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Identification of halosalicylamide derivatives as a novel class of allosteric inhibitors of HCV NS5B polymerase

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Abstract—Halosalicylamide derivatives were identified from high-throughput screening as potent inhibitors of HCV NS5B polymerase. The subsequent structure and activity relationship revealed the absolute requirement of the salicylamide moiety for optimum activity. Methylation of either the hydroxyl group or the amide group of the salicylamide moiety abolished the activity while the substitutions on both phenyl rings are acceptable. The halosalicylamide derivatives were shown to be non-competitive with respect to elongation nucleotide and demonstrated broad genotype activity against genotype 1–3 HCV NS5B polymerases. Inhibitor competition studies indicated an additive binding mode to the initiation pocket that is occupied by the thiadiazine class of compounds and an additive binding mode to the elongation pocket that is occupied by diketoacids, but a mutually exclusive binding mode with respect to the allosteric thumb pocket that is occupied by the benzimidazole class of inhibitors. Therefore, halosalicylamides represent a novel class of allosteric inhibitors of HCV NS5B polymerase.

Hepatitis C virus is the major etiological agent of non-A, non-B hepatitis. The disease is a major heath problem with an estimated 170 million people infected worldwide. HCV infection results in mild and acute liver disease, but chronic infections are common and may eventually develop into liver cirrhosis or hepatocellular carcinoma.[1–5] The current standard of care for treating HCV is a combination therapy of pegylated interferon-α (PEG-IFN-α) plus ribavirin. Response rates for HCV patients having genotypes 2 or 3 on a 24-week treatment regimen of PEG-IFN-α/ribavirin show a sustained virological response approaching 80%. Patients infected with genotype 1 HCV do not respond as well to this combination therapy demonstrating sustained virological response rates of <50% even after treatment therapies of 48 weeks in duration.[6,7]

Hepatitis C virus is a positive-sense single-stranded RNA virus. The HCV genomic RNA is 9.5 kb in length and consists of a long open reading frame, which is flanked by highly conserved untranslated regions at both the 5′ and 3′ ends. The HCV NS5B gene encodes an RNA-dependent RNA polymerase whose enzymatic activity is critical to the replication of viral RNA genome. Because of its demonstrated and vital role in viral replication, HCV NS5B polymerase has been the most studied viral protein target for small molecule HCV therapy.

Various groups have reported several classes of small molecule inhibitors of hepatitis C virus NS5B polymerase (see Fig. 1). Among the numerous non-nucleoside inhibitors, compounds with either thiophene, phenylalanine, and dihydropyranone scaffolds have been shown to bind to HCV polymerase in the lower thumb allosteric pocket by X-ray crystal structures.[8,9] A series of compounds based on a benzimidazole scaffold have been reported.[10–13] Recently, the X-ray crystal structures of the bound benzimidazole scaffold have also been solved and revealed these inhibitors bind to a site on the surface of the upper thumb domain that is 14 Å away from the lower thumb allosteric pocket that is occupied by compounds of either thiophene or phenylalanine scaffolds.[14] A series of benzothiadiazine derivatives have also been reported as initiation specific inhibitors.[15–19] More recently, acylpyrrolidine,[20–22] proline sulfonamide,[23] phenoxyphenyl acrylic acid,[24,25] thiazolidine[26] have also

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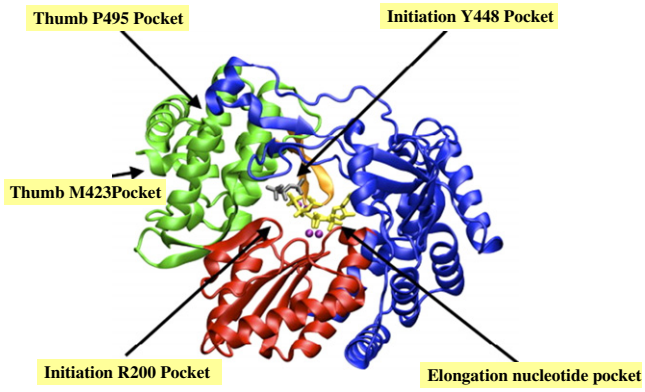
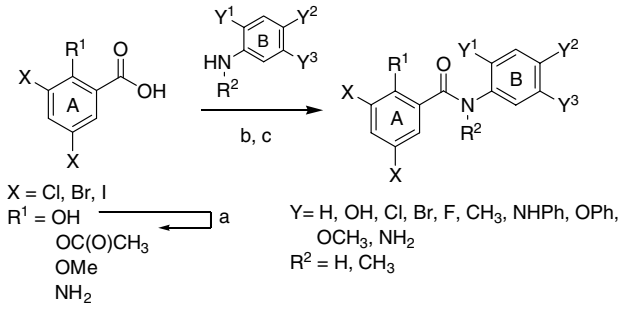


Figure 1. Ribbon diagram of the HCV RNA-dependent RNA polymerase. The fingers, palm, and thumb subdomains are colored in blue, red, and green, respectively. The catalytic site lies within the center of the polymerase domains. The β-loop is colored in orange. PDB accession code: 1GX6. Four target sites for antiviral molecules have been identified to date, including the elongation nucleotide pocket and initiation nucleotide inhibitor pocket, and allosteric thumb P495 and thumb M423 pockets (reproduced with permission).

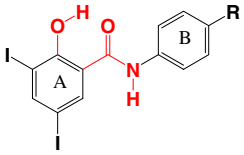
been shown to bind to this initiation pocket A. The benzofuran carboxamide[27,,28] and anthranilic acid[29] have been shown to bind to initiation pocket B that overlaps with initiation pocket A. The diketoacid derivatives have been reported as product mimics that possibly interact with the catalytic metal ions in the elongation nucleotide pocket of the enzyme active site.[30,31]

Halosalicylamide derivatives were identified from high-throughput screening as potent inhibitors of HCV NS5B polymerase. The initial structure–activity relationship of the screening hits suggested that 4,6-diiodo substitution on the phenol ring is better than dibromo and dichloro substitution. We carried out further SAR analysis on the effect of various R groups on all three substituents. The synthesis of these analogs was accomplished by amide formation from commercially available acids and anilines as illustrated in Scheme 1. The acid chlorides were formed from the halo salicylic acids and then condensed with the appropriate aniline to give the final analogs. The *O*-acetyl compound, **23**, was synthesized by first treating the salicylic acid with acetyl chloride, followed by amide formation. Compound **20**



Scheme 1. Reagents and conditions: (a) acetyl chloride, TEA, DCM, rt, 4 h; (b) $SOCl_2$, cat DMF, toluene 90 °C 1.5 h; (c) substituted aniline; dioxane 85 °C, 2 h.

Table 1. IC_{50} values against genotype 1b polymerase: effects of various R groups on the inhibition potency



Compound	R	IC_{50} (μM)
1	–H	46
2	$-CH_3$	37
3	$-NH_2$	30
4	–OH	54
5	$-OCH_3$	46
6	–F	33
7	–Cl	11
8	–Br	10
9	–OPh	5.6
10	–NHPh	1.9

was purchased from an outside vendor (Chembridge Corp.).

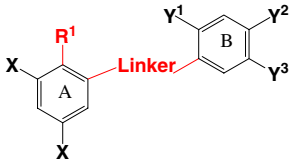
We found bulky hydrophobic groups such as phenyl ether (**9**) and phenyl amine (**10**) have the best activity as summarized in Table 1 for the diiodo substituent series. We then decided to evaluate the importance of the phenol group of the salicylamide moiety. As shown in Table 2, four pairs of compounds (**11**–**18**) with variation of R_1 as either hydroxyl group or amino group are compared. The anilines (**12**, **14**, **16**, and **18**) were about 12- to 19-fold weaker than the phenols (**11**, **13**, **15** and **17**). Substitutions on the B ring did not change activity significantly. The amide linkage in **19** can be replaced with the imine in **20** without loss of activity.

The effects methylation of the phenol hydroxyl group and the amide group of the salicylamide moiety are also investigated in Table 3. The methylation of the amide group yielded a compound (**22**) that is more than 40-fold weaker than the parent compound (**21**). A similar loss was observed for compound (**25**), with methylation of amide compound (**24**). Methylamine of the phenol group in **23**, **26**, and **27** completely abolished the activity (Scheme 2).

Our structure–activity relationship studies of halosalicylamide derivatives revealed the absolute requirement of the salicylamide moiety for optimum activity. Methylation of either the phenol group or the amide group of the salicylamide moiety abolished activity while substitutions on both phenyl rings were generally acceptable. The carboxyamide can be replaced with imine without loss of activity.

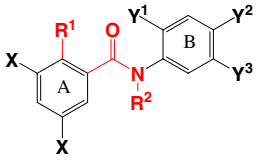
The specific binding of the halosalicylamide derivatives to HCV NS5B polymerase was confirmed by affinity selection and NMR. We then decided to characterize the mechanism of inhibition of this class of compounds. We titrated a soluble analog compound **21** with increasing concentrations of the uridine triphosphate substrate.[32] The halosalicylamide demonstrated

Table 2. IC_{50} values against genotype 1b polymerase: effects of amine replacement of the phenol hydroxyl group and the amide linkage replacement of the salicylamide moiety



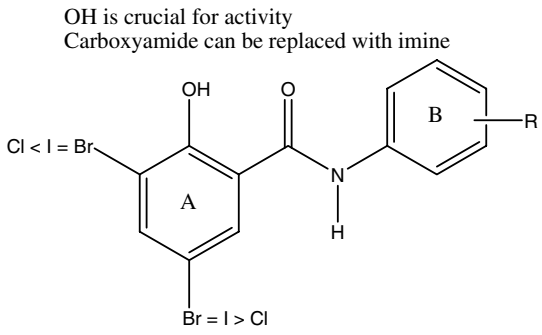
Compound	X	Y^1	Y^2	Y^3	R^1	Linker	IC_{50} (μM)
11	–Cl	–H	–Cl	–Cl	–OH	–CONH	8.6
12	–Cl	–H	–Cl	–Cl	$–NH_2$	–CONH	102
13	–Cl	–H	–Cl	$–CH_3$	–OH	–CONH	9.0
14	–Cl	–H	–Cl	$–CH_3$	$–NH_2$	–CONH	108
15	–Br	–H	–Br	–H	–OH	–CONH	6.7
16	–Br	–H	–Br	–H	$–NH_2$	–CONH	98
17	–Br	–H	–H	–Br	–OH	–CONH	6.9
18	–Br	–H	–H	–Br	$–NH_2$	–CONH	131
19	–I	–OH	–Cl	–H	–OH	–CONH	8.6
20	–I	–Cl	–Cl	–H	–OH	–CHN	8.8

Table 3. IC_{50} values against genotype 1b polymerase: effects of methylation of phenol group and the amide group of the salicylamide moiety



Compound	X	Y^1	Y^2	Y^3	R^1	R^2	IC_{50} (μM)
21	–I	–H	–Cl	–Cl	–OH	–H	4.7
22	–I	–H	–Cl	–Cl	–OH	$–CH_3$	>200
23	–I	–H	–Cl	–Cl	–OAc	–H	>200
24	–Br	–H	–Br	–H	–OH	–H	6.7
25	–Br	–H	–Cl	–H	–OH	$–CH_3$	135
26	–Cl	–H	–Br	–H	$–OCH_3$	–H	>200
27	–Cl	$–OCH_3$	–H	–H	$–OCH_3$	–H	>200

non-competitive inhibition with respect to nucleotide substrates. Competition studies using inhibitors of known binding sites indicated an additive binding mode for halosalicylamides to both the thiadiazine initiation nucleotide pocket and the diketoacid elongation



Scheme 2. SAR summary of halosalicylamides.

nucleotide pocket, but a mutually exclusive binding mode with respect to the benzimidazole thumb allosteric pocket as demonstrated in Figure 2.[32] More importantly, this class of compounds demonstrated broad genotype activity and replicon cell culture activity as shown in Table 4. However, the poor solubility of this class of compounds poses challenges for the structural biology work. Further improvement in activity and physical properties for this class of compounds is desired.

The high HCV copy number and the significant diversity of the quasispecies that are present in HCV infected patients coupled with the high potential for the development of resistance suggest that multiple drugs will likely be required to effectively treat this disease. In our high-throughput screening campaign effort, we identified halosalicylamide derivatives as a new class of allosteric inhibitors that could be used in combination with palm initiation inhibitors or

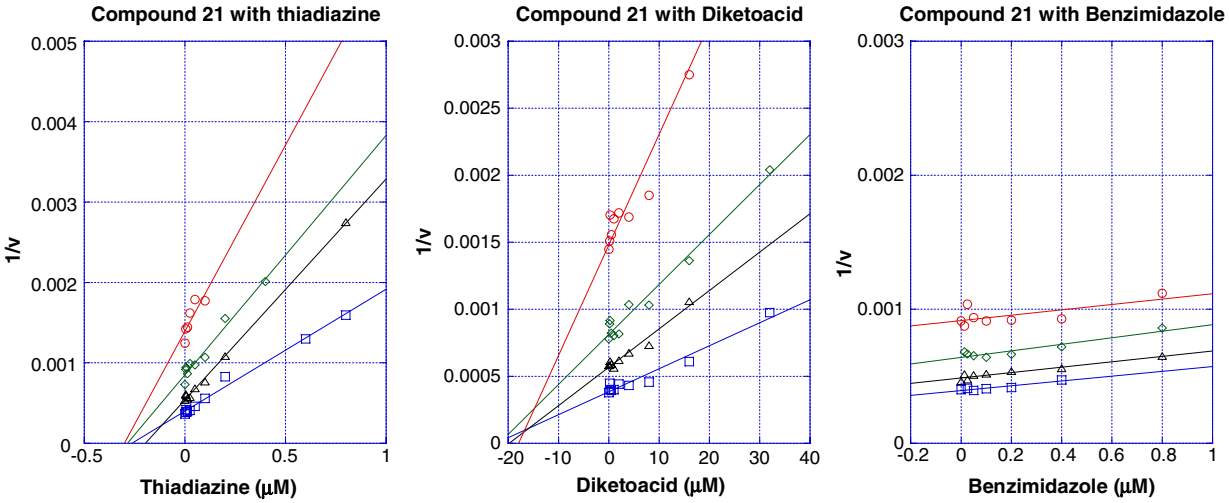


Figure 2. Inhibitor/inhibitor competition study using inhibitors of known binding sites. Various concentrations of inhibitors of known binding sites are shown on *X*-axis. Varying concentrations of compound **21** are shown as (□, Δ, ◇, and ○). The inverse of initial velocities are shown on *Y*-axis.

Table 4. Biochemical IC_{50}s against various genotypes of polymerase, replicon cell culture EC_{50} and TD_{50} of compound **21**

	1a IC_{50}	BK IC_{50}	2a IC_{50}	2b IC_{50}	3a IC_{50}	SEAP EC_{50}	MTT TD_{50}
21	5.7 μM	8.6 μM	16.2 μM	17.3 μM	14.3 μM	2.0 μM	21.8 μM

nucleosides elongation inhibitors for potential treatment of HCV infection.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.068.

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