Pharmacokinetic-Pharmacodynamic Modeling of Alpha Interferon Response Induced by a Toll-Like 7 Receptor Agonist in Mice

Neil Benson, Joost de Jongh, Jonathan D. Duckworth, Hannah M. Jones, Henry E. Pertinez, Jaiessh K. Rawal, Tamara J. van Steeg, and Piet H. Van der Graaf

Department of Pharmacokinetics, Pharmacodynamics and Metabolism, Pfizer Global Research and Development, Ramsgate Road, Sandwich, United Kingdom; LAP&PC Consultants, Leiden, The Netherlands; and School of Pharmacy and Pharmaceutical Sciences, The University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

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Recombinant alpha interferon (IFN-α) is used in the treatment of hepatitis C virus (HCV)-infected patients but is not optimal in terms of efficacy or tolerability. Toll-like 7 receptor (TLR-7) agonists stimulate the innate immune system to produce, among other cytokines, IFN-α and are being evaluated as alternative drugs to treat HCV infection. This paper describes the application of pharmacokinetic-pharmacodynamic (PK-PD) modeling to understanding the behavior of a TLR-7 agonist [9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (BHMA)] in mice, using IFN-α as a biomarker. This is the first report of such a PK-PD model, and the conclusions may be of utility in the clinical development of TLR-7 agonists for HCV infection.

An estimated 200 million people worldwide (39) are infected with hepatitis C virus (HCV). The virus replicates primarily in the liver, and 70% of those infected go on to develop chronic infection, with a further 20% progressing to serious liver disease (6). The current standard of care for HCV infection is treatment with pegylated alpha interferon (IFN-α) combined with ribavirin; however, this treatment is effective for less than 50% of patients (26). Furthermore, significant side effects, including flu-like symptoms, depression, injection site reactions, and hemolytic anemia, are observed (10). The need to develop more available, better-tolerated, and more effective medicines is therefore clear. To this end, one of the areas of interest has been the targeting of IFN inducers (13, 18) and medicines is clear. To this end, one of the areas of interest has been the targeting of IFN inducers (13, 18) and

MATERIALS AND METHODS

Human replicon in vitro antiviral assay. The human replicon assay was carried out as described in reference 34; briefly, human hepatoma cells (Huh-7) with the persistent replicon PFKI389 Luc Ubi Neo NS3-3′/EF (ReBLikon GmbH) were used. The replicon cells were plated into tissue culture-grade white 96-well plates (PerkinElmer) at 10^5 cells/well in a volume of 90 μl. Following TLR stimulation, 10 μl of peripheral blood mononuclear cell supernatant was added to the HCV replicon cells, and the cells were incubated for 48 h. Replicon levels were...
quantified using a Promega Bright Glo luciferase detection kit according to the manufacturer’s instructions.

Compounds. BHMA was synthesized as described elsewhere (21). Unless stated otherwise, all other compounds were purchased from Sigma (Poole, United Kingdom).

In vivo experiments with BHMA. BHMA was orally administered to male cd-1 outbred mice (Charles River Laboratories UK, Margate, Kent, United Kingdom) in a vehicle of 5% dimethyl sulfoxide, 50% polyethylene glycol 200, and 45% water. For the PD groups, the following dose levels (with numbers of animals per group in parentheses) were used: 0.1 mg/kg of body weight (3), 0.3 mg/kg (2), 0.5 mg/kg (2), 1 mg/kg (10), 2.5 mg/kg (4), 5 mg/kg (20), and 10 mg/kg (5). At designated time points, two 50-μl saphenous vein blood samples were taken, and a terminal 1-ml blood sample was obtained. A matrix sampling system was employed to capture composite biomarker and PK profiles. For the PK-only groups, dose levels of 5, 1, and 0.1 mg/kg were used and plasma samples were taken at 0.1, 0.25, 1, 2, 6, 8, and 10 h for the 5-mg/kg-dose group and at 1, 2, 6, 8, and 10 h for the 1- and 0.1-mg/kg-dose groups. In total, 25 animals were dosed and subjected to sampling for PK analysis. Plasma samples were prepared by centrifugation and stored at −20°C until analysis.

All animal studies were conducted according to the guidelines set by the United Kingdom Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of plasma samples for BHMA. All samples were analyzed for BHMA quantification by using a PE-Scieix API 4000 (Turbo V Ionspray source) quadrupole mass spectrometer. Samples (180 μl) were injected with the use of a PAL autosampler (CTC Analytics, Zwingen, Switzerland) onto a C18 Opti-Lynx 15- by 2.1-mm, 40-μm Particle-size column (Optimize Technologies). The total run time was 2 min. Analytes were eluted using a linear gradient consisting of 10% methanol (MeOH), 90% H2O, 0.027% formic acid, and 2 mM ammonium acetate (solution A) and 90% MeOH, 10% H2O, 0.027% formic acid, and 2 mM ammonium acetate (solution B), produced by two Hewlett Packard HP1100 binary pumps. An initial condition of 0 to 100% solution A was maintained for 0.2 min, with the flow diverted to waste via a six-port valve (Valco International, Schenkon, Switzerland). The initial condition was then ramped up to an intermediate condition (0 to 100% solution B) and the flow was diverted through a Chromolith SpeedROD RP-18 50- by 4.6-mm column (Onyx Technologies) up to the 1.2-min time point. Filters (pore size, 5 μm) were placed directly in front of the analytical column, between the column and the autosampler. The intermediate condition (0 to 100% solution B) was then held for 1.9 min and returned to the initial condition to reequilibrate the column. The total run time was 2 min. During the run, the flow was maintained at 3,000 μl/min and split 5:1 post-column step by using an Acurate flow splitter. The calibration and quality control (QC) standards were prepared to yield a coefficient of variation (CV) of 20%. The limit of quantification (LOQ) in this analysis was 0.5 ng/ml.

Compound detection (bioanalysis of BHMA). Systemic plasma compound levels were assessed via a mixed-mode cationic solid-phase extraction procedure using an Oasis MCX solid-phase extraction (SPE) disk column (Waters, Inc.). The samples were first washed four times with wash buffer. One hundred microliters of a 30-ng/ml working solution of mouse IFN-α (PBL Biomedical Laboratories Inc., Piscataway, NJ) was added to each well, and the absorbance at 450 nm, with background subtraction at 650 nm, was measured using a Victor 2 multilabel counter (Wallac OY, Turku, Finland). The relationship between absorbance units and the mouse IFN-α concentration (in picograms per milliliter) was modeled using a five-parameter logistic fit. The sample IFN-α concentration was then derived by interpolation. Samples were assayed in duplicate, with five QC duplicates at each concentration level. The calculated concentration was then converted into international units per milliliter by assuming that 1 IU/ml equals 15 pg/ml. Plasma standards and QC standards were prepared identically to the corresponding samples with respect to the final composition.

The LOQ was 0.3 IU/ml, and the CV for the determination of the IFN-α concentration was 20%.

PK and PK-PD modeling. PK and PD models are based on NONMEM VI (Icon Consulting) with Compug Verifon, version 6.6. All data visualization was carried out using S-Plus (version 6.2). Murine PK was modeled using the ADVAN4 subroutine of NONMEM (two-compartmental model), and the murine BHMA PK and PD for IFN induction and elimination were modeled using ADVAN6 for numerical solution of ordinary differential equations.

The IFN-α response to drug concentrations in plasma was modeled according to an indirect response model in which the response of the biomarker to the drug is caused by stimulation of the biomarker and the baseline response is zero (11). In this model, IFN-α is synthesized in the presence of the drug with a sigmoid saturating concentration-response relationship and is degraded at a constant rate according to equation 1:

$$\frac{dR}{dt} = \frac{S_{max} \cdot C}{S_{C50} + C} - k_{out} \cdot R$$

where \(dR/dt\) is the net rate of change in the IFN-α concentration (in international units per milliliter per hour), \(S_{max}\) is the maximum rate of synthesis of IFN-α (in international units per milliliter per hour), \(S_{C50}\) is the concentration of the compound (in nanograms per milliliter) required for half-maximal synthesis of IFN-α, \(k_{out}\) is the elimination rate constant for IFN-α (expressed as a value per hour), \(C\) is the predicted plasma drug concentration (in nanograms per milliliter), and \(R\) is the plasma IFN-α concentration in international units per milliliter.

The essential features of the model are summarized in Fig. 1.

Data were also modeled according to a linear drug response equation 2:

$$\frac{dM}{dt} = M \cdot C_{S} - k_{out} \cdot R$$

where \(M\) is the linear slope of the response to the drug (in international units per milliliter per hour per nanogram per milliliter).

Statistical parameters and population and individual predictions were calculated using the first-order conditional estimation method with interaction be-
between the two levels of stochastic effects (FOCE interaction), as implemented in NONMEM (3).

**Details of mixed-effects statistical analysis.** The interindividual variability (IIV) of appropriate parameters was modeled using multiplicative exponential random effects as expressed in equation 3:

\[ P_i = \theta \exp(\eta_i) \]  

where \( \theta \) is the typical population value for the parameter, \( P_i \) is the individual prediction, and \( \eta_i \) is the random deviation of \( P_i \) from \( \theta \). The values of \( \eta_i \) were assumed to be normally distributed, with a mean of zero and a variance of \( \sigma^2 \). The derived IIV is expressed as the percent CV. The precision of parameter estimates was expressed as the percentage of the ratio of the standard error of the parameter estimate (percent CV).

Residual variability was described using additive and proportional error models (equations 4 and 5, respectively):

\[ Y_i = F_i + \epsilon_i \]  

\[ Y_i = F_i \times (1 + \epsilon_i) \]

where \( Y_i \) denotes the observation for the \( i \)th individual at time \( t_i \), \( F_i \) denotes the corresponding prediction based on the model, and \( \epsilon_i \) denotes the residual random effect assumed to have a mean of zero and a variance of \( \sigma^2 \). For further details, see reference 32.

Models were compared using the Akaike information criteria (AIC) calculated per equation 6, where \( \text{MVOF}_A \) and \( \text{MVOF}_B \) are the minimum values of the objective function and \( p_A \) and \( p_B \) are the numbers of model parameters for model A and model B, respectively. Model A was considered statistically superior if the change in the AIC (\( \Delta \text{AIC} \)) was \(<0\):

\[ \Delta \text{AIC} = \text{MVOF}_A - \text{MVOF}_B + 2(p_A - p_B) \]  

### RESULTS

**PK of BHMA in mice.** BHMA was rapidly absorbed following oral dosing, and after reaching a maximum concentration in plasma within 0.1 h, it showed some evidence of a biphasic decrease (Fig. 2). By simultaneously fitting all the data, we found that the plasma PK for BHMA was best described by a two-compartment PK model with first-order uptake from the gastrointestinal tract and linear elimination (Table 1 and Fig. 2). The absorption rate constant was fixed at 35 h\(^{-1}\), reflecting...
complete absorption prior to the collection of the earliest PK samples (at 0.1 h). In general, the PK was described well, with CVs for parameters between 16 and 36%. The IIV of the clearance was 38%, and the residual error was 46%. The \( f_u \) in plasma was found to be 0.33 (standard error = 0.006).

**PK and PD of IFN induction by BHMA in mice.** Using the IFN-\( \alpha \) assay, we found that in the absence of a TLR-7 agonist, the basal level of IFN-\( \alpha \) was, in all cases, below the LOQ of our assay (0.3 IU/ml), and hence it was fixed at zero for the purpose of PK-PD modeling. Upon oral dosing with BHMA, a rapid and dose-dependent rise in the plasma IFN-\( \alpha \) concentration was observed, with the concentration peaking at approximately 1 to 2 h around 300 IU/ml and decreasing rapidly back to zero. By using PK parameters for animals with corresponding PD parameters and elsewhere fixing the PK parameters at their typical population values, a sequential PK-PD analysis was performed using both an indirect ordinary maximum-effect (\( E_{\text{max}} \)) model (equation 1), in which the drug effect saturates, and a model in which induction was a linear function of the plasma drug concentration (equation 2). Using the AIC (equation 6) to calculate significance, we found that comparing the ordinary \( E_{\text{max}} \) model with a linear model gave a \( \Delta \text{AIC} \) of -22.9 and that, therefore, the ordinary \( E_{\text{max}} \) model had the superior fit. The indirect ordinary fit to the data was optimal with IIV implemented for the maximum synthesis rate of IFN (\( S_{\text{max}} \)), and all model parameters could be adequately identified, with CVs ranging from 0.1 to 24% (Table 2). IIV of the \( S_{\text{max}} \) was high at 70%, and the residual error was 65 IU/ml (Fig. 3 and

### Table 2. PK-PD parameters for induction of IFN-\( \alpha \) in mice after oral dosage with 0.1 to 10 mg/kg BHMA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{out}} ) (h(^{-1}))</td>
<td>0.958</td>
<td>0.1</td>
</tr>
<tr>
<td>( SC_{50} ) (ng/ml)</td>
<td>135</td>
<td>24</td>
</tr>
<tr>
<td>( S_{\text{max}} ) (IU/ml/h)</td>
<td>294</td>
<td>8</td>
</tr>
<tr>
<td>IIV of ( S_{\text{max}} ) (%)</td>
<td>70</td>
<td>26</td>
</tr>
<tr>
<td>Residual error (IU/ml)</td>
<td>65</td>
<td>19</td>
</tr>
</tbody>
</table>

*Parameters were obtained by fitting data to an indirect response model (equation 1).*

**FIG. 3.** (A) Observed plasma IFN-\( \alpha \) concentrations (solid black circles) and typical predicted values according to equation 1 (solid gray triangles) after oral dosage with 0.1 to 10 mg/kg BHMA. Data are sorted by dose group, with dose levels (AMT) per group given in micrograms per kilogram at the top of each panel. Time is expressed in hours. (B and C) Goodness-of-fit diagnostic plots showing the typical predicted values for populations (B) and the individual model-predicted values (abscissa) versus observed values (ordinate) derived from the mixed-effects fit. The solid lines are the lines of unity.
Table 2). The typical SC_{50} was 135 ng/ml total, which with correction for the free fraction equates to 125 nM.

**Human replicon assay potency.** The observed potency of BHMA in the human surrogate replicon assay of antiviral efficacy was ca. 30 nM.

**DISCUSSION**

Previously, it has been shown that TLR-7 agonists can elicit the release of antiviral cytokines in mice (21). In this study, we extend this analysis to include application of PK-PD modeling, using BHMA as a standard TLR agonist and IFN-α as a biomarker of effect. This approach confers advantages in that the structure and parameters of the model can be related to prior mechanistic data, the concentration response can be related to any in vitro measures of pharmacology, and extrapolation of preclinical findings to the clinical outcome can in principle be facilitated. For example, a mechanistic PK-PD approach was used previously to show that buprenorphine-specific PD parameters such as binding of the drug to the target were similar in rats and humans (40). In addition, it has been found that although concentration-response data can translate well between species, the dose response can be very different due to interspecies PK differences (23). Our intention was to define the concentration response in mice and to relate this to in vitro measures of pharmacology and to the existing knowledge of the behavior of TLR-7 agonists. Ultimately, this understanding could be used to improve confidence in the extrapolation of effects for patients and optimize clinical trial design.

The PK parameters of BHMA were estimated with good precision. However, without intravenous data, the bioavailability (F) cannot be estimated, and hence, the volume of distribution (V) of ca. 40 liters/kg must be regarded as the V/F ratio. The oral bioavailability of BHMA has been reported previously to be ~40% at 3 mg/kg in rats, and the V has been reported to be ca. 2.1 liters/kg (21). Due to relatively similar tissue compositions in mice and rats, V tends to scale with an exponent close to 1 (19, 27), and so the observed V at steady state (V_s) in mice would appear to be high, given that the f_s in plasma for rats and mice are 0.22 and 0.33, respectively. However, bioavailability needs to be accounted for, and a low value of ca. 5% for this parameter in this case could explain the apparent discrepancy. The solubility of BHMA was found to be low (ca. 6 µg/ml), and this may contribute to limited and dose-dependent bioavailability. Further data will be required to elucidate this point; nevertheless, the description of the data would appear to be adequate for the purpose of PK-PD modeling.

We found that the IFN-α data were better described by an indirect E_{max} model than by a linear model. Arguably, a more complex model may be more appropriate; however, our analysis shows that the ordinary E_{max} model is a reasonable description of the data without obvious bias. Such a model would also be consistent with the proposed mechanism of agonist action whereby saturable binding of a TLR-7 agonist to TLR-7 results in the synthesis and release of IFN-α (36).

In general, the PK-PD model parameters were estimated with good precision, a notable exception being the S_{max}. Conceivably, the variability may arise from residual measurement error, interindividual error, or a combination of these. Experiments to define the interexperimetal variability of the assay methodology prior to use showed that the CV of quantification of IFN-α was 20% of the typical value. In addition, the model fit yielded reasonable estimates of residual variation and IIV. Taking these observations together, it would appear that most of the observed dispersion in the maximum response is more likely to be an interindividual PD difference, rather than being due to assay variability.

The model allowed the estimation of the midpoint of the concentration response, the degradation rate constant, and the maximum velocity of production of IFN-α. The apparent degradation rate constant for IFN-α was 0.96 h^{-1}, giving a half-life (t_{1/2}) of approximately 0.7 h, and hence the majority of the induced IFN-α is cleared from the plasma in 3 to 4 h. The terminal half-life of IFN in mice has been reported previously to be ca. 50 min (29) (or given t_{1/2} = 0.693/k, then k = 0.83 h^{-1}), versus ca. 43 min determined in this study. Hence, the estimate of the IFN-α degradation rate would appear to be consistent with previous data. The typical maximum velocity of production of IFN-α was estimated to be 294 IU/ml/h, and the maximum response is given by the S_{max}/k_{out} ratio of 306 IU/ml. The maximum in vivo response, as measured using an antiviral bioassay, has been reported previously to be 2,872 IU of IFN/ml in BALB/c mice (21). It is most likely that the apparent difference derives in large part from the difference between using the single biomarker IFN-α and the more general measures of multiple IFN types and potentially other mediators in the antiviral bioassay. This would be consistent with the documented spectrum of cytokines released by peripheral blood mononuclear cells upon challenge with TLR-7 agonists (2, 34). Interestingly, a survey of the available literature revealed that across a number of studies, a typical maximum response in humans (determined via antiviral bioassays) was ~10,000 IU of IFN/ml (17, 33, 38), and therefore, maximum IFN responses appear not to be grossly different between mice and humans.

By correcting for the free fraction, the in vivo unbound SC_{50} could be calculated. A future interesting direction for this work would be to develop assays of binding at the murine TLR-7 that would allow comparison of in vivo SC_{50} and primary in vitro pharmacology measures. In turn, this should help improve understanding of the plasma and site-of-effect equilibriums. Interestingly, however, the SC_{50} estimate was approximately fourfold our estimate of observed potency in the human surrogate replicon assay of antiviral efficacy (ca. 125 nM versus 30 nM). Whether this result reflects an expectation of good cross-species translation and, indeed, whether the replicon surrogate assay is a good indicator of clinical outcome will become apparent as more clinical data for TLR-7 agonists become available.

The observed apparent burst in IFN-α raises some intriguing questions. The clear pharmacological effect implies rapid penetration of BHMA into appropriate cells, such as the plasmacytoid dendritic cells (PDCs), and the endosomes therein. The IFN-α levels then rapidly decay back to the baseline as the drug is cleared from the plasma. In contrast, it has been shown that infection of mice with DNA and RNA viruses leads to sustained IFN-α induction for at least up to 12 h and survival for up to 14 days (15). Furthermore, it has also been found that TLR-9 ligands can be retained for long periods in the endosomal vesicles of PDCs and that this retention is critical to the
robust response (14). In this model, the retention of a TLR-7 agonist in the endosome is not required; however, further data are needed to demonstrate this conclusively. Conceivably, the retention profile could potentially be an important determinant of the time of response and may differ among TLR-7 agonists. For example, if it were possible to design drugs with endosomal retention, then this feature could confer effects independent of PK, similar to the effects of designing drugs with longer residence times on the receptor as discussed recently (35); such a parameter could be a selectable property of drug candidates.

The model implies also that repeat dosing with BHMA would result in replicate bursts of IFN-α release and, with the right dosing regimen, in sustained IFN-α as observed after infection with DNA and RNA viruses. However, as the model has been constructed using single-dose data only, such a conclusion would be an extrapolation, and hence repeat dose experiments with individuals are needed to test this assumption.

One further potential utility of measuring IFN-α and developing PK-PD models of IFN-α response to TLR-7 agonists in early clinical research may be to provide a quantitative link to outcome. Equations that describe the impact of recombinant IFN-α on viral load decrease have been reported previously (28, 31). Hence, with a PK-PD model of the drug-dependent IFN-α time course in humans, a dose-antiviral response relationship could be calculated, assuming that recombinant IFN behaves similarly to the native form. The potential benefits of linking a biomarker of pharmacology (e.g., IFN-α) to a biomarker of effect (viral load) in this way may include improved clinical trial design and more rapid and cost-effective delivery of medicines to patients. For example, this method provides a quantitative method to estimate a dose around which patient clinical trials could be designed. In addition, an understanding of variability may be crucial; we have shown that the IFN-α response to BHMA in mice appears to show quite large IV, and patient responses to TLR-7 agonists have also been found to be inconsistent, for example, 370 to 23,000 U of IFN/ml in one dose group for imiquimod (38). Furthermore, patient responses to recombinant IFN itself in the reduction of viral load have also been found to be variable (4). Together, these data indicate that an understanding of the structure and population variability in IFN responses to TLR-7 agonists and the variability in the subsequent antiviral effects will be invaluable in the interpretation and extrapolation to patients of any data from IFN-α biomarker volunteer clinical trials (38). PK-PD models incorporating both structural and stochastic elements as exemplified in this study will be a valuable tool to interpret such data, to inform the design of trials, and indeed, to project the likelihood of a successful outcome.

In summary, the PK-PD methods described in this report provide the basis to improve our understanding of the behavior of BHMA and potentially other TLR-7 agonists in vivo. Hence, identification of the features of a drug that are required for success can be facilitated. Furthermore, the findings should be of utility in the development of PK-PD models of IFN-α biomarker data for humans, and ultimately, such models could be used to provide a rational basis for the selection of optimal doses and schedules for antiviral agents.

REFERENCES


