Co-administration of Tacrolimus Suppresses Pharmacokinetic Modulation of Multiple Subcutaneously Administered Human Interferon-alpha in Beagle Dogs

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Summary: Specific antibody production is an important issue in crossover pharmacokinetic (PK) studies of protein-based formulations. We recently reported that intravenous co-administration of tacrolimus with multiple human interferon-alpha (h-IFN) administrations successfully suppressed the production of anti-h-IFN antibodies in rats. Since crossover PK studies are preferentially carried out using larger animals such as dogs or monkeys that are capable of accepting the same dosage formulations as those for clinical use, we extended our study of co-administration of tacrolimus with multiple h-IFN administrations to beagle dogs in the present study. Beagle dogs were subcutaneously administered 0.5 million IU/kg of h-IFN once a week for 4 weeks. In some experiments, tacrolimus at 0.01 or 0.1 mg/kg was intravenously co-administered at the same time as the h-IFN administration. Co-administration of the lower dose of tacrolimus (0.01 mg/kg) failed to suppress the anti-h-IFN IgG responses, while co-administration of the higher dose (0.1 mg/kg) successfully suppressed these responses. Moreover, co-administration of tacrolimus had little effect on the serum creatinine concentrations, suggesting that multiple administrations of tacrolimus at the concentrations examined did not cause severe renal disorders. Taken together, the present data confirm that co-administration of tacrolimus is a promising way to assess crossover PK studies of human or humanized proteinic formulations in beagle dogs.

Keywords: proteinic formulation; pharmacokinetics; crossover study; antibody; tacrolimus; interferon-alpha; beagle dog

Introduction

The numbers of peptide and protein drugs available for clinical use have dramatically increased in recent years,1) based on recent advances in technology and science that have provided the tools and opportunities for developing peptides and proteins as drugs. Most peptide and protein drugs are generally administered by invasive routes such as intravenous, subcutaneous or intramuscular injection because of their low bioavailability and high enzymatic degradation rates in the stomach and small intestine. To increase patients’ quality of life and facilitate acceptable compliance, new delivery techniques such as iontophoresis,2) microneedles3) and needle-free injections4) are currently under investigation. Furthermore, alternative administration routes such as oral,5) nasal6) and pulmonary7) routes have been exploited. To develop formulations with novel delivery techniques and administration routes, comparative pharmacokinetic (PK) evaluations using experimental animals are necessary. Crossover studies, in contrast with parallel studies, have been extensively carried out for comparative PK evaluations. Because it is possible to omit individual differences and to minimize the number of experimental animals by administering the same number of drug formulations (reference formulation and several candidate formulations) with an appropriate wash-out period between each administration in individual experimental animals. However, peptide and protein drugs have great potential to cause specific antibody production after mul-
multiple administrations in experimental animals. It is easily imagined that such antibodies may affect the PK of subsequent doses during multiple administrations. Consequently, it is difficult to carry out crossover PK evaluations for various peptide- or protein-based formulations in experimental animals.

We recently reported that co-administration of an immunosuppressant, tacrolimus, suppressed specific antibody production following multiple administrations of a protein drug, native human interferon-alpha (h-IFN), in rats. This observation suggests that crossover PK studies of protein-based formulations can be achieved in experimental animal models under the conditions created by co-administration of tacrolimus. For preclinical studies, it is preferable to carry out PK evaluations in larger animals such as dogs and monkeys because these animals can accept the same dosage forms and dosing devices as those required for human use. To extend our previous study, in this study we investigated the issue of whether co-administration of tacrolimus could suppress specific antibody production following multiple administrations of h-IFN in experimental animals. It is easily imagined that such antibodies may affect the PK of subsequent administrations of the h-IFN solution.

**Materials and Methods**

**Materials:** Off® containing 10 million IU of native h-IFN (MW, 13000–21000) in a vial, which is the clinical lyophilized formulation for intramuscular or subcutaneous injection, was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Prograf®, which is a clinical formulation of tacrolimus for intravenous injection comprising 5 mg tacrolimus in 1 mL of solution in a vial, was purchased from Astellas Pharmaceutical Co., Ltd. (Tokyo, Japan). All other reagents were commercially available products of analytical grade.

**Animal experiments:** Male beagle dogs weighing 9–12 kg were purchased from NARC Corporation (Sanbu, Japan) and maintained at 23°C under 60% humidity. The dogs were allowed free access to water and fed 200 g of standard laboratory chow (CLEA Japan Inc., Tokyo, Japan) once a day. The study was performed after receiving approval from the local Ethical Committee at Otsuka Pharmaceutical Co., Ltd.

The injection solution of h-IFN was prepared by the addition of 1.0 mL of sterile distilled water to each formulation vial (final concentration of 10 million IU/mL). The tacrolimus solutions were prepared by the addition of an adequate volume of sterile physiological saline to each formulation vial (final concentrations of 0.1 and 1.0 mg/mL). The h-IFN solution was subcutaneously administered into the nuchal region of the dogs at a dose of 0.5 million IU/0.05 mL/kg. In some experiments, tacrolimus was intravenously injected into the cephalic vein at a dose of 0.01 or 0.1 mg/0.1 mL/kg at the same time as the h-IFN administration. To determine the h-IFN concentrations in the blood circulation, blood samples (500 μL) were withdrawn from the cephalic vein at indicated times after h-IFN administration. The obtained blood samples were centrifuged (1800 × g, 4°C, 10 min) within 30 min at the latest to obtain serum samples. All the serum samples were stored at −20°C until analysis.

To determine the anti-h-IFN IgG, anti-h-IFN IgM and creatinine concentrations, a blood sample (500 μL) was withdrawn from the cephalic vein immediately before each subcutaneous administration of the h-IFN solution. The obtained blood samples were centrifuged (1800 × g, 4°C, 10 min) within 30 min at the latest and separated serum samples were stored as described above.

**Determination of serum h-IFN concentrations:** The h-IFN concentrations in the serum samples were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the method recommended by the manufacturer (Japan Immunoresearch Laboratories, Ltd., Takasaki, Japan). Briefly, 50 μL of h-IFN standard solutions (1.56–100 IU/mL) and the dog serum samples were diluted with the buffer included in the assay kit and applied to wells of a microtiter plate that were coated with an anti-h-IFN monoclonal antibody (mAb). The plate was incubated for 2 h, and then the wells of the plate were washed five times with the wash solution included in the assay kit to remove the unbound h-IFN. A peroxidase (POD)-linked anti-h-IFN mAb solution (100 μL) was applied to each well and the plate was incubated for a further 2 h. To remove the excess POD-linked anti-h-IFN mAb, the wells were washed five times with the wash solution. Next, 100 μL of 1 mg/mL o-phenylenediamine (OPD) solution containing 0.015% H2O2 was applied to each well for colorization. After incubation for 15 min, the colorization reaction was stopped by the addition of 100 μL of 1N H2SO4 solution. The absorbances of the wells were determined at a wavelength of 492 nm using a microplate reader (Lab Systems Multiscan Bichromatic; Labsystems, Helsinki, Finland). All incubations were performed at room temperature throughout these experiments.

**PK analysis:** The PK parameters of h-IFN were calculated using non-compartment methods. The peak serum concentration (Cmax) was read directly from the serum concentration-time curves from individual animals. The serum concentration profiles of h-IFN were analyzed based on the statistical moment theory. The areas under the serum concentration-time curve (AUC) were calculated by numerical integration using a linear trapezoidal formula.

**Determination of IgM or IgG against h-IFN:** Simple procedures were employed to detect IgM and IgG against h-IFN using the Dog IgM ELISA Quantification Kit and the Dog IgG ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX). Briefly, 50 μL of 2 million IU/mL h-IFN solution was applied to the wells of a
96-well plate. The plate was then incubated for 2 h at 37 °C to coat the h-IFN onto the each well, and the wells were washed three times with the wash solution: Tris-buffered saline (50 mM Tris-HCl pH 8.0, 0.14 M NaCl) containing 0.05% Tween 20. The wells were then blocked with Tris-buffered saline containing 1% BSA for 2 h and washed three times with the wash solution. Serum samples were diluted 200-fold with Tris-buffered saline containing 1% BSA and 0.05% Tween 20. Diluted serum samples (100 μL) were applied to the coated wells, incubated for 1 h and then washed five times with the wash solution. A total of 100 μL of 50 ng/mL horseradish peroxidase (HRP)-conjugated antibody (goat anti-dog IgG-HRP conjugate or goat anti-dog IgM-HRP conjugate) in Tris-buffered saline containing 1% BSA and 0.05% Tween 20 was added to each well. After incubation for 1 h, the wells were washed five times with the wash solution. Coloration was initiated by the addition of 100 μL of 1 mg/mL OPD solution (Sigma-Aldrich, St. Louis, MO). After incubation for 5 min, the reaction was stopped by the addition of 10 μL of 2N H2SO4 and the absorbances of the wells were measured at a wavelength of 492 nm using a microplate reader (Labsystems Multiscan Bichromatic; Labsystems). All the incubations were performed at room temperature, except for the h-IFN coating step.

**Determination of creatinine in serum:** The serum creatinine concentrations were measured by an enzymatic method with an Olympus AU2700 auto-analyzer (Olympus, Tokyo, Japan) and a commercial assay kit (Kainos Laboratories, Tokyo, Japan).

**Statistics:** Results were expressed as the mean ± SE. Statistical analyses were performed by Student’s t-test or Dunnett’s test for multiple comparisons, with the minimum level of significance determined as p < 0.05.

**Results**

**PK profiles of h-IFN following repeated subcutaneous administration in beagle dogs:** The second dose of h-IFN showed a similar serum concentration-time profile to the first dose (Fig. 1); the serum concentrations achieved maximum levels at 2 h after administration and then gradually decreased up to 12 h. After the third dose, serum h-IFN concentration achieved the maximum level at 4 h after administration and then gradually decreased up to 24 h. It appears that the elimination rate of h-IFN after the third dose was slightly attenuated as compared with those of the first and second dose; ke values were 0.266 ± 0.030 for the first dose, 0.251 ± 0.013 for the second dose and 0.152 ± 0.019 for the third dose (\(^* p < 0.05\)).

**Effects of co-administration of tacrolimus on the PK of multiple administration of h-IFN:** To investigate the effects of co-administration of tacrolimus on the PK of sequentially administered h-IFN in beagle dogs, tacrolimus was intravenously co-administered at a dose of 0.01 or 0.1 mg/kg. In the presence of co-administered tacrolimus at 0.01 mg/kg (Fig. 2A), the second and third doses showed similar serum h-IFN concentration-time profiles to the first dose. The fourth dose showed lower serum h-IFN concentrations than the first, second and third doses, but relatively higher concentrations than the fifth dose. The fifth dose did not show apparent serum h-IFN concentrations even when tacrolimus was co-administered. In contrast, co-administration of tacrolimus at 0.1 mg/kg led to similar serum h-IFN concentration profiles for all the h-IFN doses (Fig. 2B). Notably, the fifth dose showed apparent serum h-IFN concentrations in the presence of the higher dose of tacrolimus, unlike the case for the lower dose of tacrolimus.

**Effects of co-administration of tacrolimus on the anti-h-IFN IgM and IgG production responses during repeated administrations of h-IFN:** The anti-h-IFN IgG and IgM concentrations were evaluated in the obtained serum samples. Repeated administrations of h-IFN caused the production of anti-h-IFN IgG, which was detected in the sera after the third administration (Fig. 3). The anti-h-IFN IgG concentration gradually increased as the number of h-IFN administrations increased. Co-administration of the lower dose of tacrolimus (0.01 mg/kg) did not attenuate the production of anti-h-IFN IgG, and the production profile was very similar to that of control animals (without tacrolimus co-administration). Co-administration of the higher dose of tacrolimus (0.1 mg/kg) led to strong diminishment of anti-h-IFN IgG production.
Fig. 2. Effects of co-administration of tacrolimus on the pharmacokinetic profiles of multiple administrations of h-IFN in beagle dogs
Filled circles: first dose (day 0); open triangles: second dose (day 7); closed triangles: third dose (day 14); open diamonds: fourth dose (day 21); and closed diamonds: fifth dose (day 28). (A) Co-administration of tacrolimus at 0.01 mg/kg. (B) Co-administration of tacrolimus at 0.1 mg/kg. Each value represents the mean ± SE of three experiments.

Fig. 3. Effects of co-administration of tacrolimus on the anti-h-IFN IgG responses during repeated administrations of h-IFN in beagle dogs
h-IFN (0.5 million IU/kg) was subcutaneously administered once a week for 4 weeks. Tacrolimus [none (control): open circles; 0.01 mg/kg: filled triangles; 0.1 mg/kg: filled diamonds] was intravenously co-administered. The anti-h-IFN IgG levels in the obtained serum samples were determined. Each value represents the mean ± SE of three experiments. ***p < 0.005 compared with the control.

The PK parameters of h-IFN with and without co-administration of tacrolimus are summarized in Table 1. The PK parameters for each h-IFN dose were very similar in the presence of co-administration of tacrolimus at the higher dose. The findings reflected the suppression of anti-h-IFN IgG production caused by the immunosuppressive effects of co-administration of tacrolimus. The repeated h-IFN administrations did not cause any anti-h-IFN IgM production under the experimental condition we tested (data not shown).

Effects of administration of h-IFN and tacrolimus on the serum creatinine level: To examine whether repeated administrations of h-IFN or h-IFN plus tacrolimus cause severe renal dysfunction, the serum creatinine concentrations were determined during the administrations (Fig. 4). There were no differences in the serum creatinine concentrations throughout the experimental period, irrespective of repeated h-IFN administration with or without co-administration of tacrolimus. These findings showed that intravenous co-administration of tacrolimus (0.1 mg/kg) with h-IFN did not cause severe renal dysfunction in beagle dogs during at least five sequential administrations at 1-week intervals.

Discussion
Assessment and control of PK profiles is one of the most important aspects of the development of new drug formulations. In the early stage of development, crossover PK studies are preferentially carried out in experimental animal models to evaluate the PK profiles of the formulations. In contrast with parallel studies, crossover studies generally have the advantages of being able to omit individual differences and reduce the number of experimental animals required. However, all protein-based formulations are made up of humanized or human proteins, which are exogenous and have great potential to cause specific antibody production in the experimental animals. The antibodies produced by primary and/or secondary doses affect the PK of subsequent doses during repeated administrations. Therefore, comparisons of the PK of the same protein-based formulation in the same experimental animal are difficult without methodological improvements. We recently reported that co-administration of the immunosuppressant tacrolimus suppressed specific antibody production following multiple administrations of protein-based drug h-IFN in rats. In general, large animal species such as dogs or monkeys, which are close to humans, are used to evaluate the PK profiles of novel drug formulations. In the present study, we therefore extended our previous study to a larger experimen-
TABLE 1. Pharmacokinetic parameters of h-IFN in beagle dogs after the first, second, third, fourth and fifth subcutaneous administrations of h-IFN (0.5 million IU/kg) with or without intravenous co-administration of tacrolimus (0.01 or 0.1 mg/kg)

<table>
<thead>
<tr>
<th>Tacrolimus (mg/kg)</th>
<th>1st (day 0)</th>
<th>2nd (day 7)</th>
<th>3rd (day 14)</th>
<th>4th (day 21)</th>
<th>5th (day 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>76 ± 65</td>
<td>687 ± 45</td>
<td>736 ± 62</td>
<td>78 ± 37***</td>
<td>24 ± 18***</td>
</tr>
<tr>
<td>0.01</td>
<td>597 ± 151</td>
<td>606 ± 61</td>
<td>713 ± 81</td>
<td>345 ± 100</td>
<td>97 ± 13*</td>
</tr>
<tr>
<td>0.1</td>
<td>704 ± 78</td>
<td>441 ± 88</td>
<td>568 ± 80</td>
<td>655 ± 47</td>
<td>693 ± 147</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.1</td>
<td>4.7 ± 0.7</td>
<td>7.2 ± 1.1*</td>
<td>5.5 ± 0.5*</td>
</tr>
<tr>
<td>0.01</td>
<td>3.2 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>2.5 ± 0.9</td>
<td>3.5 ± 1.3</td>
<td>5.9 ± 0.6*</td>
</tr>
<tr>
<td>0.1</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>2.7 ± 0.4</td>
<td>4.8 ± 1.4</td>
<td>4.8 ± 0.5</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of three experiments. *p < 0.05, **p < 0.005 compared with the first dose.

Fig. 4. Effects of repeated administration of h-IFN or h-IFN plus tacrolimus on the serum creatinine levels in beagle dogs. h-IFN (0.5 million IU/kg) was subcutaneously administered once a week for 4 weeks. Tacrolimus [none (control): open circles; 0.1 mg/kg: filled diamonds] was intravenously co-administered at the same time as the h-IFN administration. The creatinine concentrations in the obtained serum samples were determined. Each value represents the mean ± SE of three experiments.

Optimal tacrolimus dosage must be set up in advance according to the species of experimental animal involved.

Nephrotoxicity is the most common side effect of tacrolimus in several experimental animal species.14–16 h-IFN is predominantly eliminated from blood circulation by glomerular filtration, followed by tubular reabsorption and lysosomal degradation in the kidney.17 Therefore, the PK of h-IFN may be modulated in experimental animals with impaired renal function caused by multiple administrations of tacrolimus. In the present study, five sequential weekly intravenous co-administrations of tacrolimus at a dose of 0.1 mg/kg caused no significant changes in the serum creatinine concentrations (Fig. 4), which is an indication of unchanged renal function.18,19 These observations suggest that co-administration of tacrolimus would be a safe strategy in all experimental animals for carrying out a crossover PK studies of protein-based formulations, as long as the effective dose is successfully set up in advance.

It should be noted that co-administration of tacrolimus caused anaphylaxis-like symptoms at the higher dose (0.1 mg/kg) but not at the lower dose (0.001 mg/kg) in two of the three dogs examined (data not shown). These symptoms were probably induced by the solubilizer contained in the formulation, namely polyoxyethylene hydrogenated castor oil 60 (HCO-60), which is used as a solubilizer for lipophilic drugs.20 Under our experimental conditions, HCO-60 was intravenously given at respective doses of 0.4 and 4 mg/kg for tacrolimus doses of 0.01 and 0.1 mg/kg. It has been reported that intravenous administration of more than 1.25 mg/kg of HCO-60 causes anaphylaxis-like symptoms such as flush-
Taken together with our previous report on rats, we are preferentially employed for crossover PK studies. Causing severe renal dysfunction in beagle dogs, which intravenous co-administration of tacrolimus without anaphylaxis-like symptoms during the crossover study. If caused by additives such as release from mast cells in dogs, whereas these effects are not observed in monkeys, rabbits, guinea pigs or rats. These findings are consistent with our current study and previous observations. If caused by additives such as solubilizers, severe anaphylaxis-like symptoms may not be observed in monkeys, rabbits, guinea pigs or rats. If caused by additives such as solubilizers, severe anaphylaxis-like symptoms may not be observed in monkeys, rabbits, guinea pigs or rats.21) Taken together with our previous report on rats,2) we propose that co-administration of the immunosuppressant tacrolimus could be a useful strategy for evaluating the PK profiles of exogenous protein-based formulations with multiple dosing schedules in any experimental animal model.

References


