Background: This study presents preclinical data of a novel interferon (IFN)-α8 fusion protein, PF-04849285, and compares it with IFN-α2 and pegylated IFN-α2; the latter being the current standard of care for HCV.

Methods: The antiviral properties were evaluated in vitro using the HCV replication assay (replicon) and the general encephalomyocarditis virus assay. The binding affinity to both IFNR-subunits was assessed using surface plasmon resonance. Ex vivo experiments using cynomolgus monkey and human blood were used for the evaluation of induction of IFN-inducible biomarkers (interferon inducible protein 10 [IP-10], 2′-5′-oligoadenylate synthetase [OAS2] and interleukin-6 [IL-6]). The molecule was tested intravenously and subcutaneously in cynomolgus monkey in a single dose study for two weeks at 0.01, 1, 5 and 20 mg/kg. Each route and dose combination was given to a single male animal, blood samples were collected for evaluation of biomarkers and pharmacokinetics. The compound was also tested in cynomolgus monkey in a multiple dose study for four weeks, with a twice-a-week dosing prior to a three-week wash-out period for toxicokinetics, pharmacokinetics, and biomarker evaluation at 20, 50 or 100 mg/kg subcutaneously and 20 mg/kg intravenously.

Results: The molecule is 10× more potent than the pegylated IFN-α2a, with potency similar to the unmodified IFN-α2a. No unanticipated findings were observed in cynomolgus monkey when dosed up to 20 mg/kg, >10,000-fold margin over the anticipated efficacious human dose.

Conclusions: The biomarker and toxicological findings were consistent with a potent IFN molecule. The potency and pharmacokinetic properties of the molecule are consistent with dosing at least twice daily with the potential for monthly dosing.

Introduction

Interferons (IFNs) are a class of cytokines able to interfere with viral replication; as such they have been used for the treatment of certain viral infections such as hepatitis B and C, and cancer. Besides their proven efficacy as antiviral agents for the treatment of HCV, unmodified IFN preparations have a short circulating half-life (approximately 2–8 h in humans) and therefore its use requires frequent and high dosing to maintain sustained exposure, leading to variable plasma concentrations and suboptimum pharmacokinetic characteristics (high peak-to-trough ratios).

Efforts have been made to extend IFN half-life; pegylation has been the most successful modification, providing a prolonged elimination half-life (approximately 80 h) that allows reducing the administration regime to a single weekly injection. IFN-α2b was also fused to a carrier protein (albumin) to produce Albuferon (Novartis AG [Basel, Switzerland] and Human Genome Sciences [HGSI, Rockville, MD, USA]), a fusion protein that was expected to prolong the IFN half-life even further. The albumin fusion protein showed an in vitro antiviral potency roughly equivalent to pegylated...
IFN-α2a, but unfortunately, the clinical studies revealed a high incidence of serious pulmonary adverse events in the cohort receiving the highest dose (1,200 µg) tested. These findings resulted in a drug dose reduction (900 µg) that would only allow a market dose administration once every two weeks [1–4].

The type I IFN family is composed of over 13 functional polypeptides. Although the function of the different subtypes is not well understood, there is enough evidence showing that not all members of the family have the same function. Moreover, several studies have shown that they have different antiviral, anti-proliferative and apoptotic activities [5–9]. At the molecular level, all IFN-α subtypes bind to the same receptor, IFNAR, and therefore the different functionalities can be attributed to either a differential binding affinity to the IFN receptor subunits (IFNAR1 and IFNAR2) and/or to a differential downstream signalling [9–12]. All FDA approved IFNs so far belong to the IFN-α subtype (α2a, α2b) and a consensus IFN (interferon, interferon alphancon-1), a type-1 IFN linking the most common occurring amino acid sequences at each position of available natural IFN-α into one protein [13]. However, of all type 1 IFNs, IFN-α8 is the most potent antiviral subtype so far identified [14–18]. In this study, we present the preclinical results describing the safety and efficacy of a novel IFN-α molecule, PF-04849285, a recombinant fusion protein that consists of an Fc portion, IgG1 hinge, CH2 and CH3 domains from an IgG2 immunoglobulin, linked to the N-terminus of IFN-α8 via a flexible GS linker (Fc-GS-IFNα8) [19]. Two mutations have been introduced in the CH2 domain of the IgG2 portion to eliminate any residual effector functions [20]. The fusion protein, which forms a soluble dimer via the immunoglobulin domain, was designed to combine the high antiviral potency of the IFN-α8 subtype with the good pharmacokinetic properties of an Fc part of an IgG molecule, to obtain a prolonged serum half-life.

In vitro assays have shown that PF-04849285 has good antiviral properties with potency similar to IFN-α2a (Roferon-A) and a superior potency (more than 10×) than the pegylated IFN-α2a version (Pegasys). PF-04849285 was initially selected among various lead constructs because of its high antiviral potency in vitro and its pharmacokinetic properties in rats, where a half-life of 25 h was observed. Studies in macaques further indicated that our compound was superior to both the albumin-fused and the pegylated version of IFN. Overall, the preclinical efficacy, pharmacokinetic and safety data of PF-04849285 described herein are consistent with a therapeutic IFN that has the potential to be administered at a more convenient, once per month, dosing regimen, with an efficacy and safety profile that is at least comparable to the current standard of care, thus making it a candidate for a best-in-class IFN.

Methods

Materials

Commercial IFNs, IFN-α2a (Roferon-A; Hoffmann-La Roche, Nutley, NJ, USA) and pegylated IFN-α2a (Pegasys; Hoffmann-La Roche) were used as control throughout this study. Mouse anti-human IFN-α8 antibody was from PBL InterferonSources (Piscataway, NJ, USA), and goat anti-human sulfo-tagged antibody was from MSD (Gaithersburg, MD, USA). HEK293 were from Clontech (Mountain View, CA, USA). Huh7 cell lines harbouring subgenomic RNA from HCV genotype 1a, 1b and 2a were licensed from RebLikon (Baden-Württemberg, Germany), Apath LLC (St Louis, MO, USA) and Toray Inc. (Tokyo, Japan), respectively.

Fusion protein

PF-04849285 is an Fc fusion of IFN-α8 protein, where the IFN domain is fused to the C-terminus of an Fc via a (GGGGS)3 linker. Fc portion comprises a hinge sequence of IgG1 and CH2 and CH3 sequences of IgG2.

Expression and purification

HEK293 cells were transfected with the pcDNA3.1 (+) vector (Invitrogen, Paisley, UK) containing the desired sequence using Lipofectamine (Invitrogen). Transfected cells were grown for five days during which the protein was expressed at ~30 mg/l, as measured on an analytical protein A column. The purification steps were carried out using an AKTA system (GE Lifesciences, Little Chalfont, UK) as described elsewhere [19]. The final product was >95% pure and run as a dimer (MW=91690 Da) under non-reducing conditions on SDS-PAGE and size exclusion chromatography.

In vitro assays

Replicon cells

Huh7 cell line harbouring a replicative subgenomic RNA from HCV genotypes 1a, 1b, 2a (termed here Replicon) and encoding Firefly (genotype 1b) or Renilla (genotypes 1a and 2a) luciferase reporter gene and neomycin phosphotransferase gene were propagated in Dulbecco’s modified Eagles medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Cölbe, Germany), 1 mM sodium pyruvate, 1× non-essential amino acids, 2 µg/ml blasticidin (Invitrogen), 1× penicillin-streptomycin and G418 (Geneticin) at 500 µg/ml for replicon cells selection pressure. For
activity assays, replicon cells were trypsinized, washed and resuspended with PBS. Viability was determined using a Cedex Counter (Innovatis, Bielefeld, Germany).

Replicon activity determination
All compounds were diluted in DPBS containing 2% FBS to 100 ng/ml and typically a nine point 1:5 serial dilution was performed in a 96-well plate. After viability assessment, cells (1×10⁴) resuspended in G418 supplemented medium, were added to each well of a 96-well plate containing 10 μl of the serially diluted compounds. After 48 h incubation at 37°C and 5% CO₂, luciferase activity was determined using the Firefly Luciferase Bright-Glo Luciferase Assay System for the replicon harbouring HCV genotype 1b and the Renilla Luciferase Assay System from Promega (Promega, Southampton, UK) for replicon genotypes 1a and 2a. The luminescence signal was measured using an Analyst plate reader (Molecular Devices, ON, Canada). The results were expressed in relative luminescent units (RLU). The percentage of inhibition was calculated using 1,000 IU/well of Roferon-A as a positive control and DPBS containing 2% FBS as a negative control. Assays were carried out in duplicate.

Cytotoxicity
Replicon cell viability was assessed using the tetrazolium salt metabolism assay, WST1 (Roche Applied Sciences, Penzberg, Germany). Cells were plated in 96-well plates at 1×10⁴ cells/well and incubated for 24 h. Compounds were serially diluted (1:5) at starting concentrations of 600 ng/ml (10 nM) for Pegasys, and 400 ng/ml (8.7 nM) for PF-04849285 and added to the cells. Following 48 h incubation (37°C, 5% CO₂), the medium was removed and replaced with 100 μl of medium containing WST-1 (1:5 diluted). The plates were incubated 1 h and the absorbance (450 nm) was determined with an EnVision reader (Perkin Elmer, Cambridge, UK). The percentage of inhibition was calculated using 40 μM cyclohexamide solution as a positive control and DPBS containing 2% FBS as a negative control.

EMCV assay
The antiviral activity was determined using Huh7 cells challenged with the encephalomyocarditis virus (EMCV) ATCC VR-129B (Rockville, MA, USA). In total, 100 μl of a cell suspension containing 6×10⁵ cells/ml were seeded in a 96-well plate, and after 3–4 h incubation, the medium was removed and 100 μl of the compound solution was added. IFNs were prepared in DPBS containing 2% FBS to an initial concentration of 100 ng/ml (PF-04849285) and 800 ng/ml (Pegasys and Roferon-A). After incubation for 24 h, the EMCV stock was diluted in cell culture media and the cells were challenged with approximately 3×10⁴ PFU/well.
Viable cells were quantified after incubation for 22–24 h by adding 100 μl of CellTitre Glo Luminescent Cell Viability Assay (Promega) per well. Relative luminescent units measured were used to calculate the percentage of protection. The antiviral activity was expressed as 50% effective concentration (EC_{50}).

**Antiproliferative assay**

The antiproliferative activity was assessed using Daudi Burkitt’s lymphoblast cell line (ATCC, Manassas, VA, USA) and AlamarBlue (Invitrogen). Cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 20% FBS and 2 mM l-glutamine. Cells were seeded in a 96-well plate at 5×10^4 cell/well, and serial dilution of compounds was added. Cultures were incubated for 4 days, and cell viability was determined by adding 10 μl of AlamarBlue per 100 μl of media during the last 24 h of incubation. The fluorescence signal was determined using an EnVision plate reader (Perkin Elmer) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

**EC_{50} calculation**

The activity of compounds was calculated from the experimental data and expressed as EC_{50} (pM) using an statistical four-parameter sigmoid fitting model plugged-in MS Excel 2007 (Microsoft, Seattle, WA, USA).

**IFN receptor binding affinity**

The receptor binding affinity was determined as described in Lata and Piehler in 2005 [21] and Lata et al. in 2006 [22]. As a negative control, binding of 100 nM and 1 μM of each IFN to surfaces without the IFN receptors was measured. For probing the formation of the ternary complex, IFNAR1-H10 and IFNAR2-H10 were sequentially tethered onto a membrane containing 5% tris-NTA lipids, which was assembled in the flow cell by vesicle fusion on a clean transducer slide [21–24]. After injection of the ligand, all proteins were removed with 250 mM imidazole.

In silico immunogenicity predictions

*In silico* immunogenicity was predicted using IEDB MHCII binding prediction tool [25]. To analyse the immunogenicity of the region containing the linker, the following sequence was used: lhnhytqkslslspgkGGGGSGGGGGSGGSGGSGGSGGSLpqtqthslgnrralilla.

**Animal and ex vivo studies**

Pharmacokinetic and toxicology studies were performed in monkeys in compliance with US national legislation and subject to local ethical review. At all stages, consideration was given to experiment refinement, reduction in animal numbers and replacement with *in vitro* techniques. Blood from cynomolgus monkey was collected into lithium heparin using manual restraint. Human blood was collected from healthy volunteers with informed consent. All blood samples were collected following ethical guidelines.

**Ex vivo cytokine induction**

**Whole blood induction**

Test compounds were diluted to 20× final assay concentration in PBS containing 2% FBS. Aliquots (10 μl) were transferred to a 96-well plate. Heparinized blood from healthy donors was analysed for cytokine induction as follows: 190 μl were added to each compound-containing well. Assay plates were incubated (37°C, 5% CO₂) for 1, 2, 3 or 4 h, and centrifuged at 2,000 rpm for 5 min to separate plasma from the blood cells. The plasma layer was transferred to a clean replica plate and stored at -20°C. Monkey blood was treated as described above with incubation times of 2, 3 and 4 h.

**IP-10 and IL-6 immunoassay**

Mesoscale immunoassays (Meso Scale Discovery, Gaithersburg, MA, USA) were used for cytokine quantification. Frozen plasma samples were thawed to room temperature and diluted 1:2 with MSD human plasma cytokine assay diluent. A total of 25 μl of the diluted sample was added per well, and the assay performed following manufacturer’s instructions. Plates were read on an MSD Sector Imager 6000. The standard curve was fitted using a 5-parameter logistic model including a weighting function (1/Y²).

**Transcriptional profiling**

The High Capacity cDNA Reverse Transcription Kit was used for all the experiments. Multiplex qPCR reactions were performed using 2.5 μl cDNA using TaqMan Universal Master Mix and Assay On Demand (FAM, target specific) reagents following manufacturer’s instructions (Applied Biosystems, Carlsbad, CA, USA) reagents. Species-specific primers were used for the human and cynomolgus qPCR studies. Human samples OAS2, Hs00159719_m1 (FAM) with human ACTB (beta-Actin) endogenous control (4352935E, VIC/MGB Probe, Non-Primer Limited) were used. Cynomolgus samples OAS2 (HS00942650_m1) with 18S RNA endogenous control for eukaryotes (VIC), were used. All reagents were from Applied Biosystems (Carlsbad, CA, USA). Fold induction was calculated using the formula 2-ΔΔCt, where the ΔΔCT= mean ΔCT (concentration compound) - mean ΔCT (no compound) and the ΔCT is the difference in CT between the CT of the gene of interest and the CT of the housekeeping gene.

**Cynomolgus monkeys studies**

The studies described below were conducted in accordance with the current guidelines for animal
welfare (ILAR Guide for the Care and Use of Laboratory Animals, 1996; Animal Welfare Act, 1966, as amended in 1970, 1976 and 1985, 9 CFR Parts 1, 2 and 3). The procedures used in these studies have been reviewed and approved by the Institutional Animal Care and Use Committee.

Single-dose pharmacokinetics and toleration studies in cynomolgus monkeys
Monkeys were intravenously or subcutaneously dosed with vehicle or PF-04849285 at 0.01, 1.5 and 20 mg/kg, with dose volumes of 2 ml/kg. Each route and dose combination was given to a single male animal. Samples were collected pre-dose, immediately post-dose, and at 2, 4, 8, 24, 48, 72, 120, 168 and 336 h post-dose. Blood samples (2 ml) were collected into lithium heparin and processed into plasma, and analysed by ELISA for pharmacokinetics studies and for anti-drug antibody responses. Clinical signs were monitored: food intake, body temperature (daily), heart rate, electrocardiogram, and blood pressure measurements (pre- and post-dose), haematology, coagulation and clinical chemistry (pre-treatment and day 14 or 15), liver weight and macroscopic and microscopic observations (day 14 or 15).

Compound analysis in plasma samples
Mouse anti-human IFN-α8 antibody was from PBL InterferonSource. In total, 25 µl of 1 µg/ml in PBS containing 0.015% Triton x-100 was added to each well of an MSD plate and incubated in a shaker at 25°C for 2 h. After three washes with 250 µl PBS containing 0.05% Tween-40, human serum cytokine diluent (25 µl, MSD) was added and the plates incubated for 1 h at 25°C. Pharmacokinetic samples were diluted in cynomolgus monkey plasma. Duplicated samples and controls were added to the antibody-coated plate and incubated 20 h at 8°C. Following three washes, 25 µl goat anti-human sulfo-tagged antibody (0.5 µg/ml in MSD antibody diluent) was added to each well. Following 2 h incubation at 300 rpm, 25°C, the plate was washed before addition of MSD read buffer (150 µl). The plate was read using an MSD sector 6000 plate reader.

Anti-drug antibody analysis
Cynomolgus monkey plasma samples were diluted 1:10 prior to addition to an MSD plate pre-coated with PF-04849285. Plates were incubated 1 h at room temperature with shaking. Following further washes, a RU labelled PF-04849285 solution (25 µl of a 1 µg/ml solution in 3% BSA, 0.05% Tween-20 in PBS) was added to each well and the plates incubated 1 h at room temperature with shaking. After washing, MSD read buffer (150 µl of 2×) was added and the plate read on the MSD 6000 imager. A positive anti-drug antibody (ADA) was defined as a sample that exceeded the assay light unit cutoff by three standard deviations of forty treatment-naive monkey plasma samples. Mouse anti-hIFN-α8 monoclonal antibody (0.25 µg/ml) was used as positive control and buffer and three individual cynomolgus plasma samples, diluted 1:10 as negative controls. Selected samples positive for ADA were spiked with PF-04849285 (10 µg/ml); inhibition of signal greater than 30% was considered to discriminate specific ADA responses.

One month intravenous and subcutaneous dose range study in monkey with a three week recovery period
The compound was dosed to one male and one female animal at 20, 50 or 100 mg/kg subcutaneously and 20 mg/kg intravenously twice a week for 25 days prior to a three-week wash out period. Clinical signs (daily): body weights prior to dosing and weekly during the recovery phase, food intake (daily), body temperatures, haematology, coagulation, clinical chemistry (pre-treatment and day 51). Additional samples were collected on days 11, 25, and 36 for haematology and clinical chemistry parameters only; on day 18, a sample was collected for coagulation parameters only. Blood samples were collected pre-dose, 4 and 8 h post-dose for subcutaneous and pre-dose, immediately post-dose, and 4 h post-dose for intravenous on days 1, 4, 8, 11, 15, 18, 22, 25, 29, 36, 43 and 50 and analysed for toxicokinetics and ADA (pre-dose on days 1, 15, 18, 22, 25; single arm collection on days 29, 36, 43 and 50) analysis as described above. Blood was used for biomarkers determination (OAS2, IP-10 and IL-6). Samples for biomarker were collected once weekly during the recovery phase for all animals. Animals were euthanized on day 52 and a limited set of tissues were collected and examined grossly and microscopically.

Results
In vitro studies
The in vitro antiviral activity was tested using the HCV replicon system, a standard surrogate assay for HCV infection, and the EMCV assays, a general antiviral assay that determines the degree of cytopathic protection obtained when Huh7 cells are challenged with the virus [26,27]. Table 1 shows the antiviral potency of PF-04849285 in HCV replicon cell lines compared to Pegasis and Roferon-A. The results indicate that the IFN-α8 fusion protein, PF-04849285, is 13.4-fold more potent than Pegasis on a molar basis (PF-04849285 EC_{50}=0.05 pM; Pegasis EC_{50}=0.67 pM), and approximately twofold higher than Roferon–A (EC_{50}=0.09 pM). However, a comparison of the antiviral potency using HCV replicon from genotypes 1a and 2a indicates that PF-04849285 is more potent than Pegasis, but less potent than Roferon-A on a molar basis (Table 1). Note that due to the differences in molecular weight among the different IFNs compared here, the potency is
Table 1. Antiviral and antiproliferative activity of interferon

<table>
<thead>
<tr>
<th>Compound (MW)</th>
<th>Rep 1a</th>
<th>Rep 2a</th>
<th>Rep 1b</th>
<th>EMCV</th>
<th>Antiproliferative*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roferon-A (19241)</td>
<td>0.69 (0.37, 1.29)</td>
<td>0.61 (0.37, 1.00)</td>
<td>0.09 (0.06, 0.15)</td>
<td>8.68 (5.03, 14.98)</td>
<td>1.2 (0.43, 3.2)</td>
</tr>
<tr>
<td>Pegasys (60000)</td>
<td>8.13 (4.38, 15.12)</td>
<td>8.53 (5.23, 13.91)</td>
<td>0.67 (0.42, 1.07)</td>
<td>123.13 (71.1, 213.15)</td>
<td>98.71 (36.94, 263.8)</td>
</tr>
<tr>
<td>PF-04849285 (181690)</td>
<td>1.32 (0.67, 2.45)</td>
<td>1.11 (0.68, 1.82)</td>
<td>0.05 (0.03, 0.08)</td>
<td>10.00 (5.80, 17.27)</td>
<td>3.65 (1.33, 10.0)</td>
</tr>
</tbody>
</table>

The 50% effective concentration (EC₅₀) is expressed in molar basis (pM), as geometric mean (95% CI) of six independent experiments. *Antiproliferative activity measured using Daudi-Burkitt cells. EMCV, encephalomyocarditis virus; MW, molecular weight in Da; Rep, Huh7 cells harbouring a subgenomic replicative HCV RNA from HCV genotypes 1a, 1b and 2a.

Table 2. IFN binding parameters to IFN receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>IFNR1</th>
<th>IFNAR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α2</td>
<td>kₐ 1/M.s</td>
<td>kₐ 1/s</td>
</tr>
<tr>
<td>Pegasys</td>
<td>3×10⁴</td>
<td>0.015</td>
</tr>
<tr>
<td>PF-04849285</td>
<td>3×10⁴</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Receptor-binding studies of interferon (IFN) compounds were done using plasmon resonance. All rate constants were measured by heterogeneous phase detection. The standard error for the parameters is: association constant (kₐ; M⁻¹.s⁻¹) 15%, dissociation rate constant (k_d; s⁻¹) 25%, binding affinity constant (K_D; nM) 30%. *Determined as K_D=kₐ/k_d. Recombinant IFN-α2 protein was expressed in E. coli.

expressed on a molar basis with the molecular weight for PF-04849285 corresponding to the stable dimeric form (91690 Da). The relative potencies differ if the EC₅₀ are compared in mass concentration (that is, ng/l): in that case for replicon genotype 1b, PF-04849285 is 8.7-fold more potent than Pegasys, but approximately 2.6-fold less potent than Roferon-A (data not shown).

The ability of PF-04849285 to inhibit the cytopathic effect of the EMCV infection in Huh7 cells is also shown in Table 1. The potency of PF-04849285 is 12-fold higher than Pegasys (EC₅₀=123.1 pM) but similar to that of Roferon-A (EC₅₀=8.7 pM). It is worth pointing out that the antiviral activity of PF-04849285 relative to Pegasys and Roferon, determined in replicon genotype 1b, correlates well with the one obtained using the EMCV assay. However, IFN-α2 appears to be slightly more potent when measured in this assay. No toxic effects were detected for any of the molecules up to 10 nM when assay in Huh7 cells using the WST1 viability assay (data not shown).

As IFNs are known to inhibit proliferation, we evaluated the anti-proliferative activity of the molecule using Daudi Burkitt’s lymphoma cell line, a cell line that is highly sensitive to IFN [28]. The results are shown in Table 1; it can be seen that PF-04849285 anti-proliferative activity (EC₅₀=3.65 pM) is lower than that displayed by IFN-α2a (EC₅₀=1.2 pM). PF-04849285 has a higher anti-proliferative activity, with an EC₅₀ 27-fold lower than Pegasys.

As mentioned above, IFN-α and -β signalling occurs through binding to the ubiquitously expressed IFN-receptor, which consists of two subunits, IFNR1 and IFNR2 [29]. To understand the differences observed in antiviral and proliferation potency for the different compounds, we determined the binding affinity of PF-04849285, Pegasys and recombinant IFN-α2a to both IFNR subunits. Recombinant IFN-α2a expressed in E. coli instead of Roferon-A was used in those experiments. The results showed that PF-04849285 has a relatively strong binding affinity to IFNAR2, with a K_D value lower than both Pegasys (5-fold), and the recombinant IFN-α2a (2.5-fold). In addition, all compounds showed a similar association constant (kₐ) to IFNAR2, and particularly for PF-04849285 it should be noticed that kₐ is lower than that reported for the unmodified IFN-α8 (kₐ=1.33×10⁷ 1/M.s) [18]. PF-04849285 showed a slow dissociation rate from IFNAR2 with a k_d=0.005 1/s, 10-fold lower than the one reported for the unmodified version of INF-α8 [18]. Previous reports have indicated that IFNs show a weaker binding to IFNAR1. Our findings also reflect this behaviour, with the strongest binding affinity for PF-04849285 (Table 2). Interestingly, the binding affinity of PF-04849285 towards IFNAR1 is much higher than that reported for unmodified IFN-α8 and is comparable to the values reported for unmodified IFN-α8 mutants [18] and IFN-β [30]. Strong binding to IFNAR1 has been associated with the anti-proliferative activity of IFNs, and in the case of PF-04849385, the antiproliferative activity is similar to that determined for Roferon-A using Daudi Burkitt cells [24].

It has also been suggested that the binding affinity to the ternary complex, rather than the specific binding affinity to the specific receptor subunits, dictates the activity of the different IFN subtypes [24]. However,
IFN-α8 fusion protein for the treatment of HCV

Antiviral Therapy 17.5

due to the strong non-specific binding of PF-0489285
and the strong binding affinity that this compound
presents towards IFNR2 alone, the analysis of the ternary
complex binding was not possible.

Ex vivo studies
To further assess the efficacy of the molecule we also
performed experiments to determine its capability to
induce IFN-inducible genes in human and cynomologus
monkey whole blood. Human and cynomologus
blood was challenged with PF-04849285 and Pegasys;
the capability of the two molecules to induce relevant
biomarkers such as IP-10, IL-6 and OAS2 that are
indicative of an antiviral IFN response was evaluated [31]. Preliminary kinetics studies indicated 4 h
as an appropriate interval for the end point experiments
to determine biomarkers induction (Figure 2). The
induction levels in human and cynomologus blood
for OAS2 and IP-10 are shown in Table 3. The EC50
values represent the mean results of 9 (human) and 6
(monkey) independent experiments, using blood from
five different donors.

Table 3. OAS2 and IP-10 induction following stimulation of human and cynomologus monkey blood with PF-04849285
and Pegasys

<table>
<thead>
<tr>
<th>Species and compound</th>
<th>IP-10</th>
<th>OAS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pegasys</td>
<td>1,650 ±367</td>
<td>4,327 ±1,259</td>
</tr>
<tr>
<td>PF-04849285</td>
<td>462 ±131</td>
<td>645 ±253</td>
</tr>
<tr>
<td>Cynomologus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pegasys</td>
<td>61,700 ±39,900</td>
<td>18,642 ±13,732</td>
</tr>
<tr>
<td>PF-04849285</td>
<td>379 ±136</td>
<td>643 ±303</td>
</tr>
</tbody>
</table>

Values expressed as concentration required to induce 50% of cytokine levels, expressed in pg/ml as geometric mean of nine (human) and six (monkey) independent experiments. IP-10, interferon inducible protein 10; OAS2, 2′-5′-oligoadenylate synthetase.

The data shows that PF-04849285 in human whole blood is more potent than Pegasys, with approximately sevenfold higher potency for OAS2
induction and fourfold for IP-10 induction. Interestingly, Pegasys appears from these studies to be less potent in cynomologus monkey blood than in human
blood for both IP-10 and OAS2, while the potency of PF-04849285 is conserved across the species. The resulting data from these experiments has shown that
donor-to-donor variation is observed in response to

Figure 2. Time course of IP-10 and OAS2 induction during ex vivo cynomolagus monkey whole blood stimulation experiments

Interferon inducible protein 10 (IP-10; upper panels) and 2′-5′-oligoadenylate synthetase (OAS2; bottom panels) stimulation by (A) Pegasys and (B) PF-04849285 at 2 h (represented by a diamond); 3 h (represented by a circle); 4 h (represented by a triangle). Values are average of three independent experiments. Error bars represent the standard error of the geometric mean.
PF-04849285 and Pegasys, as demonstrated by the wide CI. However, good concordance between data sets from the same individual assayed on different days was seen, suggesting that any observed donor-to-donor variation is due to natural variation in the response.

The relative OAS2 induction of PF-04849285 over Pegasys in human ex vivo blood experiments correlates well with the in vitro antiviral potency of the compound.

IL-6 induction, in both human and cynomolgus monkey blood challenged with IFN is similar to that of IP-10, which would suggest that induction of this proinflammatory cytokine is linked to the IFN mechanism (data not shown). However, it should be mentioned that the levels of IL-6 release are low and variable, with the values for Pegasys not being reliably determined in human blood assay.

Single-dose pharmacokinetics and toleration studies in cynomolgus monkeys
PF-04849285 showed a redistribution phase over the first few hours when delivered intravenously, followed by a linear elimination phase through to 168 h (Figure 3). Only the animals receiving the highest dose (20 mg/kg) had detectable plasma levels after 14 days (336 h). The accelerated clearance (1.2–1.8 ml/h/kg) and the lack of detectable drug at the lower doses were caused by the induction of ADA as demonstrated herein and previously for other IFN preparations [32,33]. During the elimination phase the terminal half-life ranges from 21 h for the lowest dose (0.01 mg/kg), where the sensitivity of the assay may have limited the collection of sufficient time points to describe the true terminal half-life, to a range of 54–94 h for the higher doses prior to the onset of ADA (Table 4). The reported half-life for Pegasys is 26 h when dosed intravenously [34].
Table 4. Pharmacokinetic parameter values following single-dose intravenous and subcutaneous administration of PF-04849285 in cynomolgus monkeys

<table>
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<tr>
<th>Route and dose, mg/kg</th>
<th>Half-life, h</th>
<th>V, ml/kg</th>
<th>Cmax, ng/ml</th>
<th>Tmax, h</th>
<th>CL, ml/min/kg</th>
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Parameters were calculated from the profiles using a PKIN add-in to Excel (Pfizer Inc., New York, NY, USA). *Insufficient data to determine accurate clearance when PF-04849285 was delivered at doses of 1 and 0.01 mg/kg. AUC, area under the curve; CL, clearance; Cmax, peak plasma concentration; F, fractional bioavailability; IV, intravenous administration; ND, not determined; SC, subcutaneous administration; Tmax, time to peak concentration; V, volume of distribution.

When delivered subcutaneously, PF-04849285 reached maximal concentration after 8 to 48 h with a linear clearance phase through to 168 h. Similarly, as for the intravenous dosing, subcutaneously delivered PF-04849285 was undetectable after 14 days (336 h) due to an accelerated clearance by ADA. During the elimination phase the terminal half-life ranged from 50 to 130 h. The values at the lower doses are 30% longer than the ones reported for Albuferon for a similar dose (0.03–0.3 mg/kg) and 5× longer than the 30–34 h for subcutaneous administration reported for Peg-Intron (Schering–Plough). The bioavailability of PF-04849285 ranged from 17–37% and was likely to have been underestimated due to the ADA response.

The induction of ADAs was investigated in plasma samples of all animals, except all receiving the highest dose, at 336 h. None of the animals had anti-IFN antibodies prior to receiving PF-04849285 (data not shown). All subjects tested positive for ADA during the study. Since drug tolerance could not be determined, a negative ADA response does not mean the absence of ADA. Measurements of ADA are shown (Figure 3).

The clinical signs assessed, such as minimal drop in food consumption without any weight loss, were nonspecific. Decreases in heart rate (QTc) were noted at 5 and 20 mg/kg subcutaneously, and a slight increase in body temperature was noted in all dose groups, but the relation to treatment is uncertain. Changes in blood pressure were sporadic and not considered to be related to the compounds. Most of the haematologic and clinical parameters (transient reduction in reticulocytes, total leukocyte numbers, neutrophil and lymphocyte numbers) were altered from baseline on day 8 with partial or complete resolution by the end of the study (day 14/15). Furthermore, the severity of changes noted in most of these clinical pathology parameters did not follow a clear dose–response relationship. No animals died during this study. The results from the single-dose study aided the selection of doses for the one month multiple dose study.

One month study with a 3-week recovery phase
Three cohorts of monkeys, one male and one female per cohort, received 20, 50 or 100 mg/kg PF-04849285 subcutaneously twice weekly during 25 days. The concentration of compound and biomarkers induction in plasma was measured and the results are presented in Figure 4. Overall, none of the doses showed significant accumulation of PF-04849285 in plasma. From day 14, all animals had reduced exposure consistent with the induction of ADA, as seen in the single dose study, with an onset between day 14 and day 25. At 50 and 100 mg/kg, ADA response could not be confirmed due to residual drug levels. After three weeks recovery period the compound was completely cleared from all surviving animals. Induction of IFN-inducible genes (IP-10 and OAS2) was similar for all doses, and biomarker levels maintained during the repeating dose period (Figure 4A, 4B and 4C) consistent with a similar level of compound exposure for all doses. Compound-related mortality was observed: one female at 20 mg/kg (intravenous) died on day 25 and other animals at ≥50 mg/kg were euthanized between days 20 and 44. Significant clinical signs that preceded death were observed. A complete description is provided in Additional file 1.

Discussion
We present here a preclinical study with a modified IFN-α8 fusion protein, PF-04849285, for the treatment of HCV, designed to combine the high antiviral activity from IFN-α8 subtype with a prolonged...
serum half-life driven by the Fc fusion part. Throughout this study the results obtained with the fusion protein were compared with the current standard of care, pegylated IFN-α2a (Pegasys) and the unmodified IFN-α2a subtype (Roferon-A). PF-04849285 was initially selected among different fusion proteins based on its enhanced in vitro activity and its pharmacokinetic properties in rats.

PF-04849285 is >10× more potent than Pegasys and than the albumin fusion IFN-α2b protein Albuferon [33,35]. Results measured in a similar EMCV antiviral assay, indicated that the latter fusion protein is 20× less potent than IFN-α2a. Consistent with the cell-based potency, PF-04849285 shows a strong binding affinity towards the two IFN receptors determined by surface plasmon resonance, with an affinity for IFNR2 (Kd) of

Figure 4. PF-04849285 plasma concentration levels and biomarkers induction during twice-weekly subcutaneous administration of PF-04849285 into cynomolgus monkeys.

Pharmacokinetics and biomarkers induction of PF-04849285 dosed in cynomolgus subcutaneously at (A) 20 mg/kg, (B) 50 mg/kg and (C) 100 mg/kg. Symbols indicate plasma fold change for interferon inducible protein 10 (IP-10; represented by a triangle) and 2′-5′-oligoadenylate synthetase (OAS2; represented by a square), and plasma concentration for PF-04849285 (ng/ml; represented by a circle). The data represent the median from one male and one female per cohort, error calculated as one standard deviation. Biomarkers data have been normalized to the pre-dose read out. Anti-drug antibody (ADA) was only measurable in the 20 mg/kg cohort. Bar plot indicates ADA response in arbitrary light units.
2 nM, 5× higher than the value found for Pegasys. On the whole, the binding experiments indicate that there is a good correlation between the antiviral activity of the compound (replicon and EMCV) and the binding affinity to IFNR2, and that the strong antiviral potency may be driven by the strong binding affinity against the IFNR2 subunit.

The *ex vivo* studies in human and cynomolgus monkey blood have demonstrated that IP-10 and OAS2 are valuable pharmacological biomarkers for PF-04849285. Moreover, the relative induction of IP-10 and OAS2 for Pegasys and PF-04849285 correlates well with the antiviral activity seen in the replicon genotype 1b and in the EMCV assay. Therefore, this data set enables the dose prediction for the *in vivo* studies and can be used for future clinical studies as a guide to define the exposure required to obtain a sustained virological response in HCV-infected patients.

The *in vitro* receptor binding and enzyme profile done by CEREP (study number 75760144) reveals a similar profile for both the control compound, Pegasys and PF-04849285 (data not shown).

PF-04849285 was tested in cynomolgus monkeys in a 2-week single-dose study and in a 4-week multiple-dose study with a recovery phase. No unanticipated findings were observed up to 20 mg/kg (subcutaneously) in a single dose study, which represents >10,000-fold margin over anticipated efficacious human dose (92–100 μg/month). Pharmacological and toxicological findings were consistent with a potent IFN molecule.

In the one month study the 20 mg/kg subcutaneous dose was reasonably well tolerated, as deaths occurred at 20 mg/kg (intravenously) and in all higher dose levels. Alterations in immune responses and corneal pigmentation and degeneration were significant adverse findings at subcutaneous doses >50 mg/kg or intravenous doses of 20 mg/kg. It is worth mentioning that the presence of anti-drug antibodies in non-human primate studies that can neutralize the molecule and its activity could of course have an effect on the low toxicity findings. In previous IFN studies, findings in animal did not clearly reflect the subsequently observed adverse effects in clinical trials. Therefore, extrapolation of the safety finding in animal for IFNs have to be taken with caution, as the low frequency in adverse effects found preclinically do not correlate with the clinical findings [32].

It is known that IFNs are immunogenic, but the response seen in monkeys is not predictive of the immunogenicity in human. In order to mitigate immunogenicity, in this work, human sequences and a well-characterised linker sequence (G4S) were used, which was shown previously not to induce an antibody response [36]. However, the junctions where the fusion partners meet are technically novel sequences and, as such, subject to the potential for an immune response. *In silico* epitope predictions using algorithms aimed at uncovering T-cell epitope binding have not identified any extra sequence that may suggest binding to the class II MHC (data not shown). However, the current state of knowledge of preclinical immunogenicity prediction is such that the risk cannot be eliminated. The Fc part of PF-04849285 is based on an IgG2 molecule and has been engineered to remove any sequences associated with ADCC or Complement Activation [20]. In *in vitro* tests to predict cytokine release, PF-04849285 showed the induction of clinically acceptable levels of IL-6 and no significant cytokine release in a solid phase cytokine release assay [37].

The pharmacokinetics of this molecule in monkey is consistent with dosing at least bi-weekly, and due to its high antiviral potency and low adverse safety found in monkeys, it has a potential for a monthly regimen, as predicted by a pharmacokinetic/pharmacodynamic model based on clinical data from Pegasys (data not shown).

It is unclear yet whether IFN will remain part of the standard of care therapy, as specific targeted antiviral therapies for Hepatitis C (STAT-C) are in development, and two small molecule inhibitors have been recently approved. Promising results have been obtained when combining these molecules with pegylated IFN plus ribavirin [38]. However, resistance may represent a major burden for STAT-C therapy, making it necessary to develop combination treatments composed of multiple STAT-Cs with non-overlapping resistance profiles. In contrast, it still remains to be evaluated whether sustained virological response can be achieved by an IFN-free regime. Therefore, it may be envisaged that IFN will remain part of the standard of care therapy for HCV until new regimens and/or therapies, such as a combination of STAT-Cs, are developed. In that scenario, the fusion protein described herein could lead to an improved dose regimen for HCV and other IFN-based therapies.

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Disclosure statement
This work was performed at Pfizer Laboratories, UK. All authors were Pfizer employees at the time that this work was carried out.

Additional file

Additional file 1: Details of a one month study with interferon alpha-2b dosed every two or four weeks in interferon-naive patients with genotype 1 chronic hepatitis C. Hepatology 2008; 48:407–417.

References


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