

Acknowledgements We thank M. Kivimäki, K. Kainulainen, A. Hakonen, J. Immanen, Q. Yu, F. D. Rodriguez, G. Resch and M. Ilijin-Jug for technical assistance and plant cultivation; E. Jokitalo for TEM; M. Laxell and S. Tähtiharju for material; I. Bancroft and K. van de Sande at GARNet/GeTCID; N. Sauer for the AtSUC2::GFP line; E. Mellerowicz, F. Rook and M. Saarna for advice; and P. N. Benfey, B. Scheres and C. Alonso Blanco for comments on the manuscript. Financial support was provided by the Academy of Finland, Tekes, EU, EMBO and the University of Helsinki.

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

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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 mortality^{1–3}. Current interferon-based therapies⁴ are suboptimal especially in patients infected with HCV genotype 1, and they are poorly tolerated, highlighting the unmet medical need for new therapeutics^{5,6}. The HCV-encoded NS3 protease is essential for viral replication^{7,8} 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 model⁹ in support of drug discovery. The HCV-infected chimpanzee, an impractical model for routine studies of HCV biology, was until recently¹⁰ 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 inhibitors^{12,13} and the weak hexapeptide inhibitor Asp-Asp-Ile-Val-Pro-Cys (K_i of 79 μ M) served as the starting lead molecule for design efforts. Our initial lead optimization capitalized on maintaining the carboxy-terminal carboxylate functionality. This carboxylate 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 derivatives^{16,17} that, in combination with optimal groups at P3 to P6, led to very specific and potent hexapeptide 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 information^{17,19,20}, tripeptide mimetics were identified that maintained potent and specific activity against the NS3 protease²¹. 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 properties²² 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

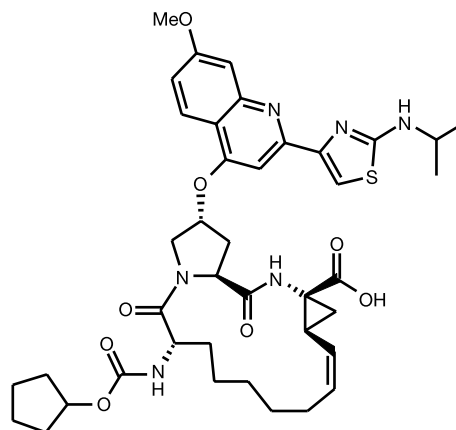


Figure 1 Chemical structure of BILN 2061. The synthesis of the macrocyclic inhibitor BILN 2061 has been described³⁰ and further details will be published elsewhere.

low-nanomolar cellular potency and an adequate oral pharmacokinetic profile in animals. BILN 2061 (Fig. 1) was selected from the optimized macrocyclic inhibitor series for further development.

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 μ 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 4 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% cytotoxic concentration (CC_{50}) of 33 μ 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 μ 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.

BILN 2061 was evaluated in a broad spectrum of pharmacological tests in order to assess its overall pre-clinical safety profile (Supplementary Information 2). These results showed that BILN 2061 is well tolerated in a broad safety pharmacology screen composed of various *in vitro* and *in vivo* assays. The pharmacokinetic and *in vitro* metabolic properties of BILN 2061 were determined in various animal species (Supplementary Information 3). BILN 2061 showed a low to moderate oral biological availability after single-dose pharmacokinetic studies, and was slowly metabolized by hepatic microsomes in all species tested.

In a randomized, double-blind, single-dose escalation study with placebo controls BILN 2061 was administered to healthy volunteers over a range of 5 to 2,400 mg (13 dose levels or placebo) as a drinking solution in polyethylene glycol 400 (PEG 400) and ethanol (20% w/w). BILN 2061 was well tolerated up to 2,000 mg but unspecific intestinal adverse events were observed at the highest dose (2,400 mg), probably due to local gastrointestinal irritation

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 reported²³ 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_{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_{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 ($C_{min} = C_{12h}$) in a twice-daily chronic dosing regimen. The predicted C_{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.

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

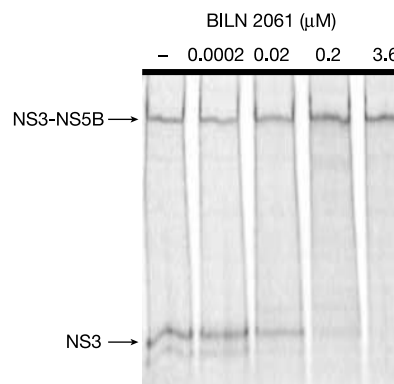


Figure 2 BILN 2061 inhibition of HCV polyprotein processing. Inhibition of NS3-protease-mediated polyprotein processing in cells containing an HCV 1b subgenomic NS2-NS5B replicon. To detect HCV non-structural protein precursors and NS3 mature protein, replicon-containing cells treated with increasing amounts of BILN 2061 were pulse-labelled for 2 h with ³⁵S-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 described¹⁸. The position of the NS3-NS5B precursor and the NS3 mature protein are indicated.

Table 1 Biological profile of BILN 2061

Assay type/secondary activity	Inhibition values
NS3-NS4A protease assays	
HCV 1b enzyme	$K_i = 0.66$ nM
HCV 1a enzyme	$K_i = 0.30$ nM
Mechanism	Competitive and reversible
Surrogate cell-based assays	
HCV replicon 1b	$EC_{50} = 3$ nM
HCV replicon 1a	$EC_{50} = 4$ nM
Serum shift assay (50% human serum)	<Tenfold increase in EC_{50}
Cytotoxicity MTT assay	$CC_{50} = 33$ μ M
Secondary activity	
Human leukocyte elastase	$IC_{50} > 30$ μ M
Cathepsin B	$IC_{50} > 30$ μ M

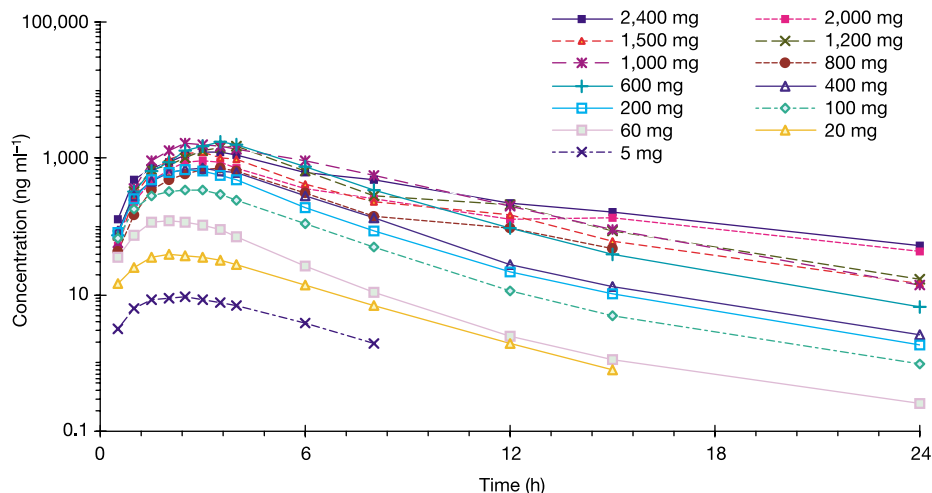


Figure 3 BILN 2061 concentration in human plasma after single-dose oral administration. BILN 2061 was exposed to healthy volunteers in a randomized, double-blind, escalating single-dose study with placebo controls. The pharmacokinetic profiles of the compound in

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.

with interferon (IFN), as well as those with either minimal or advanced liver disease^{24,25}. 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^{26–28}. 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

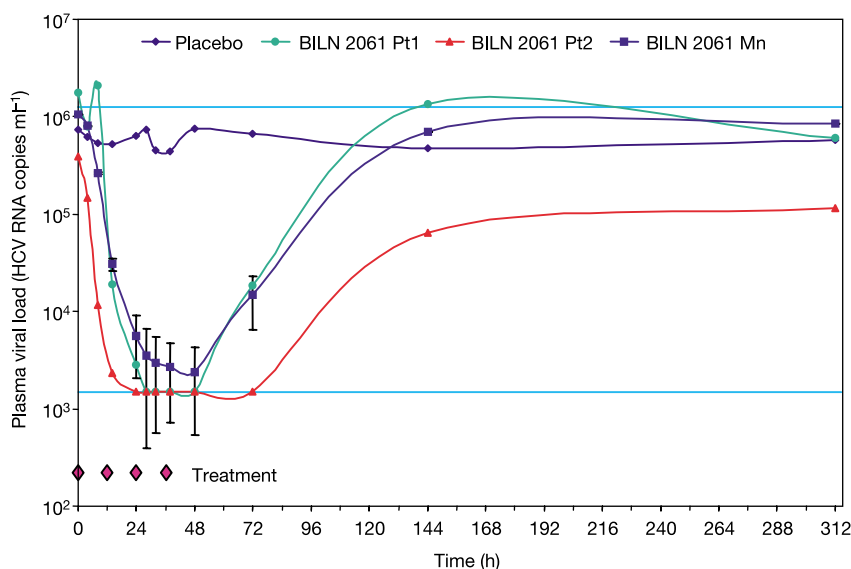


Figure 4 Antiviral efficacy of BILN 2061 in HCV-infected patients. Plasma virus load of individual patients treated with BILN 2061 (BILN 2061 Pt1, BILN 2061 Pt2), placebo (placebo) and geometric mean (BILN 2061 Mn) are shown, with standard deviation of eight patients treated with 200 mg of BILN 2061 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 reviewed and received approval from the local Institutional Review Board (IRB) or an Independent 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 generated as a result of this trial are available on 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 described¹⁸ except for the use of the fluorogenic substrate anthranilyl-Asp-(D)Glu-Ile-Val-Pro-NVal[C(O)-O]-Ala-Met-Tyr(3-NO₂)-Thr-Trp-OH. *In vitro* specificity assays were performed as previously described²² using the representative serine (human leukocyte elastase) and cysteine (human liver cathepsin B) protease. For the dose-dependent inhibition of subgenomic HCV RNA levels, HCV-specific RNA copy number was quantified as previously described¹⁸ 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 I377/NS2-3' wt⁹. In the serum shift assay, inhibitory activity of BILN 2061 was determined using replicon 1b in the presence of 50% extracellular human serum. In the presence of BILN 2061 the percentage of inhibition was determined by reduction in HCV RNA levels, which is expressed as genome equivalents per microgram of total cellular RNA recovered relative to a DMSO control without compound. The percentage of inhibition is then plotted against the compound concentration and a nonlinear curve was fitted (Hill model) to the percentage inhibition-concentration data. The calculated percentage inhibition values were then used to determine EC₅₀ using a nonlinear regression routine procedure of SAS and the following equation: inhibition (%) = $I_{\max}[\text{inhibitor}]^n / ([\text{inhibitor}]^n + EC_{50}^n)$.

The cytotoxicity of BILN 2061 (CC₅₀) was determined in Huh-7 cells using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] metabolic assay as previously described¹⁸.

Phase I and proof-of-concept clinical studies

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 400:ethanol (80%:20%) drinking solution per dose level taken on an empty stomach. Administrations were given orally as a single morning dose. The pharmacokinetic profiles of the compound in 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 a 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 A_{0-∞} and C_{max} was assessed by regression analysis.

In 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 twice daily for 2 days as an oral solution in a PEG 400:ethanol (80:20 w/w) mixture. Plasma samples were drawn at various times and plasma HCV RNA levels were determined using the Cobas Amplicor 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 range 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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Supplementary Information accompanies the paper on www.nature.com/nature.

Acknowledgements We wish to thank F. Dô, J. Duan, M. Garneau, N. Lapeyre-Paquette, S. Lefebvre, F. Liard, M. Marquis, R. Maurice, G. McKercher, A. Pause, C. Pellerin, L. Pilote, C. Plouffe and M. Rhéaume from the Biological Sciences Department, and N. Aubry, J. Bordeleau, C. Boucher, Y. Bousquet, C. Brochu, C. Chabot, R. Déziel, J.-S. Duceppe, G. Fazal, J.-M. Ferland, E. Ghro, J. Gillard, V. Gorys, S. Goulet, C. Grand-Maitre, B. Haché, T. Halmos, P. Lavallée, M. Little, E. Malenfant, R. Plante, M. Poirier, S. Valois and D. Wernic from the Chemistry Department. We also thank B. Willems and the Centre de Recherche Clinique André Viallet, Hôpital St-Luc, CHUM. We acknowledge the BILN 2061 development team and members of the departments of Drug Metabolism and Pharmacokinetics, Pharmacology, Pharmaceuticals, Analytical Sciences, Process Chemistry, Toxicology, Drug Regulatory Affairs and Medical at Boehringer Ingelheim Pharmaceuticals, Inc., Connecticut, USA; J. Croenlein, G. Nehmiz and the clinical research teams at Boehringer Ingelheim Pharma KG, Biberach, Germany; and Academisch Medisch Centrum, The Netherlands. We thank the volunteers and patients who participated in the clinical trials of BILN 2061. R.E.S. was clinical investigator and received honorarium from Boehringer Ingelheim. All other authors are or were employees of Boehringer Ingelheim.

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

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