Rat synovial tissue and blood rapamycin pharmacokinetics after intra-articular injection of free solution or nanoparticles vs. free rapamycin intravenous shot

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Abstract:	Intra-articular (IA) injection of a chondroprotective candidate may delay the osteoarthritis (OA) course, but its rapid absorption into systemic circulation may limit efficacy and produce untoward effects. We compared the pharmacokinetics (PK) of IA rapamycin injected as sustained release in nanoparticles (NPs) versus a free rapamycin suspension in the rat knee, compared to an intravenous (IV) free rapamycin shot, taken as a reference. Rats received either a single IV injection of free rapamycin (10 µM) or an IA of free or NPs-loaded rapamycin. After sequential exsanguination (15, 30, 60, 180, 360 min, D1, and D7), knee synovial tissue (ST) and cartilage histology were performed. Blood and ST concentrations (LC-MS/MS), PK parameters (area under the curve: AUC; mean residence time: MRT; elimination half-life: T 1/2) and IA biocompatibility were assessed. AUC IV was significantly higher for IV than for both IA injections (AUC IA free and AUC IA NPs), with 4248 vs. 28 and 74 µg.min.L -1 . For ST parameters, we observed a significant difference between AUC IA free and AUC IA NPs with 3735 and 10513 µg.min.L -1 correspondingly. Articular T 1/2 and MRT were higher after NPs than after free rapamycin injection: 57.8 and 5.0 h for T 1/2 and 80.6 and 5.5 h for MRT, respectively. Histological analysis revealed no chondral injuries and a slight transient synovitis only 3 hours after NPs administration. In the rat knee, rapamycin-loaded NPs delivery via a single IA injection is biocompatible and prolongs synovium joint residency, diminishes blood levels, and reduces detrimental systemic exposure.

Authors Contributions:

Pierre GILLET and Astrid PINZANO helped design the study.

The experiments were assisted by Astrid PINZANO and Christel HENRIONNET.

Analytic tools were provided by Julien SCALA-BERTOLA and Nicolas GAMBIER (PK analysis).

Elise PAPE carried out the experiments, analyzed the raw data, and wrote the first draft of the manuscript (please note that this work is part of her Ph.D.)

Julien SCALA-BERTOLA and Nicolas GAMBIER assisted data analysis.

Pierre GILLET made significant contributions to the article's revision.

All authors have read and approved the manuscript.

Graphical Abstract (for review)

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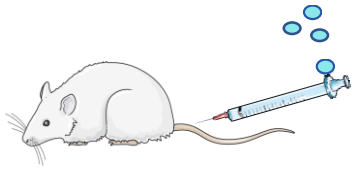
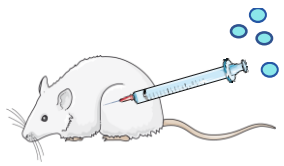
Experimental design



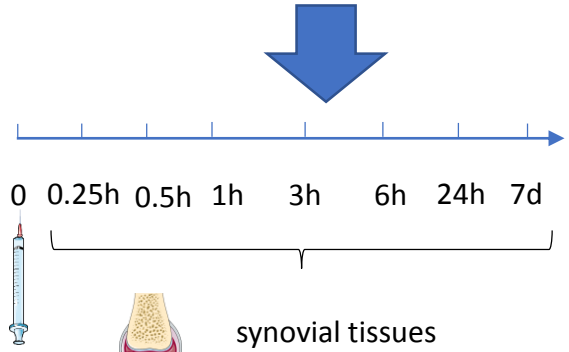
Intravenous (iv) 10µM
Free Rapamycin

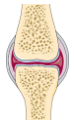


Intra-articular (ia) 10µM
Free Rapamycin

Intraarticular (ia) 10µM
Rapamycin-loaded NPs

Kinetic



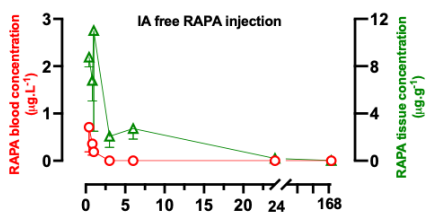


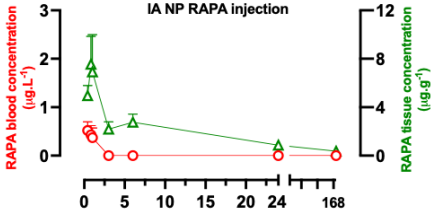
synovial tissues
cartilage

Blood samples

Results

Blood and synovium Pharmacokinetics

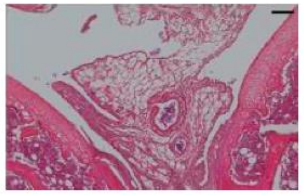


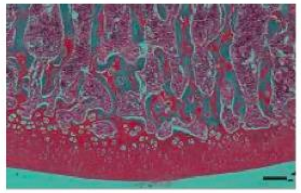


Decrease of Area Under Curve in blood with free and NPs

Increase of Area Under Curve in synovium tissue with NPs vs. Free

Synovial and chondral biocompatibility



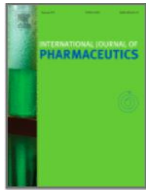


✓ No deleterious effects on synovium and articular cartilage

Summary

- RAPA-loaded NPs joint delivery is biocompatible and extends synovium retention residency vs. free RAPA injected intra-articularly.
- Intra-articular injection of RAPA-loaded NPs allows low blood levels and reduces significantly harmful systemic exposure.

In the rat knee, 10µM rapamycin-loaded NPs delivery via a single IA injection is biocompatible and significantly delays synovium joint residency and diminishes blood levels without detrimental systemic exposure.



E. Pape. (2022)

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Abbreviations:
AUC: Area Under Curve, **C_{max}**: maximal Concentration, **C_t**: concentration at t time, **DMSO**: dimethyl sulfoxide, **HPLC**: high performance liquid chromatography, **IA**: intra-articular , **IV**: intravenous, **K_{el}**: elimination rate constant, **LLOQ**: lower limit of quantification, **LOD**: limit of detection, **MRT**: mean Time Retention, **MS/MS**: mass spectrometry in tandem, **mTOR**: mammalian Target Of Rapamycin, **NPs**: nanoparticles, **OA**: osteoarthritis, **PK**: pharmacokinetic**, PLGA**: poly(lactic-co-glycolic) acid, **ST**: synovial tissue, **RAPA**: Rapamycin, **T_{max}** time to achieve C_{max}, **$T_{1/2}$** : elimination half-life

Highlights

- Rapamycin seems chondroprotective, but its distribution may produce side effects.
- Articular injection of tailored nanoparticles (NPs) may avoid systemic diffusion.
- We injected 10μM of free rapamycin (RAPA) vs. RAPA-loaded NPs in rat knees.
- RAPA-loaded NPs joint delivery is biocompatible, extending synovium residency
- Joint RAPA-loaded NPs lower blood levels and reduce harmful systemic exposure.

Abstract

Intra-articular (IA) injection of a chondroprotective candidate may delay the osteoarthritis (OA) course, but its rapid absorption into systemic circulation may limit efficacy and produce untoward effects. We compared the pharmacokinetics (PK) of IA rapamycin injected as sustained release in nanoparticles (NPs) versus a free rapamycin suspension in the rat knee compared to an intravenous (IV) free rapamycin shot taken as a reference. Rats received either a single IV injection of free rapamycin (10 μM) or an IA of free or NPs-loaded rapamycin. After sequential exsanguination (15, 30, 60, 180, 360 min, D1, and D7), knee synovial tissue (ST) and cartilage histology were performed. Blood and ST concentrations (LC-MS/MS), PK parameters (area under the curve: AUC; mean residence time: MRT; elimination half-life: $T_{1/2}$), and IA biocompatibility were assessed. AUC_{IV} was significantly higher for IV than for both IA injections ($AUC_{IA\ free}$ and $AUC_{IA\ NPs}$), with 4248 vs. 28 and 74 μg.min.L^{-1}. For ST parameters, we observed a significant difference between $AUC_{IA\ free}$ and $AUC_{IA\ NPs}$ with 3735 and 10513 μg.min.L^{-1} correspondingly. Articular $T_{1/2}$ and MRT were higher after NPs than after free rapamycin injection: 57.8 and 5.0 h for $T_{1/2}$ and 80.6 and 5.5 h for MRT, respectively. Histological analysis revealed no chondral injuries and slight transient synovitis only 3 hours after the administration of NPs. In the rat knee, rapamycin-loaded NPs delivery via a single IA injection is biocompatible and prolongs synovium joint residency, diminishes blood levels, and reduces detrimental systemic exposure.

1 Introduction

Rapamycin is an inhibitor of the mTOR pathway currently used as an immunosuppressant to prevent renal transplant rejection (Groth et al., 1999). Blocking the mTOR signaling pathway is a potential therapeutic approach to osteoarthritis (OA) by restoring autophagy and diminishing the advanced stages of osteoarthritis in various experimental models (Sasaki et al., 2012). Thus, local and systemic administration of rapamycin in rodent osteoarthritic models decreases chondral lesions (Liu et al., 2020; Matsuzaki et al., 2014; Takayama et al., 2014; Xu et al., 2019). However, rapamycin's numerous biological side effects cause undesirable systemic and paradoxical off-target articular adverse consequences, like reducing bone thickness, when administered systemically (Minton et al., 2021). It induces lipid and carbohydrate dysmetabolism and hematological disorders (anemia, thrombocytopenia), which may increase morbidity and mortality in patients suffering from systemic diseases more severe than osteoarthritic related pain (Nguyen et al., 2019).

Intra-articular (IA) administration is an exciting alternative to prevent systemic adverse effects targeting only the affected joint. For this reason, IA is currently used to inject corticosteroids, steroidal anti-inflammatory drugs, or hyaluronic acid during knee OA (Edwards, 2011). However, IA can lead to pain, infection or inflammation, and patient non-compliance to treatment. Thus, the number of IA injections should be limited in time. Furthermore, due to the structure of synovial tissue (ST), which allows small molecules (10 kDa) to diffuse through the synovial membrane and blood capillaries, many drugs are rapidly effluxed from the joint cavity after injection. Free rapamycin, which is highly hydrophobic and fragile, is not suitable for IA use because its solubility in water is 2.6 μg/ml at 25°C, and the opening of its macrolide core occurs under aqueous conditions.

To avoid the pitfalls of IA administration and to optimize its benefits, the use of drug delivery systems (microparticles, nanoparticles...) seems appropriate (Cao et al., 2021; Kraus et al., 2018; Maudens et al., 2018; Mohammadinejad et al., 2020). Thus, nanoparticles are an attractive tool for intra-articular delivery in OA treatment (Brown et al., 2019b; Mancipe Castro et al., 2021). Nanosystems seem suitable for IA delivery within these systems, particularly polymeric-loaded nanoparticles (NPs). We have recently developed dedicated rapamycin-loaded poly (lactic-co-glycolic) acid (PLGA) NPs suitable for intra-articular administration that are biocompatible in vitro with osteoarticular cells (Pape et al., 2021): the main characteristics of our NPs were i) size > 250 nm to avoid rapid clearance across blood capillaries, ii) slightly anionic charge to obtain NPs which are not sensitive to modifications in synovial fluid iii) encapsulation efficiency > 70% to limit polymer quantity in the joint cavity. This study describes the in vivo pharmacokinetic profile of rapamycin administered intra-articularly to rats' knees, in its free form or NPs shape, compared to intravenous (IV)

administration of free rapamycin taken as a reference. We wanted to validate that PLGA nanoparticles would increase the retention time of rapamycin in the joint cavity without inducing local adverse effects while reducing its systemic exposure.

2 Materials and Methods

2.1 Materials

Rapamycin powder (purity > 99%) was purchased from LC Laboratories (Woburn, Massachusetts, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (St Louis, Missouri, USA). Precipitation and extraction buffers from the MassTox® Immunosuppressants One Minute Kit and rapamycin-d3 were obtained from Chromsystem Instruments and Chemicals GmbH (Gräfelfing, Germany). Tacrolimus (5 $mg.ml^{-1}$) was received from Astellas Pharma (Levallois-Perret, France).

2.2 Animals

Five-week-old male Wistar rats (288 ± 15 g) were used in this study (Janvier Lab, Le Genest-Saint-Isle, France). Experiments were performed according to the protocol approved by the Ethics Review Committee for Animal Experimentation of Lorraine University (#2019 052909186551). During a period of acclimation (1 week), they were divided into cages of 4 rats and housed at 22±1.5°C with a 12 h light/dark cycle in plastic cages and allowed free movement. They were fed a standard rodent diet and had access to water *ad libitum*. Additionally, due to the 3R rule, only four rats per period and batch were used for the *in vivo* pharmacokinetic study, as requested by our local ethical committee.

2.3 Doses and Administration

Solutions of free rapamycin were obtained from stock solutions (20 $mg.mL^{-1}$ in dimethyl sulfoxide) and diluted in 0.9% saline buffer for a final concentration of 0.5% DMSO. Rapamycin-loaded poly(lactic-co-glycolic) acid nanoparticles were prepared as previously described using the emulsion solvent evaporation method (Pape et al., 2021). The main characteristics of NPs were an average size at 393 nm (±33)) by dynamic light scattering method, adapted to i.a. administration, an average of zeta potential at −9.02 mV (±2.41), and high encapsulation efficiency (92.0 ± 2.0%) by indirect methods.

Rats were administered: (i) free rapamycin (200 µl of 2.5 µM) via intravenous injection in the tail vein, (ii) free rapamycin (50 µl of 10 µM) by IA infrapatellar injection (right knee), and iii) rapamycin-loaded NPs (50 µl of 10 µM) by IA infrapatellar injection (right knee). Each group enrolled 28 rats (seven time-points: see infra). Injections were performed under isoflurane (2%) anesthesia. A preliminary study conducted on four naive rats injected IV with solvent checked that there were no analytical interactions between endogenous biological parameters, dimethyl sulfoxide, saline, and isoflurane, with rapamycin.

2.4 Samples and measurement of rapamycin concentrations

After parenteral injection, exsanguinations were performed sequentially (15, 30, 60, 180, 360 min, 24 h, and seven days) under general anesthesia (isoflurane 2%). Blood samples were collected in EDTA anticoagulant tubes. Then, the knees (synovial tissue, tibia, femur, and patella) were collected during necropsy. Four rats were used at each time point of the kinetic. Until the test, the blood and synovium tissue (ST) were kept at -20 °C. Rapamycin concentrations in blood and synovial tissues were assessed by HPLC-MS/MS analysis.

For blood samples, extraction was performed with 50 µL of blood, 100 µL of precipitation buffer, and 25 µL of internal standard (IS) (rapamycin 50 $\mu g.L^{-1}$). Then, 250 µL of the extraction buffer was added, and the mix was vortexed for 1 min and centrifuged (5 min, 13500 rpm). The HPLC-MS/MS system used LC20AD and LC30AD pumps (Shimadzu Corporation) for the chromatography system and a Qtrap 6500+ tandem mass detector (Life Sciences Holding France SAS, Villebon Sur Yvette, France). After that, 15 µL supernatant was injected into the chromatography system. Rapamycin was eluted in the analytic column (65 °C) using an isocratic gradient (0.6 $mL.min^{-1}$) after online extraction (2 $mL.min^{-1}$). The monitored transitions (m/z) were 931.6/864.4 and 821.5/728.5 for rapamycin and internal standard in the positive-ion mode, respectively. A calibration curve was used from 1 to 20 $\mu g.L^{-1,}$ and the lower limits of quantification (LLOQ) and limit of detection (LOD) were 0.4 $\mu g.L^{-1}$ and 0.1 $\mu g.L^{-1}$, respectively. Inter-day and intra-day precisions were respectively 2.4 and 11.5% for low level and 5.8% and 4.0% for high level.

The previous method was adapted for rapamycin measurement in the synovium: synovia were crushed in 100 µL of extraction buffer and 25 µL of IS (rapamycin-d3), then 200 µL precipitation buffer were added and vortexed before centrifugation. We used the same chromatographic conditions, except the injected volume (30 µL). The monitored transition was 934.7/864.4 (m/z) for IS. The calibration curve was from 20 to 300 $\mu g.L^{-1}$ and the LOD and LLOQ were 3 $\mu g.L^{-1}$ and 10 $\mu g.L^{-1,}$ respectively. Results were expressed in the amount of rapamycin reported to the weight of synovium. Inter-day and intra-day precisions were respectively 7.7% and 3.4% for low level and 8.2% and 0.8% for high level.

Nota Bene. Please note that we collected concomitantly synovial fluid from rat knees during necropsy with calibrated pieces of blotting paper (identical size). However, the harvested amount of synovial fluid was too small, probably because of the absence of synovitis. There are very few synovial fluids in a normal knee as in humans. So, even with this adapted extraction method and a very sensitive quantification method with a low limit of quantification, HPLC could not quantify the amount of rapamycin in such a poor volume of synovial fluid.

2.5 Pharmacokinetic (PK) analysis

We used a bi-exponential model and calculated the area under the curve from 0 to infinity ($AUC_{0\text{-}inf}$) of blood or synovial rapamycin concentration versus time curves: AUC from 0 to the last time t ($AUC_{0\text{-}t}$) was calculated using the trapezoidal method, and $AUC_{t\text{-}inf}$ was determined by C_t / K_{el}, with the K_{el} elimination rate constant estimated from the final part of the curve until infinity (classically obtained by determining the slope of the line on the last part of the elimination). Finally, $AUC_{0\text{-}inf}$ was estimated by adding $AUC_{0\text{-}t}$ and $AUC_{t\text{-}inf}$. The elimination half-life, $T_{1/2}$, was estimated by $\ln(2)/k_{el}$, and the maximal concentration (C_{max}) and the time to achieve C_{max} (T_{max}) were obtained graphically. Mean residence time (MRT) in the joint cavity represents how rapamycin remains in the articular compartment. The MRT is calculated as the area under the first moment curve (AUCM)/AUC, where AUMC is the area under the curve of concentration x time versus time, and $AUCM_{(0\text{–}inf)} = AUCM_{(0\text{–}t)} + AUCM_{(t\text{–}inf)}$. AUC and MRT were determined for the three administration conditions (IV, IA free, and IA NP) for blood and synovial compartments.

2.6 Histology and scoring

For ten days, the tibia, femur, and patella were fixed in formalin and then decalcified in formic acid (5%). After dehydration, they were embedded in paraffin and cut into 5 µm-thick sections. Cartilage injury and synovial inflammation were evaluated with modified Mankin's **(Table 1)** and Rooney's **(Table 2)** scores, respectively, using safranin-O-fast green, Sirius red, and hematoxylin-erythrosine-Saffron stainings. Two independent observers performed the scores blindly.

2.7 Statistical analysis

We performed a two-way ANOVA test or Student t-test to compare AUCs and a one-way ANOVA test with Tukey's test to compare histologic scores using GraphPad Prism version 9.40 (GraphPad Software, la Jolla, California, USA). The results are expressed as a mean and standard deviation for each time (n=4 rats) excepted for some PK parameters (standard errors in **Tab 3**). A significant difference was obtained for $p<0.05$.

3 Results

3.1 Blood and synovium PK parameters

Blood. C_{max} peaked at 13.8 $\mu g.L^{-1}$ after a single free rapamycin IV injection (10 µM) and then decreased over the next 24 hours (**Fig 1a**). We did not detect rapamycin in blood seven days after the IV injection. Furthermore, free or NPs-loaded rapamycin IA injection (**Figs 1b and 1c**) resulted in a lower systemic C_{max} (0.7 and 0.5 $\mu g.L^{-1}$ respectively) at 0.25 h. Blood concentrations fell to LOD one hour post-IA injection for both formulations. The $AUC_{0\text{-}inf}$ values were 4248, 28, and 78 $\mu g.min.L^{-1}$ for free IV, free, and NPs IA injections, respectively. $T_{1/2}$ was 14, 0.4, and 1.6 h, and MRT was 15.8, 1.1, and 2.8 h, respectively, for IV, free IA, and NPs IA injections.

Synovium. Rapamycin was not identified in ST after IV free rapamycin administration (below LOD, **Fig 1a**). After a single IA injection, C_{max} peaked at 1 vs. 0.5 h and became undetectable at 24 h vs. seven days for free and NP rapamycin, respectively (**Figs 1b and 1c**). $AUC_{0\text{-inf IA NP}}$ was greater than $AUC_{0\text{-inf IA free}}$ (10513 vs. 3735 $\mu g.min.g^{-1}$). $T_{1/2}$ and MRT with IA NPs were also increased by 11.5-fold (57.8 vs. 5.0 h) and 14.6-fold (80.6 vs. 5.5 h), respectively, compared to IA-free rapamycin. **Table I** summarizes pharmacokinetic blood and synovial parameters.

3.2 Histological scoring

We did not notice any chondral lesions (**Fig 2**) or significant synovitis (**Fig 3**) with intravenous and intraarticular injections, except for a slight transient inflammatory synovial reaction 3 hours after the injection of the intraarticular NPs. Modified Rooney's score was transiently higher only 3 hours after an IA NPs injection than with free rapamycin, although this score was still relatively low. (**Figs 2&3).**

4 Discussion

Our present work confirms the interest in rapamycin-loaded NPs for local action and low systemic diffusion, as described previously with corticosteroids (Kraus et al., 2018). In previously published studies on rapamycin in nano or microparticles, most of them have been developed for extra-rheumatological clinical applications. Among the works on rapamycin by intraarticular injection, very few have focused on its articular and systemic fate (Chen et al., 2020; Ma et al., 2019; Matsuzaki et al., 2014; Takayama et al., 2014). A pioneering work provided details on the synovial retention time of rapamycin microparticles using fluorescent cyanine labeling, persisting for up to 19 days after an intraarticular injection (Dhanabalan et al., 2020). Nevertheless, these data did not obtain pharmacokinetic parameters, ensuring that NPs present still contained the active principle or assess possible systemic diffusion. Synovial tissue (ST) consists of 80% discontinuous synoviocytes layers with intercellular junctions (20%) and no basement membrane lining the joint capsule. It consists of intracellular junctions of 0.1 to 5.5 µm (Ho et al., 2019) and acts as a size-dependent barrier. Small active pharmacological or biological agents undergo clearance from the joint into the bloodstream immediately after IA (Brown et al., 2019a; Kim et al., 2019; Zhang and Huang, 2012). In our study, the blood concentrations observed following intraarticular administration (free or NP) are rapidly lower than the limit of detection, and the blood AUCs obtained are 57- and 151-fold lower than those obtained after intravenous administration (AUC IV), respectively (**Table I**). These results suggest a significantly decreased systemic passage after intraarticular administration.

Rapamycin has low solubility and bioavailability. Local administration allows for vectorization within the joint (Butoescu et al., 2009). However, it is essential to evaluate its systemic distribution and local clearance from NPs for potential clinical

use (Partain et al., 2020). Thus, based on concentrations measured over time within synovium, T_{max} was earlier with NPs than with free rapamycin (**Table I**), consistent with the *in vitro* release profile of rapamycin-loaded PLGA NPs and their initial "burst release" effect (Pape et al., 2021). Then, higher synovium concentrations were observed with NPs compared with the free form. The kinetics of a molecule administered within the joint achieves elimination in two stages: diffusion out of the joint and elimination from the systemic circulation. Elimination from the synovial membrane is the limiting step (Sterner et al., 2016) and the retention time within the joint is under the control of k_{el} (elimination constant within the synovial membrane). Our study observed a ten-fold lower synovial membrane k_{el} for NPs than for free rapamycin. Thus, the ability of NPs to release rapamycin in a prolonged manner at the site of action of rapamycin NPs allows them to be defined as long-acting systems (Elbrink et al., 2021). These $T_{1/2}$ agree with the literature since specific sustained-release systems increase the half-life by 10 to 30 times compared to the free form (Rahimi et al., 2021). This makes it possible to limit concentration fluctuations by spacing out administrations and thus promoting better osteoarthritis (OA) evolution (Sterner et al., 2016). Besides, rapamycin quantification in the synovial fluid will be indispensable to avoiding underestimation of rapamycin amount in the whole joint in OA-induced rats.

Two essential characteristics of nanoparticles are size and charge, which can improve retention time in the articular joint. Indeed, surface charge affects NPs uptake by phagocytosing with a reduction in uptake rate for negative surface charge (Alexis et al., 2008). Then, the size of nanoparticles is implicated in their clearance from joints after intra-articular injections. Small particles (< 250 nm) have a fast clearance from the joint cavity across blood capillaries (Barua and Mitragotri, 2014; Hoshyar et al., 2016; Pape et al., 2021), and should have improved articular retention compared to free rapamycin due to these properties. Finally, incorporating rapamycin in a polymer of NPs could protect it from degradation in the articular environment and could play a role in this better retention of rapamycin (Li et al., 2021). We observed such protection during in vitro study (Pape et al., 2021) for rapamycin-loaded poly(lactic-co-glycolic) acid NPs compared to free rapamycin.

Synovial tissue (ST) is highly reactive, and an IA injection often leads to an inflammatory reaction, for example, with hyaluronic acid or even sometimes corticoids (Edwards, 2011). Some authors have observed a moderate local inflammation following the administration of poly(lactic-co-glycolic) acid microspheres. This transient inflammatory reaction, during the first few days following IA administration, disappears rapidly (Zhang and Huang, 2012). We have also previously observed such a transient effect of NPs *in vivo* (Riffault et al., 2015). However, our results clearly show in rats that IA administration of 10 µM, in free form or as NPs, does not cause a deleterious effect on a healthy rat knee. The 10 µM in 50 µL administration is based on previous publications showing a chondroprotective effect in rodent osteoarthritic knee (Takayama et al., 2014). It should be noted that

different concentrations of rapamycin may result in other pharmacodynamic pleiotropism effects depending on the cell type exposed (Mukhopadhyay et al., 2016).

We have also to consider that nanoparticles are expected to be cleared via the lymphatics rather than blood. Interestingly, the homing of immunosuppressants directly to joint draining lymph nodes and to pro-inflammatory immune cells residing in the lymphatics may contribute to the therapeutic benefit in inflammatory flares of rheumatic diseases while minimizing systemic toxicities. Intra-articular injection allows targeting of afferent lymph nodes draining the joints while others are spared, which has not been observed with other parenteral administrations (Lam et al., 2022). In addition, tailored nanoformulations have many advantages, such as sustained-release, targeted delivery, and improved pharmaceutical properties (e.g., half-life, stability, and solubility) of cargoes (Spitters et al., 2019). Age and osteoarthritis in rats also appear to be essential factors in the joint retention of nanoparticles (Partain et al., 2020). Combining nanotechnology and homing, which targets nanoparticles in the lymphatic system, may achieve a more effective and less toxic combo-therapy (Peng et al., 2021).

Finally, vectoring nanoparticle therapeutics to the lymphatics draining painful joints could improve access to pro-inflammatory immune cells residing in the lymphatics, improve localized efficacy, and allow for lower drug doses with no off-target adverse effects.

Limitations of the study. This study evaluated the distribution and clearance of rapamycin nanoparticles after IA articular administration in healthy and young rats. This will need to be confirmed in rat knee OA models, given the possible variable tropism of NPs in normal or pathological synovial or chondral tissues. In addition, rapamycin adsorption in cellulose filters (0.22 μM) led us to not use this membrane for free rapamycin or rapamycin NPs sterilization. As no available gamma sterilization method was available, we prepared rapamycin and control solutions with sterile compounds under a laminar flow hood and with DMSO, which avoids microbiological proliferation. In injected animals, as during our previous in vitro release studies, we did not see inflammatory markers or microbiological contamination, clinically or histologically, but a microbiological stability study could be performed in future experimentations for long-term/repeated use.

5 Conclusion

These articular and plasma PK observations are consistent with a slow release of rapamycin into ST and reduced absorption into the systemic circulation following a single IA injection of rapamycin-loaded NPs, yielding sustained IA residency of NP-loaded versus free rapamycin in the rat knee.

Authors Contributions:
Pierre GILLET and Astrid PINZANO helped design the study.
The experiments were assisted by Astrid PINZANO and Christel HENRIONNET.
Analytic tools were provided by Julien SCALA-BERTOLA and Nicolas GAMBIER (PK analysis).
Elise PAPE carried out the experiments, analyzed the raw data, and wrote the first draft of the manuscript (please note that this work is part of her Ph.D.)
Julien SCALA-BERTOLA and Nicolas GAMBIER assisted data analysis.
Pierre GILLET made significant contributions to the article's revision.
All authors have read and approved the manuscript.
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Data Statement: The datasets taken during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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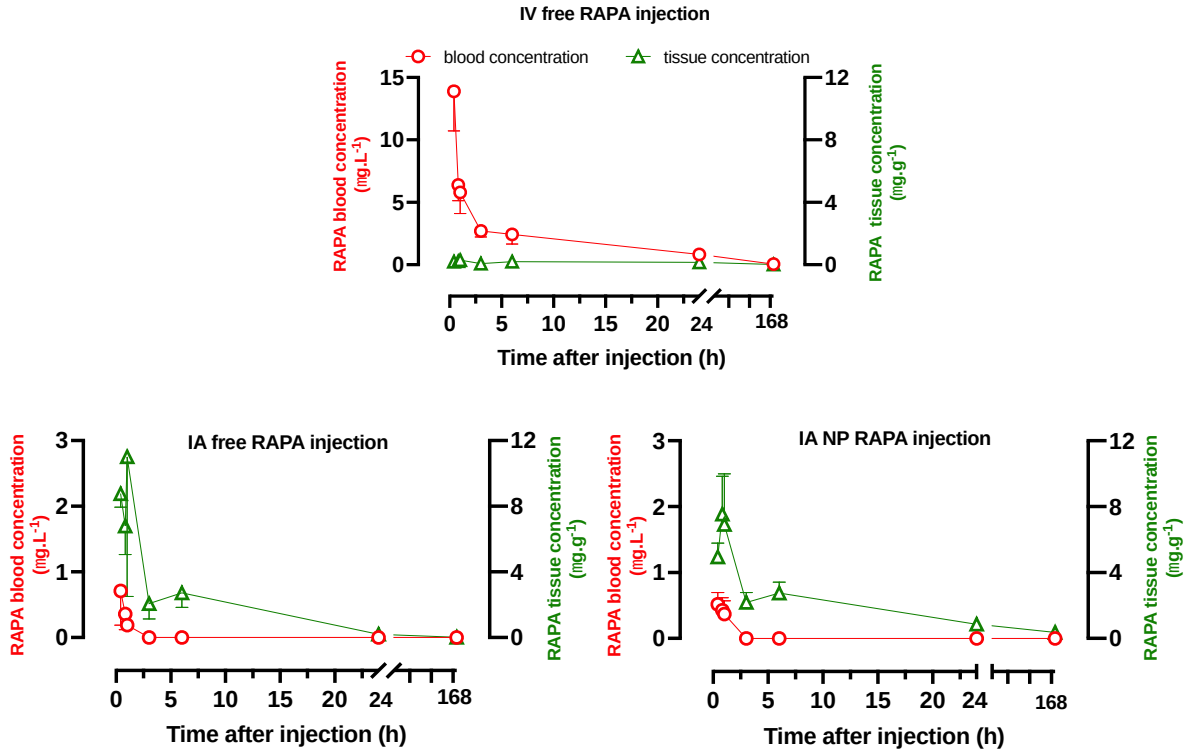

Figure 1: Blood (red) and synovial tissue (green) concentrations overtime after i) a single free rapamycin intravenous (IV) injection, ii) intra-articular (IA) free rapamycin injection, or rapamycin-loaded NPs IA injection in rats (10µM at T0, n =4/time points, mean ± SD). Please note the linear-linear scale for the sake of clarity of the figure, and the different scales for blood and tissue, for a better interpretation.

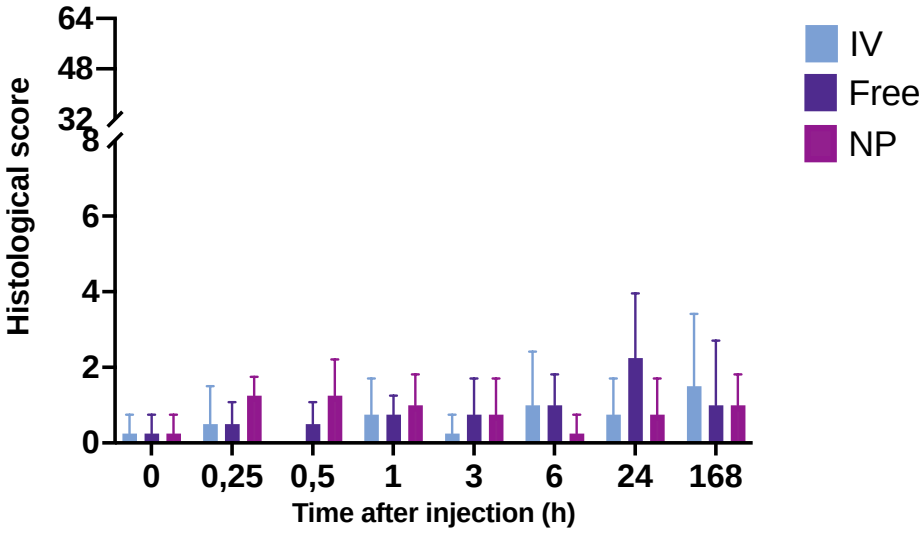

Figure 2. Sequential histological scoring of rat knee femoro-tibial cartilage (Mankin's Score) after i) a single free rapamycin (RAPA) intravenous (IV) injection, ii) intra-articular (IA) free RAPA injection (Free), or RAPA-loaded NPs IA injection (NP) in rats (10 μM at T0, n = 4/time-points). No statistical difference is observed for both IA injections versus IV shoot taken as control (one-way ANOVA followed by Tukey's post hoc test at each time point). Data are mean ± SD.

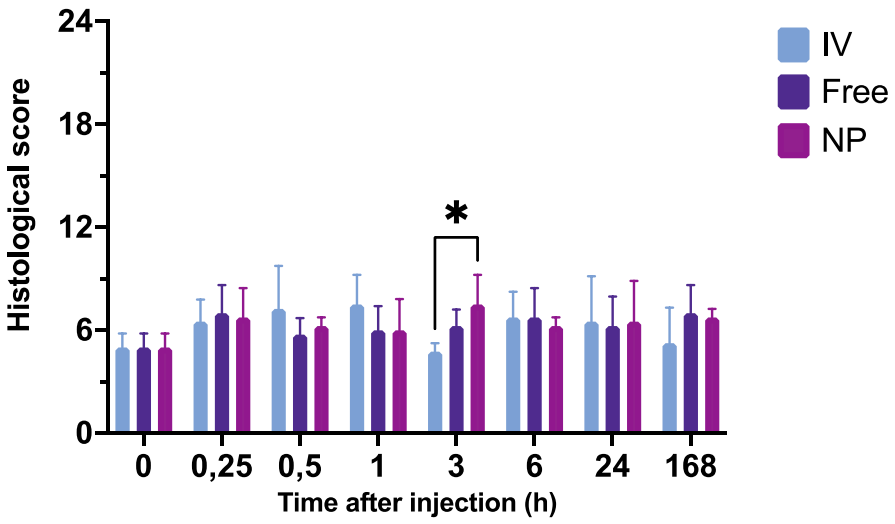

Figure 3. Sequential histological scoring of rat knee synovium (Rooney's Score) after i) a single free rapamycin (RAPA) intravenous (IV) injection, ii) intra-articular (IA) free RAPA injection (Free), or RAPA-loaded NPs IA injection (NP) in rats (10 µM at T0, n = 4/time-points). No statistical difference is observed for both IA injections versus IV shoot taken as control (one-way ANOVA followed by Tukey's post hoc test at each time point). Data are mean ± SD.

Table 1: Scoring chondral system with a modified Mankin's histological score performed in medial, lateral, femoral, and tibial compartments (coronal sections, sum of each compartment). Two independent observers performed the scores blindly (the maximal score is 64).

Structure	Score	Toluidine blue staining	Score
Normal	0	Normal	0
Surface irregularities	1	Slight reduction	1
Surface irregularities with fibrosis	2	Mild reduction	2
Clefts to transitional zone	3	Severe reduction	3
Clefts to radial zone	4	Non-staining	4
Clefts to calcified zone	5		
Complete disorganization	6		
The thickness of hypertrophic chondrocyte layer	**Score**	**Cells**	**Score**
Normal	0	Normal	0
Moderate decrease	1	Diffuse hypercellularity	1
Severe decrease	2	Cloning	2
		Hypocellularity	3
Sirius Red Staining	**Score**	**Collagens organization in polarized light microscopy**	**Score**
Homogeneous and moderate at the surface area	0	Homogeneous and moderate at the surface area	0
Severe at the surface area	1	Severe at surface area	1
Severe to mild zone	2	Severe to mild zone	2
Severe to calcified zone	3	Severe to calcified zone	3
Bone remodeling	**Score**	**Bone osteolysis**	**Score**
None	0	None	0
Present	1	Present	1

Table 2: Scoring synovial membrane system with modified Rooney's mean histological score performed (coronal sections, mean of 4 localizations per knee, HES staining). Two independent observers performed the scores blindly. The maximal score per knee is 24.

Number of synovial lining cells	Score	Surface fibrin deposition	Score
Normal	0	None	0
2-3	1	Slight	1
4-5	2	Mild	2
5-10	3	Marked	3
> 10	4	Severe	4
Fibrosis	**Score**	**Blood vessel proliferation**	**Score**
< 10 % of surface	0	2-3	0
10-25%	1	3-9	1
25-50%	2	9-15	2
50-75%	3	15-22	3
>75%	4	>22	4
Perivascular infiltrates of lymphocytes	**Score**	**Diffuse infiltrates of lymphocytes**	**Score**
None	0	None	0
0-25%	1	1-25%	1
25-50%	2	25-50%	2
50-75%	3	50-75%	3
>75%	4	>75%	4

Table(s)

Table 3. Pharmacokinetic (PK) parameters in the blood and synovial fluid after a single free rapamycin IV, free rapamycin IA, or rapamycin-loaded NPs IA injection in rats (10µM at T0, n =4/time points; [SE: standard error] calculated with GraphPad® 9.4].

PK parameters	Blood parameters			Synovial parameters (right knee with IA injection)		
	IV	IA free	IA NPs	IV	IA free	IA NPs
C_{max} (blood µg.L^{-1} synovium µg.g^{-1}) [SE]	13.8 [1.6)-]	0.7 [0.26]	0.5 [0.09]	< LOD	11 [5.4]	7 [1.5]
T_{max} (h)	0.25	0.25	0.25	NA	1	0.5
$AUC_{0\text{-}inf}$ (blood µg.min.L^{-1} synovium µg.min.g^{-1}) [SE]	4 248 [713]	28 [a] [16]	74 [a] [118]		3 735 [827]	10 513 [b] [2757]
K_{el} (min^{-1})	0.0008	0.028	0.0078		0.0023	0.0002
$T_{1/2}$ (h)	14	0.4	1.6		5.0	57.8
MRT (h)	15.8	1.1	2.8		5.5	80.6

[a] Signifies a statistically significant difference ($p < 0.05$) between IV and IA (two-way ANOVA); [b] Denotes a statistically significant difference ($p < 0.05$) between IA-free and IA NPs (Student's t-test). LOD stands for the limit of detection, SE for standard error, and NA for not applicable. Please note that all groups had undetectable rapamycin concentrations in the synovium of their left uninjected knees. *Nota bene*: since T_{max} is visually calculated, and K_{el}, $T_{1/2}$, and MRT are mathematical extrapolations, no standard errors can be calculated (see materials and Methods).