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Reference


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Perivascular sustained release of atorvastatin from a hydrogel-microparticle delivery system decreases intimal hyperplasia

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A combination of a sustained (from microparticles) and fast (from hydrogel) release of atorvastatin is necessary to inhibit intimal hyperplasia. The formulation is placed perivascularly, and atorvastatin permeates the vascular wall.
Abstract

Intimal hyperplasia (IH) is the major cause of grafted vessel occlusion and occurs frequently after bypass intervention. No pharmaceutical formulation is currently available to prevent this pathology. Local perivascular delivery of an appropriate active compound released in a time-dependent manner (from day one up to 4 weeks) is necessary for an efficient single-administration preventive therapy. To this aim, we propose the combination of gel and microparticles delivery system containing atorvastatin (ATV). The incorporation of ATV in a cross-linked hyaluronic acid gel, provided in vitro a fast release over 3 days, while ATV-loaded poly-lactic-co-glycolic acid (PLGA) microparticles dispersed in the gel gave a sustained release over 4 weeks. In vivo, ATV formulations were applied perivascularly in mice undergoing carotid artery ligation. IH was significantly reduced (-68 %) in presence of ATV incorporated in hyaluronic acid gel and encapsulated in microparticles compared to control. No significant IH alteration in IH was observed when ATV was incorporated only in the gel (fast release) or only in the microparticles (slow release) demonstrating that a biphasic release of ATV is essential to interfere with the development of IH.

ATV was detected in adjacent tissues 28 days after the intervention, showing the sustained presence of the drug in vivo. After four weeks ATV was not detected in remote tissues, except at a very low concentration (0.044 ng/mg) in the liver, suggesting a very low risk of systemic toxicity of locally delivered ATV. Additionally, the ex vivo data showed that ATV in solution permeates through isolated human saphenous veins and thus is a good candidate for perivascular delivery.

Our data demonstrate that a local biphasic ATV release on the mice ligated carotid efficiently prevents the development of IH without apparent toxicity.

Keywords

Intimal hyperplasia; Microparticle; Hyaluronic acid hydrogel; Perivascular administration; Atorvastatin
1. Introduction

Despite the improved results of endovascular treatment (balloons, stents), open surgical revascularization remains the gold standard for the treatment of vascular occlusive diseases. It is performed in over one million patients per year worldwide [1]. Autologous saphenous vein graft is the conduit of choice for peripheral bypasses. However, graft failure may occur within up to five years following the intervention, leading to a redo surgery, amputation and increased mortality rate [2]. Indeed, the combination of hemodynamic factors, such as turbulent blood flow due to a mismatch between graft and artery mechanical properties as well as surgical manipulation, result in a progressive lumen occlusion due to cell proliferation (restenosis) [3]. This clinical occurrence is mainly driven by intimal hyperplasia (IH), which is the thickening of the inner layer of the vessel due to vascular smooth muscle cell (VSMCs) migration and proliferation as well as high extracellular matrix secretion (Fig. 1 upper panel) [4-6]. Even if aspirin and statins are prescribed, in the vast majority of patients, for secondary prevention of IH, the restenosis rate after open surgical bypass revascularization remains high [1, 7-9].

Figure 1. Representation of the vein graft bypass surgery with the development of the intimal hyperplasia responsible for graft failure (upper panel) and the atorvastatin (ATV) delivery system proposed as a preventive treatment to preclude intimal hyperplasia development (lower panel).
A preventive therapy applied locally during the intervention, as a single administration, ensuring adequate dose of drug delivered on site can be a promising approach [10, 11]. The application of the drug around the vessel (perivascular) is easily achieved during surgery as the vessel is exposed and accessible. Several strategies have been developed for the perivascular administration of drugs aiming at preventing graft failure. For instance, sheaths [12], wraps [13], meshes [14], membranes [15], cuffs [16], particles [17, 18] and gels [19-24] have been explored. To our knowledge, none of these technologies are used in clinical practice due to a lack of efficacy [25]. The limitations of these approaches include difficulty in handling and applying the formulations, as well as the need for repeated administrations due to a non-optimal drug release profile and the fast degradation of the polymers [21, 26].

A cross-linked hyaluronic acid (clHA) hydrogel was selected as the vehicle for the local delivery. The selection of clHA was initially motivated by essential mechanical properties that would ensure ease of handling and conformable covering of the graft. Additionally, clHA is enzymatically degraded by hyaluronidases, avoiding the need for a second surgery to remove the implant [27]. Nevertheless, its cross-linked nature slows the degradation. When a drug is loaded in a gel, a fast diffusion-driven release in less than 3 days is observed [25]. However, ideally, a sustained release for at least 4 weeks is required to control IH progression [1]. Therefore a biphasic system is proposed, consisting of a clHA gel phase to provide a fast release of the compound and ensure the position of the formulation around the vessel and drug loaded-polyactic-co-glycolic acid (PLGA) microparticles to ensure the slow release of the drug (Fig. 1 lower panel).

Appropriate selection of the drug is essential to limit the inhibition of the vessel's re-endothelialization, which could lead to thrombus formation [25]. Inspired by the success of endovascular drug eluting stents, the recent literature on perivascular administration focuses on the delivery of antiproliferative compounds (paclitaxel, sunitinib, sirolimus) [12, 13, 17, 18, 23, 28, 29]. However, these compounds tend to inhibit the re-endothelialization of the vessel [18, 30]. For this reason, we selected a non-antiproliferative pharmacological approach, atorvastatin (ATV). Although statins are traditionally thought to exert their beneficial effects on saphenous vein graft patency by reducing the blood’s lipid content, it was also demonstrated that they inhibit IH in arterialized vein grafts by limiting macrophage infiltration and VSMC migration and proliferation [24, 31-34].

We previously showed that when ATV is released from the clHA gel, it maintains its activity and inhibits cell proliferation and migration of hVSMC in vitro [35]. The objectives of the present study are: a) to prepare formulations with various in vitro release profiles; b) to demonstrate in
vivo the efficacy of the formulations; and c) to explore whether ATV is able to permeate human vein grafts ex vivo from the perivascular side to the lumen.

2. Materials and methods

2.1. Materials
Poly(D,L-lactic-co-glycolic acid) (PLGA; Resomer® RG503 molecular weight 42.000 Da Boheringer Ingelheim; Ingelheim; Germany), atorvastatin calcium (Chemos GmbH; Regenstauf; Germany), atorvastatin-d5 sodium salt, (TRC, Canada), PVA or polyvinyl alcohol (Mowiol® 4-88; Kuraray Europe; Hattersheim am Main; Germany), chloroform (Chromasolv® plus for HPLC, ≥ 99.9 %, 0.5 - 1.0 % ethanol as stabilizer, Sigma- Aldrich Chemie GmbH, Steinheim, Germany), Toluidine Blue O (Merck, Germany) and Poloxamer 407 (Pluronic® F127, BASF, Germany) were used as received. Cross linked hyaluronic acid hydrogel (Fortelis extra®) was a generous gift from Anteis, Meyrin, Switzerland. All other chemicals were of analytical grade. Nitex® 03 with a 100-µm mesh opening was purchased from Sefar, Heiden, Switzerland. MF-Millipore GSWP02500 filters (0.22 µm) were purchased from Millipore Merck, Darmstadt, Germany. Vertical Franz's diffusion cells with an internal diameter of 20 mm were manufactured on demand by Glass Technology, Meyrin, Switzerland.

2.2. Microparticle preparation
Microparticles were prepared by an oil-in-water (o/w) solvent emulsion-evaporation process. We dissolved 375 mg of PLGA and 37.5 mg of ATV in 7 g of chloroform before emulsification in a PVA 2 % aqueous solution at 1500 rpm for 20 minutes using a paddle stirrer (Eurostar digital, IKA-Werke, Staufen, Germany). The emulsion was added to 50 mL of water, and chloroform was evaporated at room temperature overnight at a stirring rate of 500 rpm. Unloaded microparticles were prepared similarly without ATV. The particles were washed and concentrated by successive steps of centrifugation/re-suspension before freeze-drying (Alpha 2-4 LSC, Christ, Kuhner, Switzerland). ATV-loaded microparticles are referred to as Matv and unloaded microparticles as M.

2.3. Microparticle size analysis
Freeze dried particles were suspended in water, and their size distribution was measured by laser diffraction on a Mastersizer S Long Bench equipped with a small volume sample dispersion unit and a dispersion unit controller set at 1100 rpm (Malvern Instruments Ltd, Worcestershire, UK). The analysis was performed using the Fraunhofer model. The refractive index was set at 1.5295 for microparticles and 1.33 for water.
2.4. Quantifying microparticle drug loading

Approximately 4 mg of microparticles were dissolved in 3 mL of acetonitrile. Then, 3 mL of ethanol were added to ensure ATV dissolution. The samples were analyzed by reversed phase HPLC (LC module I plus, Waters corporation, Milford, USA), and ATV was measured with a UV spectrometer set at $\lambda = 245$ nm, retention time 7.8 min. The column was a Nucleosil CC 125 / 4 120-5 C$_{18}$ (Macherey-Nagel GmbH & Co. KG, Oensingen, Switzerland), maintained at 25°C. The mobile phase was 55 % acetate buffer (10 mM, pH 3) and 45 % acetonitrile, at a flow rate 1 ml/min. The calibration curve was constructed by consecutive dilutions of ATV in acetonitrile/ethanol containing PLGA (0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 µg/ml). The method has been fully validated, and a limit of quantification of 500 ng/mL and limit of detection of 50 ng/mL were obtained. A trueness of 98 to 102 % was determined, and the intermediate precision was 2 %; moreover, the three replicates injected on three different days demonstrated the repeatability of the method. The quantification was conducted in triplicate. The results are presented as actual drug loading, corresponding to the percent mass ratio of the encapsulated drug to the mass of the microparticles recovered.

2.5. Incorporation of microparticles and/or ATV in the gel

Freeze dried particles were dispersed in water. ATV was dissolved in a hydroalcoholic solution of 30 % ethanol. The gel was mixed with the suspension of microparticles (94 mg per gram of gel) and/or ATV in solution using a spatula and immediately freeze-dried in a Telstar LyoBeta 15 (Telstar, Terrassa, Spain) with primary drying at -40 °C under 0.2 mBar for 10 h followed by 10 h of secondary drying at 20 °C. Prior to use, the cake was re-hydrated with a sufficient amount of sterile water to obtain the initial weight. To minimize the microbial load, the preparation was performed in a hood under laminar flow (Steag LFH 07.15, Luftechnik + Metallbau AG, Wettingen, CH), solutions were filtered prior to use and glassware was sterilized. The commercial cLHA gel was sterile. ATV-loaded gels are referred to as Gatv and unloaded gels as G.
The composition of the various formulations is presented in Table 1. The GatvMatv was prepared by merging the amounts of ATV contained in Gatv and the microparticles Matv.

**Table 1.** Amount of ATV loaded in the gel and/or the microparticle formulations applied *in vivo.*

G: gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV

<table>
<thead>
<tr>
<th>Formulation</th>
<th>clHA Gel</th>
<th>Microparticles</th>
<th>Total ATV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM</td>
<td>no ATV</td>
<td>Unloaded microparticles</td>
<td>-</td>
</tr>
<tr>
<td>G_{atv}M</td>
<td>1mg ATV /g of formulation</td>
<td>Unloaded microparticles</td>
<td>1mg/g of formulation</td>
</tr>
<tr>
<td>GM_{atv}</td>
<td>no ATV</td>
<td>1mg ATV /g of formulation</td>
<td>1mg/g of formulation</td>
</tr>
<tr>
<td>G_{atv}M_{atv}</td>
<td>1mg ATV /g of formulation</td>
<td>1mg ATV /g of formulation</td>
<td>2mg/g of formulation</td>
</tr>
</tbody>
</table>

**2.6. Scanning electron microscopy**

For microscopic observations, an atorvastatin ethanolic solution (50 % w/v) was lyophilized and rehydrated with distilled water at a concentration of 1 mg/mL. Particles were suspended in distilled water. A drop of the suspension was placed onto stubs, dried under vacuum, covered with a 15 - 20 nm layer of gold and examined by scanning electron microscopy (SEM) using a JSM - 7001FA (JEOL, Tokyo, Japan) at 5.0 kV. To obtain microparticles cross-sections, dry particles were immersed in common wood glue and cut using a razor blade. These sections were further processed as mentioned above.

**2.7. Rheological measurements**

The rheology of the preparations described in Table 1; clHA as received or after lyophilization (G) and Poloxamer 407 at 20 % w/w were characterized at 37°C with a Haake Rheostress 1, Thermo Scientific fitted with a 35 mm / 2° cone-plate geometry. The instrument was set to perform the measurements with three repetitions and deliver the results as the mean. Rotation was performed over a shear rate ranging from 0.01 to 500 s⁻¹. Oscillation was performed with a frequency ranging from 0.628 to 31.4 rad/sec.

**2.8. Swelling measurements**

Approximately 50 mg of clHA or Poloxamer 407 were allowed to swell in MilliQ water at 37°C. After 24 hours, the swollen gel was filtered under moderate pressure through a 100-µm mesh. The toluidine blue staining test was used to confirm the absence of hyaluronic acid in the filtrate. The swelling ratio was determined as the ratio of the weight of the swollen gel to the weight of lyophilized gel.
2.9. In vitro ATV release from the formulations

ATV release from the gels was performed using Vertical Franz’s diffusion cells. In this system, the gel is kept separated from the release medium by a 0.22-μm filter. The filter allows the free diffusion of ATV. With this set up, the gel is not in direct contact with the medium, avoiding its dilution. If the gel was placed directly into an aqueous medium, it would form a gel-particle suspension [36]. Briefly, 500 mg of each formulation were placed in the donor compartment and 10 mL of PBS (0.1 M, pH 7.4) / SDS 0.1 % in the receptor compartment (ensuring sink conditions, solubility of ATV 600µg/ml). The receptor compartment was under constant magnetic stirring. Franz’s cells were placed in an incubator at 37° C (EG 110 IR, Jouan, Saint-Herblain, France). At predetermined time intervals, 0.5 mL were withdrawn from the receptor compartment and replaced with fresh medium. The samples were analyzed by reversed phase HPLC as previously described (paragraph 2.4). The calibration curve was constructed by consecutive dilutions of ATV in PBS (0.1 M, pH 7.4) / SDS 0.1 % (0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 µg/ml). Each experiment was conducted in triplicate, and the values are expressed as the mean ± standard deviation. To assess whether the GatvMatv curve was similar to the sum of GatvM and GMatv, we used the similarity factor $f_2$, as recommended by the United States Pharmacopeia, for dissolution profile comparison [37].

2.10. In vivo model

Six weeks old male mice (C57BL/6J WT strain) weighing 20-25 g were used. All in vivo experiments were performed in compliance with the Swiss Federal Law on the Protection of the Animals, following a protocol accepted by the canton of Vaud Authority (Service cantonal de la consommation et des affaires vétérinaires), authorization n° 2523.

A mouse model generating IH by ligation of the carotid artery (CAL model) was used for the in vivo tests [38]. The procedure is depicted in figure 2. Animals were anesthetized with spontaneous breathing of 1 % isoflurane (Forene®, AbbVie AG) followed by an intraperitoneal injection of a mixture of Ketamine 100 mg/kg (Ketalar®, Pfizer AG) and Xylazine 20 mg/kg (Rompun® 2 %, Bayer). The animals were installed on a platform heated at 38° C. Using a microscope, a left longitudinal neck incision was made, and the left common carotid artery was carefully exposed between the sternocleidomastoid and digastic muscles. A ligation of this vessel was performed proximally to the bifurcation site with 7.0 polypropylene suture threads (Fig. 2, upper panel). When formulations were tested, 50 µl of gel was applied at the ligation site (Fig. 2, lower panel). The skin was then sutured with 4.0 polypropylene suture threads. The animals were maintained on a standard chow diet for a period of 4 weeks. Euthanasia was
performed at the 28th post-operative day by cervical dislocation after inhalation of 99 %
isoflurane. Following sacrifice, each mouse was perfused with an intracardiac injection of 5 mL
of a 1 % PBS solution at physiological pressure for 5 minutes and 5 mL of 4 % formaldehyde
(DKK Italia). In total 68 animals were used (sham n=8, GM n=18, GatvM n=8, GMatv n=8,
GatvMatv n=18, healthy n=8).

Common carotid arteries were harvested bilaterally from the mice at sacrifice (28 days),
fixed in formalin and paraffin-embedded. To observe the IH formation, the left and right carotid
arteries were transversely sectioned in 6-µm thick cuts at 0.1 mm from the ligation. Hematoxylin
and eosin (HE) as well as Van Gieson Elastin (VGEL) staining were performed for histologic and
morphometric analysis. A Panoramic MIDI slide scanner (3DHistech Kft., Hungary) was used to
digitize the sections. Snapshots were extracted using Panoramic Viewer software. Upon first
observation of the sections, about 10% of the slides showed the presence of thrombi, therefore,
these samples were not taken into account. For intimal and medial thickness, as the intima is not
always homogenous in thickness, 72 measurements (12/cross section on 6 cross sections) were
performed as recently published [39]. Neointima thickness was defined as the distance between
the lumen and the internal elastic membrane. For each carotid artery, the intima and media
thickness were measured. The media thickness was defined as the distance between the
internal elastic lamina and the external elastic lamina. Similar methodology and measurements
were performed on contralateral carotids. Each carotid section was divided by a 2x2 grid. The
thicknesses of intima and media were defined as the mean of the thickness of four
measurements, randomly chosen in each square of the grid.

For immunofluorescence, five-micrometer sections of carotids were prepared from
paraffin-embedded tissues. In brief, after deparaffinization and heat-mediated antigen retrieval,
sections were incubated with a primary antibody against SMA (rabbit polyclonal, ab5694; Abcam
1:100), in combination with biotinylated CD31 (rat polyclonal; 553371; BD Pharmingen, 1:100) in
blocking buffer (PBS, 2% BSA, 0.3% Triton X-100) at 4°C overnight. Samples were then
incubated for 1 h at room temperature with either Alexa-Fluor-594- or -488-coupled secondary
antibodies (Life Technologies), diluted 1:500 in PBS, covered with PBS containing 50% glycerol
and 0.4µg/mL DAPI, and observed by fluorescence microscopy (Leica Leitz DMRB, Nidau,
Switzerland). Two controls, in which we omitted the primary or secondary antibody, were
included in each experiment.

At sacrifice at 28 days, we collected adjacent tissues (thymus, sternocleidomastoid
muscle, and neck skin) as well as remote tissues (biceps femoris muscle, heart, lungs and liver).
Tissues were rinsed with PBS and preserved at -80°C. The concentration of ATV was measured
by LC/MS-MS. Tissues were homogenized in PBS (Omni TH Tissue Tearor, Kennesaw, USA) and centrifuged. Stock solutions of ATV calcium salt and ATV-d5 (internal standard) stock solutions were prepared in methanol and diluted with MeOH/H2O 1:1. Working solutions were prepared by homogenizing tissue blank in PBS and adding ATV solutions (0.002, 0.005, 0.010, 0.020, 0.050, 0.100, 0.200, 0.500, 1.000, 2.000, 5.000, 10.000 ng per mg of tissular suspension). Twenty microliters of the supernatant were injected in an LC/MS-MS Triple stage quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fischer Scientific, Inc., equipped with an electro-spray ionization (ESI) interface and operated with Xcalibur 2.0.7 software (Thermo Fischer Scientific Inc., Waltham, MA). The column used was a Waters Acquity UPLC HSS T3 2.1 x 50 mm, 1.8 μm, at +5° C. The mobile phase ‘A’ consisted of 10mM ammonium formate and 0.1 % formic acid, while mobile phase ‘B’ of acetonitrile + 0.1% formic acid. At flow rate of 300 µl/min, run a gradient of A from 60% to 20%. Retention time was 2.6 min. The limit of quantification (LOQ) (i.e., the lowest level of calibration) was 0.2 ng /mL, corresponding to 0.002 ng ATV per mg tissue.

**Figure 2.** Main steps of the carotid artery ligation procedure for generating intimal hyperplasia: exposure of the carotid segment (upper, left panel), ligation (upper right panel). ATV-loaded formulation is deposited onto the anastomosis site (lower left panel) before closure. At sacrifice, after 28 days (lower right panel) gel is still found perivascularly.

### 2.11. Ex vivo permeability model

Four segments of non-varicose human great saphenous veins (hGSV) were used under patient written consent. The hGSV were cut longitudinally and mounted on a watertight Franz’s diffusion cell (intima facing the receptor compartment). In total, 11 pieces of hGSV were
obtained. The medium used to fill the Franz’s diffusion cells was RPMI 1640 containing 5 % fetal bovine serum (Gibco, Life Technologies) and 1 % antibiotic-antimycotic solution (10,000 U/mL penicillin G, 10 mg/mL streptomycin sulfate, 25 mg/mL amphotericin B, and 0.5 μg/mL gentamycin). Four-hundred microliters of ATV (16 μg/mL) in solution in the medium were placed in the donor compartment. The receptor compartment was filled with 1 mL of medium and was kept under magnetic agitation at 37° C and 5 % CO₂.

At predetermined time intervals (1 h, 24 h, 36 h, 48 h, 60 h, 72 h), 50 μL of culture medium was withdrawn from the receptor compartment. In a separate set of experiments, 50 μL of solution was withdrawn from the donor chamber at 24 h and 72 h. The samples were analyzed by reversed phase HPLC as described earlier. At 24 h or 72 h, veins were collected, washed with medium and stored at -80° C to measure the ATV concentration. The concentration of ATV in tissue was measured by LC/MS-MS as described above.

The hGSVs were fixed in formalin before and after being mounted on Franz’s diffusion cell. They were then transversely sectioned in 6-μm thick cuts and stained with HE and VGEL. Histomorphometric analysis was performed as described above.

2.12. Statistical analyses
All statistical analyses were performed using GraphPad Prism 6.05. Outliers were removed by ROUT Q = 1 %.

2.12.1. In vivo histomorphometry
Ordinary one-way ANOVA with Tukey’s multiple comparison test was performed to compare the groups.

2.12.2. Biodistribution
A two-tailed Mann-Whitney t-test was performed to compare GMatv and GatvMatv groups for adjacent tissues.

2.12.3. Ex vivo permeation
A two-tailed Mann-Whitney t-test was performed to compare vein content in ATV at 24h and 72h. Ordinary one-way ANOVA with Dunn’s multiple comparison test compared donor content in ATV at 0h, 24h and 72h.

3. Results
We prepared three different formulations to evaluate the most efficient treatment for IH. These formulations had various release profiles in vitro: a) fast release within a few days, b) sustained release over several weeks, and c) combination of fast and sustained release. A control formulation was also prepared containing an unloaded gel and microparticles.
3.1. Preparation and characterization of the formulations

The microscopic and macroscopic aspects of the formulations are shown in Fig. 3. After dispersion in water and drying, ATV was in the form of a nanosuspension of amorphous filaments (Fig 3a). Spherical PLGA microparticles of a mean diameter of 15 μm (span = 1.453) were obtained. Their cross-sections had a plain, matrix aspect (insert in Fig 3b). The ATV actual drug loading was 1.5 ± 0.18 % (w/w).

Poloxamer 407 as a frequently used excipient for gel formulation was included in the early tests. Poloxamer 407 at 20 % w/w and clHA had a similar appearance at 37°C (Fig. 3 c and d). The clHA lost its transparency after lyophilization and became opaque when ATV and/or microparticles were incorporated (Fig. 3 f-h).

![Figure 3. Microscopic and macroscopic photographs of the formulations. SEM pictures of a: ATV nanosuspension, b: ATV-loaded microparticles and cross section in the insert. Gel pictures of c: poloxamer 407 at 37°C, d: crosslinked HA as received, e: lyophilized rehydrated crosslinked HA (G), f: G containing free ATV and unloaded microparticles (GatvM), g: microparticles encapsulating ATV in G (GMatv), h: G containing free ATV and microparticles encapsulating ATV. Gel volume presented on photographs ≈ 0.1-0.3 ml.](image)

Although poloxamer 407 and clHA have comparable viscosities, their elasticity parameters are markedly different (Table 2). The weak elasticity of the poloxamer 407 exhibited a ten-fold higher tan(δ) value compared to clHA. Little swelling potential was observed for poloxamer 407 compared to clHA.
The viscosity of the gel decreased with the incorporation of the ATV ethanolic solution (GatvM) or increased when microparticles were added in absence of ATV in the gel (GM, GMatv). For all of the formulations, excepted for the poloxamer, the elastic properties were practically unaltered as the viscosity modulus ($G''$) remained well below the elastic one ($G'$) throughout the measurement ($\tan(\delta) < 1$)

### Table 2. Rheological and swelling characteristics of the formulations. Values of $\tan(\delta)$, $G'$ and $G''$ at a 1 Hz oscillation. Viscosity at a shear rate of 10 s$^{-1}$. Swelling was determined only for clHA and poloxamer. G: lyophilized gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV, nd: not determined

<table>
<thead>
<tr>
<th>Gel</th>
<th>$\tan(\delta)$ (°)</th>
<th>$G'$ (Pa)</th>
<th>$G''$ (Pa)</th>
<th>$\eta$ (Pas)</th>
<th>Swelling ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>clHA (as received)</td>
<td>0.450</td>
<td>127.0</td>
<td>57.2</td>
<td>47.6</td>
<td>2693 ± 123</td>
</tr>
<tr>
<td>Poloxamer 407 20% w/w</td>
<td>3.39</td>
<td>10.5</td>
<td>35.5</td>
<td>42.7</td>
<td>51 ± 17</td>
</tr>
<tr>
<td>G</td>
<td>0.397</td>
<td>208.5</td>
<td>82.9</td>
<td>39.3</td>
<td>nd</td>
</tr>
<tr>
<td>GM</td>
<td>0.457</td>
<td>241.9</td>
<td>110.6</td>
<td>53.5</td>
<td>nd</td>
</tr>
<tr>
<td>GatvM</td>
<td>0.497</td>
<td>217.1</td>
<td>107.9</td>
<td>22.3</td>
<td>nd</td>
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<tr>
<td>GM_{atv}</td>
<td>0.475</td>
<td>289.7</td>
<td>137.5</td>
<td>72.3</td>
<td>nd</td>
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<tr>
<td>GatvM_{atv}</td>
<td>0.432</td>
<td>243.8</td>
<td>105.3</td>
<td>70.4</td>
<td>nd</td>
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</tbody>
</table>

### 3.2. In vitro drug release profile

The three formulations presented different release kinetics in vitro (Fig. 4). ATV diffused out of GatvM within 3 days (fast release), GMatv release extended over 4 weeks (sustained release). It showed a triphasic release profile with a lag time between seven and twelve days. GatvMatv demonstrated a combination of a fast release (60% of ATV) in the first 3 days and a sustained release over 4 weeks. The GatvMatv curve was similar to the curve obtained by the addition of the data from GatvM and GMatv, as indicated by the similarity factor $f_2 > 50$. 
Figure 4. ATV release from the formulations in vitro (PBS (0.1 M, pH 7.4) / SDS 0.1 %). Left panel: Cumulative release expressed as amount (left axis) or concentration (right axis). The dash curve corresponds to the calculated sum of releases of GatvM and GMatv. Right panel: Non-cumulative release of ATV at five-day intervals. Each point is a mean ± SEM (n=3). G: gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV.

3.3. In vivo efficacy on the CAL model

The in vivo efficacy of the formulations containing ATV (GatvM, GMatv, GatvMatv) was compared to the unloaded gel and microparticles (GM) on a carotid artery ligation model of mice generating IH. Controls comprised sham operated and healthy mice. Four of 68 mice were found dead a few hours after the operation due to internal bleeding. Five mice presented luminal thrombi on histological sections and were excluded from the measurements. After 28 days, the gel and microparticles applied perivascularly were still observed (Fig. 2, lower right panel). No evidence of ATV toxicity such as necrosis was observed macroscopically.

Representative histological samples of carotid artery sections are presented in Fig. 5. In the sham mice, we observed the presence of the typical hyperplastic disordered arrangement of VSMCs on the sub-intimal area of the vessel (Fig. 5). The intimal thickness increased up to almost complete occlusion of the lumen in the carotids for these animals. The intima/media ratio alteration for GM (+5 %, p > 0.9999) and GatvM (-24 %, p = 0.941) showed no significant difference compared to sham operated animals. When microparticles encapsulating ATV were present in the formulation (GMatv), the intima/media ratio decreased compared to sham operated animals (-39 % p = 0.6840). However, the values reached significance only for GatvMatv, when ATV was added in the gel and was at the same time encapsulated in the microparticles (-68 %, p = 0.0415). The intima/media ratio of the animals treated with GatvMatv formulation was not significantly different from the one observed with healthy mice (p = 0.7490). The preservation of the endothelial cell lining in the vessel wall was demonstrated (Fig. 6).
α-smooth muscle cell actin was present in the intimal area revealing the presence of hyperplasia.

**Figure 5.** Effect of the different formulations on the intimal hyperplasia generated by the ligation of the carotid artery in mice. The formulation combining a fast and sustained release (GatvMatv) significantly decreased intimal hyperplasia in the mouse model. Upper panel: Representative histological sections of mice carotid arteries stained with VGEL and HE. Lower panel: Mean values of the intima thickness and intima/media ratio of carotid arteries. G: gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV. Data are presented as the mean values of 7 to 8 animals, except from GM and GatvMatv, for which 16-17 animals were used. Each bar is the mean ± SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons: P < 0.05 (*), P < 0.01 (**) compared to the sham. P < 0.01 (++), P < 0.001 (+++), compared to GM. P < 0.05 (#), P < 0.01 (##), P < 0.001(###) compared to healthy mice.
Figure 6. Representative histological sections of immunostained mice carotid arteries. No lesion of the vessel endothelium is observed with ATV, clHA as well as PLGA microparticles. Upper panel: anti-α-SMA demonstrates the presence of VSMCs. Middle panel: anti-CD-31 shows that the endothelial lining is present in all conditions. Lower panel: merge of α-SMA, CD-31 and DAPI. G: gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV.

The concentration of ATV was quantified in tissues adjacent to the site of application such as the thymus gland, neck skin and sternocleidomastoid muscle (Fig. 7). We detected 0.5 to 3.2 ng of ATV per mg of tissue in animals treated with gels containing ATV-loaded microparticles (GMatv, GatvMatv). ATV was below the detection limit for GM and GatvM formulations. ATV was not detected in remote tissues such as biceps femoris, heart, lungs and serum. In the liver, we measured 0.1 ng/mg in animals receiving Matv.
Figure 7. Tissues concentration of ATV in organs at 28 days. Concentration of ATV in adjacent tissues (left panel) and remote tissues (right panel). ud: undetected. G: gel, M: microparticles, Gatv: gel containing free ATV, Matv: microparticles encapsulating ATV. Each bar is the mean ± SEM in ng of ATV per mg of tissue (n = 8). Two-tailed Mann-Whitney t-test compared GMatv with GatvMatv: P < 0.05 (*).

3.4. Ex vivo diffusion of ATV through human saphenous veins

We investigated the diffusion of ATV in solution through a hGSV wall using a Franz’s cell set-up. First, we evaluated the microstructural changes of the tissue after 3 days of exposure to high ATV concentrations (Fig. 8, upper panel). No microscopic alteration was observed on the vessel wall in terms of nuclei integrity and overall organization both for VSMCs and endothelial cells. ATV in the donor compartment gradually diffused through the vein wall and was detected in the receptor compartment (Fig. 8, lower panel). Moreover, ATV impregnated the vein during the first 24 hours. The total ATV concentration in the tissue at 72 h (46.8 ± 12.3 ng ATV /mg) was not different compared to 24 h (40.5 ± 5.6 ng ATV /mg tissue). We observed a 25% decrease in the total amount of ATV in the 72 h time course. No ATV degradation peaks appeared on the HPLC chromatogram (data not shown).
**Figure 8.** *Ex vivo* diffusion of ATV through human saphenous veins. Upper panel: Human vessel histology is unaltered after 72 h both in a Franz cell with VGEL and H&E staining. Lower panel: Atorvastatin permeates the human vein. Distribution of ATV in the Franz cell between donor (light grey), human vein (grey) and receptor (black) compartments. Amounts of ATV are written in the bar. Each bar is the mean ± SD (n=11). Two-tailed Mann-Whitney t-test gave no significant difference between vein content in ATV at 24h and 72h. Ordinary one-way ANOVA with Dunn’s multiple comparison test for donor content in ATV showed $P < 0.05$ between 0h and 24h, and $P < 0.001$ between 0h and 72h. No significant difference was observed between 24h and 72h.
4. Discussion

The aim of this study was to explore the potential of a single administration of an ATV formulation to prevent the development of IH, which is responsible for bypass graft failure. The local perivascular route of administration was selected. We address the preparation and the in vivo test of a drug delivery system containing ATV gathering the following components: a) a clHA gel for the ease of application, slow biodegradability [27], a proven track record of safety in humans providing a fast release of ATV; b) PLGA microparticles for providing sustained drug release kinetics; and c) atorvastatin as the active compound, which permeates the vein and reduces VSMC proliferation and migration [35].

In perivascular delivery, biodegradable sheaths, wraps, meshes, membranes and cuffs have been tested. They demonstrate the advantage of complete coverage of the graft [12-16]. However, many polymers turn stiff and brittle after time passes in vivo and/or do not match the vascular tissue elasticity [12]. In the clinical setting, a gel would be more convenient in terms of ease of application and can ensure a full coverage of the vessel. Gels such as poloxamer 407 (Pluronic®) [17, 23, 40] or PLGA-PEG-PLGA (ReGel®) have already been described for this route of administration [19-22]. However, the rheological profile of the poloxamer 407 demonstrated a complete lack of elasticity (Table 2). Non-elastic gel is also prone to flow away from the site of application. Additionally, these gels are known to rapidly dissolve in vivo [41], precluding their use for our goal of a release kinetic from day 0 to day 28.

Thus, we selected a cross-linked gel that would remain in vivo for the long term. Commercially available clHAs are used in rheumatology (Synvisc®), ophthalmology (Anikavisc™) and for aesthetic interventions (Fortélis Extra®). Due to their cross-linked nature, the residence time of these formulations in vivo can extend from hours to months. In the present study, the clHA gel was still detected at the site of application 28 days after the intervention (Fig. 2). This finding is consistent with its elastic rheological behavior (Table 2).

A sustained release strategy is crucial for the prevention of IH, that was shown to progress up to 49 days after intervention in an arteriovenous polytetrafluoroethylene graft model [42]. In fact, the acute stage of IH development occurs during the first 30 days following the intervention [1]. As clHA doesn’t offer the possibility of delivering the drug for a long period of time (Fig. 4 GatvM), PLGA microparticles were added in the gel. Zhu et al. have used PLGA particles incorporated in ReGel® releasing dipyridamole over 35 days [20] Owen et al. dissolved rapamycin in ReGel® using DMSO or methanol with a release kinetic over 15 days. [21]. Unfortunately, these non-cross-linked gels, which were used as matrices for particle support, are
known to wash away rapidly. Moreover, it was also mentioned that a series of injections might be necessary to maintain an effective drug concentration [21].

To determine the optimal release kinetics inhibiting IH, formulations were ranked as “fast” and/or “sustained” according to their in vitro release kinetics. We used the in vitro curves to support the comparison of the in vivo results obtained with the different formulations. Fast release corresponds to GatvM (3 days in vitro) when only the ATV nanosuspension was present in the gel. This result is related to the diffusion time of the slightly water soluble ATV [13]. A typical sustained release is observed when PLGA microparticles are present in GMatv (4 weeks in vitro) [43]. The combination of fast and sustained release profiles was ensured with GatvMatv.

In vivo drug biodistribution in the surrounding tissues will also be modulated by drug accumulation, drug clearance and excretion, parameters not evaluated in this study.

The in vivo efficacy of the formulations was assessed on the CAL model in mice. The CAL model is a well-known and well described model for IH development in small animal triggering the formation of VSMC-rich neointima layer [38, 39, 44, 45]. Recently, using this very same CAL model we showed that the ligation of the mouse carotid triggered the formation of a VSMC-rich neointima layer which was increased in hypertensive mice in association with an increased cell proliferation and recruitment of CD45-positive inflammatory cells compared to normotensive mice [39]. The sham operated carotids presented a radical lumen occlusion with the increase of the intimal region and VSMC proliferation (Fig. 5 and 6). The formulations varied in efficacy in vivo (Fig. 5). We observed that unloaded cHA and microspheres had no effect on inhibiting IH. The literature on this topic is controversial, as some studies report that HA is able to reduce IH formation [46, 47], had no effect [48], or an increased cellular response was observed [49]. In these studies, the types and concentrations of HA gels vary, and thus, a case by case study should be performed to conclude on the efficacy of HA over IH.

The most efficient ATV loaded formulation in vivo was the combination of a fast and sustained release (GatvMatv). GatvM had little effect in decreasing the development of IH. This might be related to the short time frame during which ATV is available in the proximity of the carotid artery (3 days in vitro). ATV-loaded microparticles (GMatv) showed a higher anti-restenotic effect (-39 % I/M) compared to GatvM (-24 %), suggesting that a sustained release is mandatory for the formulation to be efficient. The in vitro release profile of GatvMatv is the sum of the release profiles of GatvM and GMatv (Fig. 4). However, in vivo, IH prevention effect of GatvMatv (I/M ratio 0.32) was higher than the effect observed with GatvM (I/M ratio 0.76) combined with the effect of (I/M ratio 0.61) in absence of any interaction (combination effect I/M ratio 0.46, corresponding to a 54% decrease). This suggests that the concomitant presence of
ATV in both gel and microparticles leads to a synergistic rather than an additive effect as a 68% decrease was observed in the presence of ATV in gel and microparticles.

The efficacy of the combined formulation is thought to be related to the time course of the development of IH which progresses mainly as a hyperacute (hours to days) and acute stages (days to weeks) [42, 50]. Indeed, the release profile of GatvMatv matches this time course well; a) a high amount of ATV is made available within the first few days to prevent the high proliferation rate of VSMC on the media and the adventitia and b) when the migration of the VSMC and subsequent proliferation takes over later as a constant amount of ATV is delivered from the microparticles.

In vitro, we previously established that the EC50 of ATV for the proliferation and migration of hVSMCs is 10 ng/μL [35]. The amounts that were detected in adjacent tissues in vivo 4 weeks after local administration of formulations GMatv, GatvMatv are in the same order of magnitude (Fig. 7). Such high ATV concentration is 3 order of magnitude higher than the ATV plasma concentration typically obtained after oral administration [51]. Instead, the maximum plasma concentration achieved after oral administration is 0.01 ng/μL [51]. Therefore, only a local formulation can achieve high, bioactive ATV concentrations.

These high local concentrations of ATV may raise concerns for potential local toxicity. Particularly, the endothelial lining could be affected, generating life-threatening thrombogenic events. We showed that ATV, as well as clHA and PLGA microparticles, had no destructive effect on the vessel’s endothelium (Fig. 6). As the endothelium was intact in all conditions, either the endothelium is not affected by the intervention or healing occurred during the 4 weeks following the intervention. These results are in agreement with the observations of Zheng et al. and stress the advantages of ATV compared to cytotoxic treatments [30, 52, 53].

Biodistribution data showed that ATV was not detected at day 28 in the serum, and low concentrations were found in remote tissues except for the liver (Fig. 8). Although ATV is widely used in clinical practice; hepatotoxicity and severe myopathy are commonly described as side effects [54]. Meanwhile, it was reported that oral doses of ATV as high as 2.5 and 5 mg/kg/day are necessary to inhibit IH in animal models [31, 32, 55]. Those doses are close to the toxicity zone, as an administration of 10 mg/kg/day demonstrated extensive liver necrosis in rabbits. Therefore, local administration limits the risk of systemic toxicity by reducing the drug distribution in remote tissues after four weeks. In this study, mouse tissues were macroscopically intact upon harvesting. Even for the ex vivo model, when the tissues were exposed to an extremely high ATV concentration, no structural alteration was observed on hGSV histological sections (Fig. 8).
The rodent model employed has the disadvantage of slim vasculature compared to human vasculature. As shown by Lovich & Edelman, the diffusive barrier imposed by the vessel to the drug depends greatly on the adventitia thickness [56]. Thus, we had to ensure that the in vivo effect on the slim mice carotids could be reproduced on thicker vessels such as the grafts used for bypass interventions in humans. Therefore, we investigated the ex vivo permeation of ATV through the hGSV which are the material of choice for bypass grafts. We showed that ATV applied perivascularly would reach its site of action - the intimal layer - in human vessels (Fig. 8). A high concentration of ATV rapidly impregnated the hGSV after 24 h, confirming the relevance of our choice of a lipophilic statin. Creel et al. who worked on the arterial distribution of paclitaxel ex vivo reached a similar conclusion [57]. However, compared to our results, their 4 h-long perivascular exposure of the vessel to the drug was probably not sufficient for the drug to reach the intima. Our extended time course on hGSV over 3 days allows us to conclude to a slow diffusion process over days.

5. Conclusion

We developed an easily applicable drug delivery system for the prevention of IH, through a single administration of a cross-linked HA hydrogel / PLGA microparticle formulation loaded with ATV. The carotid artery ligation model allowed us to validate an in vivo proof-of-concept. The combination of a fast and sustained release of ATV provided a synergistic effect, which efficiently inhibited the development of IH. No evidence for toxicity was observed on harvested tissues. Limitations of this in vivo model compared to the physiological conditions in humans include low shear stress environment, triggering of arterial instead of vein tissue differentiation and use of healthy non-atherogenic, non-hypercholesterolemic animals. Further studies on larger animals, such as porcine models are warranted.

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References


