Bioorganic & Medicinal Chemistry Letters 19 (2009) 1779–1783

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and SAR of piperazinyl-*N*-phenylbenzamides as inhibitors of hepatitis C virus RNA replication in cell culture

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ARTICLE INFO

Article history:
Received 14 November 2008
Revised 20 January 2009
Accepted 22 January 2009
Available online 27 January 2009

This paper is dedicated to the memory of Dr. Giovanni Migliaccio—an inspirational scientist and sorely missed colleague.

Keywords:
Hepatitis C virus
Modulation of the dimerization of NS5A
Substituted piperazinyl-*N*-(aryl)benzamides

ABSTRACT

The RNA replication machinery of HCV is a multi-subunit membrane–associated complex. NS5A has emerged as an active component of HCV replicase, possibly involved in regulation of viral replication and resistance to the antiviral effect of interferon. We report here substituted piperazinyl-*N*-(aryl)benzamides as potent inhibitors of HCV replication exerted via modulation of the dimerization of NS5A.

Hepatitis C virus (HCV) is the most frequent cause of chronic viral hepatitis, with about 3% of the world's population infected.[1] In the majority of acutely infected individuals, HCV evades the immune response and establishes a chronic infection associated with liver cirrhosis and in some cases hepatocellular carcinoma.[2] There is currently no broadly effective therapy, making the development of HCV-specific antiviral agents an urgent need.[3,4]

HCV is a small, enveloped, single stranded positive RNA virus in the Flaviviridae family. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3000 amino acids. This polyprotein comprises the structural (C, E1 and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins that are required for replication and packaging of the viral genomic RNA. The RNA replication machine of HCV is a multi-subunit membrane-associated complex. The nonstructural protein NS5A is an active component of HCV replicase[5,6], possibly involved in regulation of viral replication and resistance to the antiviral effect of interferon.[7,8] NS5A is a large phosphoprotein (56–58 kDa) organized into three domains, including an amphipathic α-helix at its amino terminus that promotes membrane association and a zinc binding domain. Mutations disrupting either the membrane anchor[9] or zinc binding[10] of NS5A have been shown to be lethal for RNA replication. Thus, NS5A is a potential target for antiviral therapy.

Whilst HCV replicative enzymes, such as NS3 protease and NS5B polymerase, have been extensively explored, efforts to target NS5A have largely been hampered by an incomplete understanding of the role of this multifunctional protein in replication—and a resultant lack of a screening protocol for enzyme inhibition.

A few years ago, the development of subgenomic replicons that replicate in a human hepatoma cell line provided a system to test or screen compounds that inhibit genome replication[11,12], and thus make it possible to identify structural classes that disrupt the function of any of the viral proteins involved in replication—including those with no known enzymic activity (such as NS5A).

In this report, we describe a class of piperazinyl-*N*-(aryl)benzamides (identified from screening the in-house compound collection) as potent inhibitors of HCV replication in such a surrogate cell-based assay (Fig. 1). Resistant replicon mutants raised to this class show mutations that map to domain IA of NS5A—implicating modulation of NS5A function as a potential MOA for this class.

The compounds described herein were assessed for their ability to inhibit replication of subgenomic HCV RNA, measured in HUH-7 cells using a modification of the procedure of Bartenschlager.[13]

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doi:10.1016/j.bmcl.2009.01.066

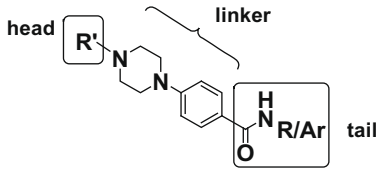


Figure 1. HCV replication inhibitors: (**1**) piperazinyl-*N*-(aryl)benzamides.

Unless otherwise stated, cell-based data (EC_{50}) were measured in the presence of 10% fetal calf serum.

The piperazinyl-*N*-(aryl)benzamide scaffold proved readily amenable to SAR in the head and tail regions. Thus, compounds reported in Table 1 were prepared from commercially available methyl 4-aminobenzoate, as outlined in Scheme 1. Heating methyl 4-aminobenzoate at reflux with bis(2-chloroethyl)amine hydrochloride in *n*-butanol for 7 days afforded arylpiperazine **3** which could be readily isolated by filtration. Following capping of the secondary amine with a BOC group, base mediated hydrolysis of the ester functionality afforded the free carboxylic acid **5**. Amide bond formation was then mediated either by standard peptide coupling reagents, or by reaction of the carboxylic acid with a 1:1 mixture of thionyl chloride/benzotriazole[14] in dichloromethane. Filtration to remove benzotriazole hydrochloride then afforded a stock solution of the acid chloride, which could be readily aliquoted and reacted in parallel with a set of amines—facilitating SAR in the tail region. Final products were typically isolated either via automated direct phase column chromatography or reverse phase HPLC.

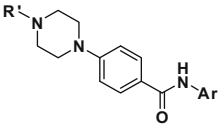
In the instance where Ar was *para*-hydroxyaniline **7**, protection of the phenolic OH as an acetate ester and removal of the BOC group gave **9** as a convenient common intermediate for SAR in the head region. Urethanes, ureas and amides were prepared through reaction of the secondary amine with, respectively, appropriate chloroformates, isocyanates, and acid chlorides, in the presence of an organic base. Excess reagent was scavenged, permitting the *O*-acetate protected intermediates to be isolated by simple filtration and evaporation of volatiles. Base mediated hydrolysis of the acetate protecting group followed by acidification yielded the target molecules, which frequently could be isolated via simple filtration and washing with aq HCl or alternatively by automated reverse phase HPLC (Scheme 2).

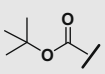
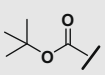
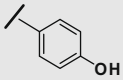
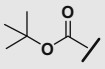
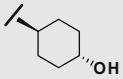
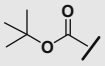
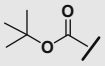
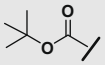
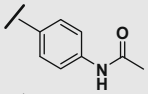
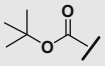
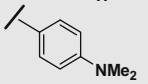
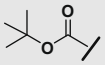
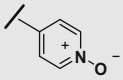
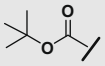
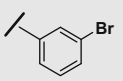
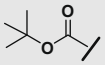
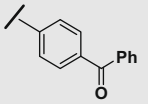
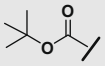
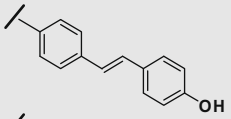
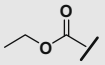
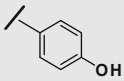
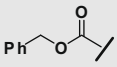
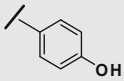
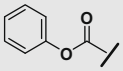
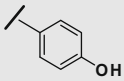
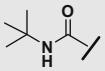
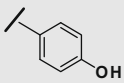
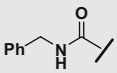
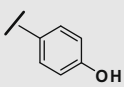
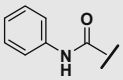
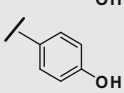
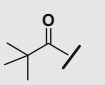
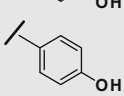
Compounds with modifications to arylpiperazine core were prepared according the Scheme 3. The amide fragment **28** using methyl 4-bromobenzoate, a commercially available starting material. At first the methyl ester was hydrolysed with aq NaOH and the resulting free acid was coupled with 4-aminophenol in DMF, using PyBOP as coupling agent in the presence of *N*-methylmorpholine. Protection of the hydroxyl group was affected with TBDMSCl in the presence of imidazole. This fragment was then used in the palladium mediated N-arylation of amines. Following work-up and chromatography, the desired *O*-de-silylated material was isolated directly, without the need for a separate deprotection step to give **29** or **30**.

Clearly, for inhibitors of replication in cell culture, the absence of a biochemical assay complicates the interpretation of SAR. For instance, a structural change might have an impact on either intrinsic potency on an enzyme target or features pertinent to the assay context or indeed both.

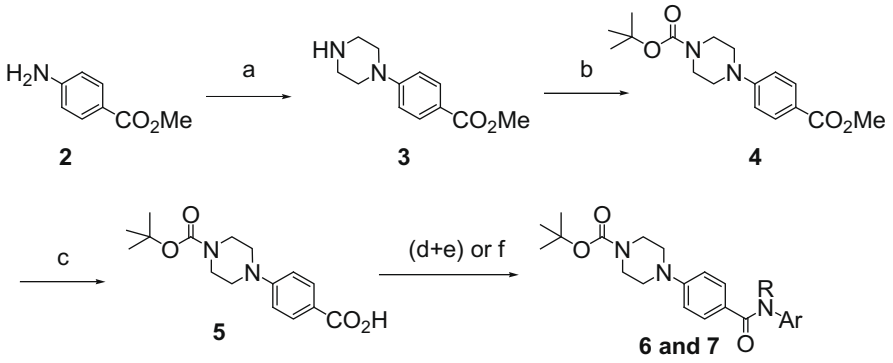
With this in mind, Table 1 illustrates our findings. In the tail region, aromatic functionality was preferred over aliphatic (entries **7** and **11**). Substitution of the aromatic moiety was critical, with *para*-hydroxy **7** proving particularly efficacious. Replacing the OH with electron withdrawing (isoelectronic F **12**, CN **13**) or donating (amide **14**, amine **15**) or pyridine *N*-oxide isostere **16** all proved deleterious. Increasing the bulk of the substituent (e.g., *meta*-Br **17**, acetophenone **18**) brought activity back into the sub-μM range, with vinylphenol **19** proving equipotent to **7**.

Table 1
Head/tail group SAR: cell-based activity

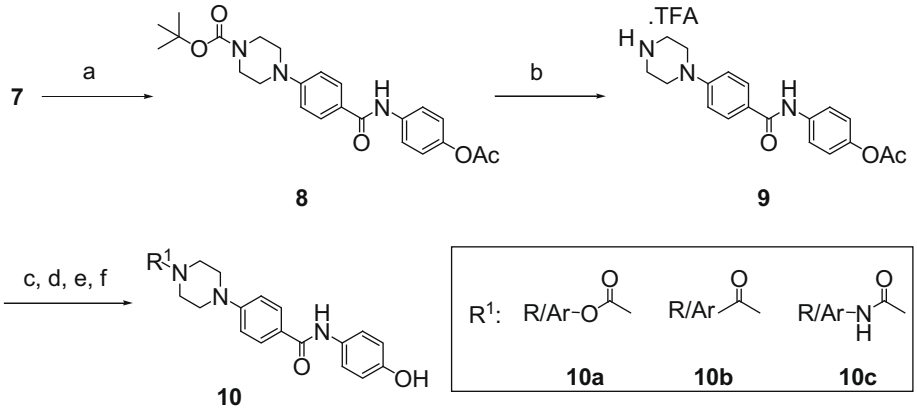


Compound	R^1	Ar	EC_{50} (μM)[a]
6			>10
7			0.16
11			>10
12			>10
13			5.6
14			>10
15			>10
16			>10
17			0.58
18			0.69
19			0.14
20			9.6
21			0.55
22			4.1
23			0.47
24			2.7
25			19.6
26			0.78

[a] EC_{50} values are the mean from at least 2 experiments.



Scheme 1. Synthesis-SAR in the tail region. Reagents and conditions: (a) bis(2-chlroethyl)amine hydrochloride salt (1.0 equiv), *n*-butanol, reflux, 7 days; (b) $(Boc)_2O$ (1.1 equiv), Et_3N (1.2 equiv), DMF, rt; (c) 1 N NaOH (aq): EtOH (1: 2), 60 °C; (d) $SOCl_2$-benzotriazole 1.5 M stock solution (1.05 equiv), DCM, rt; (e) Ar/R-NH_2 (1 equiv), NMM (1.5 equiv), DMF, 40 °C; (f) PyBOP (1.5 equiv), NMM (3.0 equiv), 4-amino-phenol (1.1 equiv), DMF, rt.



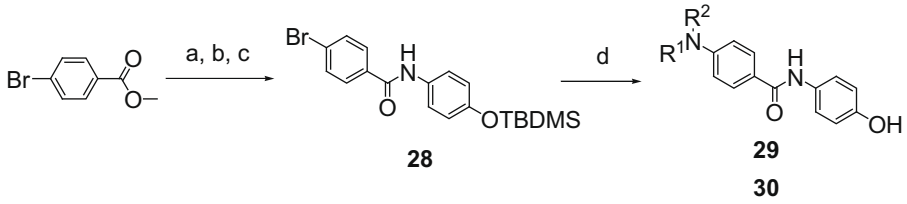
Scheme 2. Synthesis-SAR in the head region. Reagents and conditions: (a) Ac_2O (2.0 equiv), Et_3N (2.2 equiv), DCM, rt; (b) DCM: TFA (1:1), rt; (c) chloroformate or acychloride, or isocianide, or sulfonyl chloride (2.0 equiv), Et_3N (3.5 equiv), THF, rt; (d) PS-trisNH$_2$ resin scavenging; (e) 1 N NaOH (aq) (10.0 equiv), THF; (f) 1 N HCl (aq).

In the head region, carbamates, ureas and amides all afforded sub-μM inhibitors (**21**, **23** and **26**). Bulky substituents proved best (e.g., **7** and **23**), with alkyl preferred over benzyl and aryl across all 3 classes (see compounds **7** and **21**–**26**) (see Table 1).

For the most part, modifications to the arylpiperazine core proved incompatible with maintaining cell-based activity (Table 2). Deletion of either of the piperazine nitrogens, **31** (made as in the Scheme 1 starting from commercially available *N*-Boc-4-(4-carboxyphenyl) piperidine) and **29** resulted in a 10-fold loss of activity with respect to **7**. Opening of the piperazine ring **30**, positional isomer **32** (made starting from methyl 3-amino-benzoate) and simple *N*-methylation of the anilide **33** all led to structures essentially devoid of activity.

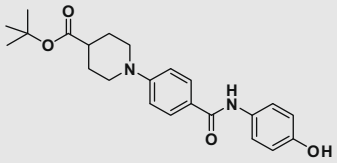
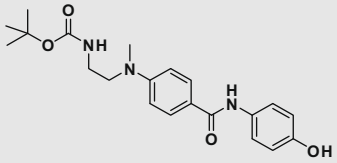
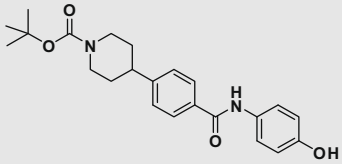
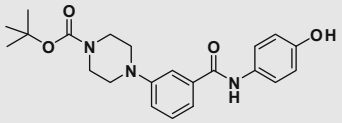
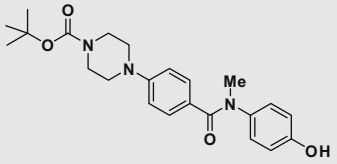
To investigate the mechanism by which this chemotype inhibits HCV replication, **7** was selected as a representative example for further profiling. The activity of this compound on the viral enzymes was evaluated in cell-free assays performed with the purified proteins. At concentrations up to 10 μM, **7** did not inhibit the serine protease, ATPase or helicase activities associated with the NS3-NS4A protein using either the full length NS3-4A complex or the protease and helicase domains. Similarly, this compound did not inhibit the activity of the ΔC55 and ΔC21 forms of the NS5B polymerase. Lastly, the compound did not show any measurable activity in a cell-based assay for the NS3-4A protease, ruling out the possibility that the compound was indeed an inhibitor of this enzyme but required metabolic activation by host cells. These results strongly suggest that the activity of **7** on replicon replication is not exerted through inhibition of the viral enzymes for which cell-free assays are available.

In the attempt to identify potential molecular target(s) for this class, using escalating concentrations of **7** replicons resistant to **7** were selected and characterized. Several drug-resistant cell clones were isolated, phenotypically similar to parental cells and expressing comparable levels of viral RNA and proteins but exhibiting reduced susceptibility to **7** and its active analogues. These clones were still sensitive to inhibition by interferon-α as well as



Scheme 3. Synthesis-SAR in the head region. Reagents and conditions: (a) NaOH (1.1 equiv), THF, rt; (b) 4-aminophenol (1.1 equiv), pyBOP (1.5 equiv), NMM (3 equiv), DMF, rt; (c) TBDMSCl (1.2 equiv), imidazole (2.5 equiv), DMF, rt; (d) Cs_2CO_3 (1.4 equiv), $Pd_2(dba)_3$ (0.02 equiv), BINAP (0.03 equiv), R^1R^2NH (1.2 equiv), toluene, rt.

Table 2
Modifications to arylpiperazine core: cell-based activity

Compound	Structure	EC_{50} (μM)[a]
29		1.6
30		>10
31		1.7
32		>10
33		>10

[a] EC_{50} values are the mean from at least 2 experiments.

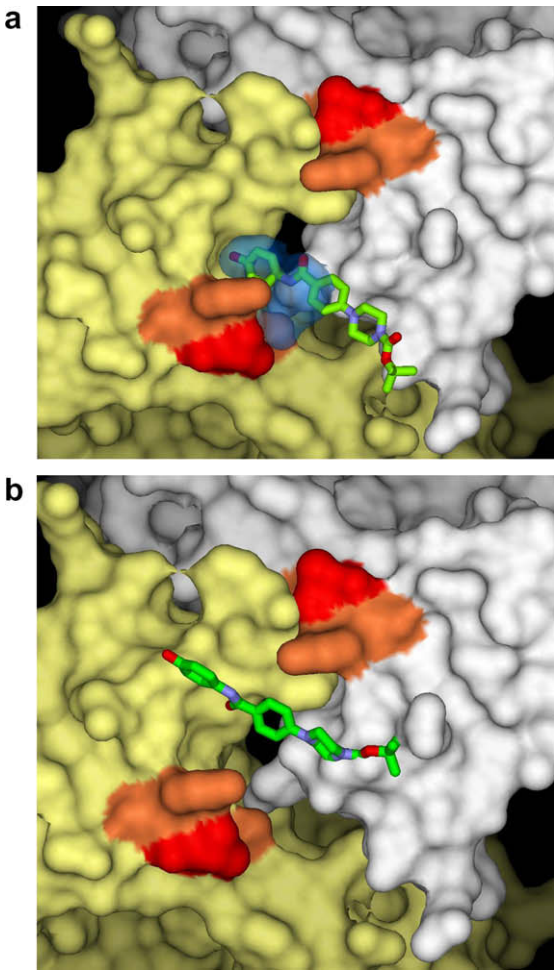


Figure 2. Resistance mutations mapped in the crystal structure of NS5A: red A92 V, orange Y93H and R157 W, (a) binding model of **7** and NS5A monomer and (b) binding model of **7** with NS5A dimer.

inhibitors of the NS5B polymerase and NS3/4A protease, demonstrating that resistance was specific for compound **7**.

Analysis revealed that the mutations responsible for resistance were located in the NS5A coding region. More specifically, replicons containing three different single mutations in the NS5A protein (A92 V, Y93H and R157 W) were clearly resistant to compound **7**.[15] As expected, the replicons carrying these mutations were cross-resistant to active analogues in the piperazinyl-*N*-arylbenzamide class, but not to other replication inhibitors, including NS5B polymerase and NS3/4A protease inhibitors.

Taken together, these results point towards the inhibitory activity of this structural class being exerted by directly or indirectly interfering with the function(s) of NS5A. In this regard, it is interesting to note that mapping the resistance mutations (A92 V, Y93H and R157 W) in the crystal structure of NS5A (Fig. 2) domain IA homo-dimer, shows them to lie at the interface of the two subunits. Thus, if one assumes a biological relevance for the dimerization process, then one might hypothesize that these analogs could exert their anti-HCV activity via modulation of the dimerization of NS5A.

Modelling studies, docking **7** to the NS5A domain IA homo-dimer, show two potential binding modes (Fig. 2). Binding to one subunit of the dimer, as in Figure 2(a), could perturb dimerization, whilst binding (Fig. 2(b)) across the dimer interface could exert a stabilizing effect. In this regard, it is tempting to interpret recent work in the patent literature[16], describing potent symmetrical and pseudosymmetrical replication inhibitors (with NS5A as their putative target) as favouring hypothesis (b), whereby this class might bind across the dimerization interface.

We have identified a novel class of potent inhibitors of HCV replication. SAR in head and tail regions afforded sub-μM activity in the replicon in diverse structural classes, **7** and **20** in the 100 nM range. Resistant replicon mutants raised to this class show mutations in domain IA of NS5A – implicating modulation of NS5A function as a potential MOA for this class. These mutations map to the homo-dimer interface in the crystal structure of domain IA of NS5A and suggests a possible biological relevance for the dimerization process of the protein.

Acknowledgments

We thank Giacomo Paonessa and Laura Pacini for enzymology; Nadia Gennari and Mauro Cerretani for biological testing, and Francesca Naimo for RP-HPLC purifications. This work was funded in part by a grant from the MIUR.

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