defined as blood flow being stopped for at least 30 seconds.

RNA Extraction and quantitative RT-PCR

For each sample, mouse livers were recovered, flash frozen, and a fraction was grinded in 1 mL of Qiazol (Qiagen, France) with an Ultra Thurax (Imlab, France). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, France), and 250 ng were reverse transcribed using the Verso cDNA Synthesis Kit (ThermoFisher Scientific, France) according to the manufacturer's instructions. The transcript levels were determined by real-time quantitative PCR using the CFX 96TM Real-Time System (Biorad, Hercules, CA, USA) and the SYBR Green Master Mix PCR kit as recommended by the manufacturer (ThermoFisher Scientific, France). PCR reactions were carried out in duplicates in 96-well plates (15 µL per well) in a buffer containing 1x SYBR Green mix (including Taq polymerase, dNTPs, SYBR Green dye), 280 nM forward and reverse primers and 1:10 dilution of reverse transcript RNA. After denaturation at 95°C for 15 min, amplification occurred in a two-step procedure: 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C, with a total of 30 cycles. Identical thermal cycling conditions were used for all targets. Specific primers were designed using the NCBI Primer Blast interface and are presented in Table 1. PCR efficiency of the primer sets was calculated by performing real-time PCR on serial dilutions. For each experiment, PCR reactions were performed in duplicate and 3 independent experiments were analyzed. Relative gene expression was determined with the formula fold induction: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t \text{ GI}$

[unknown sample]-Ct GI [reference sample])-(Ct reference genes [unknown sample]-Ct reference genes [reference sample]). GI is the gene of interest. RPS26 and 36B4 are used as reference genes. The reference samples are ND mice and the unknown samples are HFD mice.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). *In vitro* and *ex vivo* parametric data were compared with two-tailed Student *t*-test. *In vivo* non-parametric data were compared with two-tailed Mann-Whitney *U* test. Multiple comparisons were done by one-way ANOVA followed by Dunnett's test. P-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, USA).

224

225 **Results**

226 **LDLR^{-/-} mice fed with a HFD for 12 weeks display type 2 diabetes and dyslipidemia**

227 LDLR^{-/-} mice were fed with HFD (HFD mice) and compared to ND-fed mice (ND mice).
228 After 12 weeks of diet, HFD mice showed a significant higher body weight than ND mice
229 (31.9±1.1 vs 23.3±0.9) (Fig 1A) and were intolerant to glucose (Fig 1B). In addition, HFD
230 mice showed significant increased blood glucose (8.40±0.78 vs 4.33±0.18 mmol/L), HbA_{1c}
231 (25.0±0.4 vs 20.4±1.4 mmol/mol), and insulin (0.128±0.024 vs 0.057±0.015 µg/L)
232 concentrations (Fig 1C, D, E). The HOMA-IR index was also significantly increased in HFD
233 mice (1.02±0.12 vs 0.34±0.09) (Fig 1F). Total cholesterol (24.57±1.50 vs 15.88±0.79
234 mmol/L), HDL- (5.07±0.16 vs 3.22±0.12 mmol/L) and LDL-cholesterol (15.65±0.87 vs
235 10.15±0.53 mmol/L) concentrations were also significantly increased compared to ND mice
236 (Fig 1G, H, I). Unexpectedly, no significant difference was found in free fatty acids and
237 triglycerides plasma levels between HFD and ND mice (Supplemental Digital Content 1).
238 Taken together, these results demonstrated that HFD mice are dyslipidemic and present all the
239 characteristics of type 2 diabetes (hyperglycemia, glucose intolerance, insulin resistance).

240

241 **HFD mice display elastic fibers fragmentation and increased aortic pulse wave velocity**

242 We next evaluated the impact of HFD on extracellular matrix remodeling in the aorta of mice
243 by measuring elastic fibers fragmentation. Elastin has autofluorescent properties and emits
244 fluorescence at 500- 560 nm when excited at 488 nm (31) (Fig 2A). In HFD mice, the main
245 number of breaks per elastic lamella was significantly increased compared to ND mice
246 (1.4±0.1 vs 0.3±0.1 breaks, Fig 2B). Moreover, the repartition of the number of breaks per
247 elastic lamella was also strongly different. In ND mice, up to 2 breaks per elastic lamella was
248 observed with the following repartition: no break (72%), 1 break (23%) and 2 breaks (5%)

(Fig 2C). In contrast, up to 6 breaks per elastic lamella was observed in HFD mice with the following repartition: no break (31%), 1 (30%), 2 (18%), 3 (12%), 4 (6%), 5 (2%) and 6 breaks (<1%) (Fig 2C). Taken together, these results demonstrated that HFD induced vascular extracellular matrix remodeling and fragmentation of elastic fibers in the aorta of mice. Finally, we monitored the evolution of aPWV in HFD mice (Fig 2D). At baseline, aPWV was calculated at 0.365 ± 0.024 cm/ms. No major modification was observed between baseline and 9 weeks of HFD (0.377 ± 0.024 cm/ms). After 12 weeks, aPWV was significantly increased to 0.449 ± 0.021 cm/ms in HFD mice.

Administration of oseltamivir phosphate in HFD mice has limited effects on type 2 diabetes and dyslipidemia

The impact of OP administration was next evaluated in LDLR^{-/-} mice fed with the same HFD during 12 weeks. OP (50 mg/kg, + OP) or vehicle (- OP) were administrated weekly by intraperitoneal injections, starting at the onset of HFD. At this concentration (50 mg/kg), OP was previously shown to completely ablate tumor vascularization, tumor growth and spread to the lungs in a mouse model of heterotopic xenografts of MDA-MB-231 tumors (13). As shown in Fig 3A, B, C, no significant difference was observed on glycemia, insulinemia, and HOMA-IR index between OP- and vehicle-treated groups, although a trend for increased insulinemia and HOMA-IR index was noted. Body weights (Fig 3G), HbA_{1c}, free fatty acids and triglycerides concentrations (Supplemental Digital Content 2) were also comparable between OP- and vehicle-treated mice. When considering plasma cholesterol (Fig 3D, E, F), a significant decrease of total-cholesterol concentrations was observed in OP-treated group when compared to vehicle-treated group (19.95 ± 1.29 vs 24.57 ± 1.50 mmol/L). This was associated with a significant reduction of LDL-cholesterol concentrations in OP-treated mice (12.98 ± 0.78 vs 15.65 ± 0.67 mmol/L).

Administration of oseltamivir phosphate in HFD mice decreases elastic fibers fragmentation without modification of pulse wave velocity

We next evaluated the effects of OP treatment on elastic fibers fragmentation and aPWV. As shown in Fig 2B, HFD induces a significant increase in elastic fibers breaks compared to ND. In HFD mice, OP administration was able to significantly decrease the main number of breaks per elastic lamella compared to vehicle-treated mice (0.9 ± 0.1 vs 1.7 ± 0.3 , Fig 4A). This was associated with major differences in the repartition of the number of breaks per elastic lamella (Fig 4B). In vehicle-treated mice, up to 7 breaks per elastic lamella was observed. In OP-treated mice, no more than 4 breaks were observed. The repartition of elastin breaks was as follows for the vehicle-treated group: no break (18%), 1 (39%), 2 (21%), 3 (14%), 6 (4%) and 7 breaks (4%). For the OP-treated group, the repartition was no break (40%), 1 (35%), 2 (17%), 3 (6%) and 4 breaks (2%). However, no major impact on the aPWV parameter was observed between the 2 groups (Fig 4C).

Administration of oseltamivir phosphate in HFD mice has no effect on atherosclerosis and carotid thrombosis

After 12 weeks of HFD, LDLR^{-/-} mice developed large atherosclerotic lesions in aortic roots (Fig 5A, left panels). After ORO staining and quantification, the mean lesion size was estimated at 0.36 ± 0.03 mm² for vehicle-treated mice and 0.41 ± 0.02 mm² for OP-treated mice (Fig 5A, right panels). Together, these data demonstrated that, in our experimental conditions, OP treatment had no effect on atherosclerosis development. We also evaluated the effects of OP treatment on arterial thrombosis by using the FeCl₃-induced carotid artery thrombosis model (34). Thrombosis was triggered by 7.5% FeCl₃ in both vehicle- and OP-treated HFD mice and time to complete thrombotic occlusion was monitored using real-time

videomicroscopy and defined as an observed arrest in blood flow during at least 30 seconds. No difference in the mean occlusion time was observed between vehicle- and OP-treated mice (8.5 ± 0.4 vs 8.9 ± 0.5 min) (Fig 5B). Although not significant, a trend for a decrease of the number of embols released during thrombus formation was observed in the OP-treated group (11.5 ± 3.5 vs 5.9 ± 1.4 embols) (Fig 5C). Taken together, these data demonstrated that, in our experimental conditions, OP treatment had no effect on carotid thrombosis but may lead to reduced embolization.

Administration of oseltamivir phosphate in HFD mice induces liver inflammation and fibrosis

After 12 weeks of HFD, the liver weight of LDLR^{-/-} mice had more than doubled compared to mice fed with ND diet (1.34 ± 0.07 vs 0.61 ± 0.19 g). No difference was observed between vehicle- and OP-treated HFD mice (Fig 6A). To evaluate potential toxic effects of OP on the liver, expression of different inflammation (F4/80, IL-1 β , TGF- β 1) and extracellular matrix remodeling (MMP-2, -9, -12, collagen) markers were assessed by quantitative RT-PCR. As shown in Fig 6B, LDLR^{-/-} mice fed with HFD showed a moderate but significant increase by 1.6- and 1.3-fold of IL-1 β and TGF- β 1 mRNA expression levels, respectively, compared to ND mice. No significant difference was observed for the macrophage F4/80 marker. In OP-treated HFD mice, F4/80, IL-1 β , TGF- β 1 mRNA expression levels were further significantly increased by 1.7-, 1.7- and 1.8-fold, respectively, compared to vehicle-treated HFD mice (Fig 6B). When considering markers of extracellular matrix remodeling, HFD mice induced a significant increase by 30-fold of MMP-12 mRNA expression levels compared to ND mice, and no significant difference was observed for MMP-2, -9 and type I collagen (alpha 1 chain) (Fig 6C). However, OP treatment further significantly increased by 1.7- and 2.5-fold MMP-12 and collagen mRNA expression levels, respectively, compared to vehicle-treated HFD mice

(Fig 6C). Together, these results showed that OP administration to HFD-fed LDLR^{-/-} mice could trigger liver inflammation, extracellular matrix remodeling and fibrosis.

Discussion

Together with zanamivir, OP form the basis of clinically available flu antiviral therapeutics (35). Whereas viral sialidase inhibitors have also broad specificity for bacterial NEUs, studies that have assessed the activity of zanamivir and OP against human NEUs (hNEUs) have reported weaker efficacy (9, 10). In a recent study, IC_{50} values for OP were estimated at more than 500 μ M against the four hNEUs, and the only viral NEU inhibitor tested with notable activity against hNEU2, hNEU3 and hNEU4 was zanamivir (10). However, despite this low inhibitory potency of OP for hNEUs, several reports have evaluated its use as potential inhibitor of hNEU1 sialidase activity and related signaling pathways in *in vitro* and *in vivo* studies. Among the four hNEUs described so far, NEU1 is indeed the most studied sialidase. Firstly identified in lysosomes (2), NEU1 is also found at the plasma membrane of cells and regulates a myriad of membrane receptors by desialylation resulting in either activation or inhibition of the receptors (36-44). At the plasma membrane, NEU1 also associates with the elastin-binding protein (EBP) and the carboxypeptidase protective protein/cathepsin A (PPCA) forming the elastin receptor complex (ERC) (45). NEU1 is known to be required for elastogenesis and signal transduction through this receptor (45-47), and for the biological effects mediated by the elastin-derived peptides (48, 49). Implication of NEU1 in atherosclerosis is supported by different studies. High expression of NEU1 has been reported in peripheral blood mononuclear cells of patients with acute myocardial infarction and in macrophages present in the intima layer, in calcified regions and within the adventitia of the plaque region in human carotid arteries (24). Enhanced NEU1 expression induces a pro-inflammatory phenotype, triggers production and release of cytokines and chemokines in monocytes, and promotes phagocytosis and cytokine expression in macrophages (24). In human macrophages, NEU1 also modulates the sialylation level of CD36 receptors and enhances oxidized LDL uptake in response to elastin-derived peptides

(41). Direct evidence for NEU1 involvement in atherosclerosis came from the works of Gayral et al (23). Using chimeric LDLR^{-/-} mice devoid of NEU1 activity in the haematopoietic lineage, Gayral et al showed decreased atheroma plaque size progression in aortic sinus and leucocytes infiltration. Moreover, in ApoE^{-/-} mice expressing hypomorphic levels of NEU1 (*Neu1^{hypo}Apoe^{-/-}*), White et al have reported reduced serum levels of very-low-density lipoprotein (VLDL) and LDL-cholesterol, decreased infiltration of inflammatory cells into lesions, and significant reduction of atherosclerosis (25). Consistent with these findings, administration of the broad spectrum sialidase inhibitor DANA had anti-atherogenic effect in the ApoE^{-/-} mice (25). Taken together, all these studies suggest that targeting NEU1 expression or catalytic activity may represent a promising target for managing atherosclerosis. In light of these promising data, we evaluated in this study the effects of OP administration on atherosclerosis development in HFD-fed LDLR^{-/-} mice, at a concentration (50 mg/kg) that completely ablates tumor vascularization, tumor growth and spread to the lungs in a mouse model of heterotopic xenografts of MDA-MB-231 tumors (13) and twice lower than the one used by Gilmour et al in a mouse model of heterotopic xenografts of human pancreatic cancer (12). From the results presented here, it is suggested that OP treatment has no protective effect on atherosclerosis development since comparable atheroma plaque size was observed in aortic sinus of vehicle- and OP-treated LDLR^{-/-} mice. If these contradictory results rely on differences between diet formulation, diet duration and/or mice genotype remain to be further evaluated. In the present study, LDLR^{-/-} mice were fed for 12 weeks with high-fat, high-carbohydrate diet, and one may consider that the protocol used here was too drastic to see any potential benefit of OP treatment. Moreover, OP was administrated weekly and not daily as described previously (13), and one may speculate that the time between two injections has to be shorten. However, using this protocol, OP was shown to be effective in reducing plasma LDL-cholesterol levels, in agreement with the works of White et al showing that ApoE^{-/-} mice

399 expressing hypomorphic levels of NEU1 had reduced serum levels of LDL-cholesterol (25).
400 Taken together, these results show that inhibition of NEUs by OP modulates lipoprotein
401 metabolism and reduces LDL-cholesterol levels. Modification of sialic acids on
402 apolipoproteins is known to affect lipoprotein metabolism (50-52), and advanced
403 desialylation of LDL by sialidases is considered as a primary step of atherogenic modification
404 of lipids (53).
405 Here, we also observed that OP-treated HFD mice are prone to develop insulin resistance.
406 Even if the difference failed to be significant between vehicle- and OP-treated groups, a near
407 2-fold increase of the HOMA-IR index was observed for OP-treated mice. Importantly,
408 insulin receptor activation has been shown to be under the regulation of NEU1 (37, 38) and
409 mice deficient in NEU1 rapidly develop glucose intolerance and insulin resistance after being
410 fed with HFD (38). Further works have uncovered a crosstalk between NEU1 and MMP-9 in
411 alliance with G-protein coupled receptor (GPCR), which was essential for insulin-induced IR
412 activation and cellular signaling (21, 22).
413 Another interesting finding from this study comes from the protective effect of OP on elastic
414 fibers integrity in aorta. Elastic fibers are fundamental for mechanical properties of the arterial
415 wall, and elastic fibers fragmentation has major consequences on atherosclerosis progression
416 (26). Degradation of elastin involves elastases such as MMPs (MMP-2, -7, -9, -12), cysteine
417 cathepsins (L, S, K, V), and/or the neutrophil elastase, (54). Whether OP reduces expression
418 or activity of elastases in aorta remains to be further evaluated. Damaged or degraded elastic
419 fibers are generally not repaired and replaced by collagens and proteoglycans that stiffen the
420 arterial wall. Even if no effect was observed on aPWV, OP treatment may help in limiting
421 arterial stiffness occurring with atherosclerosis. Decreasing elastin fragmentation may also
422 limit the generation of elastin-degradation products, which are critical players of
423 atherosclerosis development (26).

One major issue of atherosclerosis is plaque rupture and thrombosis. Since desialylation regulates platelet activation and aggregation (55, 56), we hypothesized that OP treatment may decrease atherothrombosis. However, we did not observe any difference in our chemically-induced carotid thrombosis model between vehicle- and OP-treated groups. In contrast, and even if the difference failed to be significant, a near 2-fold decrease of the number of embols released during thrombus formation was observed in OP-treated HFD mice. These results, that remain to be further confirmed, may suggest that OP could reduce embolization and hence increase thrombus stability.

The use of OP in patients has been associated with adverse effects such as nausea, vomiting, psychiatric effects, renal events (57), and liver toxicity (9, 27-29). Here, we report that OP treatment has adverse effects on the liver and significantly increased mRNA expression levels of typical inflammation markers such as the pan-marker of murine macrophages F4/80, and the pro-inflammatory cytokines IL-1 β and TGF- β 1. Together with increase in mRNA expression levels of MMP-12 and type I collagen, our results suggest that OP treatment also triggers ECM remodeling in the liver and fibrogenic responses. Long-term consequences on potential steatohepatitis development associated with OP treatment remain to be evaluated.

In conclusion, the present study shows that, in these experimental settings, OP treatment has limited effects on atherosclerosis and related thrombotic events in LDLR^{-/-} mice. Although higher concentrations and daily injections have to be considered before stating that OP has no protective effect on atherosclerosis and thrombosis, these data suggest that caution must be taken concerning its side effects on liver. Even if promising effects of OP have been reported in different animal models of human diseases, benefits versus harms of OP remains to be further evaluated before potential application in human.

Acknowledgements

This work was supported by funding from CNRS, URCA, and the Fondation de France. We thank Jean-Luc Breda for animal handling and Caroline Fichel for aorta cryosections.

Conflict of interest

The authors declare that they have no conflict interest.

Author contributions

PM conceived, designed, and supervised the study. LD, SB and ML helped in designing the study. OB, AW, TS, EV, CS, AF, SG, AH, SB, BRC and PM performed the experiments. AB, HS, SJ, PG and LM contributed to discussion. PM and LD were involved in writing the manuscript and PM and CS prepared the figures.

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Figure legends

Figure 1. Impact of HFD on body weight gain and metabolic parameters in LDLR^{-/-} mice.

Body weight (A), glucose tolerance test (B), plasma glucose (C), HbA_{1c} (D) plasma insulin (E), HOMA-IR index (F), and plasma total- (G), HDL- (H), and LDL- (I) cholesterol concentrations were measured from fasted LDLR^{-/-} mice. White bars (A, C-I) and white circles (B) correspond to mice fed with normal diet (ND), and black bars (A, C-I) and black squares (B) correspond to mice fed with HFD. Data represent the mean $\pm$ SEM of 4 to 5 mice. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 2. Impact of HFD on elastin fragmentation in aorta and aortic pulse wave velocity in LDLR^{-/-} mice.

(A) Autofluorescence of aortic elastic fibers in normal diet (ND) and high fat diet (HFD) mice. White arrows indicate zone of elastin breaks. (B) Quantification of the number of breaks per elastic lamella in ND (white bar) and HFD (black bar) mice. Data represent the mean $\pm$ SEM of 4 to 5 mice per group (***, $p < 0.001$). (C) Percent repartition of the number of breaks per elastic lamella in ND and HFD mice. The total circle represents 100%. (D) Evolution of aPWV every 3 months in HFD mice. Data represent the mean $\pm$ SEM of 7 to 9 mice. (*, $p < 0.05$ compared to baseline).

Figure 3. Effect of oseltamivir phosphate treatment on body weight and metabolic parameters in HFD-fed LDLR^{-/-} mice.

Plasma glucose (A), insulin (B), HOMA-IR index (C), and plasma total- (D), HDL- (E), and LDL- (F) cholesterol concentrations were measured from fasted LDLR^{-/-} mice fed with high fat diet (HFD) for 12 weeks, and treated (+OP) or not (-OP) with oseltamivir phosphate (50

mg/kg, weekly). (G) Body weight. White bars (A-F) and white circles (G) correspond to vehicle-treated mice, and black bars (A-F) and black squares (G) correspond to OP-treated mice. Data represent the mean $\pm$ SEM of 4 to 5 mice. (ns, non-significant; *, $p < 0.05$).

Figure 4. Effect of oseltamivir phosphate treatment on elastin fragmentation in aorta and aortic pulse wave velocity in HFD-fed LDLR^{-/-} mice.

(A) Quantification of the number of breaks per elastic lamella in mice treated with vehicle (-OP, white bar) or OP (+OP, black bar). Data represent the mean $\pm$ SEM of 4 to 5 mice per group (**, $p < 0.01$). (B) Percent repartition of the number of breaks per elastic lamella in mice treated with vehicle (-OP) or OP (+OP). The total circle represents 100%. (C) Evolution of aPWV every 3 months in HFD mice treated with vehicle (-OP, white circles) or OP (+OP, black squares). Data represent the mean $\pm$ SEM of 7 to 10 mice per group.

Figure 5. Effect of oseltamivir phosphate treatment on atherosclerosis in aortic sinus and carotid thrombosis in HFD-fed LDLR^{-/-} mice.

(A) Quantification of atherosclerotic lesion sizes in the aortic roots of mice treated with vehicle (-OP) or OP (+OP). Left panel shows a representative photomicrograph of ORO-stained fatty streaks for each group (original magnification $\times 50$). Right panel shows quantitative analysis of atherosclerotic lesion sizes (mm^2) for mice treated with vehicle (-OP, white circles) and OP (+OP, black squares). The mean $\pm$ SEM is shown. (B) Thrombotic occlusion time of mice treated with vehicle (-OP, white circles) or OP (+OP, black squares) after chemical injury of carotid arteries by 7.5 % FeCl_3 . The mean $\pm$ SEM is shown. Top photographs show, as an illustration, a representative progression of thrombus formation in mice injected with vehicle (-OP) or OP (+OP). (C) Number of embols released during thrombus formation in the carotid arteries of mice treated with vehicle (-OP, white bar) and

OP (+OP, black bar). Data represent the mean $\pm$ SEM of 6 to 7 carotids per group. (ns, non-significant).

Figure 6. RT-qPCR analysis of gene expression in the liver of ND- and HFD-fed LDLR^{-/-} mice and impact of oseltamivir phosphate treatment.

(A) Liver weight of mice fed with normal diet (ND, white bar) or high fat diet (HFD) treated with vehicle (-OP, black bar) or OP (+OP, hatched black bar). Data represent the mean $\pm$ SEM of 4 to 5 mice per group. (B, C) Total RNA was extracted from mice livers and analyzed by RT-qPCR as described in the Materials and Methods. Each gene expression was normalized using 2 reference genes (36B4 and RPS26) and results are presented as fold induction of ND mice. White bars, ND mice; black bars, HFD mice treated with vehicle; hatched black bars, HFD mice treated with OP. Data are expressed as mean $\pm$ SEM of 4 to 5 mice per group. For each mouse, PCR reactions were performed in duplicate from 3 independent experiments. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).