Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 38 (2003) 339–343
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

The design of potent, non-peptidic inhibitors of hepatitis C protease

David M. Andrews [a,*], Helene M. Chaignot [a], Barry A. Coomber [a], Mike D. Dowle [a], S. Lucy Hind [a], Martin R. Johnson [a], Paul S. Jones [a], Gail Mills [a], Angela Patikis [b], Tony J. Pateman [b], J. Ed Robinson [a], Martin J. Slater [a], Naimisha Trivedi [a]

[a] *Department of Medicinal Chemistry, GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage, SG1 2NY, UK*
[b] *Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage, SG1 2NY, UK*

Received 19 September 2002; received in revised form 16 December 2002; accepted 22 January 2003

Abstract

The pyrrolidine-5,5-*trans*-lactam template was used to design small, neutral, mechanism-based inhibitors of hepatitis C NS3/4A protease displaying potent activity in the replicon cell-based assay. The activity of this series is not dependent upon its chemical reactivity and molecules have been synthesised which combine enhanced biochemical potency with improved plasma stability. Promising initial pharmacokinetic data indicating the potential for further optimisation of this series into low molecular weight, drug-like inhibitors is presented.
© 2003 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Hepatitis C; NS3; 4A protease; 5,5-*trans*-lactam; Replicon; Mechanism-based; Serine protease

1. Introduction

Hepatitis C virus (HCV) infects chronically an estimated 3% of the global human population [1], often leading to cirrhosis, hepatocellular carcinoma and liver failure in later life [2–6]. It has been estimated that of those currently infected, 20 and 4% are likely to develop liver cirrhosis and liver cancer respectively in the next decade [7,8]. Current therapies are based upon interferon-α alone or in combination with ribavirin. Although sustained response rates are markedly improved using combination therapies, at least 50% of patients fail to show a sustained response. Additionally, current therapies have the disadvantage of frequent and severe side-effects [9–11]. The development of new therapies to treat HCV infection effectively is thus of paramount importance, and is currently an intensive area of research [12].

HCV is a small, enveloped virus, the genome of which is a 9.5 kb single stranded RNA that encodes for a single polyprotein of 3010–3030 amino acids. This polyprotein is processed by cellular signal peptidases to produce the structural viral proteins (C, E1, E2, p7), whereas viral proteases (NS2, NS3) are responsible for the production of mature non-structural replicative proteins. The multifunctional 70 kD NS3 protein is the most extensively studied viral protein [13,14]. The amino-terminal third of the protein is a trypsin-like serine protease that cleaves the NS3-4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions. Although isolated NS3 is enzymatically active, it forms a heterodimer with the NS4A co-factor which is believed to be the most physiologically-relevant form of the enzyme [15,16]. It has been reported that when appropriate mutations were introduced into the NS3 protease region of the HCV genome, the infectivity of these RNAs in chimpanzees was abolished [17]. NS3 protease is thus an essential viral function and should prove to be an excellent target for the development of novel anti-HCV agents.

A number of peptidic and non-peptidic inhibitors of the serine protease have been reported [18]. The majority of the former have been competitive inhibitors designed from peptide substrates or cleavage products, whilst non-peptidic molecules have emerged through random screening and have displayed non-competitive mechan-

* Corresponding author.
E-mail address: david.m.andrews@gsk.com (D.M. Andrews).

0223-5234/03/$ - see front matter © 2003 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.
doi:10.1016/S0223-5234(03)00050-3

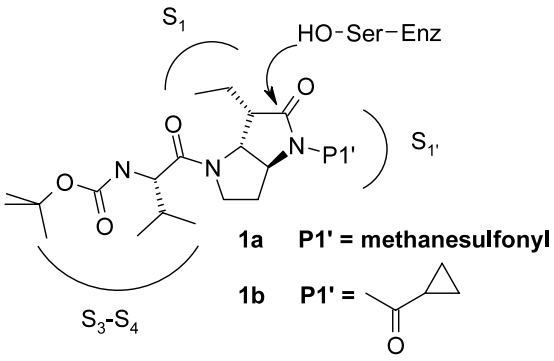


Fig. 1. Ethyl pyrrolidine-5,5-*trans*-lactam template: potential interactions with protease subsites.

isms of action. As a starting point for the design of inhibitors we sought to take advantage of the previously-reported pyrrolidine-5,5-*trans*-lactam template which allows access to the S1, S1′ and S3-S4 specificity pockets [19], and traps the active site serine hydroxyl group by acylation (Fig. 1).

2. Results and discussion

The *trans*-lactam template has been developed into inhibitors of human neutrophil elastase [20]; intracellular activity, plasma stability, and oral activity have been demonstrated. The first report concerning the application of this template to HCV NS3/4A protease, described α- and β-ethyl-substituted *trans*-lactams [21]. The first generation inhibitors (e.g. **1**) suffered from modest hydrolytic stability in plasma (mediated by non-specific esterases/hydrolases—c.f. analogous to experience in the elastase area [22,23]) and limited synthetic accessibility. We employed an analogous synthetic strategy utilising the robust and flexible methodology previously described (Fig. 2) [21], using disubstituted silyl ketene acetals **4** in the acyl iminium coupling step to prepare the α,α-disubstituted *trans*-lactam compounds **8**–**13** (Table 1) [24].

Following on from these initial studies, we selected **11**, **14** and **15** as exemplifying a range of human plasma stability and determined their stability in dog and rat plasma. It was apparent immediately that dog and human plasma stability showed good correlation with each other, but all compounds examined showed very high turnover in rat plasma (90–100% turnover at 4 h—data not shown). We felt that it was highly unlikely that compounds with such poor plasma stability would show acceptable in vivo pharmacokinetic properties in the rat. Since dog plasma appeared to more closely resemble human plasma from a metabolism standpoint, we investigated the i.v. pharmacokinetics of **11**, **14** and **15** in the dog (see Table 2). We were interested to establish whether in vitro plasma stability measurement would constitute a useful pre-screen for dog in vivo studies. A

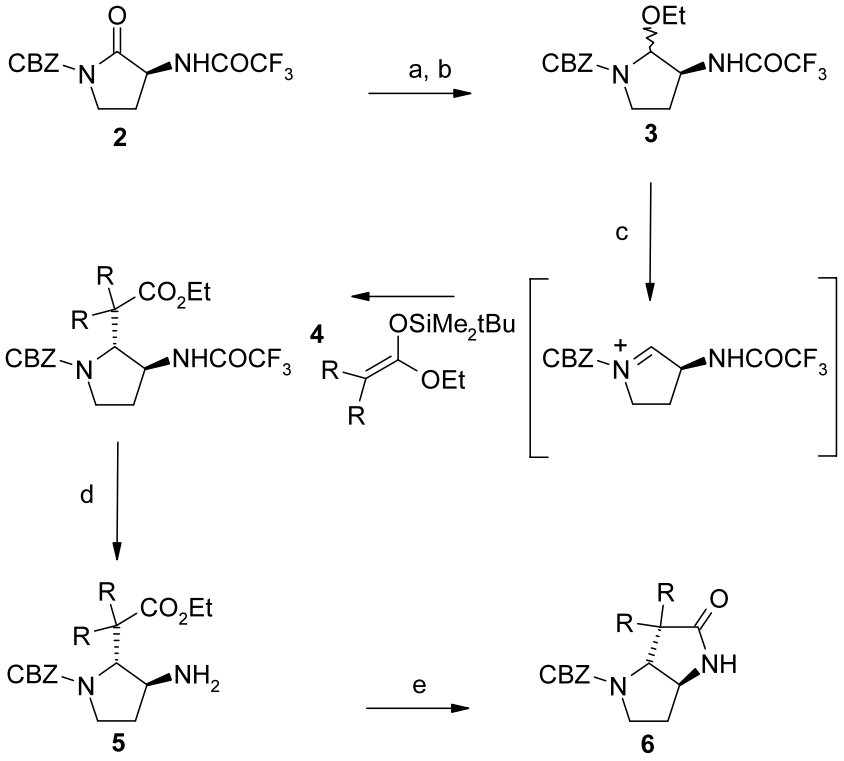
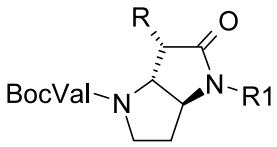


Fig. 2. Reagents and conditions: (a) $LiBH_4$/THF; (b) EtOH/c H_2SO_4; (c) $BF_3 \cdot OEt_2$, DCM; (d) K_2CO_3, EtOH; (e) tBuMgCl, THF. Yield **3**–**6**, 78% overall.

Table 1
HCV NS3/4A protease inhibitory activity and plasma stability of pyrrolidine-5,5-*trans*-lactams



R=	R^1=methane–sulfonyl	HCV protease IC_{50} (μM)	Human plasma stability (% turnover at 4 h)	R^1=cyclopropyl–carbonyl	HCV protease IC_{50} (μM)[2] [a]	Human plasma stability (% turnover at 4 h) [b]
α-Ethyl	**1a**	30	66	**1b**	8.9	64
α-Methyl	–	–	–	**7**	97	42
Dimethyl	**8**	34	35	**9**	3	21
Cyclobutyl	**10**	4.4	90	**11**	0.51	28
Cyclopentyl	**12**	31	58	**13**	2.5	39

[a] Chromogenic assay: enzyme NS3 protease domain only, 0.56 μM final concentration; 4A concentration 0.8 μM; substrate (Ac-EDVVPC-pNA) final concentration 1.5 mM; substrate Km = 1.85 mM; absorbence read at 405 nm. Test compounds were pre-incubated with enzyme for 4 h before the addition of substrate.

[b] Each compound was incubated in fresh human plasma at a concentration of 25 μM and a temperature of 37 °C; an aliquot was withdrawn and deproteinated with acetonitrile at 4 h. The samples were assayed by LC-MS on an API-300 using APCI source and single-ion monitoring. Results were expressed as percentage turnover.

plot of in vitro stability vs. in vivo clearance shows the overall correlation to be poor (Fig. 3) and we therefore concluded that in vitro plasma stability determination was of little utility in selecting compounds for further study.

A combination of crystallographic and modelling studies, as well as analysis of SAR trends led us to synthesise **16**–**18** wherein the urethane function which links the *tert*-butyl and valyl substituents is replaced by a urea (see Table 3). Replacement of the hydrophobic *tert*-butyl urethane with an analogous cyclopentyl urea leads to an improvement in potency of about 3-fold. Further elaboration of the urea substituent to introduce an additional valyl unit (**17**) further improves potency. This is rationalised by crystallographic studies which indicate that the isopropyl sidechain of the valine makes a more favourable interaction with the S4 pocket on the enzyme surface than that made by the *tert*-butyl of **11** (Fig. 4). Serendipitously, the replacement of the ethyl ester by an amide substituent bearing a solubilising group, led to the biochemical potency being further enhanced to about 20-fold that of **11**. A good correlation between increased biochemical potency and activity in the replicon cell-based assay is observed [1].

[1] The replicon system consists of a modified liver cell line (Huh7) supporting cytoplasmic replication of (sub)genomic HCV RNA (i.e. replicons). When the cells divide, replicons are distributed between progeny cells by cytoplasmic inheritance. It is unable to produce infectious virus particles since no structural proteins are encoded. In the absence of a true cellular system for HCV replication, it is the cell-based assay of choice for the HCV molecular targets: protease, polymerase and helicase. The potent peptidic inhibitor Ac–E–D–V–V–LV–C–Tic–Nle–S–Y–NH2 (HCV protease $Ki = 0.34$ μM) [16] is inactive in this system.

The lead urethane **11** and ureas **16**–**18** were also examined by i.v. cassette dosing in the dog. It is notable that all the ureas presented show reduced clearance compared to the urethane, in conjunction with higher volume of distribution. Ester **17** is of particular interest since it demonstrates clearance approximately 50% of dog liver blood flow, good volume of distribution and modest elimination half-life. These properties were particularly notable in view of the fact that **17** bears a terminal, metabolically vulnerable ethyl ester side chain. We hypothesised that potency and pharmacokinetics properties could be further optimised by the synthesis of stabilised analogues at this position and the progress made in this area will be the subject of future communications.

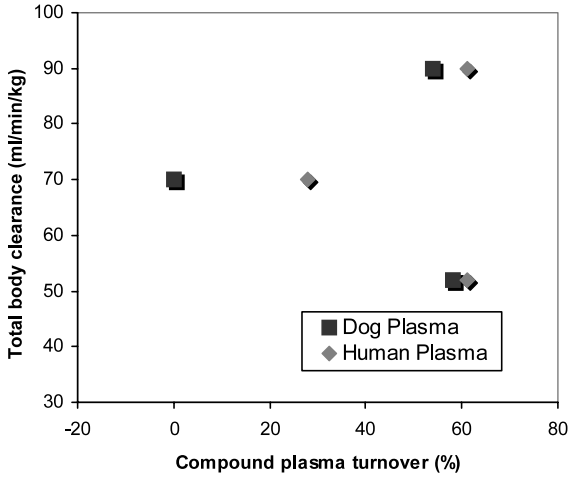
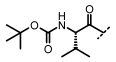
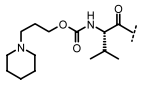
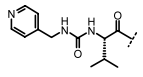


Fig. 3. Scatter of compound plasma stability (% turnover at 4 h) vs. total body clearance following i.v. administration to dog.

Table 2
Biochemical potency, in vitro and in vivo pharmacokinetic parameters compared

	R	HCV Protease kobs/I ($M^{-1}s^{-1}$) [a]	Dog Turnover (%) Dog plasma stability (% turnover at 4h)	HumanTurnover (%) Human plasma stability (% turnover at 4h)	Dog Tot Bdy Clr (ml/min/kg)
11		400	0	28	70
14		81	58	61	52
15		248	54	61	90

[a]Fluorogenic assay: enzyme NS3-4A full-length, 20 nM final concentration; substrate (Aminobenzoyl–E–D–V–V–P–C–S–M–S–Y(3-NO_2)-NH_2) 25 μM final concentration (signal increase at Em_{420} nm (Ex320 nm)); Kobs/I values were obtained at 3 concentrations (ca. 5-fold apart); the Kobs/I value was calculated by dividing the Kobs value by the molar concentration used. Units of measurement are $M^{-1}\,s^{-1}$.

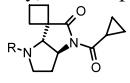
3. Conclusions

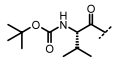
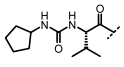
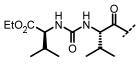
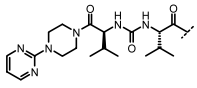
The present studies have shown that exploitation of the *trans*-lactam template is capable of yielding non-peptidic inhibitors of HCV protease displaying potent activity in the replicon cell-based assay. Moreover, we have demonstrated that the potency of this series does not rest upon its chemical reactivity since we have been able to synthesise molecules which combine enhanced biochemical potency with improved plasma stability. We have confirmed the utility of structure-based design in the elaboration of the urea linked compounds; and we have presented promising initial pharmacokinetic data indicating the potential for further optimisation of this series into low molecular weight, drug-like inhibitors.

Acknowledgements

We thank Drs B.C. and G.H. for support and encouragement; Drs G.B., S.B. and M.E. for provision

Table 3
Biochemical and cellular potency, in vivo pharmacokinetic properties of **11**, **16**–**18**



	R	HCV Protease kobs/I ($M^{-1}s^{-1}$)	ELISA Replicon IC_{50} (uM) [a]	C_{max} (ng/ml) [b]	T_{max} (min)	Tot Bdy Cl (ml/min/kg)	Vd_{ss} (L/kg)	$AUC_{O\text{-}inf}$ (ng.hr/ml)	t1/2 (hr)
11		300	9.0	800	5	65	0.3	51	0.33
16		912	3.7	114.6	5	20	0.87	170	0.55
17		2292	0.30	209.5	5	19	2.07	178	1.76
18		7760	0.45	70.7	5	45	1.32	74	0.77

[a]Assay carried out in 5–15 subline of Huh-7 cells (Lohmann, V., Korner, F., Koch, J-O., Herian, U., Theilmann, L. Bartenschlager, R., 1999, Science, 285, pp. 110–113). Compounds applied from 40 mM stock solutions of compound, serially diluted. 100 μl of Huh-7 5–15 cell suspension 2×10^5 cells mL^{-1}) was added to all wells. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. Visualisation was by ELISA using an anti-NS4 primary antibody. GRAFIT software was used to plot the curve of percentage inhibition against compound concentration and derive the compound IC_{50}. In-assay cytotoxicity was assessed by microscopic examination of the stained cell sheets, and expressed as the lowest compound concentration at which any cellular effect was visible.[b]Compounds (dosed as a cassette of five test compounds plus an internal standard) were administered intravenously via the femoral vein at a dose level of 0.2 mg kg^{-1} body weight per compound to male beagle dogs in 20% DMSO, 50% PEG200, 20% water. Blood was sampled from the jugular vein over the time period 5 min to 24 h post dose. Plasma samples were prepared by solid phase extraction. The extracts were analysed by HPLC and detected by single ion monitoring on a Finnigan TSQ mass spectrometer. The analytical column was a Capital Inertsil ODS3 silica 5 m, 50 × 2.1 mm. The mobile phase was 0.1% formic acid in water:methanol (70:30). The flow rate was 0.4 mL min^{-1}.

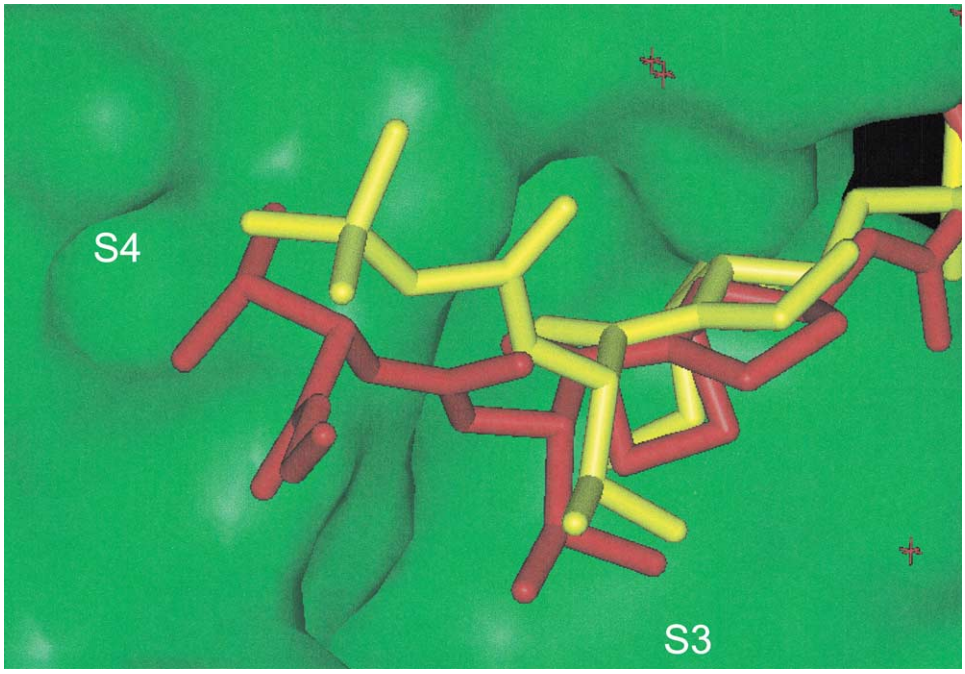


Fig. 4. X-ray crystal structure of *tert*-butyl urethane **11** (yellow) and bis-valyl urea **17** (red) bound to HCV NS3 protease. Note the more favourable interaction of the isopropyl sidechain with respect to the S4 pocket in **17**.

of NS3 protease protein and initial assay systems; D.E. for provision of intermediates; Drs T.S., D.S. and A.G. for X-ray crystallography and molecular modelling; N.G. and S.C. for biochemical potency data; and Dr N.P. and L.A. for replicon test results.

References

[1] World Health Organisation Weekly Epidemiological Record 72, (1997) 65.
[2] M.A. Gerber, J. Hepatol. 17 (Suppl. 3) (1993) 108.
[3] M.J. Alter, Hepatology 26 (1997) 62.
[4] J.H. Hoofnagle, Hepatology 26 (1997) 15S.
[5] A.D. Kwong, Antiviral Res. 40 (1998) 1.
[6] J.H. Hoofnagle, Digestion 59 (1998) 563.
[7] A.M. Di Bisceglie, Lancet 351 (1998) 351.
[8] J.L. Brown, Lancet 351 (1998) 83.
[9] O. Reichard, R. Scvarcz, O. Weiland, Hepatology 26 (1997) 108S.
[10] G.L. Davies, R. Esteban-Mur, V. Rustgi, J. Hoefs, S.C. Gordon, M.L. Shiffman, S. Zeuzem, A. Craxi, M.H. Ling, J. Albrech, N. Eng. J. Med. 339 (1998) 1493.
[11] J.G. McHutchinson, et al., N. Eng. J. Med. 339 (1998) 1485.
[12] Q. May Wang, M.X. Du, M.A. Hockman, R.B. Johnson, X.-L. Sun, Drugs Future 25 (2000) 933.
[13] B.E. Clarke, J. Gen. Virol. 78 (1997) 2397.
[14] M.E. Major, S.H. Feinstone, Hepatology 25 (1997) 1527.
[15] J.L. Kim, K.A. Morgenstern, C. Lin, T. Fox, M.D. Dwyer, J.A. Landro, S.P. Chambers, W. Markland, C.A. Lepre, E.T. O'Malley, S.L. Harbeson, C.M. Rice, M.A. Murcko, P.R. Caron, J.A. Thomson, Cell 8 (1996) 344.
[16] J.A. Landro, S.A. Raybuck, Y.P.C. Luong, E.T. O'Malley, S.L. Harbeson, K.A. Morgenstern, G. Rao, D.J.A. Livingston, et al., Biochemistry 36 (1997) 9340.
[17] A.A. Kolykhalov, K. Mihalik, S.M. Feinstone, C.M. Rice, J. Virol. 74 (2000) 2046.
[18] C. Steinkuhler, U. Koch, F. Narjes, V.G. Matassa, Curr. Med. Chem. 8 (2001) 919.
[19] I. Schechter, A. Berger, Biochem. Biophys. Res. Commun. 27 (1967) 157
For a description of protease nomenclature
[20] S.J.F. MacDonald, G.D.E. Clarke, M.D. Dowle, L.A. Harrison, S.T. Hodgson, G.G.A. Inglis, M.R. Johnson, P. Shah, R.J. Upton, S.B. Walls, J. Org. Chem. 64 (1999) 5166.
[21] M.J. Slater, D.M. Andrews, G. Baker, S.S. Bethell, S. Carey, H. Chaignot, B. Clarke, B. Coomber, M. Ellis, A. Good, G. Hardy, E. Robinson, Bioorg. Med. Chem. Lett. 12 (2002) 3359.
[22] S.J.F. Macdonald, M.D. Dowle, L.A. Harrison, P. Shah, M.R. Johnson, G.G. Inglis, G.D. Clarke, R.A. Smith, D. Humphreys, C.R. Molloy, A. Amour, M. Dixon, G. Murkitt, R.E. Godward, T. Padfield, T. Skarzynski, O.M. Singh, K.A. Kumar, G. Fleetwood, S.T. Hodgson, G.W. Hardy, Bioorg. Med. Chem. Lett. 11 (2001) 895 (reference cited therein).
[23] P.E. Finke, S.K. Shah, D.S. Fletcher, B.M. Ashe, K.A. Brause, G.O. Chandler, P.S. Della, K.M. Hand, A.L. Maycook, D.G. Osinga, J. Med. Chem. 38 (1995) 2449.
[24] D.M. Andrews, S.K. Shah, B.M. Ashe, K.A. Brause, G.O. Chandler, P.S. Dellea, K.M. Hand, A.L. Maycock, D.G. Osinga, Org. Lett. 4 (2002) 4475.