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Non-nucleoside inhibitors of HCV polymerase NS5B. Part 3: Synthesis and optimization studies of benzothiazine-substituted tetramic acids

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ABSTRACT

Benzothiazine-substituted tetramic acids were discovered as highly potent non-nucleoside inhibitors of HCV NS5B polymerase. X-ray crystallography studies confirmed the binding mode of these inhibitors with HCV NS5B polymerase. Rational optimization of time dependent inactivation of CYP 3A4 and clearance was accomplished by incorporation of electron-withdrawing groups to the benzothiazine core.

Hepatitis C virus (HCV) was first characterized in 1989 as the major cause of non-A and non-B hepatitis infections.[1] An estimated 3% of the global human population is infected by HCV, the leading cause of chronic liver disease and liver transplants.[2] Currently approved therapies involve pegylated interferon-α-2a as a single agent or in combination with ribavirin. These therapies provide a sustained virological response in approximately 50% of patients infected with genotype 1, and have the disadvantage of frequent and severe side effects.[3] The HCV RNA-dependent RNA polymerase, NS5B is a well characterized enzyme, essential for viral replication, with no known mammalian equivalent, and thus represents an attractive target for the development of novel anti-HCV agents.[4]

Previous work described our lead optimization studies of benzothiazine-substituted quinolinedione series as non-nucleoside inhibitors of HCV NS5B polymerase.[5] While compounds such as **1** were found to be potent inhibitors of HCV polymerase both in enzymatic and replicon assays (Fig. 1), they suffered from poor exposure after oral administration due to low solubility. Thus, we sought to reduce the planarity of compounds such as **1** by replacing the quinolinedione ring by a non-planar chiral tetramic acid (**2**).

Herein, we report a novel series of inhibitors of HCV polymerase in which the quinolinedione ring is replaced by a C-*tert*-butyl substituted tetramic acid leading to benzothiazine-substituted tetramic acids.[6,7] In the present study, we focused on optimization of lead structure **2** via substitutions of the benzothiazine ring and modulation of the lipophilic substituent.

The synthesis of these compounds commenced from 6-nitrobenzothiazole **5** (Scheme 1). The thiazole ring of **5** was opened

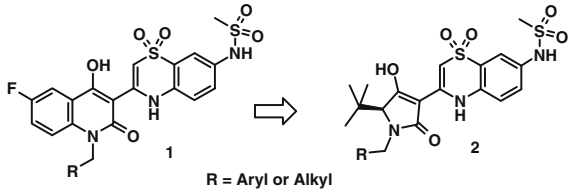

Figure 1. Previous lead compound **1** and novel tetramic acid congener.

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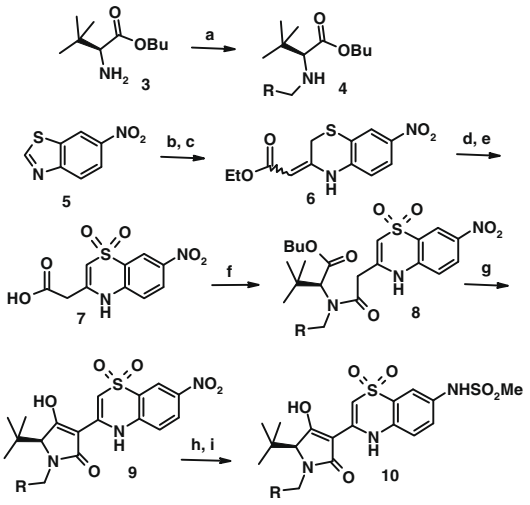


Scheme 1. Reagents and conditions: (a) $NaBH(OAc)_3$, RCHO, DCM, AcOH, rt; (b) KOH, EtOH, 100 °C; (c) $EtO_2CCH_2COCH_2Cl$, AcOH, AcOEt, 70 °C; (d) $KMnO_4$, HCOOH, acetone, THF, 0 °C, 90%; (e) LiOH, THF, 0 °C; (f) DIC, DMF, 25 °C; (g) KO*t*Bu, EtOH, 25 °C; (h) H_2, Raney-Ni, 25 °C; (i) MsCl, Py, 25 °C.

under basic conditions and then doubly alkylated by treatment with ethyl 4-chloroacetoacetate, providing the benzothiazine core as a 1:1 mixture of exocyclic olefin isomers **6**. After selective sulfur oxidation with potassium permanganate, the ester was carefully hydrolyzed with lithium hydroxide at low temperature to prevent acid decarboxylation. The benzothiazine-substituted acetic acid fragment **7** was coupled to L-*tert*-butylglycine butyl ester derivatives **4**, which was readily obtained by reductive alkylation of **3**.[8] A Dieckman condensation of ester **8** led to the backbone structure of the benzothiazine-substituted tetramic acid scaffold (**9**). Reduction of the nitro group with Raney-nickel was followed by treatment with methanesulfonyl chloride in pyridine, leading to functionalized benzothiazine-substituted tetramic acids **10**.

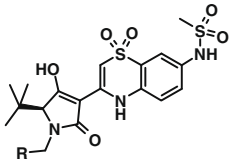
The replacement of the original quinolinedione by a tetramic acid could change the orientation of the benzyl lipophilic group. Thus, our original plan was directed toward the optimization of this substituent. The in vitro data of this study are reported in Table 1. The in vitro potency was measured against the NS5B enzyme[9] and in an HCV subgenomic replicon (GT-1b)[10,11] cell based assay in the presence of 5% fetal bovine serum (FBS). The effect of protein binding on the inhibitors potency was evaluated in the HCV subgenomic replicon cell based assay where 40% human serum (HS) is added.

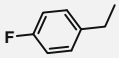
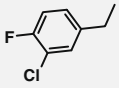
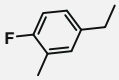
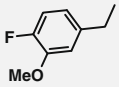
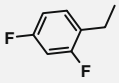
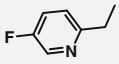
Fluorine substitution at the para position of the phenyl ring provided a threefold improvement in replicon potency (compare **11** and **12**, Table 1). Addition of small groups such chloro (**13**), methyl (**14**) or methoxy (**15**) at the meta position maintained or increased enzyme and replicon potency, but these groups had a detrimental effect on the compound potency in the replicon assay performed in the presence of 40% HS. The installation of an additional fluorine group at the ortho position (compound **16**) did not improve potency. With the aim to reduce protein binding of **16** by adding polarity, an isosteric pyridyl replacement (**17**) was evaluated, but did not provide any improvement. The isoamyl group (**18**) is equipotent with the *p*-fluorobenzyl (**12**), though losses potency in the presence of HS. To our surprise, the replacement of the isopropyl group by a *tert*-butyl group enhanced the replicon potency and reduced protein binding (compare compounds **18** and **19**).

To better understand the interactions of the different benzothiazine inhibitors with the NS5B protein, a co-crystal structure of compound **20** complexed with the enzyme was obtained (Fig. 2).[12,13] Analysis of this structure, overlapped with a benzothiazine-substituted quinolinedione (**1a**), illustrates the similarities between these two scaffolds. Both the quinolinedione and tetramic acid are expected to have an ionized hydroxyl group (**1a** cpKa 4.77, **20** cpKa 5.89) interacting with the backbone NH of Tyr448 and a conserved water molecule. As predicted, the methyl *tert*-butyl group interacted with the surface of Met555 in a region previously filled with a substituted phenyl ring on the quinolinedione scaffold. The position of the methyl sulfonamide interacting with Asp318 and Asn291 and the location of the lipophilic tail were nearly identical with **1a**.

Tetramic acid **12** achieved our objective of very good potency in the serum shifted HCV replicon assay and was selected for PK

Table 1
Enzyme and replicon data for benzothiazine-substituted tetramic acid derivatives **11–19**



Compds	R	NS5B IC_{50}[a] (μM)	Replicon EC_{50}[a,b] (μM)	
			5% FBS	40% HS (FS)[c]
11		0.008	0.032	0.139 (4.3)
12		0.007	0.012	0.033 (2.7)
13		0.005	0.009	0.163 (18.1)
14		0.003	0.012	0.122 (10.2)
15		0.004	0.028	0.422 (15.1)
16		0.013	0.035	0.226 (6.5)
17		0.042	0.201	1.718 (8.5)
18		0.005	0.03	0.66 (22)
19		0.008	0.016	0.065 (4.1)

[a] IC_{50} and EC_{50} values for inhibition calculated from two independent determinations.
[b] CC50 >50 μM for all analogues.
[c] EC_{50} fold-shift values (40% human serum/5% FBS) shown in parentheses.

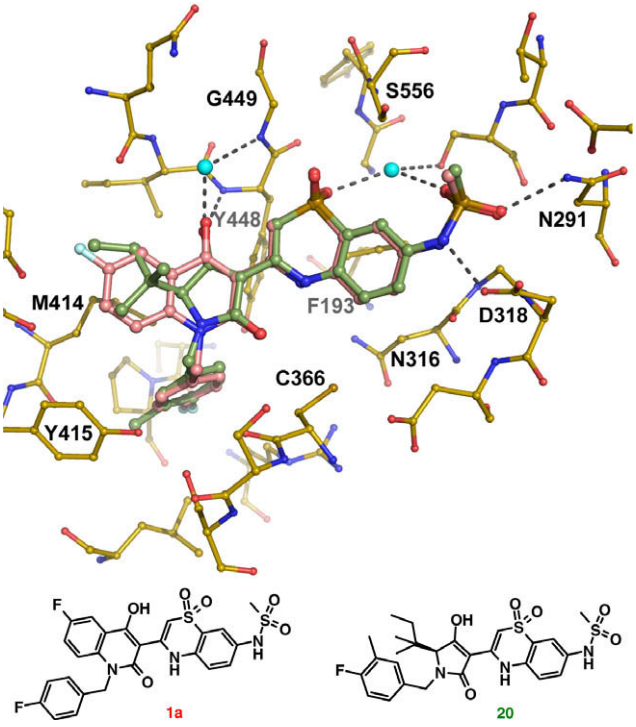


Figure 2. X-ray crystal structure overlap of compounds **20** and **1a** bound to the palm site of CΔ21 NS5B polymerase.

studies. In vivo oral rat PK data of **12** showed slow absorption (T_{max} = 1.3 h) and high clearance (iv Cl = 101 mL/kg/min) resulting in low exposure (AUC = 189 ng × h/mL, 5 mg/kg dose). Despite this poor exposure, compound **12** displayed a moderate oral bioavailability (22%) in rats. To better understand the high clearance, the data in Table 2 were collected. We measured rat iv clearance and microsomal stability of other potent analogues that also showed reasonable permeability (Table 2). These closely related analogues showed rat iv clearance that did not correlate to in vitro microsomal stability. In order to unravel the mechanism of clearance, benzothiazine **12** was C-14 radio-labeled at the stable methyl sulfonamide carbon and then dosed iv in rat. To our surprise, benzothiazine **12** was cleared mainly via biliary excretion, with ~90% of the C-14 radio-label recovered from rat bile with ~70% as unchanged parent.

Our off target screening also revealed that these compounds caused time dependent inactivation (TDI) of CYP 3A4. A metabolite ID study of **12** showed primarily metabolic oxidation at the *tert*-butyl group leading to alcohol **21a** (Fig. 3). In vitro debenzylation (**21b**) was also detected.

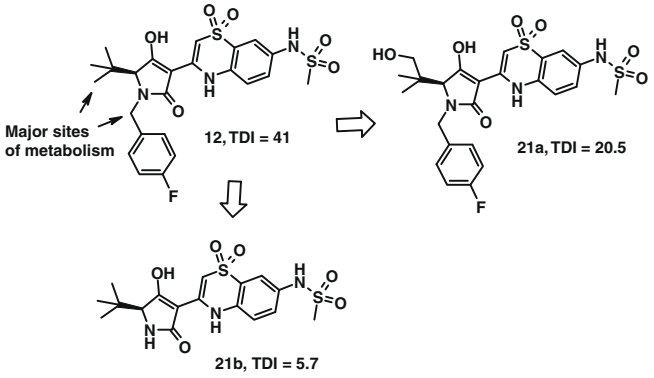
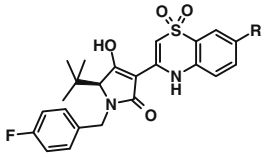


Figure 3. In vitro metabolites (**21a** and **21b**) of compound **12** and their corresponding TDI values.

Both metabolites **21a** and **21b** were synthesized and shown to still display TDI signals (Fig. 3). The TDI liability was attributed to phase I metabolism associated with either the 1,4-diaminophenyl or the olefin functionality present in the C7-methylsulfonylamino substituted benzothiazine scaffold. To test this hypothesis, a series of C-7 substituted benzothiazines were evaluated (Table 3). Interestingly, a reduction in the electron density of the benzothiazine ring by incorporation of electron-withdrawing groups at the C-7 position led to a significant reduction in TDI (**22–24**).

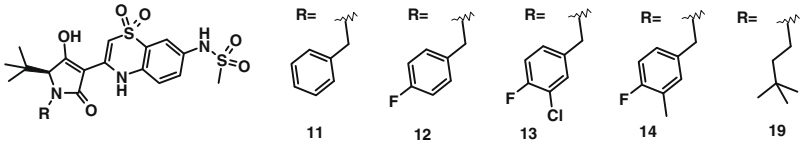
Table 3
Enzyme, replicon and TDI data for C-7 substituted benzothiazine derivatives **12**, **22–24**



Compds	R	NS5B IC_{50}[a] (μM)	Replicon EC_{50}[a,b] (μM)	TDI 3A4 (%)
12	$NHSO_2Me$	0.007	0.005	41
22	F	0.338	1.87	7.6
23	CN	1.2	6.85	10.9
24	NO_2	1.8	16.5	0.4

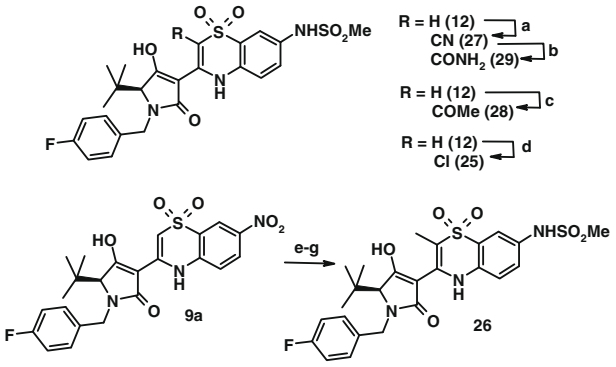
[a] IC_{50} and EC_{50} values for inhibition calculated from two independent determinations.
[b] CC50 >50 M for all analogues.

Table 2
In vitro permeability, rat microsomal stability, iv clearance, and TDI data for selected compounds following single dose administration to rats



Compds	Caco-2 A to B (ER)[a]	PAMPA (cm/sec×10^{-6})	RLM[b] (μL/min/mg)	Cl[c] (mL/kg/min)	TDI 3A4 (%)
11	0.5 (11)	1.8	nd	144	19
12	0.2 (25)	1.4	32	101	41
13	—	0.98	84	86	41
14	—	1.62	327	101	39
19	0.2 (14)	1.8	79	>100	23

[a] Caco-2 assay run for 21 days. Efflux ratio, ER = (B-to-A)/(A-to-B).
[b] RLM: in vitro liver microsomal data.
[c] Rat PK study: dose iv = 2 mg/Kg.

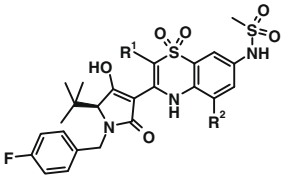


Scheme 2. Reagents and conditions: (a) TsCN, pyridine, 0 °C, 24%; (b) 1 N HCl, THF, rt, 15%; (c) Ac_2O, pyridine, 25 °C, 6%; (d) CF_3SO_2Cl, pyridine, 0 °C, 43%; (e) CH_2O, MeCN, rt, then $NaCNBH_3$, rt, 41%; (f) H_2, Raney-Ni, 25 °C, 48%; (g) MsCl, Py, 25 °C, 72%.

To further test our TDI hypothesis by blocking likely metabolism at the C2–C3 double bond, small groups such a Cl (**25**), Me (**26**) and CN (**27**) were introduced at the C-2 position of the benzothiazine ring. The synthesis of C-2 substituted benzothiazine compounds **25–29** is shown in Scheme 2.[14] The C2–C3 double bond was found to be reactive towards electrophiles. The nitrile group was installed by a direct electrophilic addition of tosyl cyanide to fully elaborated benzothiazine **12**. Nitrile **27** was then converted to the corresponding primary amide **29** by acid hydrolysis. The acetyl and chlorine groups were incorporated at the C-2 position similarly by reaction with acetic anhydride and trifluoromethylsulfonyl chloride respectively. With the aim to install a methyl group at the 2 position, benzothiazine **12** was reacted with methyl iodide, but the methyl group was incorporated at the thiazine nitrogen instead. In light of this result, intermediate **9a** was selectively methylated at the C-2 position upon treatment with formaldehyde followed by sodium cyanoborohydride. The nitro group was then converted to the desired methyl sulfonamide **26** as described previously.

Incorporation of these groups maintained replicon potency (Table 4), even as C-2 was extended to bigger electron-withdrawing groups such as acetyl (**28**) and carboxamide (**29**). However, these groups only somewhat reduced the TDI signal. In order to block possible metabolism at the C-4 nitrogen, a fluorine atom was incorporated at the C-5 position of the benzothiazine, which led to a noted reduction in TDI (**30**). Furthermore, the incorporation of a carboxamide at C-2 (**29**), or a fluorine at C-5 (**30**),[14] of the benzothiazine template also had a beneficial effect on iv clearance.

Table 4
Enzyme, replicon, TDI and clearance data for benzothiazine-substituted tetramic acid derivatives **25–35**



Compds	R^1	R^2	NS5B IC_{50}[a] (μM)	Replicon EC_{50}[a,b] (μM)	TDI 3A4 (%)	Cl[c] (mL/kg/min)
12	H	H	0.007	0.005	41	101
25	Cl	H	0.002	0.004	31	nd
26	Me	H	0.01	0.078	21.9	nd
27	CN	H	0.006	0.016	24.7	nd
28	MeCO	H	0.004	0.02	15.1	nd
29	NH_2CO	H	0.011	0.016	17.8	5
30	H	F	0.009	0.036	8.5	36

[a] IC_{50} and EC_{50} values for inhibition calculated from two independent determinations.
[b] CC50 >50 M for all analogues.
[c] Rat PK study: dose iv = 2 mg/Kg.

In conclusion, we have described a novel series of benzothiazine-substituted tetramic acids as non-nucleoside inhibitors of HCV polymerase NS5B. An initial structure–activity relationship was established for this new class of compounds and their binding mode was confirmed by X-ray crystallography. Our initial lead structure **12** showed an inhibitory activity of 33 nM in our serum shifted replicon assay. This compound suffered from high time dependent CYP3A4 inactivation and biliary clearance. These liabilities were successfully optimized by incorporation of electron-withdrawing groups at the C-2 and C-5 positions of the benzothiazine ring. A reduction of electron density in the thiazine ring simultaneously altered biliary transporter recognition and reduced formation of reactive metabolites while still retaining good cellular potency, as demonstrated by analogues **29** and **30**.

Acknowledgments

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8. The enantiomeric purity of these compounds was retained through the synthetic sequence.
9. For a detailed description of the enzyme assay see: Ma, H.; Jiang, W.-R.; Robledo, N.; Leveque, V.; Ali, S.; Lara-Jaime, T.; Masjedizadeh, M.; Smith, D. B.; Cammack, N.; Klumpp, K.; Symons, J. *J. Biol. Chem.* **2007**, *282*, 29812.
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11. A representative number of benzothiazine-substituted tetramic acids (**11**, **12**, **14**, **16**, **27**, **29**, **30**) were also evaluated in a GT-1a replicon assay. This class of compounds had a minimal loss in inhibitory activity of four to 10-fold relative to our standard GT-1b replicon assay.
12. Atomic coordinates of the structure **20** bound to CΔ21 NS5B polymerase were deposited with the RCSB Protein Data Bank (PDB) under the accession code **3H59**. The crystal structure was determined following previously described procedures: Le Pogam, S.; Kang, H.; Harris, S. F.; Leveque, V.; Giannetti, A. M.; Ali, S.; Jiang, W.; Rajyaguru, S.; Tavares, G.; Oshiro, C.; Hendricks, T.; Klumpp, K.; Symons, J.; Browner, M. F.; Cammack, N.; Najera, I. *J. Virol.* **2006**, *80*, 6146.
13. Compound **20** was the only compound in this series that provided a high resolution crystallographic data. This analogue is closely related to compound **14** but with the addition of an extra methyl group at the *tert*-butyl group. This modification had minimal impact in enzyme and cell activity: compound **20** NS5B IC_{50} 0.013 μM; replicon EC_{50} 0.024 μM.
14. The synthesis of C-5 fluoro substituted benzothiazine **30** was accomplished following the sequence shown in Scheme 1 but starting from 4-fluoro-6-nitrobenzothiazole.