Identification of *N,N*-Disubstituted Phenylalanines as a Novel Class of Inhibitors of Hepatitis C NS5B Polymerase

Laval Chan,* T. Jagadeeswar Reddy, Mélanie Proulx, Sanjoy K. Das, Oswy Pereira, Wuyi Wang, Arshad Siddiqui, Constantin G. Yannopoulos, Carl Poisson, Nathalie Turcotte, Alexandre Drouin, M. Hicham Alaoui-Ismaili, Richard Bethell, Martine Hamel, Lucille L'Heureux, Darius Bilimoria, and Nghe Nguyen-Ba

Shire BioChem Inc., 275 Armand-Frappier Boulevard, Laval, Quebec H7V 4A7, Canada

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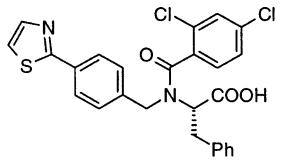
Abstract: The HCV NS5B RNA dependent RNA polymerase plays an essential role in viral replication. The discovery of a novel class of inhibitors based on an N,N-disubstituted phenylalanine scaffold and structure−activity relationships studies to improve potency are described.

Introduction. Infection by the hepatitis C virus (HCV) has been recognized as one of the leading causes of liver impairment such as cirrhosis and hepatocellular carcinoma. It is estimated that 3% of the world's population or about 170 million people are seropositive, and there is compelling evidence that within 10−20 years, progression to cirrhosis and hepatocellular carcinoma will occur in about 20% and 5%, respectively, of those afflicted.[1] Other factors such as alcohol consumption can also be important determinants for disease progression.[2] Currently, HCV infection is one of the leading causes of liver transplantation in the U.S. and it is estimated that 9000 deaths per year result from HCV infection. The recommended treatment, interferon α-2b (or a poly(ethylene glycol) conjugate) in combination with ribavirin, provides a sustained response in only a portion of the treated patients, and furthermore, side effects can be severe.[1] The lack of an effective and well-tolerated treatment has therefore spurred intense research efforts to develop anti-HCV agents.

HCV is a 9.6 kb positive strand RNA virus of the flaviviridae, genus *Hepacivirus*, and it contains a single open reading frame coding for a ∼3000 amino acid polyprotein, which is further processed into various structural (core, E1, and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) viral proteins by host and viral proteases.[2] The NS3 chymotrypsin-like protease and the NS5B RNA dependent RNA polymerase are probably the most studied targets for anti-HCV therapy because they are crucial for viral replication.[3] Extensive structure−activity relationship studies (SAR) of several classes of NS3 protease inhibitors have been reported.[4] Various institutions and pharmaceutical companies, including ours, have disclosed patents describing structurally diverse low molecular weight inhibitors of NS5B polymerase.[5] Examples of a nucleoside triphosphate as well as other low molecular weight inhibitors are shown in Figure 1. The crystal structure of the NS5B polymerase has also been independently resolved by three groups,[6] and the enzyme has the characteristic right-handed "fingers−palm−thumb" domain of polymerases. The active site that resides in the palm region contains the conserved GDD motif of polymerases and is partially enclosed by the fingers and thumb domains. An NS5B polymerase inhibitor (JTK-002/JTK-003) from Japan Tobacco Inc.[6b] has been reported to be undergoing phase II clinical trials.[7] The structure of this compound has not been disclosed, but it may be structurally related to **JTI** (Figure 1).

To identify NS5B inhibitors, an assay using a full-length recombinant polymerase expressed from baculovirus-infected Sf9 insect cells was developed. A FlashPlate scintillation proximity assay was used to measure the incorporation of radiolabeled UTP in a polyrA/biotinylated-oligo dT template-primer captured on the surface of streptavidin-coated microtiter plate.[8] Our screening campaign resulted in the identification of a novel phenylalanine derivative **1** with an IC_{50} of 5.7 μM.



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This lead compound provided a good starting point for our optimization study because it was also found to be selective against human DNA polymerases. The IC_{50} for α, β, and γ polymerases was found to be greater than 50 μM.

Synthesis.[9] All the compounds described in this study were prepared according to one of the three routes depicted in Scheme 1. Reaction of the amino acid (as the *tert*-butyl ester) with a suitable aldehyde under reductive amination conditions yielded an N-alkylated amino acid that was then transformed to a tertiary amide followed by acid deprotection to give the desired compounds (method A). An alternative approach was to first install the benzamide moiety followed by abstraction of the amide proton with sodium hydride in DMF and alkylation with a suitable electrophile (method B). Last, alkylation of a *N*-MTR[10] protected amino acid followed by release of the amine with TFA/EtSMe, amidation and basic hydrolysis of the methyl ester (method C) was also used to provide the desired compounds. This last method was used to ascertain that alkylation of the amide moiety provided the requisite N-alkylation instead of O-alkylation. The biaryl unit in **21** was constructed using standard Suzuki conditions.

Discussion. From preliminary structure−activity analysis of inhibition of NS5B polymerase, it became apparent that the potential for optimization resided primarily in the *N*-benzylic portion of the phenylalanine derivative. For example, evaluation of the bromo derivatives **2**, **3**, and **4**, which were to be used as palladium cross coupling intermediates, showed that NS5B inhibi-

* To whom correspondence should be addressed. Phone: (450) 978-7802. Fax: (450) 978-7777. E-mail lchan@ca.shire.com.

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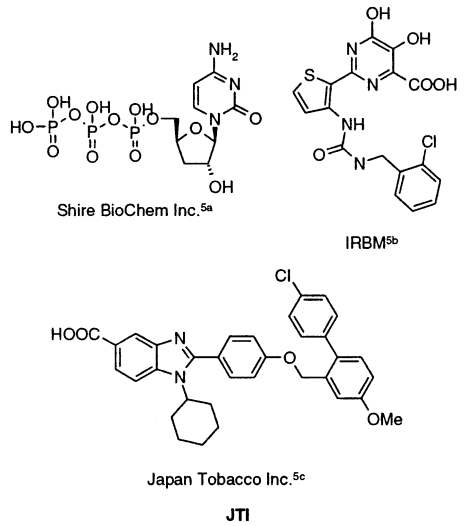
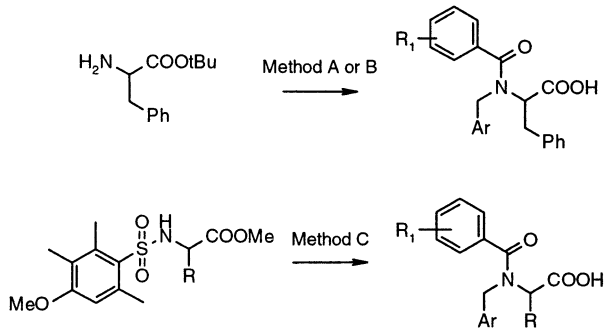


Figure 1. HCV NS5B Polymerase Inhibitors.

Scheme 1. Synthesis of Compounds **2**−**21**[a]

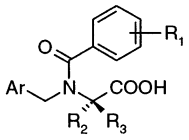


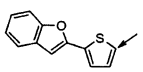
[a] Reagents. Method A: (a) Aldehyde, $NaCNBH_3$, $ZnCl_2$, MeOH; (b) acid chloride, *N*-methylmorpholine, DMAP, CH_2Cl_2; (c) TFA/CH_2Cl_2. Method B: (a) 2,4-dichlorobenzoyl chloride, NEt_3, CH_2Cl_2; (b) alkyl bromide, NaH, DMF; (c) TFA/CH_2Cl_2. Method C: (a) NaH, alkyl bromide, DMF, 0 °C; (b) TFA/CH_2Cl_2, EtSMe; (c) acid chloride, NEt_3, DMAP, CH_2Cl_2; (d) LiOH, THF/MeOH/water. Suzuki reaction: boronic acid, $Pd(PPh_3)_4$, DME, Na_2CO_3(aq), 60 °C.

tion was retained in these analogues and the meta isomer, which had an IC_{50} of 3.5 μM, was virtually equipotent to screening lead **1** (Table 1). Changing the phenylalanine moiety to glycine analogue **5** significantly decreased activity, indicating a requirement for a hydrophobic group at this part of the molecule. It appears that there may be preference for the L-amino acid because the D-phenylalanine analogue **6** was slightly less active with an IC_{50} of 8.6 μM. All subsequent analogues were therefore prepared using L-phenylalanine as the starting material. The monochlorobenzamides **7** (para) and **8** (ortho) were less active than their 2,4-dichloro counterpart, suggesting that chloro groups at both these positions are important.[11] On the basis of these findings, the 2,4-dichlorobenzamide together with the L-phenylalanine moieties were retained and a systematic exploration of the *N*-benzylic part of the molecule was initiated with emphasis on the meta position.

A 2-fold decrease in activity was observed in the unsubstituted phenyl **9** compared to *m*-bromo-**3**, underlying the importance of having a substituent at this position. Both the *m*-chloro-**10** and the fluoro derivatives **11** were also active with **10** (IC_{50} of 1.4 μM), being 4-fold more potent than the screening lead **1**. Other small

Table 1. HCV NS5B Polymerase Inhibitory Activity of N-Substituted Phenylalanine Analogues[a]



Compound	Method	R_1	Ar	R_2	R_3	IC_{50}(μM)[a]
1						5.7
2	B	2,4-Cl_2	2-BrPh	CH_2Ph	H	8
3	A	2,4-Cl_2	3-BrPh	CH_2Ph	H	3.5
4	A	2,4-Cl_2	4-BrPh	CH_2Ph	H	9.1
5	C	2,4-Cl_2	3-BrPh	H	H	53
6	A	2,4-Cl_2	3-BrPh	H	CH_2Ph	8.6
7	C	4-Cl	3-BrPh	CH_2Ph	H	11
8	C	2-Cl	3-BrPh	CH_2Ph	H	15
9	A	2,4-Cl_2	Ph	CH_2Ph	H	8
10	B	2,4-Cl_2	3-ClPh	CH_2Ph	H	1.4
11	B	2,4-Cl_2	3-FPh	CH_2Ph	H	3.2
12	B	2,4-Cl_2	3-MePh	CH_2Ph	H	1.8
13	A	2,4-Cl_2	3-CF_3Ph	CH_2Ph	H	1.7
14	B	2,4-Cl_2	3-NO_2Ph	CH_2Ph	H	0.7
15	B	2,4-Cl_2	3-CNPh	CH_2Ph	H	0.8
16	B	2,4-Cl_2	2-CNPh	CH_2Ph	H	6
17	B	2,4-Cl_2	4-CNPh	CH_2Ph	H	8
18	A	2,4-Cl_2	3-pyridyl *N*-oxide	CH_2Ph	H	2.7
19	A	2,4-Cl_2	3-pyridyl	CH_2Ph	H	11
20	A	2,4-Cl_2		CH_2Ph	H	3.1
21		2,4-Cl_2		CH_2Ph	H	8.6
JTI						0.62

[a] IC_{50} values were determined singly from dose−response curves using 11 concentrations for each compound. Curves were fitted to data points using nonlinear regression analysis, and IC_{50} values were interpolated from the resulting curves using GraphPad Prism software, version 2.0 (GraphPad Software, Inc., San Diego, CA). A positive control was included as an internal standard in each set of experiments, and results were considered accurate only when the IC_{50} value of the positive control was within 0.45 ± 0.16 μM.

substituents at the meta position such as methyl **12** and trifluoromethyl **13** are tolerated and provided analogues that inhibited NS5B polymerase with IC_{50} values of about 1.8 μM. The most potent compounds identified in this study were the *m*-nitro-**14** and *m*-cyano-**15**. Both had IC_{50} values of about 0.7 μM and retained selectivity against human DNA polymerases α, β, and γ (IC_{50} > 50 μM). The meta preference was again demonstrated because the *o*-cyano and *p*-cyano analogues **16** and **17** were almost 10-fold less potent than the meta counterparts. Interestingly, the 3-pyridyl *N*-oxide **18** had an IC_{50} of 2.7 μM, whereas the 3-pyridyl derivative **19** was 4-fold less potent, again underlying the necessity of having a substituent at the 3 position. Finally, bioisosteric replacement of the phenyl ring with a thiophene provided analogues that retained some inhibition. For example, the 5-bromothiophene **20** and benzofuran analogue **21** had IC_{50} values of 3.1 and 8.6 μM, respectively, indicating that there is probably more scope for this type of modification. We have also evaluated one of the most potent inhibitors (**JTI**) described by Japan Tobacco Inc. In our assays, **JTI** displayed an IC_{50} value of 0.62 μM,[12] which is similar to the IC_{50} values of our best analogues **14** and **15**.

Conclusion. We have identified a novel class of potent HCV NS5B polymerase inhibitors that are also selective against human DNA polymerases. The phenylalanine analogues **14** and **15** have NS5B inhibitory

activities that compare favorably with an inhibitor reported by Japan Tobacco Inc. This study has delineated the structural requirements for potency, and it suggests that small substituents at the meta position of the *N*-benzyl group are optimum. In addition, crystal structures of **13** and **21** bound to HCV NS5B polymerase have been generated to a resolution of 1.9 and 2.9 Å, respectively. The binding site is the same for both compounds and is located in a region about 30 Å from the active site.[13] Further optimization and mechanistic and structural studies to elucidate the mode of action of this series are ongoing.[14]

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Supporting Information Available: General synthetic procedures, ^{1}H NMR, HPLC and HRMS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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