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Note

Correlation of in vivo and in vitro release data for rh-INFα lipid implants

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ABSTRACT

Previous in vitro experiments had shown that rh-INFα releasing tristearin implants feature promising properties making them an excellent tool for the delivery of therapeutic proteins. Sustained release for periods up to one month could be achieved, associated with high protein stabilization. The objective of this study was to investigate for the first time the in vivo release properties of these implants in rabbits and to establish an in vivo–in vitro correlation. Computer modeling was used to simulate rh-INFα serum levels based on pharmacokinetic data. Protein serum concentrations on therapeutically relevant nearly constant levels could be detected for 9 days. Modeling revealed that in vivo release correlated closely with the release monitored in vitro.

1. Introduction

Since pharmaceutical proteins given by injection must be repeatedly administered in short intervals to reach and maintain therapeutic useful concentrations in the blood plasma, low patient compliance and high hospital costs are resulting. To overcome these problems polymer-based delivery systems for therapeutic proteins have been developed. Although application of these new delivery devices is very promising most of the used polymer systems such as poly(lactic-coglycolic acid) (PLGA) [1] show some major drawbacks, i.e., interference with the protein stability like protein–polymer interaction, pH-shift and interface formation during formulation [2]. Recently, a new model depot system for the controlled delivery of rh-INFα has been developed in our working group [3]. This implant consists of a lipid component, rh-INFα, hydroxypropyl-β-cyclodextrin (HP-β-CD) or trehalose as a stabilizer and PEG 6000 as a release modifier. The use of lipids known as matrix materials with a high biocompatibility [4,5] allows to generate implants capable of a sustained protein release. The application of solvent-free processing techniques like compressing the lipid mass satisfies the necessity for both a high biocompatibility and a high protein stabilization. For long-time storage especially HP-β-CD can provide a high stability of the embedded therapeutic protein within the matrix [6].

In vitro release experiments have shown a continuous release over 30 days with a close to linear release phase for the first 2 weeks. Overall protein liberation up to 95% can be achieved within 4 weeks.

This controlled release is a consequence of the interaction of PEG with the incorporated rh-INFα, leading to a reversible precipitation of the protein within the matrix, hence the resulting retardation of dissolution of the INFα strongly contributes to the controlled release [7]. The aim of this work was to elucidate whether the release profile of the implants in vitro can be verified in vivo, i.e., in rabbits. We wanted to confirm whether the release mechanism was under total physiochemical control, independent of enzymes and other components of body fluids and mechanical stress. Further, we wanted to prove the expected excellent biocompatibility of the lipid implants.

2. Materials and methods

Rh-interferon α-2a (rh-IFNα, Roche Diagnostics, Penzberg, Germany; protein conc. 1.7 mg/ml in a 25 mM acetate buffer of pH 5.0, 120 mM sodium chloride) was lyophilized in a 1:3 ratio with hydroxypropyl-β-cyclodextrin (HP-β-CD, Merck, Darmstadt, Germany).

Tristearin (Dynasan 118) was purchased from Condea Chemie, Witten, Germany and polyethylene glycol 6000 (PEG 6000) is a product from Clariant, Gendorf, Germany.

All other materials (from Merck) were of high purity grade.

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2.1. Implant manufacturing

Implants were prepared by using a 5 ton hydraulic press (Maassen, Eningen, Germany).

The implant components – tristearin powder 80%, PEG 6000 10% and INFα-lyophilisate 10% – were ground in an agate mortar. The resulting mixture was compressed with a pressure of 2 tons for 30 s. The obtained implants had an average weight of 50 mg and an average height of approximately 2.3 mm. The drug load of the implant was 2.5% of the implant weight, accounted for the rh-INFα dose.

2.2. Animal experiment setup

In order to investigate the rh-IFNα release rate of the delivery device, lipid implants were administered to a group of five young female rabbits. Rabbits were chosen as experimental animals, because in contrast to rats or mice rabbits show a distinctive subcutaneous fat tissue which is quite similar to the human one. Under ketamin/xylazin anaesthesia one implant per animal was placed subcutaneously between the omoplates and the insertion site was sewn up. Samples of approx. 0.5–1 ml blood – gained from the ear vein – were taken daily for the first week, afterwards every three days.

Blank values were taken before the implantation.

To gain basic pharmacokinetic data, another group of three rabbits received an injection of unretarded rh-INFα solution (10^7 IU, i.e 37 μg rh-INFα per kg bodyweight).

Samples were taken every hour for at least 9 h, and after 12 and 24 h.

2.3. In vivo release studies

Blood samples were centrifuged and the resulting serum was frozen at −80 °C until analysis.

Analysis was performed with a 96-well INFα-ELISA (Bender MedSystems, Vienna, Austria).

Rh-INFα in the rabbit blank serum solutions of known concentrations (8.0–1600.0 pg/mL) were used to generate calibration curves. Analysis was conducted according to the ELISA test protocol. The absorbance of the colored product was measured using a CS-930-1PC plate reader (Shimadzu, Kyoto, Japan). The detection limit of the rh-INFα-ELISA was 4 pg/ml.

2.4. Rabbit anti-rh-INFα-antibody determination

Repeatedly administered injections of rh-INFα were reported to cause antibody generation in rabbits [8]. As no rabbit anti-rh-INFα-antibody-ELISA was available spiking experiments with serum samples were conducted in order to investigate whether rabbit antibodies against rh-INFα were present in the serum.

Aliquots of the samples drawn on day 9 or later were analyzed by INFα-ELISA and showed no detectable amount of rh-INFα. An exact amount (1.600 or 3.200 ng/mL) of rh-INFα solution was now added to these rabbit serum samples, and the mixture was incubated for 1 h at 40 rpm in a horizontal shaker. After such spiking, the recovery of the added rh-INFα was determined via the rh-INFα-ELISA kit from Bender.

It was expected that the antibodies generated in the rabbits against the recombinant human protein can also capture the added rh-INFα, and the analysis would therefore lead to a low recovery of the added rh-INFα.

2.5. Histological studies

At the end of the animal experiment (after 28 days) the implants were surgically removed. Slices of the implant and surrounding tissue were stained with a hematoxylin and eosin (H and E) stain. Image data were collected through a Leica DFC 320 camera (Leica Microsystems, Wetzlar, Germany) mounted on an Orthoplan microscope (Leica, Wetzlar, Germany).

2.6. In vitro release studies

Studies were conducted by incubating the implants ($n = 3$) at 37 °C in TopPac® vials containing 2.0 ml isotonic 0.01 M phosphate buffer, pH 7.4. The samples were shaken at 40 rpm (Certomat® IS, Braun Biotech International, Melsungen, Germany). Samples were taken daily the first 7 days, afterwards every 3 days. Sample volumes were replaced with fresh buffer. Analysis was conducted using a Thermo Separation Products HPLC system equipped with a Tosoh TSK-Gel G3000 SWxl column. 120 mM disodium hydrogen phosphate dehydrate, 20 mM sodium dihydrogen phosphate and 4 g/L sodium chloride, adjusted with hydrochloric acid to a pH of 5.0 were used as mobile phase. The flow rate was set to 0.6 mL/min, UV detection was performed at 210 nm wavelength.

2.7. Computer modeling

To model virtual rh-INFα serum levels, a single compartment model was used. Basic pharmacokinetic data and data gained by the in vitro release studies were collected to calculate the virtual rh-INFα blood levels by using the in vitro release curve as input function and the basic pharmacokinetic (PK) data for modeling distribution and elimination.

Input functions were created using an Inverted Gaussian (IG) function covering the experimentally obtained data [9]. The invasion rate of the rh-INFα into the rabbit blood described by the IG-function of the control group was neglected and therefore was not included into the final modeling. Variables of the IG-functions were chosen to allow a good fit on the particular curve. Any other data necessary for PK-calculations and modeling, i.e., volume of distribution and bioavailability of rh-INFα were provided by the literature [10].

Calculations were conducted with a self-programmed program and obtained data transferred into Microsoft Excel® for diagram generation. The pharmacokinetic analysis program WinNonlin® was used to determine the half-life and the elimination rate of rh-INFα.

3. Results and discussion

3.1. Pharmacokinetic data

The reference group with non-retarded rh-INFα-solution applied as s.c. injection provided the basic PK-data. The Cmax was reached after 2.5 h. Rh-INFα in serum was eliminated with an average terminal elimination half-life of 3.5 h ($k_{el} = 0{,}2$). After 12 h no rh-INFα was detectable any longer. The rh-INFα serum curve gained from the reference group is shown in Fig. 1. The modeled curve covering the rh-INFα serum curve depends on two functions: an IG-function describing the inflow and a first-order kinetics curve describing the elimination of rh-INFα used for PK-data determination.

The IG-function was calculated using the following formula [9]:

$$Y = \mathrm{Gain}^{*}\sqrt{\frac{\mathrm{MT}}{2^{*}\pi^{*}\mathrm{cv}^{2*}t^{3}}} * \exp - \left(\frac{(t - \mathrm{MT}^{2})}{(2^{*}\mathrm{cv}^{2*}\mathrm{MT}^{*}t)}\right)$$

where MT = 2.423, $cv^2 = 1.908$ and Gain-value = 9.209.

Goodness-of-fit was calculated using the following formula:

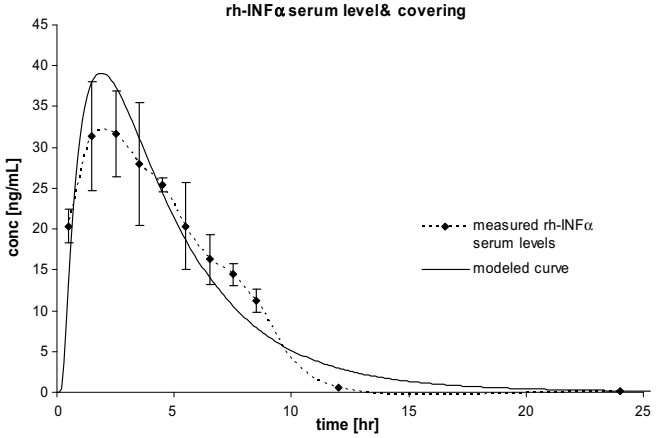

Fig. 1. Rh-INFα-serum levels after injection of 37 μg rh-INFα per kg body mass to a collective of three female rabbits. The obtained curve was covered with a modeled curve providing necessary PK-data.

$$X^2 = \sum \frac{(O - E)2}{E}; \quad [\mathrm{O} = \text{observed value}, \ \mathrm{E} = \text{expected value}].$$

Condition for the calculation of an IG-function was at $t = 24$ h $O/E = 0.5$.

The goodness-of-fit (GOF)-value for the fitting was 11.13.

This pharmacokinetic data reveal high similarity to the data gained in human clinical trials with rh-INFα containing releasing systems [10]. The human half-life of rh-INFα was reported to be about 5 h, and therefore the chosen model is considered as appropriate and plausible.

3.2. In vitro release studies and modeling

As shown in Fig. 2 the implants revealed continuous sustained protein release for 4 weeks. The initial drug release was below 25% of the incorporated protein for the first 24 h.

Over 14 days the protein release was almost linear and overall protein liberation from the implant was higher than 90%. An IG-function was generated covering the in vitro release curve. The IG-function was calculated using the following formula:

$$Y = \mathrm{Gain}^* \sqrt{\frac{\mathrm{MT}}{2^*\pi^*\mathrm{cv}^{2*}t^3}} * \exp - \left(\frac{(t - \mathrm{MT}^2)}{(2^*cv^{2*}\mathrm{MT}^*t)} \right)$$

where MT = 1077.2, $cv^2 = 7.798$ and Gain-value = 48.69.

The GOF-value was 0.227.

This IG-function served as an input function for the calculation of virtual rh-INFα serum levels.

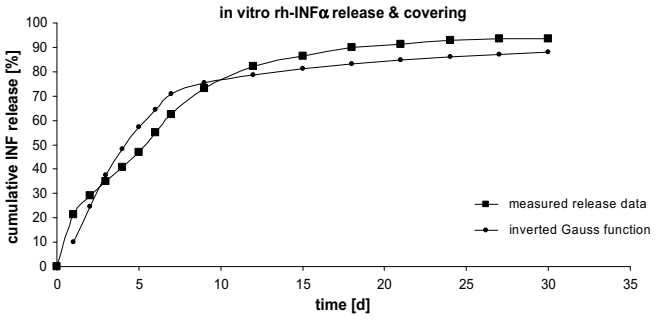

Fig. 2. Cumulative rh-INFα in vitro release from tristearin implants. The release curve was covered with an inverted Gaussian function serving as input function for computer modeling.

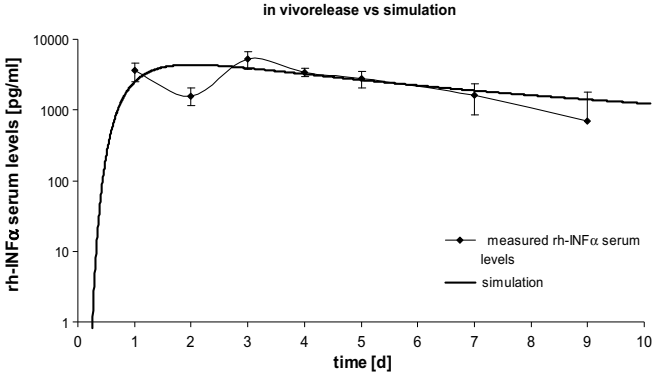

Fig. 3. In vivo rh-INFα curve obtained from a collective of five young rabbits. Curve was covered with the simulated rh-INFα curve obtained by computer modeling.

3.3. In vivo release studies and modeling

Serum samples revealed high rh-INFα-contents on an almost constant level over 9 days after the insertion of the implant. Fig. 3 shows the obtained protein serum curves. High and therapeutic rh-INFα levels were still detectable on day 9, protein levels abruptly wear off from day 10 on. This phenomenon is most likely attributed to the antibodies generated in rabbits against human recombinant INFα because neither in vitro data nor the in vivo serum level curve, or the histology data allow any other explanation.

In order to corroborate this hypothesis, spiking experiments with serum samples after day 9 were conducted in a way that has already been described in the methods section.

These experiments clearly revealed that serum components, i.e., antibodies captured large amounts of the spiked rh-INFα. From the data shown in Fig. 4 it can be concluded that a massive production of antibodies against the rh-INFα had taken place. Presumably these antibodies were capable of masking all the protein released from the implants. This leads to the presumption that rh-INFα is still continuously liberated from the implant after 9 days but the released rh-INFα in the serum was quantitatively captured by the rabbit antibodies.

As explained above in the methods section a simulated animal model was developed. This model consists of a serum compartment with an inflow function described as an inverted Gaussian distribution and first-order kinetics for the outflow.

First-order kinetic data, i.e., the elimination rate was obtained by the analysis of the rh-INFα-injection experiment of the control group. Cumulative release levels from the in vitro experiments were calculated from virtually diluted concentrations based upon the volume of distribution and bioavailability provided by the literature [10]. The IG-function was then fitted on these concentrations and provided virtual serum concentrations. The IG-function was calculated with the same formula and variables used for the in vitro release experiment.

The modeling of the rh-INFα rabbit serum levels revealed that the theoretical rh-INFα serum levels were in accordance with the experimentally obtained serum levels. Both the curve progression and the quantity of the modeled rh-INFα levels correlated very closely with the experimentally obtained rh-INFα serum level curve and values (Fig. 3).

The obtained GOF-value for the fit of the modeled curve on the experimentally obtained curve was 4.37.

Of course, the early outrun of the in vivo data after 9 days does not allow correlations after that time point but it can be presumed that without the interference of rabbit antibodies against the rh-INFα, release of rh-INFα would have been still detectable for many days.

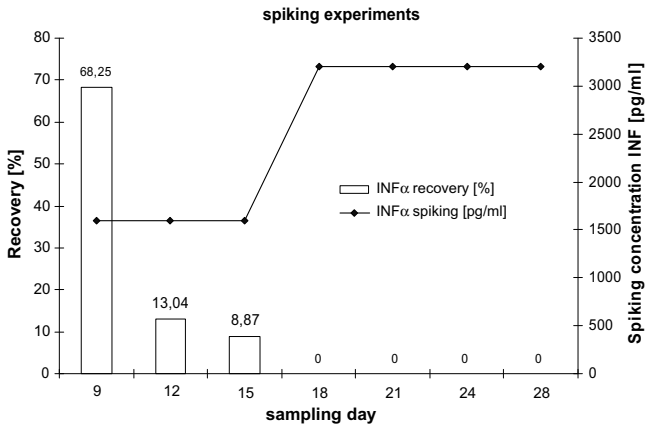

Fig. 4. Spiking experiment data: analysis revealed an increasing rabbit-anti-rh-INFα-antibody titer masking large amounts of rh-INFα in the rabbit serum.

3.4. Biocompatibility and degradation

After implantation of the device, the rabbit's body temperature, the behavior, as well as the eating habit were under steady observation. However no fever or other signs of inflammation and illness like food repulsion could be noticed. After a few days the tissue at the insertion site was healed and it was hardly visible that an implantation had taken place there (Fig. 5). At the end of the animal experiment the implants were surgically removed whereas no macroscopic encapsulation of the implant was noticeable (Fig 6). The surrounding tissue was unaffected by the implant and showed only low signs of inflammation. The implant maintained its structure and appeared to be intact and not eroded; migration of immune cells into the lipid matrix did not appear.

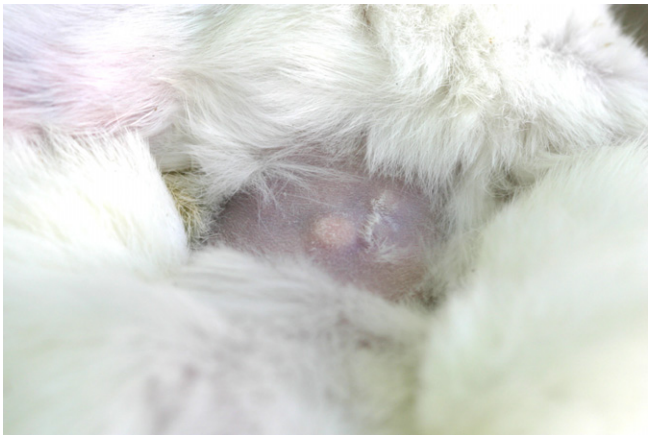

Fig. 5. The implantation site before surgical removal of the implant for histological studies. The imprint of the implant is visible through the subcutaneous tissue.

Fig. 6. Dissection of the implantation area: the implant maintained its structure and was located loosely beneath the subcutis without any macroscopic noticeable encapsulation.

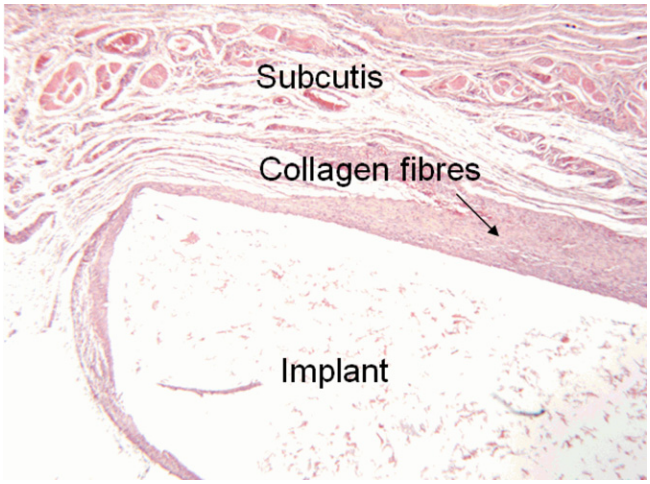

Fig. 7. Twenty-five times magnified immune-histological H&E staining of the subcutaneous tissue surrounding the implant: the tristearin implant is located on the lower half of the picture, surrounded by some collagen fibres.

Microscopic investigations and tissue staining (Fig. 7) revealed that the implantation provoked only weak immune reactions leading to a migration of granulocytes into the surrounding tissue at the insertion site. Cicatrisation of the subcutis was noticed, indicating a quick healing rate and low irritation of the tissue.

4. Conclusion

The rh-INFα release in vitro from a sustained delivery system was compared to in vivo data in rabbits, a relevant model for the potential s.c. application in humans.

It can be stated that during the first 9 days protein release in vivo is in surprisingly quantitative accordance with the data gained by in vitro experiments.

Computer modeling using PK-data obtained in injection experiments revealed that the release properties observed in the animal experiment are in very close causal correlation to the simulated data. The modeled rh-INFα serum levels show the same curve progression for the first 9 days like the rh-INFα serum levels obtained in this animal experiment. Further on the quantity of the modeled rh-INFα levels is in accordance with the measured rh-INFα serum levels. Goodness-of-fit value for the modeled curve was 4.37. This almost exact match of the curves concerning the quality and the quantities of the rh-INFα serum levels allows us to presume that without rabbit rh-INFα-antibodies the rh-INFα release after day 9 would be still ongoing on therapeutic relevant levels for several days. The virtual elimination half-life of rh-INFα could be extended from approx. 4 h up to more than 50 h by means of the depot system. Thus, it can be stated that in terms of biocompatibility and controlled release the implant used in these experiments is a very suitable device for the delivery of rh-INFα and potentially for other pharmaceutically relevant drugs too. Studies to adjust the geometrical form of such devices towards convenient rod-like or micro particulate application forms as well as studies for other protein drugs incorporated into depots of the presented type are underway. Biodegradation studies are under way too. Based on the

in vivo experience presented here, the clinical use in animals and humans may be achievable in the near future.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.05.010.

References

[1] V.R. Sinha, A. Trehan, Biodegradable microspheres for protein delivery, J. Control. Release 90 (2003) 261–280.
[2] M. Morlock, H. Koll, G. Winter, T. Kissel, Microencapsulation of rh-erythropoietin, using biodegradable poly(D,L-lactide-co-glycolide). Protein stability and the effects of stabilizing excipients, Eur. J. Pharm. Biopharm. 43 (1997) 29–36.
[3] S. Mohl, G. Winter, Continuous release of rh-interferon alpha-2a from triglyceride matrices, J. Control. Release 97 (2004) 67–78.
[4] H. Reithmeier, J. Herrmann, A. Göpferich, Lipid microparticles as a parenteral controlled release device for peptides, J. Control. Release 73 (2001) 339–350.
[5] W. Vogelhuber, E. Magni, M. Mouro, T. Spruss, C. Guse, A. Gazzaniga, A. Göpferich, Monolithic triglyceride matrices: a controlled-release system for proteins, Pharm. Dev. Technol. 8 (1) (2003) 71–79.
[6] S. Mohl, G. Winter, Continuous release of rh-interferon (alpha-2a) from triglyceride implants: storage stability of the dosage forms, Pharm. Dev. Technol. 11 (2006) 103–110.
[7] S. Herrmann, S. Mohl, F. Siepmann, J. Siepmann, G. Winter, New insight into the role of polyethylene glycol acting as protein release modifier in lipidic implants, Pharm. Res. 24 (8) (2007) 1527–1537.
[8] P.W. Trown, R.J. Wills, J.J. Kamm, The preclinical development of Roferon – A. Roche Res. Cent., Nutley, NJ, USA, Cancer (NY, United States) 57 (Suppl. 8) (1986) 1648–1656.
[9] A. Ring, Einfluß der Modellwahl auf die Zuverlässigkeit der Schätzung pharmakokinetischer Parameter, Dissertation, S. 25 (2001). Available from: <http://sundoc.bibliothek.uni-halle.de/diss-online/01/02H026/prom.pdf>.
[10] BPI Service GmbH, Fachinfo-Service. Postfach 1255, 88322 Aulendorf; Roferon-A, Summary of Medicinal Product Characteristics, 2003.