Predicting drug release and degradation kinetics of long-acting microsphere formulations of tacrolimus for subcutaneous injection

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Abstract

Today, tacrolimus (FK506) represents a cornerstone of immunosuppressive therapy for liver and kidney transplants and remains subject of preclinical and clinical investigations, aiming at the development of long-acting depot formulations for subcutaneous injection. One major challenge arises from the inability of meaningful IVIVC methods predictive for the in vivo situation, together with a strong impact of multiple kinetic processes on the plasma concentration-time profile. In the present approach, two microsphere formulations were compared with regards to their in vitro release and degradation characteristics. A novel biorelevant medium provided the physiological ion and protein background. Release was measured using the dispersion releaser technology under accelerated conditions. A release of 100% of the drug from the carrier was achieved within 7 days. The capability of the in vitro performance assay was verified by the level A in vitro-in vivo correlation analysis. The contributions of in vitro drug release, drug degradation, diffusion rate and lymphatic transport to the absorption process were quantitatively investigated by means of a mechanistic modelling approach. The degradation rate, together with release and diffusion characteristics provides an estimate of the bioavailability and therefore can be a guide to future formulation development.

1. Introduction

Today, tacrolimus (FK506) represents a cornerstone of immunosuppressive therapy after organ transplantation as well as in the treatment of immunological disorders [1,2]. The drug selectively inhibits the phosphatase calcineurin, thereby reducing transcription of various cytokines including interleukins, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, and interferon-γ [3]. Due to a high interindividual and intraisindividual variability in pharmacokinetics, combined with a narrow therapeutic index, the blood concentration needs to be carefully monitored [4]. In the past, non-adherence with the standard dosing regimen has been major cause of graft failure especially in adolescent patients [5]. The administration of tacrolimus once a day resulted in a 6-month persistence rate of 81.5% compared to 71.9% with a dosage form given two times a day [6].

As an alternative, dose-related toxicities can be controlled by using long-acting injectables [5,7–18]. Recently, two formulation prototypes were developed using poly-lactide-co-glycolide (PLGA) as the matrix...
Tacrolimus was embedded into the biodegradable polymer from where it is slowly released into the subcutaneous tissue. PLGA has a solid track record of preclinical and clinical safety and enables the release rate to be precisely controlled. Over the years, a wide variety of in vitro tests has been developed, to measure the drug release from microparticle dispersions. A reliable separation from physiological media, together with a meaningful quantitative assessment of the physiologically relevant release mechanisms still poses a challenge to the scientific community. As a result, and with few exceptions, drug release is almost exclusively tested in buffer media without further evaluating the influence of biomolecules on the release pattern. The dispersion releaser (DR) is a state-of-the-art performance assay and has been applied to the many, was used for all the experiments. The DR devices were fabricated at the Goethe University (Frankfurt am Main, Germany). The DR is a dialysis-based technology developed by our group for testing drug release from nanoparticle and microparticulate systems. More information on the design, specifications and performance of the device have been published previously.

### 2.2. Quantification of tacrolimus

A Chromaster high performance liquid chromatography (HPLC) system (VWR Hitachi, Tokyo, Japan) was used to quantify tacrolimus. It consisted of an HPLC pump (5160), a column oven (5310), an auto sampler (5260), and a UV–Vis detector (5420). A reversed phase C8 column (Nucleosil, 4.6 mm × 150 mm, 5 μm) was kindly provided by Astellas. A mobile phase comprising 60% (v/v) of acetonitrile and 40% (v/v) of an aqueous 0.2% (v/v) of phosphoric acid in ultrapure water was pumped at a flow rate of 1 mL/min and column was maintained at 50 °C using a column oven.

All samples comprising proteins were subjected to pre-treatment. In brief, samples were diluted with acetonitrile and then agitated at 14000 rpm at 20 °C for 15 min (Thermomixer comfort, Eppendorf AG, Hamburg, Germany). Precipitated protein contents were separated through centrifugation at 14,000 rpm for 15 min (Centrifuge 5430 R with rotor FA-45-30-11, Eppendorf AG, Hamburg, Germany). The supernatant was suitable for HPLC analysis without further treatment. The concentration of tacrolimus in each sample was measured by detecting the absorbance at a wavelength of 215 nm with an injection volume of 60 μL. Linearity was demonstrated in a concentration range between 0.12 μg/mL and 24 μg/mL. The limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 33 ng/mL and 105 ng/mL, respectively.

### 2.3. Determination of drug solubility in different media

The equilibrium solubility of tacrolimus was determined by adding an excess of tacrolimus to 5 mL of each medium. The resulting suspensions were incubated at constant temperature (37 °C ± 0.5 °C) and stirred at 100 rpm. After 24 h, suspensions were filtered through polytetrafluoroethylene (PTFE) syringe filters with a pore size of 0.45 μm (13 mm Syringe filter, VWR International GmbH, Darmstadt, Germany) to separate the undissolved and dissolved drug fractions. Syringe filters were pre-saturated using drug solution by discarding the first milliliter of the filtrate. The filtrates were analyzed by HPLC.

### 2.4. Characterisation of tacrolimus-loaded microparticles

The particle size distributions of TAD-10 and TAD-21 were determined using a DIPA 2000 analyzer (Donner Tech, Or Akiva, Israel) using the principle of laser obscuration time. The freeze-dried particles were dispersed in ultrapure water to obtain a suspension with a concentration of 1 mg/mL. Two milliliters of the suspension were transferred into a disposable plastic cuvette. For each sample, three measurements were performed under continuous agitation. Number and volume-weighted
size distributions were summarized and reported by the DIPA software (Donner Tech, Or Akiva, Israel). To understand the morphology of the tacrolimus microparticles, scanning electron microscopy (SEM) was used. For the SEM analysis, freeze-dried powder of each formulation was homogeneously distributed onto SEM-sample holder and sputtered with gold by an Agar Sputter Coater (Agar Scientific, Essex, UK). A Hitachi S4500 (Hitachi, Tokyo, Japan) equipped with a field emission electron gun was used to obtain high-resolution micrographs.

2.5. Biorelevant drug release testing

To perform the in vitro release test, the DR device was mounted into a USP dissolution apparatus II (Pharma Test Apparatebau AG, Hainburg, Germany) equipped with a mini-vessel configuration. A detailed description of the specifications of the DR device has been published previously [24,30–32,36]. A CE membrane with a MWCO of 1000 kDa was selected for the dialysis procedure. The membrane was pretreated according to the instructions of the manufacturer. The subcutaneous interstitial buffer (SIB) was used to mimic a physiologically relevant ion background for testing the drug release. Simulated subcutaneous interstitial fluid (SSFIF) comprises SIB supplemented with serum proteins, providing a biorelevant background to simulate the physiological microenvironment [37,38]. The biorelevant release of tacrolimus from the microspheres was tested in SIB with a total protein content of 1% (w/v) of FBS, i.e. SSIF, and an amount of 3% (w/v) of MbCD to account for the absorption of the drug from the administration site (sink conditions). SIB supplemented with the same amount of MbCD (SIB) served as a reference. The donor compartment was filled with 4 mL of each bio-relevant release medium containing 3.27 mg of the microparticle formulation. The acceptor compartment was filled with 136 mL of either bio-relevant or non-biorelevant release medium. The experiment was conducted at 37 ± 0.5 °C at an agitation rate of 75 rpm in both compartments. Samples were collected from the acceptor compartment at the following predetermined time points: 2, 4, 6, 8, 10, 20, 24, 30, 40, 50, 72, 96, 120, 144 and 168 h. The sampling volume (0.5 mL) from the acceptor compartment was replenished with fresh medium. Samples were then treated and analyzed according to the method described above. Further details on this procedure can be found in the supplementary materials (S2-S3).

2.6. In vivo pharmacokinetic study in cynomolgus monkeys

The in vivo study was conducted at Shin Nippon Biomedical Laboratories, Ltd. (SNBL, Tokyo, Japan). Astellas Pharma Inc., Yaizu Pharmaceutical Research Center and SNBL Drug Safety Laboratories have been awarded accreditation status by the AAALAC International. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. [39]. For each formulation, the freeze-dried microspheres were dispersed in an aqueous solution containing 0.5% (w/v) carboxymethyl cellulose sodium and 10% (w/v) sucrose. The well-dispersed suspensions were administered subcutaneously to 38–45 months old male cynomolgus monkeys at a single dose of 0.9 mg/kg (body weight: 2.9–3.2 kg). As a control, tacrolimus solution (Prograf® injection diluted with saline to 0.3 mg/mL) was injected intravenously at a single dose of 0.3 mg/kg. At predefined time points, blood samples were collected from the femoral vein using a heparinized syringe (n = 3 each). From each collected sample, 0.5 mL was mixed with 0.1 mL of distilled water and 0.1 mL of internal standard (50 ng/mL of ascomycin, Wako Pure Chemical Industries, Ltd., Osaka, Japan) in methanol. Then, 3 mL of deproteinization solution (2.8% (w/v) zinc sulfate/methanol/acetonitrile (20:12:8)) was added before centrifugation (1870 ×g, 15 min). The supernatant was further purified using a solid-phase extraction C18 reversed phase column (Bond Elut C18, 100 mg, 1 mL, Agilent Technologies, Inc., CA, USA) and washed with 1 mL of 40% methanol and 1 mL of hexane. Adsorbed drug on the column was eluted with 2 mL of methanol. The eluent was evaporated and dissolved in 0.4 mL of 50% acetonitrile, which was then measured using an HPLC-MS (CBM-20A, LC-20 CE, DGU-20A5, SIL-20 AC, CTO-20 AC, Shimadzu Co., Kyoto, Japan and TSQ Quantum Access Max, Thermo Fisher Scientific Inc., MA, USA) equipped with a C18 column (2.1 mm × 150 mm, 5 μm) at 55 °C. A gradient flow of ammonium acetate aqueous solution (2 mM) and ammonium acetate methanol solution (1 mM) was used as the mobile phase [19]. Non-compartmental analysis (NCA) for each pharmacokinetic profile was performed using Phoenix® software, formerly WinNonlin® (Certara USA, Inc., Princeton, USA). Essential non-compartmental pharmacokinetic parameters (Cmax, Tmax and AUCCl) were calculated for each data set. Further, the drug-related parameters of tacrolimus were extracted from the pharmacokinetic profile obtained after intravenous administration of the drug to cynomolgus monkeys. A comparison of different compartmental models indicated the data to be well-represented by a two-compartment model. Phoenix® software (Certara USA Inc., Princeton, USA) was used for this comparison including a visual inspection of the data as well as the coefficients of variation (CV), the Bayesian information criterion (BIC) and the Akaike information criterion (AIC).

2.7. In vitro-in vivo correlation

In vitro-in vivo correlations (IVIVC) were performed using the Phoenix® software including the IVIVC toolkit® (Certara USA, Inc., Princeton, USA). A unit impulse response (UIR) function was determined as a weighting function for the body system. Pharmacokinetic data from the intravenous administration of tacrolimus solution (0.3 mg/mL) at a single dose of 0.3 mg/kg served as input for the UIR function. The fraction absorbed was determined from the whole blood concentration-time data, while the Weibull equation was fitted to the in vitro release data to obtain an apparent continuous fraction dissolution profile. The correlations are presented as Levy plots, whereby the fraction of the drug input into the system in vivo against the fraction dissolved in vitro. The unity line (slope = 1) serves as a reference line, where the dissolution rate equals the system input rate.

2.8. Development of the physiologically-based biopharmaceutics model

To provide a compartmental framework for the mechanistic analysis of the pharmacokinetic profile, a PBPK model of the subcutaneous tissue developed (Fig. 1). The model was designed using Stella® Architect (isee systems, Lebanon, USA). While microspheres with a large diameter remain at the injection site and release tacrolimus into the interstitial fluid, small particles (<100 nm) are taken up by the lymph capillaries from where they enter blood circulation [40–46]. Free tacrolimus released at injection site undergoes a degradation process. The diffusion rate of the drug (km) is responsible for the residence time of the free drug before it appears in the blood plasma and, therefore, sets the time frame for in vivo degradation and bioavailability. The processes involved in the in vivo absorption of tacrolimus are illustrated in Fig. 1 (green part). A global optimization of the model was performed to estimate all relevant parameters. The mechanism of drug release was determined using the biorelevant in vitro release assay. A scaling factor (xscal) was used to account for the differences between the assay design (accelerated conditions) and the real in vivo release.

\[ \text{Release} = x_{\text{scal}} \cdot \text{in vitro cumulative release} \]

The degradation mechanism of tacrolimus was investigated in bio-relevant medium. Drug degradation is well-described by an exponential function using the degraded fraction (Adeg) and the half-life of the drug (t1/2) in the subcutaneous tissue.
Degradation \( t_{\text{rc}} \) can be expressed as:

\[
\text{Degradation}_{t_{\text{rc}}} = A_{\text{deg}*} \left( 1 - e^{-\left( \frac{t_{1/2}}{t_{1/2}} \right)} \right)
\]  

The lymphatic uptake of PLGA nanoparticles into the lymph capillaries does not follow a first-order kinetic process as reported in literature \([40, 41]\). Therefore, a time-dependent uptake was assumed. For all other processes including drug diffusion as well as distribution and elimination, first-order transport was assumed. More information on the calculation of the lymphatic transport and the in vivo diffusion rate has been included in the supplementary materials (S4).

All the parameters were optimized using the differential evolution method provided by Stella Architect, including \( x_{\text{rel}}, A_{\text{deg}}, t_{1/2}, k_{\text{lymph}}, k_{\text{diff}}, X_{\text{deg}}, \) and \( V \) \([47]\). The optimization process was run simultaneously for both formulations and the absolute average fold error (AAFE) was calculated as a simple measure for the difference between the profiles (supplementary materials, S5).

2.9. Partial sensitivity analysis

The sensitivity of PBB model to the variations of each individual input parameter was studied by carrying out a partial sensitivity analysis. The perturbations in the model output caused by 10% and 20% changes were examined. The local sensitivity was quantified as relative sensitivity coefficient \( R_{ij} \) \([48]\):

\[
R_{ij} = \frac{\partial O_i}{\partial I_j} \times \frac{I_j}{O_i}
\]  

where \( R_{ij} \) represents the relative sensitivity coefficient of an output parameter \( j \) to an input parameter \( i \) at their local, baseline values \( O \) and \( I \). The term \( \frac{\partial O_i}{\partial I_j} \) represents the alteration rate of \( O_i \) upon the change of \( I_j \). This partial derivative is then normalized by both the output variable and model input parameter. If the relative sensitivity coefficient is higher than 0.1, it indicates a sensitive influence of a model input parameter on an output variable.

2.10. Data presentation and statistical analysis

All experiments were repeated at least in triplicate \((n \geq 3)\). Data from all the experiments are presented in form of arithmetic mean ± standard deviation (SD), whereby the calculation was performed using Microsoft Excel (Microsoft, Redmond, USA). Several analysis were conducted by programming in software R\(^{\text{®}}\) (www.r-project.org), including running the drug recovery model, the determination of membrane permeability coefficient, normalization of cumulative release as well as calculation of \( \text{f2} \) value and AAFE value. Further details on the \( \text{f2} \) and AAFE value calculation are provided in the supplementary materials (S5). All the figures were created with the software OriginPro 2020 (OriginLab Corporation, Northampton, USA).

3. Results and discussion

More than three decades after the discovery of tacrolimus, controlling the blood levels in drug therapy still poses a challenge to pharmaceutical formulation development. A high variability in the pharmacokinetic profiles, together with a narrow therapeutic index mandate the design of long-acting depot formulations.

In the present approach, the impact of serum proteins and physiologically relevant ion concentrations on stability and release of the compound from the two formulation prototypes TAD-10 and TAD-21 was evaluated using the DR technology. The recovery and membrane permeation model was applied to calculate the drug release. A level A IVIVC provides evidence for a direct relationship between the in vitro data and the pharmacokinetic profile obtained after administration of the formulations to cynomolgus monkeys. Furthermore, the contributions of drug release, drug degradation, lymphatic transport, and drug diffusion to the wholeblood concentration-time profile were investigated by means of a PBB model.

3.1. Solubility of tacrolimus in different release media

As expected, drug solubility in aqueous media was comparably low, achieving a maximum value of 0.24 ± 0.08 μg/mL after 24 h (SIB, 37 °C ± 0.5 °C). Consequently, the solubility of the drug was considered rate-limiting for the overall absorption process. To compensate for the flow equilibrium created by continuous absorption of the drug from the site of administration, MbCD was added to the medium (to provide sink conditions). The cyclodextrin has been used for solubilization of tacrolimus previously \([49-51]\). After addition of 3% (w/v) of MbCD to the
medium, a solubility of 19.06 ± 0.73 μg/mL was achieved. In presence of serum proteins, a slight but not significant increase in drug solubility was observed (20.64 ± 1.23 μg/mL). However, the adsorption of serum proteins to the surface of microparticles may still be an important parameter with regards to the release kinetics. Consequently, a protein background was added to the medium to monitor these effects on microparticle formulations.

3.2. Characterization of microparticle formulations

Initially, the drug load of each formulation was determined. TAD-10 exhibited a drug load of 26.3%, while TAD-21 comprises 26.5% of tacrolimus.

Additionally, the microspheres were analyzed for their particle size and morphology. The particle diameter and cumulative size distribution is presented in Fig. 2.

In the micrometer range, there was no significant difference observed between the size distributions. Also, the morphology of both formulations was comparable, as indicated by the micrographs (Fig. 3). Both particle species were characterized by a rough, non-porous surface and spherical in shape. Consequently, the release from TAD-10 and TAD-21 can be assumed to be mainly driven by the degradation of the polymeric materials Resomer® RG752H (PLGA, PLA/PGA ratio: 75/25) and Resomer® R202H (PLA). Under very similar test conditions, the degradation rate of Resomer® RG752H was observed to be much faster compared to Resomer® R202H [52-55]. The presence of PLA lowers hydrolytic degradation of the polymer as reflected by the release profiles (section 3.4).

3.3. In vitro degradation kinetics

The degradation of tacrolimus was monitored in presence and in absence of serum and a very similar degradation profile was observed (Fig. 4, left). The elevated temperature, together with a pH of 7 and the presence of divalent cations such as magnesium and calcium are likely to be responsible for this outcome [56].

Consequently, a non-enzymatic degradation mechanism was assumed. The overlay of both degradation profiles in absence and in presence of FBS (Fig. 4, left), together with a summarized degradation profile (including data points collected in absence and in presence of FBS) is presented in Fig. 4 (right). A first-order kinetic process was determined with a coefficient of determination of 0.9827.

Based on the experimental data, the degraded fraction and the half-life of the compound (t1/2) were calculated:

\[ F_D = 90.56 \times \left(1 - \exp \left(\frac{-t}{0.68}\right)\right) \]  

(4)

Under biorelevant conditions, tacrolimus exhibits a degradation half-life of 11.3 h. The degradation function (Eq. (4)) was further used in the permeation recovery model (supplementary materials, S2).

3.4. In vitro release kinetics in non-biorelevant and biorelevant media

The release profiles were obtained by quantifying the amount of tacrolimus in the acceptor compartment of the DR at each sampling time point (Fig. 5). According to the concentration profiles, the initial release was followed by a decrease in the tacrolimus concentration for both formulations. The highest concentration was detected at day 1 for TAD-10 and at day 2 for TAD-21.

Evidently, the release is a result of the overlapping kinetic processes of drug release and drug degradation in both media. Therefore, a correction of the drug release profiles (for the influence of drug degradation) was accomplished using the recovery model (supplementary materials, S2). For this purpose, tacrolimus was assumed to remain stable within the microspheres followed by release and degradation of the drug. The resulting drug release profiles are presented in Fig. 6.

Additionally, with respect to the dialysis-based set-up used for drug release testing, the cumulative release was normalized using the four-step model [57]. In this procedure, the drug release from the carrier is calculated from the release profile of the formulation and the membrane permeation coefficients are obtained in a reference experiment. The membrane permeation coefficients (k_m) of tacrolimus in different media...
can be found in the supplementary materials (S6, Table S1). In absence of FBS, the diffusion of tacrolimus through the membrane was accelerated by a factor of 2 indicating a high plasma protein binding of the drug.

A comparison of the non-normalized and the normalized cumulative drug release profiles is presented in Fig. 6. TAD-10 exhibits a considerable burst effect. Before normalization (Fig. 6a and c, blue), the initial release phase was accomplished after 0.5 days with a total drug release of approximately 55% followed by a sustained release. In presence of proteins, the formulation prototype exhibits a much higher cumulative release reaching a plateau at 100%. This was not indicated by the solubility experiments (section 3.1). Therefore, other mechanisms must be taken into consideration. On the one hand, in presence of proteins, a more rapid degradation of the PLGA microspheres was reported in literature [58–61]. On the other hand, no more than 90% of tacrolimus were released from TAD-10 in absence of proteins which indicates a higher absolute release when using the biorelevant medium. A physical stabilization of the dispersion with serum proteins acting as a soluble stabilizer have been reported to affect the drug release in the subcutaneous tissue [55]. Such an effect would explain both, a higher release

Fig. 3. Particle morphology of TAD-10 (A and B) as well as TAD-21 (C and D) in the scanning electron micrograph. All microparticles were characterized by a rough, non-porous surface and spherical shape.

Fig. 4. Overlay of the in vitro concentration profiles of tacrolimus in presence and in absence of FBS (left). The difference between the degradation in medium with or without protein content is not significant. Therefore, both data sets were combined, and the non-degraded fraction was calculated (right). The mean value \((n = 6)\) was plotted with standard deviation \((\pm \text{SD})\). The experimental data was fitted using a first-order function.
rate and the increase in the absolute drug release over the time of the experiment. The normalized release profiles represent the release of tacrolimus from the microparticles in the donor chamber (Fig. 6b and d). The in vitro test provides biorelevant microenvironment but, due to the hydrodynamics in the donor chamber, accelerates the release process considerably. Under these conditions a burst effect was confirmed for both formulations. For TAD-21, this effect accounted for approximately 15% of the total dose. There was no significant difference in the release rate observed over the whole time of the experiment. However, the addition of proteins to the medium still led to an increase in the release rate. Except for the burst effect, the profile was well-characterized by first-order kinetics.

In addition, the presence of proteins in test medium improved the capability of the assay to discriminate between both formulations. While the non-biorelevant medium leads to a very similar release rate for both formulations (Fig. 6b), the release profiles of TAD-10 and TAD-21 under biorelevant conditions differ significantly (Fig. 6c and d).

To quantify the difference between the two normalized release profiles, the $f_2$ factor was calculated (supplementary materials, S3) comparing the same test conditions for different formulations and the same formulations for different test conditions. The release of TAD-10 differed significantly from the release of TAD-21 in non-biorelevant ($f_2 = 39.9$) and biorelevant medium ($f_2 = 33.3$). The $f_2$ value confirms an improved capability of the biorelevant medium to discriminate between different formulations. For TAD-10 the $f_2$ values of the non-biorelevant (39.3) and the biorelevant medium (37.3) differed as well. In conclusion, the addition of proteins to the test medium had a strong effect on the capability to discriminate between the two formulations. The addition of serum proteins is therefore recommended for further investigations, particularly when investigating drug formulations for subcutaneous injection.

3.5. Primate pharmacokinetics of tacrolimus and its microsphere formulation

The whole blood concentration-time profile of tacrolimus in cynomolgus monkeys was analyzed using two data sets obtained after intravenous and subcutaneous injection of either the drug or the formulation. After visual inspection of the overlay and under consideration of the quality criteria AIC, BIC and CV, a two-compartment model was selected. The pharmacokinetic parameters obtained from this model (supplementary materials, S6 Table S2) were integrated into PBPK model-based simulations. Both formulations exhibit an initial burst effect with TAD-10 (Fig. 7, blue) reaching a second peak after 10 days, followed by continuous decrease of the drug concentration. However, for TAD-21, the initial burst was not followed by a slow increase but resulted in a rapid decrease of the drug concentration reaching a plateau after 10 days and another plateau approximately 30 days after the administration (Fig. 7, yellow).

Additionally, a non-compartmental analysis was performed (Table 1). TAD-10 exhibits a higher $C_{\text{max}}$ and lower $T_{\text{max}}$, whereas the AUCl was slightly lower compared to TAD-21. Both formulations exhibited a very similar behavior with a double peak.

However, this was more obvious for TAD-10 than for TAD-21 which was characterized by a plateau phase rather than a second maximum.

3.6. Analysis of IVIVC

At present, there are no regulatory guidelines defining procedures for the establishing an IVIVC for subcutaneously administered depot formulations. A level A IVIVC was performed based on the normalized biorelevant in vitro release (Fig. 6d). The main purpose of this investigation was a verification of the in vitro assay rather than selecting a favorable formulation candidate. The point-to-point relationships between in vitro and in vivo profiles are presented in Fig. 8. Obviously, there is a delay in absorption compared to dissolution due to the degradation and diffusion from the injection site to the nearest blood vessel. After injection, the burst release has a high impact on the pharmacokinetic profile. At later time points, there is an almost linear relationship between the in vitro and in vivo data for both formulations. This indicates the suitability of the in vitro assay for determining the physiologically relevant release rate of the formulations. An overestimation of drug absorption was observed for TAD-10 and TAD-21, respectively. An estimation of drug release based on total dose recovery is the most likely explanation. The in vivo degradation lowers the absolute bioavailability significantly and is difficult to capture in the in vitro experiment.

In general, the biorelevant release assay anticipates the in vivo release profile of tacrolimus microspheres and will be useful in formulation development. In quality control, the improved capability to discriminate between different formulations and potentially formulation qualities may mandate the addition of a low content of serum proteins or other stabilizing agents.

To further elucidate the influence of in vivo processes on drug
absorption, the PBB model provides a suitable compartmental framework. In this context, the diffusion of the drug from the injection site to the bloodstream, a certain uptake of particles or drug molecules by the lymphatic system as well as the degradation of tacrolimus were taken into consideration.

3.7. Understanding in vivo absorption based on optimized parameters

The PBB model reflects major characteristics of the in vivo absorption process. A global optimization by differential evolution was carried out to identify the in vivo parameters presented in Table 2. In this context, the in vivo release was assumed to correspond to the in vitro release after using a scaling factor to account for the time difference between the in vitro and the in vivo experiment. The scaling factor remained similar for modelling the pharmacokinetics of both formulations and, therefore, does not affect the predictive power of the model.

The estimated half-time of tacrolimus in subcutaneous tissue in vivo (9 days) was much longer than the one determined in vitro (11h) resulting in a scaling factor of 0.057 for drug degradation. Consequently, the total degradation of the free tacrolimus in subcutaneous tissue (5.8%) was much lower compared to the in the in vitro situation (90.6%).

The in silico estimation suggests that the major fraction of the drug becomes available in the blood.

The lymphatic uptake in humans averages 3 L*day\(^{-1}\), while the re-absorption rate from interstitial space into blood capillaries averages 17 L*day\(^{-1}\) [62,63]. The estimated lymphatic uptake (Table 2) highlights a considerable difference compared to human data. In this context, the differences between the two species must be taken into consideration. After optimization, a lymphatic uptake of 0.001 per day was observed using the data from cynomolgus monkeys. With 2409 mL the blood volume estimated by the model was smaller compared to a two-compartment model (3479 mL) using intravenous data. PLGA microsphere formulations may lead to such changes potentially due to the longer residence time of the drug in different tissues. A similar effect was observed in other studies investigating particle-based delivery systems as well [64–66]. Further analysis of the sites of accumulation indicated a significant fraction to remain longer in the lymphatic system before entering blood circulation, which also affects the volume of distribution in the central compartment. Besides, the drug degradation within subcutaneous tissue and during drug transport could lead to a small change of \(V_d\) as well.

As a next step, the optimized simulation was compared to the in vivo

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Fig. 6. In vitro release profiles of TAD-10 (blue) and TAD-21 (yellow) in subcutaneous interstitial buffer without protein background (a and b) as well as modified stimulated subcutaneous interstitial fluid in presence of a protein background (c and d). The profiles are presented before (a and c) and after (b and d) mathematical correction using the four-step model. All four profiles have been corrected for the impact of drug degradation using the recovery model. The data points are presented as mean values (n ≥ 3) with error bars showing corresponding standard deviation (± SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The mechanistic analysis included the drug release, the lymphatic uptake, the degradation at injection site as well as the diffusion of tacrolimus to the blood vessels (Fig. 9c and d). For both formulations, a significant lymphatic uptake was observed during the first few hours indicating the presence of free drug or small particles in the formulation [40–42,44].

Commonly, PLGA microspheres agglomerate before they disintegrate into fine particles [67]. After injection, the uptake of particles smaller than 100 nm into lymphatic system would explain the elevated plasma levels. This would be followed by the formation of agglomerates at the injection site. With further disintegration, fragments are released into the lymphatic capillaries. The absorption of tacrolimus through the lymphatic system was lower for TAD-10 indicating a relatively poor stability as compared to TAD-21 [52–55]. In fact, the matrix material used for the synthesis of TAD-10 is more sensitive to hydrolytic degradation. A stability study in biorelevant medium over 7 days confirmed the more rapid degradation and agglomeration of TAD-10 (data not shown). Additionally, the initial burst release observed for TAD-10 supports this hypothesis.

3.8. Parameter sensitivity analysis

During the parameter sensitivity analysis (PSA) the sensitivity of the model to each of the simulation parameters was investigated. The model was not only sensitive to the kinetics of drug release but also to the kinetics of the lymphatic uptake, the diffusion kinetics and the elimination rate of the drug (Fig. 10). Other rate constants such as the distribution parameters $k_{12}$ and $k_{21}$ did not have a significant effect. The related relative sensitivity coefficients were close to zero (data not shown). However, even a change of 10% in the elimination rate led to a significant change of $C_{\text{max}}$, $T_{\text{max}}$, $\text{AUC}_{\text{all}}$ and had major influence on the second peak in the pharmacokinetic profile. A change in the diffusion rate did not alter $\text{AUC}_{\text{all}}$ for both formulations, while $C_{\text{max}}$ and $T_{\text{max}}$ of the first and the second peak were affected for TAD-10 only. For TAD-21, no such effect was observed. The simulation was sensitive to a change in the lymphatic uptake which indicates a pharmacokinetic profile highly dependent on formulation parameters and materials. Therefore, an

![Figure 7](image1)

**Fig. 7.** Whole blood concentration of tacrolimus. Each of the microsphere formulation (TAD-10: blue; TAD-21: yellow) was subcutaneously injected into cynomolgus monkeys ($n = 3$). Mean values of concentrations were plotted against predetermined time points, whereas the standard deviations are shown by the error bars ($\pm$ SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

<table>
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<th>Formulation</th>
<th>Data source</th>
<th>$C_{\text{max}}$ [ng/mL]</th>
<th>$T_{\text{max}}$ [day]</th>
<th>$\text{AUC}_{\text{all}}$ [day*ng/mL]</th>
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<td>TAD-10</td>
<td>$\text{in vivo}$</td>
<td>16.13</td>
<td>0.03</td>
<td>323.12</td>
<td>14.69 / 6.98</td>
</tr>
<tr>
<td>TAD-21</td>
<td>$\text{in vivo}$</td>
<td>14.06</td>
<td>0.07</td>
<td>366.85</td>
<td>8.27 / 31.02</td>
</tr>
</tbody>
</table>

![Figure 8](image2)

**Fig. 8.** IVIVC of the TAD-10 formulation based on biorelevant in vitro release (left) and IVIVC of the TAD-21 formulation based on biorelevant in vitro release (right). The unity line has a slope of 1, which represents the same rate of dissolution and system input. Both formulations showed burst release as well as linear correlation between fraction absorbed in vivo and dissolved in vitro despite of accelerated release test.
extrapolation to other prototypes may be more challenging. The burst release of TAD-10 was approximately 5 times higher compared to the burst effect of TAD-21. Consequently, drug diffusion had a stronger influence on the in vivo absorption. For TAD-21, due to the lower drug concentration in the subcutaneous tissue, the transfer of fine particles through the lymphatic system was more dominant.

The outcome of the global optimization sheds a new light on the complex interplay between drug release and degradation and will be useful in the design of novel formulations. Both formulations exhibit a burst release, which was also confirmed by the occurrence of a double peak in the primate pharmacokinetic profiles. By analyzing the mechanisms underlying the in vivo pharmacokinetics using the PBB model, physiological processes with influence on the in vivo absorption were elucidated. The in vitro assay enables a meaningful

<table>
<thead>
<tr>
<th>Formulation</th>
<th>( C_{\text{max}} )</th>
<th>( T_{\text{max}} )</th>
<th>AUC_{\text{all}}</th>
<th>Second peak ( C_{\text{max}} / T_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAD-10</td>
<td>1.48</td>
<td>29.17</td>
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<td>1.02 / 1.62</td>
</tr>
<tr>
<td>TAD-21</td>
<td>1.58</td>
<td>11.21</td>
<td>1.05</td>
<td>1.13 / 1.58</td>
</tr>
</tbody>
</table>

Fig. 9. Overlay of the simulated pharmacokinetic profiles compared to the profiles observed in cynomolgus monkeys for TAD-10 (a) and TAD-21 (b). Also, the involvement of the simulated in vivo processes is illustrated highlighting the absolute amounts of tacrolimus undergoing a certain process. The PBB model included the lymphatic uptake, drug degradation, and drug diffusion (c, d).

Table 3
Fold error for the in silico estimates of pharmacokinetic parameters. A value below 2 is generally acceptable for an effective simulation.

4. Conclusion
In the present approach, an in vitro release and degradation assay for tacrolimus-loaded PLGA microspheres was designed. The DR technology in combination with biorelevant medium provided an acceptable prediction of the in vivo release as indicated by the IVIVC. In addition, the presence of proteins led to an improved capability of the assay to discriminate between different formulation prototypes. Both formulations exhibit a burst release, which was also confirmed by the occurrence of a double peak in the primate pharmacokinetic profiles. By analyzing the mechanisms underlying the in vivo pharmacokinetics using the PBB model, physiological processes with influence on the in vivo absorption were elucidated. The in vitro assay enables a meaningful
Fig. 10. Local sensitivity analysis for the parameters integrated in the PBB model for both formulations. The in vivo drug concentration-time profiles of both formulations were simulated by systematically altering the parameters of drug release, lymphatic uptake, diffusion, and elimination. The model was not sensitive to the 10% change of degradation at injection site and in vivo distribution parameter, i.e. $k_{12}$ and $k_{21}$ (data not shown).
prediction of the in vivo performance. Simultaneously, major influences on the pharmacokinetic profile were identified, which also suggested the potential factors that lead to the interindividual pharmacokinetic variability.

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Appendix A. Supplementary data

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References
