An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus

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Hepatitis C virus (HCV) infection is a serious cause of chronic liver disease worldwide with more than 170 million infected individuals at risk of developing significant morbidity and mortality1-3. Current interferon-based therapies are suboptimal especially in patients infected with HCV genotype 1, and they are poorly tolerated, highlighting the unmet medical need for new therapeutics4,5. The HCV-encoded NS3 protease is essential for viral replication6,7 and has long been considered an attractive target for therapeutic intervention in HCV-infected patients. Here we identify a class of specific and potent NS3 protease inhibitors and report the evaluation of BILN 2061, a small molecule inhibitor biologically available through oral ingestion and the first of its class in human trials. Administration of BILN 2061 to patients infected with HCV genotype 1 for 2 days resulted in an impressive reduction of HCV RNA plasma levels, and established proof-of-concept in humans for an HCV NS3 protease inhibitor. Our results further illustrate the potential of the viral-enzyme-targeted drug discovery approach for the development of new HCV therapeutics.

In spite of intensive efforts, many aspects of the disease and the biology of HCV remain unclear, making drug discovery an extremely challenging endeavour. Robust and practical cell culture systems that support viral replication are still lacking, necessitating the use of surrogate cellular systems such as the subgenomic HCV replicon cell model8 in support of drug discovery. The HCV-infected chimpanzee, an impractical model for routine studies of HCV biology, was until recently9 the only animal model supporting HCV replication. Target-based antiviral drug discovery—an approach exemplified by the human immunodeficiency virus (HIV) drug discovery model, which mainly relies on the use of in vitro assays—has led recently to the identification of several anti-HCV compounds awaiting clinical validation through tangible therapeutic benefit in HCV-infected patients.

A substrate-based approach to the design of active site inhibitors of viral proteases generally benefits from a genetic conservation of the substrate-binding site and gains from available structural information defining the topographies of the complementary surfaces between the substrate or inhibitor and the protease. Substrate recognition by the NS3 protease is characterized by preference for a cysteine residue at P1 (nomenclature as in ref. 11) but requires extended interactions between enzyme and substrate distributed over the minimal substrate peptide P6 to P4. This requirement is dictated by the shallow, solvent-exposed substrate-binding cleft of the enzyme. The NS3 protease therefore represented a formidable challenge for the design of small molecule inhibitors. Amino-terminal products derived from cleavage of peptide substrates are competitive inhibitors12,13 and the weak hexapeptide inhibitor Asp-Asp-Ile-Val-Pro-Cys (K1, of 79 μM) served as the starting lead molecule for design efforts. Our initial lead optimization capitalized on the carboxy-terminal carboxylic functionality. This carboxylic acid confers increased potency against NS3 protease and selectivity with respect to a large panel of proteases14. Knowing the important contribution of the P1 residue in substrate and inhibitor binding to the enzyme, a large effort was devoted to the replacement of the reactive cysteine side chain. Evaluation of different alkyl and cycloalkyl side chains15 resulted in the identification of the (1R,2S)-1-amino-2-vinylcyclopropyl carboxylic acid as a suitable and chemically stable cysteine replacement (J.R. et al., unpublished observation). Exploration of the P2 position identified key substituted proline derivatives16,17 that, in combination with optimal groups at P3 to P6, led to very specific and potent hexapeptidyl inhibitors with activity in the subgenomic HCV replicon model18. After an iterative and extensive structure–activity-relationship approach and guidance from NMR- and X-ray-derived structural information19-21,23, tripeptide mimetics were identified that maintained potent and specific activity against the NS3 protease22. Rigidification of their scaffold by intramolecular linking of the P1 side chain to the P3 side chain produced novel macrocyclic inhibitors with desirable drug-like properties24 that exhibited improved NS3 protease inhibition in cells. Subsequently, we undertook an intensive structure–activity-relationship campaign leading to a subset of compounds that achieved the targeted
BILN 2061 displayed potent and competitive inhibition of the NS3 proteases of HCV genotypes 1a and 1b with a mean $K_i$ of 0.30 nM and 0.66 nM, respectively (Table 1). The inhibition of NS3 protease of HCV genotype 1b by BILN 2061 was reversible as demonstrated by the increase in steady-state velocity after dilution of a preformed inhibitor–enzyme complex into buffer-containing substrate (Supplementary Information 1). The inhibition of BILN 2061 was highly specific to the NS3 protease as demonstrated by the lack of significant activity (half-maximal inhibitory concentration ($IC_{50}$) > 30 $\mu$M) against human leukocyte elastase and human liver cathepsin B, representatives of serine and cysteine proteases respectively. The ability of BILN 2061 to inhibit NS3 protease activity in human liver cells was evaluated using the subgenomic HCV replicon cell model (Table 1). Treatment of replicon-containing cells with BILN 2061 for 3 days resulted in a dose-dependent decrease of HCV RNA of two orders of magnitude with a mean 50% effective concentration ($EC_{50}$) of 0.30 nM and 3 nM for the HCV replicon 1a and 1b, respectively. The addition of 50% human serum to the culture medium resulted in less than a tenfold increase in $EC_{50}$. A mean 50% cytotoxicity concentration ($CC_{50}$) of 33 $\mu$M was observed for BILN 2061, resulting in an apparent selectivity index of 10,000 in Huh-7 cells when compared with the $EC_{50}$ value obtained for inhibition of subgenomic HCV RNA replication. The mechanism of inhibition of BILN 2061 was further confirmed in replicon-containing Huh-7 cells by its ability to block NS3-mediated polyprotein processing (Fig. 2). Treatment with 0.0002–3.6 $\mu$M BILN 2061 showed a dose–response inhibition of cis-cleavage occurring at the NS3–NS4A junction associated with a reduction of the mature NS3 protein and an increase in the NS3-NS5B precursor. The accumulation of NS3-NS5B precursor in the presence of BILN 2061 suggested that polyprotein processing is inhibited at all NS3-dependent cleavage sites.

In a randomized, double-blind, single-dose escalation study with placebo controls BILN 2061 was investigated in patients infected with HCV genotype 1 in a two-day, twice-daily treatment. The plasma HCV RNA virus load was measured up to 11 days after administration in patients treated orally with 200 mg BILN 2061 or placebo (Fig. 4). BILN 2061 was highly effective, inducing a rapid decline in virus load in all treated patients (geometric mean), and reaching in some patients (Pt1 and Pt2) undetectable levels within 24–28 h after administration. This substantial and impressive effect corresponds to a 2–3 log$_{10}$ or greater reduction in virus load for all patients treated with BILN 2061. The virus load was undetectable in most of the patients at 48 h after initiation, although it was positive at the detection limit of 50 HCV RNA copies ml$^{-1}$ using a qualitative transcription-mediated assay (Bayer). Finally the virus load decline was followed by a virus rebound in all patients that returned to pre-treatment levels within 6–13 days after initiation of BILN 2061 treatment. No significant reduction in virus load was observed in plasma samples of placebo-treated patients. Similar substantial virus load declines were observed in BILN 2061-treated patients that were either naive to the treatment or previously treated caused by the large drug amount. No serious clinical or laboratory findings were identified. This included the absence of any change in liver function tests at all doses. The complete safety data set of this clinical study has been reported and more details will be published elsewhere (H.N., G.S. and C.-L.Y., manuscript in preparation). Pharmacokinetics of BILN 2061 in humans after oral administration demonstrated an initial rise followed by a biphasic decline in plasma concentrations (Fig. 3). The $C_{\text{max}}$ (maximum concentration in plasma) occurred mostly within 2–4 h after administration and the mean elimination half-life was around 4 h for all dose groups. $C_{\text{max}}$ and AUC$_{0-\infty}$ (area under the plasma concentration–time curve) appear to be dose-proportional up to 1,200 mg. From data extrapolation of the 200 mg dose group, a steady-state concentration of 42 nM could be predicted as the trough plasma concentration ($C_{\text{min}} = 12$ nM) in a twice-daily chronic dosing regimen. The predicted $C_{\text{min}}$ corresponds to 14-fold the cellular efficacy of BILN 2061 ($EC_{50}$ of 3 nM). The favourable oral pharmacokinetic profile of BILN 2061 in humans and the lack of relevant adverse events in this study further support the evaluation of BILN 2061 in HCV-infected patients.

Table 1 Biological profile of BILN 2061

<table>
<thead>
<tr>
<th>Assay type/secondary activity</th>
<th>Inhibition values</th>
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<tr>
<td>NS3-NS4A protease assays</td>
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<tr>
<td>HCV 1b enzyme</td>
<td>$K_i = 0.66$ nM</td>
</tr>
<tr>
<td>HCV 1a enzyme</td>
<td>$K_i = 0.30$ nM</td>
</tr>
<tr>
<td>Mechanism</td>
<td>Competitive and reversible</td>
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<td>Surrogate cell-based assays</td>
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<tr>
<td>HCV replicon 1b</td>
<td>$EC_{50} = 3$ nM</td>
</tr>
<tr>
<td>HCV replicon 1a</td>
<td>$EC_{50} = 4$ nM</td>
</tr>
<tr>
<td>Serum shift assay (50% human serum)</td>
<td>&lt;Tenfold increase in $EC_{50}$</td>
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<tr>
<td>Cytotoxicity MTT assay</td>
<td>$CC_{50} = 33$ $\mu$M</td>
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<tr>
<td>Secondary activity</td>
<td></td>
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<tr>
<td>Human leukocyte elastase</td>
<td>$IC_{50} &gt; 30$ $\mu$M</td>
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<tr>
<td>Cathepsin B</td>
<td>$IC_{50} &gt; 30$ $\mu$M</td>
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In a randomized, double-blind, proof-of-concept study with placebo controls BILN 2061 was investigated in patients infected with HCV genotype 1 in a two-day, twice-daily treatment. The plasma HCV RNA virus load was measured up to 11 days after administration in patients treated orally with 200 mg BILN 2061 or placebo (Fig. 4). BILN 2061 was highly effective, inducing a rapid decline in virus load in all treated patients (geometric mean), and reaching in some patients (Pt1 and Pt2) undetectable levels within 24–28 h after administration. This substantial and impressive effect corresponds to a 2–3 log$_{10}$ or greater reduction in virus load for all patients treated with BILN 2061. The virus load was undetectable in most of the patients at 48 h after initiation, although it was positive at the detection limit of 50 HCV RNA copies ml$^{-1}$ using a qualitative transcription-mediated assay (Bayer). Finally the virus load decline was followed by a virus rebound in all patients that returned to pre-treatment levels within 6–13 days after initiation of BILN 2061 treatment. No significant reduction in virus load was observed in plasma samples of placebo-treated patients. Similar substantial virus load declines were observed in BILN 2061-treated patients that were either naive to the treatment or previously treated caused by the large drug amount. No serious clinical or laboratory findings were identified. This included the absence of any change in liver function tests at all doses. The complete safety data set of this clinical study has been reported and more details will be published elsewhere (H.N., G.S. and C.-L.Y., manuscript in preparation). Pharmacokinetics of BILN 2061 in humans after oral administration demonstrated an initial rise followed by a biphasic decline in plasma concentrations (Fig. 3). The $C_{\text{max}}$ (maximum concentration in plasma) occurred mostly within 2–4 h after administration and the mean elimination half-life was around 4 h for all dose groups. $C_{\text{max}}$ and AUC$_{0-\infty}$ (area under the plasma concentration–time curve) appear to be dose-proportional up to 1,200 mg. From data extrapolation of the 200 mg dose group, a steady-state concentration of 42 nM could be predicted as the trough plasma concentration ($C_{\text{min}} = 12$ nM) in a twice-daily chronic dosing regimen. The predicted $C_{\text{min}}$ corresponds to 14-fold the cellular efficacy of BILN 2061 ($EC_{50}$ of 3 nM). The favourable oral pharmacokinetic profile of BILN 2061 in humans and the lack of relevant adverse events in this study further support the evaluation of BILN 2061 in HCV-infected patients.

Figure 2 BILN 2061 inhibition of HCV polyprotein processing. Inhibition of NS3-protease-mediated polyprotein processing in cells containing an HCV 1b subgenomic NS2-NS5B replicon. To detect HCV non-structural protein precursors and NS3 mature protein, replicon-containing cells treated with increasing amounts of BILN 2061 were pulse-labelled for 2 h with $^{33}$S-labelled methionine/cysteine. After incubation, cell extracts were immunoprecipitated with a specific anti-NS3 protein antibody and products were analysed by SDS–PAGE followed by phosphor imaging as previously described. The position of the NS2-NS5B precursor and the NS3 mature protein are indicated.
with interferon (IFN), as well as those with either minimal or advanced liver disease. A detailed description of the antiviral effect, safety and tolerability of BILN 2061 will be reported elsewhere.

The efficacy of BILN 2061 in humans establishes proof-of-concept for an NS3 protease inhibitor and a new class of a selective anti-HCV agent that was specifically designed to inhibit an essential viral enzyme. The purpose of this short trial in humans was to assess the in vivo antiviral efficacy of BILN 2061, which was selected only on the basis of its in vitro potency in surrogate enzymatic and cell culture assays and its oral pharmacokinetic profile in animals. The pharmacodynamic effect of BILN 2061 was assessed with an experimental treatment of HCV-infected humans following a trial design in which a significant virus load reduction was expected based on current IFN-based therapies. In patients treated with BILN 2061 the extent of viral decline is significantly greater than that observed for IFN-treated patients and can be explained by a greater effectiveness of BILN 2061 in specifically blocking the production of HCV virions. Hence, the exceptional efficacy of BILN 2061 (200 mg dose) was evident by the suppression of virus load below the detection level (1,500 RNA copies ml⁻¹) after only one day of treatment. In IFN-treated patients, the kinetics in patients that responded to the treatment is at best characterized by an initial virus load decline of 0.5–2 log₁₀ within 48 h, and required a 2–4-week treatment with either standard or pegylated IFN to attain a 3 log₁₀ decline in virus load. It has been reported recently that HCV protease inhibition may lead to a restoration of the cellular antiviral response mediated by IFN regulatory factor 3 (ref. 29) in addition to

Figure 3 BILN 2061 concentration in human plasma after single-dose oral administration. BILN 2061 was exposed to healthy volunteers in a randomized, double-blind, escalating single-dose study with placebo controls. The pharmacokinetic profiles of the compound in six active subjects were determined at the various dose levels tested. Geometric mean plasma BILN 2061 concentration versus time profiles are depicted for the various dose levels.

Figure 4 Antiviral efficacy of BILN 2061 in HCV-infected patients. Plasma virus load of individual patients treated with BILN 2061 (BILN 2061 Pt1, BILN 2061 Pt2), placebo (placebo) and geometric mean (BILN 2061 Mn) are shown, with standard deviation of eight patients treated with 200 mg of BILN 2061 twice daily for 2 days as an oral solution in a PEG 400:ethanol mixture. Diamonds represent time of administration. The linear quantitative range of 1,500–1,250,000 HCV RNA copies ml⁻¹ of the Cobas Amplicor HCV Monitor V2.0 is indicated with horizontal blue lines.
its effects on viral replication. We cannot exclude that this may be a contributing factor in the rapid and marked virus load decline observed in patients treated with BILN 2061. The antiviral results of protease inhibitor BILN 2061 in a proof-of-concept human trial clearly demonstrate the great potential of selective and potent anti-HCV agents. BILN 2061 will require longer trials to assess sustained antiviral activity and holds great promise to markedly improve treatments of chronic HCV infection.

Methods

Clinical trials, ethical conduct and consent
Clinical trials were initiated after the protocol, informed consent and subject information form had been approved, and after administration of the protocol was approved by the Institutional Review Board (IRB) or an Ethics Committee (IEC). The IRB or IEC have performed all duties outlined by the requirements of the participating countries. The trial was carried out in accordance with the principles stated in the Declaration of Helsinki and its amendments (revised version from 1996) and in accordance with Good Clinical Practice and local laws. Data were collected on the request from the participating physicians, the sponsor’s monitors, the quality assurance auditors, by the IRB or IEC, and the regulatory health authorities. Before subject participation in the trial, written informed consent was obtained from each subject according to the regulatory and legal requirements of the participating country.

In vitro inhibitory potency of BILN 2061

Inhibition studies were performed as previously described16 except for the use of the fluorescent substrate anthranilyl-Asp-(D)Glu-Ile-Val-Pro-NVAl(C(0)-O)-Ala-Met-Tyr(3-N02)-Thr-Tyr-OH. In vitro specificity assays were performed as previously described16 using the replicon 1b system. A virus dose response study (human leukocyte elastase) and systemic (human liver cathespin B) protease. For the dose-dependent inhibition of subgenomic HCV RNA levels, HCV-specific RNA copy number was quantified as previously described16 by quantitative real-time polymerase chain reaction with reverse transcription with the ABI PRISM 7700 sequence detection system, and normalized to the total cellular RNA recovered as quantified with RiboGreen (Molecular Probes) using a HCV bicistronic NS2-NS5B subgenomic replicon 1a (G.K. et al., unpublished observation) and 1b corresponding to the described clone 1377/NS3-3′ wt. In the serum shift assay, inhibitory activity of BILN 2061 was determined using replicon 1b in the presence of 50% assay sensitivity of 50 HCV RNA copies ml−1 and a limit of detection of 1,500 HCV RNA copies ml−1.

In a randomized, double-blind, escalating single-dose study with placebo controls BILN 2061 was administered to eight male subjects (six active and two placebo) in a 10 ml PEG mixture. Plasma samples were drawn at various times and plasma HCV RNA levels were assayed in a blinded fashion using the ABI PRISM 7700 sequence detection system, and normalized to the total cellular RNA recovered as quantified with RiboGreen. Data were collected on the request from the participating physicians, the sponsor’s monitors, the quality assurance auditors, by the IRB or IEC, and the regulatory health authorities. Before subject participation in the trial, written informed consent was obtained from each subject according to the regulatory and legal requirements of the participating country. A randomized, double-blind, proof-of-concept study with placebo controls BILN 2061 was administered to eight male subjects (six active and two placebo) in a 10 ml PEG 40%ethanol solution per oral solution taken on an empty stomach. Subjects were given orally as a single morning dose. The pharmacokinetic profiles of the compound in the six active subjects were determined at the various dose levels tested: 5, 20, 60, 100, 200, 400, 600, 800, 1,000, 1,200, 1,500, 2,000 and 2,400 mg. Plasma samples obtained at various times were analysed by liquid chromatography and mass spectrometry method. The specific procedure and specification of the analytical methods will be reported elsewhere (C.-L.Y. et al., unpublished observation). Pharmacokinetic parameters (Supplementary Information 3) were calculated using a non-compartmental method. Dose proportionality of plasma BILN 2061 concentrations in terms of AUC0–∞ and Cmax was assessed by regression analysis.

A randomized, double-blind, proof-of-concept study with placebo controls the antiviral activity of BILN 2061 was investigated in ten patients infected with HCV genotype 1 (INNO-LIPA HCV II, Innogenetics) and with minimal liver fibrosis as determined histologically. Patients were treated with 200 mg BILN 2061 (n = 8) or placebo daily for 2 months as an oral solution in a PEG 40%ethanol (80:20 w/w) mixture. Plasma samples were drawn at various times and plasma HCV RNA levels were determined using the Cobas AmpliCycler HCV Monitor V2.0 (Roche Diagnostics), which has a limit of detection of 1,500 HCV RNA copies ml−1 and a linear quantitative range from 1,500 to 1,250,000 HCV RNA copies ml−1. All samples were analysed by a central laboratory. Plasma samples were also subjected to the branched DNA assay (Bayer), resulting in a linear quantitative measurement from 1,500 to 40,000,000 HCV RNA copies ml−1 and to the transcription-mediated amplification assay (Bayer), resulting in a lower order assay sensitivity of 50 HCV RNA copies ml−1.

Received 25 August; accepted 2 October 2003; doi:10.1038/nature02099. Published Online 26 October 2003.
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(100–441) and Tc1-inttron (321–566) (numbers from genomic sequence). The probe used for gfp was gfp1 (19–319) (numbered from ATG). Details of the probes used for analyses of Tc3 and Tc1 dsRNA are available on request. 5′–RACE analyses used the SmartTag kit (Clontech), SuperScriptII reverse transcriptase (GibcoBRL) and two DNA polymerases. S100II fractions were prepared as described in ref. 25. Standard procedures were used for primer-extension analyses. Sequences of all primers used are available on request.

Quantification of protected fragments (RNase protection assays) was performed using ImageQuant software.

Transgenic lines

The chimeric gfp reporter plasmids were produced by inserting various fragments into plasmid pAZ132 (ref. 26). Plasmid pAZ1 (TIR fusion) contained nucleotides 1–54 of Tc1 (genomic sequence) in the sense orientation in the SgrAI site. Plasmid pAZ4 (unc-TIR fusion) contained nucleotides 113,177–113,190 of unc-22 (spliced sequence) in the sense orientation in the SgrAI site. Plasmid pAZ132 contained nucleotides 1–54 of Tc1 in the sense orientation in the EcoRI site. To prevent transgene silencing due to the presence of high transgene copy numbers, low-copy-number transgenic lines were generated by ballistic transformation using a neomycin phosphotransferase cassette inserted into the genomic sequence inserted into the Smal site of the pBlueScript-specific probe. Crosses using pkIs1661 showed that all three transgene copies in this line reside at one locus and segregate in a mendelian manner. However, the transgenes can be lost (presumably due to recombination), as is apparent from the presence of worms with an unc-119 phenotype (PCR analyses confirmed transgene loss in these worms). Transgene loss is not uncommon for ballistic-generated transgenic and varies for the lines as follows: pkIs1662, 1%; pkIs1663, 1%; pkIs1664, 1%; pkIs1665, 1%; pkIs1666, 1%; pkIs1667, 0%; pkIs1668, 1%. Interestingly, transgene loss strongly increases upon crossing to strains defective in the sense orientation in the TIR 3 stop. By DNA blot analyses (carried out according to standard procedures), transgene copy number was determined using SacII- and BglII-digested genomic DNA and gfp- and pBlueScript-specific probes. Crosses using pkIs1661 showed that all three transgene copies in this line reside at one locus and segregate in a mendelian manner. However, the transgenes can be lost (presumably due to recombination), as is apparent from the presence of worms with an unc-119 phenotype (PCR analyses confirmed transgene loss in these worms). Transgene loss is not uncommon for ballistic-generated transgenic and varies for the lines as follows: pkIs1661, 1%; pkIs1662, 1%; pkIS1663, 2%; pkIS1664, 80%; pkIS1665, 0%; pkIS1666, 1%; pkIS1667, 0%; pkIS1668, 0%; pkIS1669, 0%; pkIS1670, 1%; pkIS1671, 0%; pkIS1672, 1%. Interestingly, transgene loss strongly increases upon crossing to strains defective in transposon silencing (mut-7 and pk732 but not rol-1); this transgene loss is not dependent on the presence of the Tc1 TIR sequence, as it also occurs upon crossing mut-7 to pkIs1665, an unc-22 fusion line.

dsRNAs

Plasmids for dsRNA production in E. coli comprised pTS302 dsRNA11 (for unc-22) and pTS303 (containing nucleotides 1–441 of the Tc1 genomic sequence inserted into the Smal site). E. coli expressing mut-16 dsRNA were obtained from well 175 of the C. elegans feeding library.

Received 28 August; accepted 23 September 2003; doi:10.1038/nature02107.


Acknowledgements We thank R. ketting for help in experiments and discussions, and E. Berezikov for help with the ballistic transformations. We acknowledge S. Fischer, N. Vastenhouw, V. Robert, E. Cuppen, R. May and M. Joosten for helpful discussions or for critically reading the manuscript. This work was supported by a VIDI fellowship from the Dutch Science Foundation (NWO) to T.S.

Competing interests statement The authors declare that they have no competing financial interests.

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In this Letter, the ‘Competing interests statement’ should be corrected to: ‘The authors declare competing financial interests: R.E.S. was the clinical investigator and received an honorarium from Boehringer Ingelheim. All the other authors are or were employees of Boehringer Ingelheim.’