Journal of Medicinal Chemistry

Article

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Optimization of Potent Hepatitis C Virus NS3 Helicase Inhibitors Isolated from the Yellow Dyes Thioflavine S and Primuline

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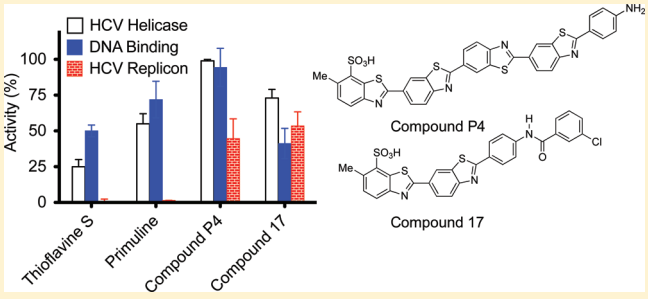
S *Supporting Information*

ABSTRACT: A screen for hepatitis C virus (HCV) NS3 helicase inhibitors revealed that the commercial dye thioflavine S was the most potent inhibitor of NS3-catalyzed DNA and RNA unwinding in the 827-compound National Cancer Institute Mechanistic Set. Thioflavine S and the related dye primuline were separated here into their pure components, all of which were oligomers of substituted benzothiazoles. The most potent compound (**P4**), a benzothiazole tetramer, inhibited unwinding >50% at 2 ± 1 μM, inhibited the subgenomic HCV replicon at 10 μM, and was not toxic at 100 μM. Because **P4** also interacted with DNA, more specific analogues were synthesized from the abundant dimeric component of primuline. Some of the 32 analogues prepared retained ability to inhibit HCV helicase but did not appear to interact with DNA. The most potent of these specific helicase inhibitors (compound **17**) was active against the replicon and inhibited the helicase more than 50% at 2.6 ± 1 μM.



INTRODUCTION

The hepatitis C virus (HCV) infects about 170 million people worldwide, causing profound morbidity and mortality.[1] HCV is typically treated with the nucleoside analogue ribavirin combined with recombinant human alpha interferon. Although such treatments are effective, therapy is poorly tolerated, expensive, and not equally effective against all HCV genotypes.[2] Better HCV treatments are therefore being modeled on other antivirals which, unlike interferon and ribavirin, directly attack proteins that HCV synthesizes in human cells. Such "direct acting antivirals" (DAAs) typically are small molecules that inhibit viral enzymes, with the most common targets being the HCV RNA polymerase and an HCV protease. Two HCV protease inhibitors, telaprevir[3] and boceprevir,[4] were recently approved for use in HCV patients, but neither alone eradicates HCV infection because HCV rapidly evolves to become resistant to these first-generation DAAs.[5] Protease inhibitors need to be administered with interferon and ribavirin and, consequently, many patients still poorly tolerate the new therapies. The overall goal of this project is to find new DAAs for HCV that might be used with telaprevir, boceprevir, or similar drugs to replace interferon and ribavirin in HCV therapy.

Telaprevir and boceprevir both inhibit the HCV nonstructural protein 3 (NS3). NS3 is one of 10 proteins derived from the ca. 3000-amino-acid-long polypeptide encoded by the HCV RNA genome. Viral and host proteases cleave the HCV polyprotein into mature structural (core, E1, E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV nonstructural proteins form four enzymes. NS5B is a polymerase that synthesizes new viral RNA. The NS2 and NS3 proteins combine to form an autocatalytic protease. NS3 and NS4A combine to form a serine protease that cuts itself, cleaves the NS4B/NS5A and NS5A/NS5B junctions and some cellular proteins. Most relevant to the present work, NS3 is also an ATP-fueled helicase that can separate and rearrange RNA/RNA, RNA/DNA, and DNA/DNA nucleic acid duplexes and displace nucleic acid bound proteins.[6]

Helicases have been widely studied as potential drug targets although progress has been slower than for other viral enzymes.[7,8] Nevertheless, HCV needs a functional helicase to replicate in cells,[9−11] and small molecules that inhibit HCV helicase-catalyzed reactions also inhibit cellular HCV RNA replication.[12−14] In this paper, we report a new class of compounds that inhibit the NS3 helicase and also act against the HCV replicon. We describe the procurement of these

Received: January 5, 2012
Published: March 12, 2012

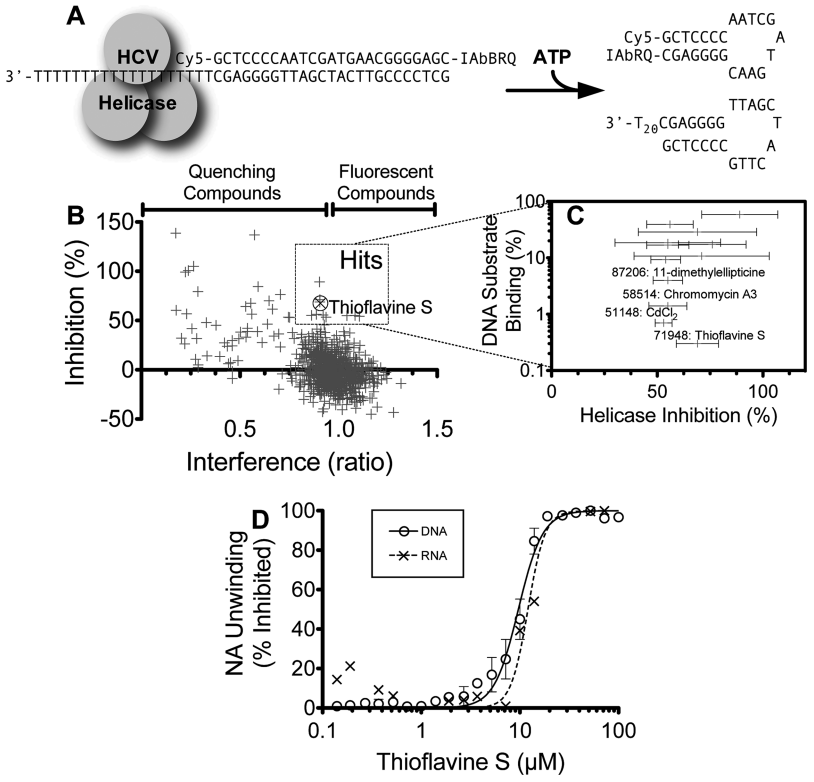


Figure 1. Discovery of thioflavine S as a HCV helicase inhibitor. (A) Schematic drawing of the MBHA mechanism. (B) The NCI Mechanistic Set of 827 compounds was screened with an MBHA, each at 20 μM. Fluorescence was read before and 30 min after ATP addition, and compound inhibition was calculated from F_{30}/F_0 ratios. Hits were defined as compounds inhibiting more than 50% and interfering less than 20%. (C) Hits from the MBHA primary screen were tested for their DNA-binding capacity with an FID counterscreen at 1.5 μM compound concentration, and percent binding was calculated. Numbers refer to NSC numbers. (D) Concentration response curves for thioflavine S when assayed in MBHAs using either a DNA or RNA substrate.

inhibitors via isolation from commercial samples of thioflavine S and primuline, two chemically related dye preparations, and through the chemical synthesis of congeners based on those isolated leads.

RESULTS

Assay Development and Screening. Numerous HCV helicase inhibitors have been reported in the literature, but many of these also bind the helicase nucleic acid substrate. HCV inhibitors that interact with DNA or RNA could also inhibit cellular nucleic acid enzymes like RNA polymerase,[15] therefore such compounds might not act like true DAAs. The goal of this study was to identify chemical probes that specifically target NS3. Such compounds are needed to better understand why the virus encodes a helicase, which may lead to better candidates for drug development. To facilitate HCV helicase inhibitor identification, Belon and Frick developed a new helicase assay that simultaneously identifies compounds that interact with the helicase substrate and compounds that inhibit helicase action.[16] This molecular beacon-based helicase assay (MBHA) uses a dual-labeled hairpin-forming DNA oligonucleotide annealed to a longer oligonucleotide, which forms a tail for the helicase to load (Figure 1A). Once ATP is added, the helicase displaces the molecular beacon, resulting in a decrease in substrate fluorescence. By comparing substrate fluorescence before ATP is added (F_0), one can identify compounds that bind the MBHA substrate. At the same time, compounds that inhibit helicase action can be identified by fluorescence changes in an MBHA before and 30 min after ATP addition (F_{30}/F_0 ratio). In other words, the MBHA can be used as an "internal" counter-screen to identify compounds that appear to affect unwinding because they interfere with the assay. The most common types of interfering compounds are those that fluoresce or absorb light at the same wavelengths as the MBHA Cy5-labeled substrate. Alternatively, other compounds may bind the DNA substrate and distort it to change how the quenching moiety on the beacon is oriented relative to the Cy5 fluorophore.

To identify HCV helicase inhibitors, the MBHA was used to screen the National Cancer Institute Developmental Therapeutics Program's Mechanistic Set Library (http://dtp.nci.nih.gov/branches/dscb/mechanistic_explanation.html). In total, 827 compounds (at 20 μM) were screened using a MBHA with a DNA substrate (Table S2, Supporting Information). When compound interference was plotted versus percent inhibition (Figure 1B), it was clear that the majority of compounds that appeared to inhibit HCV helicase also quenched fluorescence of the MBHA substrate. Compound interference in the MBHA was evaluated by comparing the fluorescence of assays containing each compound to the fluorescence of DMSO-only negative controls before the addition of ATP. Hits were defined as those compounds that did not interfere with the assays more than 20%, of which 12 were identified. These 12 hits were then subjected to a counterscreen that was designed to independently identify compounds that exhibit DNA-binding properties using a modified fluorescent intercalator displacement (FID) assay.[17] The FID counterscreen used ethidium bromide to determine a

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

Table 1. Activity for Isolated, Pure Compounds from Primuline or Thioflavine S

compd	helicase[a] IC_{50} (μM)	DNA binding (ethidium bromide)[a] EC_{50} (μM)	DNA binding (SYBR Green I)[a] EC_{50} (μM)	ATPase[a] IC_{50} (μM)
thioflavine S	24 ± 1.3	>100	61 ± 37	50 ± 17
T1	33 ± 24	>100	ND	>100
T2	26 ± 21[b]	>100	ND	ND
primuline	12 ± 1	>100	43 ± 14	67 ± 27
P1a	70 ± 31	>100	>100	>100
P1b	122 ± 5	>100	>100	>100
P2a	49 ± 45[b]	>100	ND	ND
P2b	10 ± 4.6	73 ± 36	32 ± 3	>100
P3	0.9 ± 0.1	55 ± 20	ND	15 ± 4
P4	0.9 ± 0.4[c]	15 ± 3	15 ± 8	5 ± 3

[a]Helicase (MBHA), DNA binding (FID), and ATP hydrolysis were monitored in the presence of eight different concentrations of each compound (2-fold dilution series starting at 100 μM). IC_{50} values were determined from concentration−response curves. All values are means ± standard deviations from three independent titrations with inhibitor. ND, not determined. [b]Average value from three different batches of compound. [c]Average value from two different batches of compound.

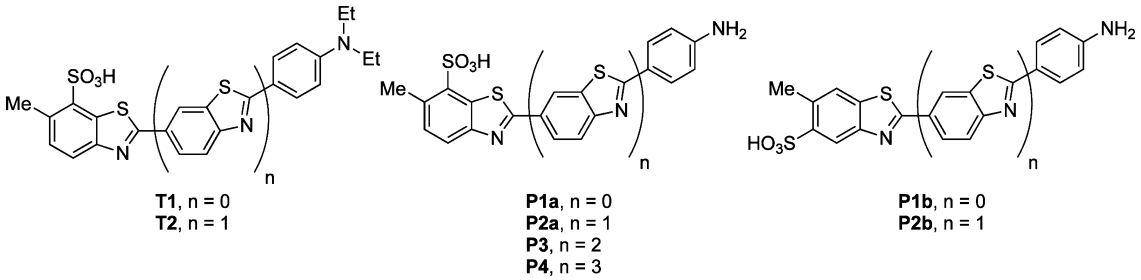


Figure 2. Structures of isolated, pure compounds from thioflavine S (**T**) and primuline (**P**) dyes.

molecule's ability to bind DNA and is based on the assumption that a DNA-binding compound displaces a fluorescent DNA intercalating agent, leading to a decrease in observed fluorescence. Compounds were tested at 1.5 μM in the presence of the 25 base pair substrate used in the helicase assays (Figure 1C). Results showed that even at a compound concentration 13 times lower than that used in the MBHA, most of the hit compounds decreased the fluorescence of an ethidium bromide−DNA complex by more than 10%, indicative of the molecule's ability to bind DNA. The DNA minor-groove-binding compound Berenil (IC_{50} = 1.6 ± 0.1 μM) was used a positive control in all FID assays.[18]

Four compounds decreased the fluorescence of DNA-bound ethidium bromide less than 8%. The first, $CdCl_2$, was a known HCV helicase inhibitor that binds in place of the magnesium ion needed for ATP hydrolysis to fuel unwinding.[19] The second, ellipticine, was found to fully quench DNA-bound ethidium bromide fluorescence at higher concentrations, and the IC_{50} value for ellipticine in MBHAs (5.6 ± 0.8 μM) was similar to its apparent affinity for DNA, suggesting that it inhibited the helicase by interacting with the substrate. Chromomycin A3 inhibited HCV helicase-catalyzed-DNA unwinding with an IC_{50} of 0.15 ± 0.03 μM, but had no effect on HCV helicase-catalyzed RNA unwinding (data not shown). This false positive can be explained by the fact that chromomycin A3 functionally resembles ethidium bromide, and they both represent fluorescent DNA-binding compounds.[20] This result also demonstrates that not all DNA binding compounds will decrease DNA-bound ethidium bromide fluorescence in an ethidium bromide-based FID assay. The final sample, thioflavine S, did not affect DNA-bound ethidium bromide fluorescence until its concentration exceeded 100 μM, at which point a 20% fluorescence decrease was observed. In concentration response experiments (Figure 1D), thioflavine S inhibited HCV helicase-catalyzed DNA-unwinding with an IC_{50} of 10 ± 1 μM, and it inhibited HCV catalyzed RNA unwinding with an IC_{50} of 12 ± 2 μM. The related dye thioflavine T, which, like thioflavine S, is used to specifically stain Alzheimer amyloid plaques,[21] had no effect on either HCV-catalyzed DNA- or RNA-unwinding (data not shown).

Purification and Characterization of HCV Helicase Inhibitors. Thioflavine S is not a single compound but rather a heterogeneous dye that is structurally related to another heterogeneous yellow dye, primuline.[22] Primuline (MP Biomedicals cat. no. 195454) inhibited HCV helicase in MBHAs with about twice the potency as thioflavine S (Table 1). To better understand how these dyes inhibit HCV helicase, both dye mixtures were separated using reverse-phase preparative HPLC or a combination of normal-phase silica gel column chromatography and reverse-phase preparative HPLC.

The structure of commercial samples of thioflavine S is not reported consistently or is left intentionally vague, creating confusion over the chemical identity of the screening hit. For instance the MSDS (Sigma Aldrich) for thioflavine S describes the compound only as "methylated, sulfonated primuline base." In the NCI and PubChem online databases, thioflavine S (NSC71948, SID550242) was reported as a mixture of methylated benzothiazoles and Packham's group reported the same structure.[23] To identify the components of commercially obtained thioflavine S responsible for the observed activity, we purified the commercial sample (Sigma cat. no. T1892) via reverse-phase preparative HPLC to obtain two compounds (**T1** and **T2**), the structures of which were assigned using NMR and LC/MS (Figure 2).

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

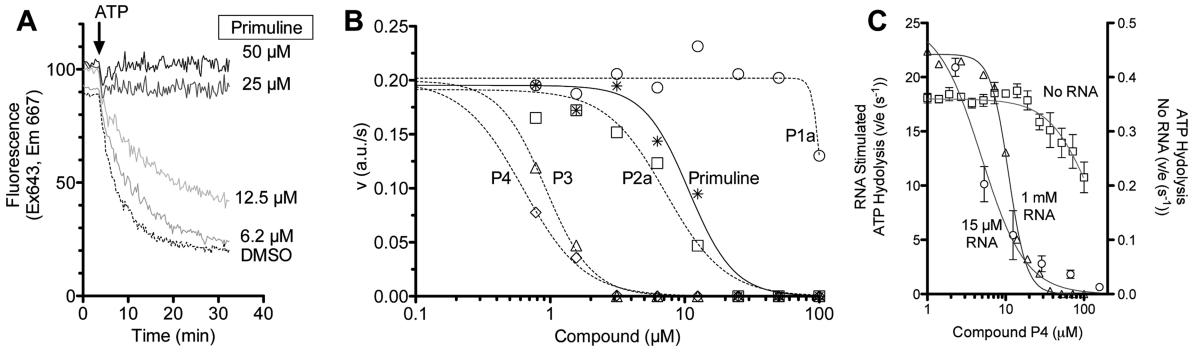


Figure 3. Effects of primuline and its components on HCV helicase-catalyzed DNA-unwinding and ATP hydrolysis. (A) MBHAs performed at various concentrations of primuline. Reactions were initiated by adding ATP at the indicated time. (B) Initial rates of DNA unwinding in MBHAs containing indicated concentrations of primuline (*), compound **P1a** (○), **P3** (△), or **P4** (◇). Data are fit to eq 3 (methods). Individual reaction time-courses and curve-fits used to calculate initial rates are shown in Figure S1 (Supporting Information). IC_{50}s listed in Table 1 are the averages from three separate titrations with each compound. (C) ATPase assays were performed in the absence of RNA (□), 15 μM (○), or 1 mM (△) poly(U) RNA (measured as μM UMP). ATP was present at 1 mM, the reactions were initiated by rapidly mixing in NS3h, and the amount of phosphate released was measured after 15 min at 26 °C. Various concentrations of **P4** were present as indicated.

Contrary to our expectation that the isolated compounds would be methylated primuline derivatives, isolated **T1** and **T2** were the *N*,*N*-diethyl products of the primuline monomeric and dimeric benzothiazoles. Both **T1** and **T2** manifested some inhibitory activity against helicase-catalyzed DNA unwinding (Figure S1, Supporting Information, Table 1), but neither was as potent as thioflavine S or primuline, suggesting that a minor component of the dye was inhibiting HCV helicase action.

In total, six compounds were purified from primuline. The two major components, **P1a** and **P2a**, were separated via reverse-phase preparative HPLC in 9.2% and 7.6% isolated yield (by weight for **P1a** and **P2a**, respectively). In the MBHA, **P2a** was significantly less potent than the primuline mixture, while **P1a** was effectively inactive. That the purified major active component, **P2a**, did not possess increased potency compared to the mixture containing the inactive **P1a** was unexpected and hinted that, perhaps, highly potent components could be present in the primuline mixture in small amounts. The direct isolation of minor components via reverse-phase preparative HPLC of the dye mixture was not successful. Hence, the purification procedure was modified, enabling the isolation of four minor components. Four chromatographic bands enriched with minor components (UV and LC-MS) were obtained from silica gel chromatography of commercial primuline upon elution with 20% DCM/MeOH. Subsequent reverse-phase preparative HPLC purification afforded the relatively minor components **P1b**, **P2b**, **P3**, and **P4**, where **P3** and **P4** represented 0.49% and 0.23% isolated yield (by weight) of the dye, respectively. All purified compounds were composed of a central benzothiazole oligomer of 1−4 units terminating with a *p*-aminobenzene group (Figure 2).

In the MBHA, all of the compounds purified from primuline were helicase inhibitors although **P1a** and **P1b** only partially inhibited unwinding at the highest concentrations tested (Figure S1, Supporting Information). Potency correlated with the length of the benzothiazole chain. For **P3** or **P4**, only about 1 μM of either was needed to reduce the rate of helicase-catalyzed DNA-unwinding by 50% (Figure 3B, Table 1).

These purified compounds resemble molecules known to bind DNA, typically along the minor groove, such as the cyanine dye known as BEBO,[24,25] but unlike many DNA-binding benzothiazoles, these helicase inhibitors are not positively charged. Instead they are anionic, due to the sulfonate group on the terminal benzothiazole rings. FID assays with the purified compounds revealed that they, indeed, possessed some ability to bind DNA. Like thioflavine S, all compounds, except **T1**, **P1a** and **P1b**, decreased the fluorescence of ethidium bromide-bound DNA by at least 10% when present at 100 μM. However, only compounds **P3** and **P4** decreased ethidium bromide-bound DNA fluorescence more than 50% at the highest concentration tested (100 μM). **P3** decreased the fluorescence of ethidium bromide-bound DNA with an EC_{50} of 55 ± 20 μM, and **P4** decreased fluorescence with an EC_{50} of 15 ± 3 μM.

Because **P3** and **P4** clearly interacted with ethidium bromide-stained DNA, we suspected that the other benzothiazoles might also bind DNA but in ways that do not displace the intercalated ethidium bromide. We therefore examined the effect of each compound on DNA stained with other dyes, and found that most compounds decreased the fluorescence of DNA stained with SYBR Green I. The affinity of primuline, thioflavine S, and the purified compounds for the MBHA substrate DNA was therefore estimated using a modified FID assay where ethidium bromide was replaced with SYBR Green I. The results were less drastic compared to those seen with ethidium bromide, with **P4** binding slightly more tightly than all other compounds (Table 1).

It should be noted that, assuming that IC_{50} values in the MBHA reflect dissociation constants for a compound-helicase-DNA complex and EC_{50} values reflect the affinity of a compound for DNA, it appears thioflavine S and primuline bind the helicase complex more tightly than they bind DNA and that **P4** binds DNA 17 times more weakly. These data suggest that little, if any, compound was bound to DNA in MBHAs at concentrations needed to inhibit helicase action, suggesting that the yellow dye-derived benzothiazoles inhibit HCV helicase directly.

If the purified benzothiazoles inhibit helicase-catalyzed nucleic acid unwinding by directly binding NS3, then they might also inhibit other functions of NS3, namely ATP hydrolysis in the presence and absence of stimulating nucleic acids. The most potent compound, **P4**, was therefore added to NS3 ATPase assays. The compound inhibited both assays in a dose-dependent manner (Figure 3C). **P4** inhibited ATP hydrolysis both in the presence and absence of RNA, indicating that the compound is not simply sequestering RNA and

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

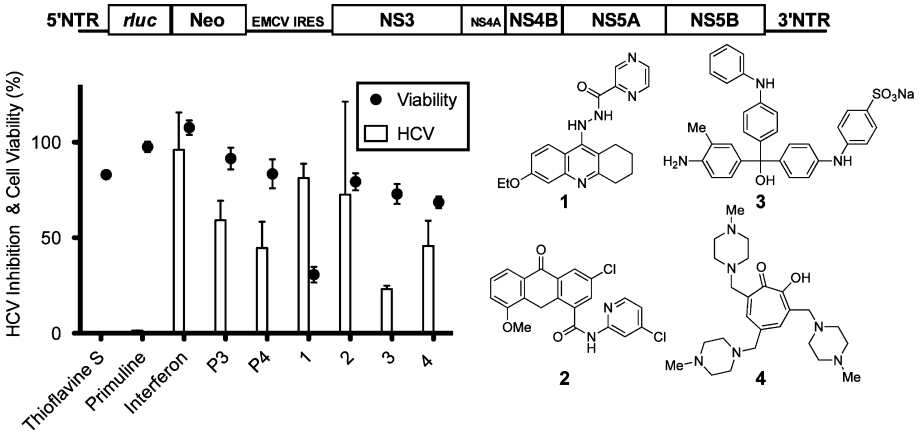


Figure 4. Effect of various compounds on Huh7.5 hepatoma cells harboring a stably transfected subgenomic *rLuc* HCV replicon. All compounds were tested at 10 μM such that cell media contained 0.5% (v/v) DMSO. Percent *Renilla* luciferase, which is proportional to HCV RNA content, is expressed with regard to cells grown in media and 0.5% DMSO. Cell viability was measured with the Titer-Glo luminescent cell viability kit (Promega) and is also expressed compared to DMSO controls.

preventing activation of ATP hydrolysis. It is also interesting to note that far more **P4** is needed to inhibit ATP hydrolysis in the absence of RNA, indicating that the presence of nucleic acid might enhance the enzyme's affinity for inhibitors (Figure 3A). It is not uncommon for helicase inhibitors to inhibit the protein's ability to hydrolyze ATP because ATP hydrolysis is needed to fuel unwinding. When ATPase assays were performed with compounds isolated from thioflavine S and primuline (Table 1), the same pattern was observed as previously seen in the MBHAs and FIDs (i.e., the longer benzothiazole oligomers were always more potent in all assays than the shorter oligomers).

Most of the compounds isolated from the two yellow dyes are fluorescent, absorbing light around 340 nm and emitting near 420 nm. Their extinction coefficients and peak absorption wavelengths increase as the length of the benzothiazole oligomer increases. Their relative fluorescence decreases with the length of the benzothiazole chain. None of the compounds absorbed light near the excitation or emission wavelengths of the Cy5-labeled MBHA substrate or the wavelengths where ethidium bromide-stained DNA, SYBR Green I-stained DNA, NS3-catalyzed peptide cleavage, or ATP hydrolysis were measured.

To test if the above compounds might be useful as HCV antiviral agents, they were added to cells harboring a HCV subgenomic RNA replicon. The HCV replicon chosen was derived from the same HCV strain (genotype 1b) as the NS3 protein used for screening and enzyme assays and was a variant of the replicon first reported by Lohmann et al.[26] with two cell culture adaptive mutations (E1202G and S2204I).[27,28] The subgenomic replicon used here also had a *Renilla* luciferase gene fused to the 5′-end of the neomycin phosphotransferase gene used for selection, so that the cellular levels of *Renilla* luciferase correlated directly with the amount of HCV RNA present in cells.[29] After replicon transfection and selection, cells were treated in parallel with one of the compounds purified from thioflavine S and primuline or one of four recently reported HCV helicase inhibitors **1**–**4**[14,30–32] in two triplicate sets (Figure 4, and Table 2). One set of cells was used for *Renilla* luciferase assays, and the other set was used to determine cell viability using a firefly luciferase-based assay, and all compounds were tested at 10 μM. While none of the compounds isolated from the yellow dyes were notably toxic to cells, only **P3** and **P4** showed any ability to decrease the amount of HCV RNA present in the cultures. The ability of **P3** and **P4** to inhibit HCV replication was similar to that of the comparison helicase inhibitors tested. None of the comparison helicase inhibitors were particularly toxic at 10 μM except for **1**. Only compound **3** inhibited MBHAs with a potency similar to the yellow dyes, although the precise effects of compounds **3** and **4** on HCV helicase action were difficult to assess because both interfered with the MBHA (Figure S2, Supporting Information).

Table 2. Activity of Reference Inhibitors under Comparison Assays

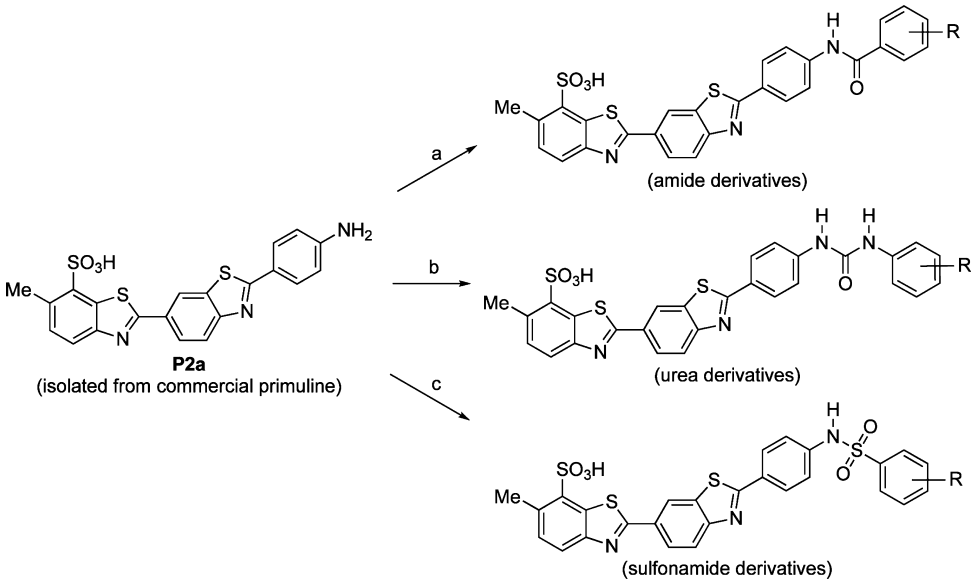
compd	helicase[a] IC_{50} (μM)	DNA binding (ethidium bromide)[a] EC_{50} (μM)	DNA binding (SYBR Green I)[a] EC_{50} (μM)	ATPase[a] IC_{50} (μM)
1	>100	>100	>100	>100
2	25 ± 6	>100	>100	>100
3	17 ± 7	4 ± 2	20 ± 2	>100
4	19 ± 8	>100	74 ± 21	>100

[a]Helicase (MBHA), DNA binding (FID), and ATP hydrolysis were monitored in the presence of eight different concentrations of each compound (2-fold dilution series starting at 100 μM). IC_{50} values were determined from concentration–response curves. All values are means ± standard deviations from three independent titrations with inhibitor. ND, not determined.

Synthesis of Primuline Analogues. The positive results obtained with some of the higher-order components of thioflavine S and primuline led us to undertake a structure–activity relationship study based on them. However, difficulties with both isolation of sufficient quantities from the commercial sources and with fully synthetic approaches necessitated that we consider more accessible bioisosteric equivalents. Accordingly, we focused on modification of the amino group at the terminus of **P2a**, which was isolable from commercial primuline in sufficient quantities to serve as a starting material. We envisioned that an appropriately rigid linker could substitute for the third benzothiazole ring of **P3**. Thus, the terminal amine of **P2a** was acylated to give amides and reacted with isocyanates or sulfonyl chlorides to give urea analogues and sulfonamides, respectively (Scheme 1). Table 3 and Figure S3 (Supporting Information), illustrate the effect of various functional groups on substituted phenyl amide derivatives. These included

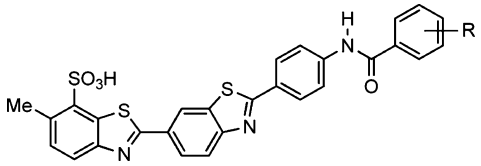
dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319–3330

Scheme 1[a]



[a]Reagents: (a) Substituted benzoyl chloride, pyridine, 80 °C; (b) arylisocyanate, DMF, 80 °C; (c) arylsulfonyl chloride, pyridine, 80 °C.

Table 3. Activity of Phenyl Amide Derivatives of P2a

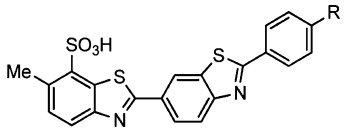


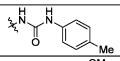
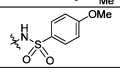
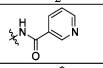
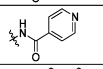
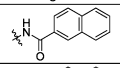
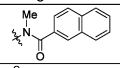
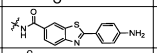
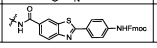
compd	R	helicase IC_{50} (μM)[a]	DNA binding (SYBR Green I) (% displaced at 100 μM)[a,b]	HCV replication (% inhibited)[a]	cell viability (% viable)[a]	assay matrix solubility[d] (μM)
5	H	11 ± 1.5	31 ± 13	45 ± 5	88 ± 2	ND[e]
6	4-NH_2	10 ± 2.4	63 ± 15	33 ± 1	93 ± 4	ND
7	4-F	5.2 ± 0.6	35 ± 15	50 ± 5	94 ± 2	129.4
8	4-OCH_3	10 ± 2.6	35 ± 10	64 ± 4	85 ± 5	ND
9	4-CO_2CH_3	9.7 ± 4.6	28 ± 10	40 ± 1	101 ± 8	ND
10	4-Cl	3.4 ± 0.3	67 ± 17	42 ± 9	84 ± 6	ND
11	4-CH_3	3.3 ± 0.3	50 ± 15	52 ± 12	87 ± 4	ND
12	4-CF_3	1.8 ± 0.4	69 ± 9	44 ± 12	90 ± 4	ND
13	4-*t*-Bu	8.2 ± 1	72 ± 19	51 ± 9	87 ± 4	ND
14	4-$N(CH_3)_2$	11 ± 6.7	44 ± 4	22 ± 2	94 ± 5	ND
15	4-Br	5.2 ± 4	70 ± 9	7 ± 18	113 ± 5	ND
16	4-NHFmoc	5.4 ± 1	76 ± 5	57 ± 21	92 ± 4	ND
17	3-Cl	2.6 ± 1	41 ± 11	54 ± 10	112 ± 4	29.2
18	3,4-di-Cl	3.7 ± 1	67 ± 12	43 ± 15	114 ± 7	2.6
19	2-CF_3	14 ± 1	30 ± 15	0 ± 9	112 ± 1	ND
20	3-CF_3	20 ± 12	46 ± 10	41 ± 8	121 ± 3	ND
21	2-F,6-CF_3	17 ± 6	66 ± 40	55 ± 7	122 ± 2	ND
22	2-F,3-CF_3	9.2 ± 3	49 ± 27	48 ± 18	122 ± 1	ND
23	3-F,4-CF_3	17 ± 17	66 ± 18	48 ± 4	129 ± 2	ND
24	3,5-di-CF_3	22 ± 4	43 ± 13	60 ± 4	122 ± 5	180.1
25	2-F,5-CF_3	6.4 ± 2	35 ± 26	39 ± 4	132 ± 14	ND
26	3-F,6-CF_3	19 ± 15	35 ± 21	61 ± 14	118 ± 4	ND
27	3-F,5-CF_3	28 ± 7	48 ± 17	51 ± 9	113 ± 1	ND

[a]Helicase (MBHA), DNA binding (SGI-FID) were monitored in the presence of eight different concentrations of each compound (2-fold dilution series starting at 100 μM). IC_{50} values were determined from concentration−response curves. All values are means ± standard deviations from three independent titrations with inhibitor. [b]Average (±SD) percent bound at 100 μM. [c]Cell viability and HCV replicon assays were performed in triplicate in the presence of 10 μM compound. Average (±SD) percent inhibition or viability is reported. [d]Solubility measurements were performed using a mock assay matrix (25 mM MOPS, 1.25 mM $MgCl_2$, 0.05 mM DTT, 5 μg/mL BSA, 0.01% v/v final [Tween 20], and 5% v/v final [DMSO]) at pH 6.5. [e]ND, not determined.

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

Table 4. Activity of Additional P2a Derivatives



cmpd	R	Helicase [a] IC_{50} (μM)	DNA binding [a,b] (% @ 100 μM)	HCV replication [c] (% inhibited at 10 μM)	Cell viability [c] (% viable at 10 μM)	assay matrix solubility[d] (μM)
28		5.3±0.9	90±9	43±9	93±1	ND[f]
29		24±2	69±4	44±5	75±3	ND
30	$NHCO_2Me$	>100	48±5	1±12	100±6	ND
31		22±2	15±10	51±22	113±6	>110[e]
32		52±20	19±7	59±13	117±5	ND
33		2.7±0.7	64±7	61±10	87±4	3.7
34		14±0.1	35±23	-30±30	98±1	ND
35		5.5±2.1	59±4	37±4	85±2	ND
36		4.0±2.4	67±5	42±5	90±4	ND

[a]Helicase (MBHA), DNA binding (SGI-FID) were monitored in the presence of eight different concentrations of each compound (2-fold dilution series starting at 100 μM). IC_{50} values were determined from concentration−response curves. All values are means ± standard deviations from three independent titrations with inhibitor. [b]Average (±SD) percent bound at 100 μM. [c]Cell viability and HCV replicon assays were performed in triplicate in the presence of 10 μM compound. Average (±SD) percent inhibition or viability is reported. [d]Solubility measurements were performed using a mock assay matrix (25 mM MOPS, 1.25 mM $MgCl_2$, 0.05 mM DTT, 5 μg/mL BSA, 0.01% v/v final [Tween 20], and 5% v/v final [DMSO]) at pH 6.5. [e]Solubility measurement was performed using MOPS buffer (25 mM MOPS, 1.25 mM $MgCl_2$, 2% v/v final [DMSO]) at pH 6.5. [f]ND, not determined.

electron-donating or -withdrawing groups as well as aliphatic moieties. While replacing the *para* substitution R═H with NH_2, OCH_3, CO_2CH_3, *t*-Bu, and $N(CH_3)_2$ had no significant effect on the potency of helicase inhibition, replacement with F, Br, and NHFmoc groups led to slightly more potent analogues (2-fold, compared to R═H). Even better analogues (3−5-fold) were obtained when Cl, CH_3, CF_3, and 2-naphthalene (**33**, Table 4, replacement of phenyl group of **5**) groups were used. The increased size of alkyl substitution from Me to *t*-Bu resulted in an ca. 3-fold activity loss. Altering the Cl position from *para* to *meta* had no effect on helicase inhibition, although the compound with *meta* substitution displayed weaker DNA binding. Increasing the number of chloro groups as in **18** showed no improvement. Moving the CF_3 group from the *para* position to the *ortho* or *meta* position resulted in a loss of inhibitory activity (7−10-fold, compounds **12**, **19**, and **20**). Introduction of additional fluorines to the derivatives also exhibited no effect on the helicase inhibition when compared to **12** (see **21**−**27**). In fact, depending on the position of fluorine substitution, a significant decrease in potency was observed. In DNA-binding assays, none of the **P2a** derivatives decreased the fluorescence of DNA-bound ethidium bromide by more than 10%, even at 100 μM. Like **P3** and **P4**, many of the derivatives appeared to bind SYBR Green I-stained DNA. However, unlike **P3** and **P4**, many of the derivatives did not displace more than 50% SYBR Green at the highest concentration tested (100 μM). Therefore, to compare the DNA-binding potential of all derivatives, the percent SYBER Green I displaced at the highest concentration tested (100 μM) was compared rather than EC_{50} values (Tables 3 and 4). Most of the amide derivatives were at least a 10-fold more potent in the MBHA than the DNA-binding assay.

Two additional classes of chemical linkers were also explored to mimic the benzothiazole moiety (Table 4). The urea analogues were synthesized from **P2a** by reacting **P2a** with different isocyanates and the sulfonamide analogues prepared via the sulfonation of **P2a** with sulfonyl chlorides. The urea analogue **28** has comparable potency in the MBHA to the amide analogue **11**, although increased DNA binding was observed (13 μM for **28** compared to >100 μM for **11**). Less potent analogues were achieved via sulfonation (e.g., **29**, 2-fold activity drop) compared to **11**. Replacing substituted phenyl with methyl resulted in complete loss of activity (**30**). Analogues targeting improved solubility by replacing the phenyl ring of **5** with the pyridine ring, produced less potent analogues (2−5-fold decrease in helicase activity, **31**, **32**). *N*-Methylation of the naphthyl analogue **33** also caused a significant drop in activity (5-fold, **34**), which could indicate the loss of a key hydrogen bond interaction.

In an effort to mimic the tetrameric structure of **P4**, the more elaborate amide derivatives **35** and **36** were synthesized. No improvements in potency were observed for the tetrameric analogues **35** and **36** over the previous trimeric analogues. The simple one-step synthesis of the trimeric analogues compared to the tetrameric analogues prompted us to focus on the former for future studies targeting more potent inhibitors of helicase function and HCV replication.

When all derivatives (**5**−**36**) were compared with the purified compounds and the recently reported helicase inhibitors (**1**−**4**), it is clear that most compounds that bound

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

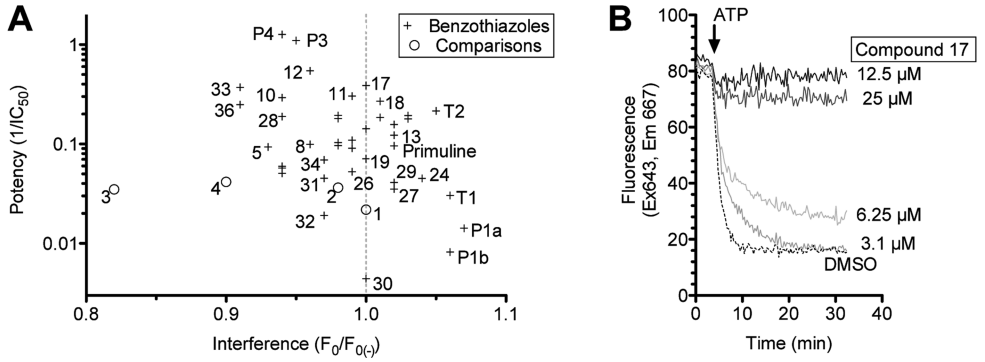


Figure 5. Comparative effects of benzothiazoles in HCV helicase assays. (A) Potency of each compound in MBHAs is plotted against the interference observed in an MBHA performed in the presence of 10 μM of each compound. Points are labeled with compound number, although some numbers are omitted for clarity. Compounds that bind the helicase DNA substrate and quench its fluorescence lie on the left side of the plot, while those that enhance substrate fluorescence are on the right. (B) Effects of various concentrations of the most potent compound that does not interfere with the MBHA (compound **17**) in standard MBHAs where Cy5 fluorescence was monitored.

Table 5. PK Evaluation of Compound 17

aqueous solubility (μg/mL)[a] (at pH)				plasma protein binding (% bound)		plasma stability[e]		hepatic microsome stability[g]		
Prisma HT buffer[a]	PBS[b]	assay matrix[c]	PAMPA Pe ($\times 10^{-6}$ cm/s)[d] (@ pH)	human 1 μM/10 μM	mouse 1 μM/10 μM	human/mouse	aqueous stability[f]	human	mouse	hepatic toxicity[h] LC_{50} (μM)
36.7 (5.0)			0 (5.0)							
>60 (6.2)	0.12 (7.4)	17.8 (6.5)	0.22 (6.2)	98/99	98/99	96.6/95.0	100	83.57	83.11	>50
>60 (7.4)			0 (7.4)							

[a]In aqueous *p*ION's Prisma HT buffer, pHs 5.0/6.2/7.4. [b]In aqueous PBS, pH 7.4. [c]In a mock assay matrix (25 mM MOPS, 1.25 mM $MgCl_2$, 0.05 mM DTT, 5 μg/mL BSA, 0.01% v/v final [Tween 20], and 5% v/v final [DMSO]) at pH 6.5. [d]In aqueous buffer; donor compartment pH's 5.0/6.2/7.4; acceptor compartment pH 7.4. [e]Percent remaining at 3 h. [f]In aqueous PBS buffer with 50% acetonitrile, pH 7.4; % remaining after 48 h at room temperature. [g]Percent remaining at 1 h. [h]Toward Fa2N-4 immortalized human hepatocytes

DNA in the FID assay also interfered with the MBHA by quenching substrate fluorescence (Figure 5A). The most potent benzothiazoles were notably more effective than the recently reported helicase inhibitors used for comparison, two of which appeared to function primarily by interacting with the DNA substrate (compounds **3** and **4**). Compound **17** (Figure 5A) was the most potent compound that did not interfere with the MBHA, and it eliminated the HCV replicon without apparent toxicity, similar to both **P3** and **P4**. Also, like **P4**, compound **17** inhibited HCV helicase-catalyzed RNA-unwinding and ATP hydrolysis (data not shown).

The pharmacokinetic (PK) properties of compound **17** were profiled using a standard panel of assays (Table 5). The most striking result is the solubility variation depending on the buffer system used. While the aqueous solubility is low in the PBS-based solvent system, in both the detergent-containing (Tween 20) assay matrix and the proprietary Prisma HT buffer system, the compound is readily soluble. The unknown identity of the components in the Prisma HT buffer system complicates further speculation into the solubility discrepancy. The solubility results have inspired us to pursue improving compound solubility through formulation with other solubilizing agents, and these experiments are currently in progress. The low solubility and permeability of compound **17** were not surprising for a polycyclic aromatic compound of molecular weight 592 g/mol. Encouragingly, compound **17** was highly stable under the various conditions screened and possessed no detectable hepatic toxicity. Analogues possessing improved solubility and PAMPA properties will be the aim of future efforts.

DISCUSSION

One of the challenges in developing chemical probes that target helicases is that potent helicase inhibitors often exert their actions by binding nucleic acid helicase substrates. Such compounds lack specificity because they can inhibit any protein that needs to access the genetic material. We attempted to discover more specific helicase inhibitors that do not target nucleic acids using high-throughput helicase and DNA-binding assays. However, even the most promising compounds, which were purified from the most active compound-library sample, interacted with the DNA substrate in the absence of protein. We have, nevertheless, been able to engineer potent analogues that interact with DNA less tightly yet still retain an ability to inhibit helicase-catalyzed nucleic-acid-strand rearrangements. Some of these compounds retain the important ability to inhibit HCV replication in cells and could therefore prove useful for antiviral drug development.

To discover helicase inhibitors that do not bind nucleic acids, we screened a compound library using a helicase assay that simultaneously detects a compound's ability to interact with the helicase substrate and its ability to interfere with the movement of HCV helicase through DNA. The results of the screen confirmed that most compound library samples that block helicase movements also interact with the helicase substrate. Using a DNA binding assay, we confirmed that most of the newly uncovered HCV helicase inhibitors interact with DNA (Figure 1C). The binding assay was based on a FID assay[17] that monitors fluorescence changes that occur when a compound interacts with DNA stained with ethidium bromide. The most active sample in the screened compound library that least

dx.doi.org/10.1021/jm300021v | J. Med. Chem. 2012, 55, 3319−3330

affected the fluorescence of ethidium bromide-stained DNA was a yellow dye called thioflavine S, and potent benzothiazole oligomers were purified from this dye and its relative, primuline. When the most active benzothiazole oligomers purified from primuline were found to interact with SYBR Green-stained DNA, we learned that the ethidium bromide-based FID failed to detect the interaction of thioflavine S with DNA. A more sensitive SYBR Green I assay was therefore developed and used to chemically optimize more specific **P2a** derivatives. The observation that DNA interactions escaped detection in the ethidium bromide-based FID reinforces the notion that care needs to be taken when using FID assays because DNA-binding compounds might not displace the bound intercalator. There is likewise still some uncertainty as to whether or not the **P2a** derivatives that failed to influence the fluorescence of SYBR Green-stained DNA interact with nucleic acids. Preliminary results using isothermal titration calorimetry support the relative affinities reported here, but more extensive DNA- and RNA-binding experiments clearly need to be done with these compounds.

The compounds reported here are more potent and specific than other recently reported HCV helicase inhibitors (Table 2, Figure 5). However, we have not been able to reproduce all of the results previously reported for the comparison helicase inhibitors. For example, the nucleotide mimic (compound **1**), which had been reported to have a K_i of 20 nM,[30] had almost no detectable effect on the MBHA at 5000 times higher concentration (Table 2 and Figure S2, Supporting Information). Compound **1** had some antiviral activity, and it was coupled with notable toxicity at 10 μM (Figure 4). The acridone (compound **2**) was about 17-fold less potent in our helicase assay than it was in a previously reported assay, yet it still was one of the most effective compounds in eliminating the HCV replicon. Similarly, although its interaction with the helicase substrate obscured effects in our MBHA, the triphenylmethane (compound **3**) displayed no inhibition at its previously reported IC_{50} of 12 μM and the compound did not appear as cytotoxic as had been reported.[32] Compound **4** had antiviral activity as previously reported, but again, compound interference in the MBHA made its effect on the helicase in vitro difficult to judge.[31] Our different results could be due to several factors, including the use of different recombinant HCV helicase proteins and assay conditions (Figure S2, Supporting Information).

CONCLUSIONS

In conclusion, we have found that the commercial dyes thioflavine S and primuline contain potent compounds for the inhibition of the NS3 helicase of HCV. We show here that minor components of primuline inhibit both the HCV helicase and HCV replicon replication. The antiviral potential of these trimeric and tetrameric benzothiazoles inspired the derivatization of the more abundant dimeric constituent. Several derivatives were found to be close in potency to the isolated trimer or tetramer in the MBHA and to possess improved DNA binding profiles. Importantly, the antiviral potential of this class of helicase inhibitors does not appear to depend entirely on either their ability to inhibit HCV helicase or bind DNA. We speculate that the ability to inhibit HCV replication results from a compound's ability to enter Huh7.5 cells, which can be monitored by examining compound fluorescence when they are administered to cells. In cultures, both primuline and thioflavine S mainly stain membranes, but preliminary data suggest that some of the derivatives might enter cells. Effects of the most potent replicon inhibitors on HCV replication (e.g., **11**, **17**, **24**, and **26**) are presently being examined in more detail. The results of these studies will be used to inform additional chemistry efforts toward helicase inhibitors, which is an ongoing concern of our laboratories.

EXPERIMENTAL SECTION

Materials. Thioflavine S and primuline were purchased from Sigma (cat. no. T1892, lot no. 048K1656) and MP Biomedicals (cat. no. 195454, lot no. 7792J), respectively. The Mechanistic Diversity Library was obtained from the National Cancer Institute (NCI, http://dtp.cancer.gov/repositories.html). All other reagents were purchased from commercial suppliers and used as received. Methylene chloride, acetonitrile, toluene, ethyl ether, and THF were dried by being passed through two packed columns of anhydrous, neutral alumina prior to use. HPLC/MS analysis was carried out with gradient elution (5% CH_3CN to 100% CH_3CN) on an Agilent 1200 RRLC with a photodiode array UV detector and an Agilent 6224 TOF mass spectrometer. Compound purity was determined using RP HPLC and was measured on the basis of peak integration (area under the curve) from UV/vis absorbance (at 214 nm), and compound identity was determined on the basis of exact mass analysis. All compounds used for biological studies have purity >95% (Table S1, Supporting Information) except for the following compounds: **P3** (89.2%), **P4** batch 2 (85.6%), **6** (90.0%), **11** (89.1%), **14** (80.4%), **17** (93.6%), **28** (93.2%), **32** (94.4%), and **36** (88.6%).

All oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The partially duplex DNA substrates used in MBHAs consisted of a helicase substrate forming 25 base pairs and consists of a 45-mer bottom strand 5′-GCT CCC CGT TCA TCG ATT GGG GAG C$(T)_{20}$-3′and the 25-mer HCV top strand 5′-Cy5-GCT CCC CAA TCG ATG AAC GGG GAG C-IBRQ-3′. The 19 base pair RNA substrate used in MBHAs consisted of a 39 nucleotide long bottom strand 5′-AGU GCC UUG ACG AUA CAG $C(U)_{20}$-3′ and the 24 nucleotide long top strand 5′-Tye^{665}-AGU GCG CUG UAU CGU CAA GGC ACU-IBRQSp-3′. Underlined areas denote hairpin-forming regions. DNA and RNA substrates were annealed and purified as described previously.[16]

The cloning, expression, and purification of His-tagged recombinant HCV NS3 protein have been described previously.[33−36]

Helicase Assays. All molecular beacon-based helicase assays (MBHAs) were performed as described before.[16,36] For screening the NCI library, MBHAs contained 25 mM MOPS pH 6.5, 1.25 mM $MgCl_2$, 5.0 nM MBHA substrate, 12.5 nM NS3h_1b(con1), 5 μg/mL BSA, 0.01% (v/v) Tween20, 0.05 mM DTT with 20 μM each test compound (2% v/v final DMSO). In each flat, black 384-well plate, 56 compounds were tested, in triplicate, along with three negative controls (DMSO only), three positive controls (500 nM dT_{20}), and two wells with no enzyme. Fluorescence was read before ATP (F_0) addition and 30 min after ATP was added to 1 mM (F_{30}) using a Tecan Infinite M200 fluorescence microplate reader with excitation and emission wavelengths set to 643 and 670 nm, respectively. Percent inhibition was calculated with eq 1, and compound interference in the MBHA was calculated with eq 2.

$$\text{inhibition}(\%) = ((Fc_0/Fc_{30}) - (F(-)_0/F(-)_{30}) / (1 - (F(-)_0/F(-)_{30}))) \times 100 \quad (1)$$

$$\text{interference (ratio)} = (Fc_0/F(-)_0) \quad (2)$$

In eqs 1 and 2, Fc_0 is the fluorescence of the reactions containing the test compound before adding ATP, Fc_{30} is the fluorescence of the test compound reaction 30 min after adding ATP. $F(-)_0$ is the average of three DMSO-only negative control reactions before adding ATP and $F(-)_{30}$ is the average of three DMSO-only reactions 30 min after adding ATP.

To monitor helicase reaction kinetics and to calculate IC_{50} values, MBHAs were performed in 60 μL in white 1/2 area 96-well plates and

measured in a Thermo Varioscan Multimode reader (Thermo Scientific) using the 643 nm excitation wavelength and 667 nm emission wavelengths. Reactions were again performed by first incubating all components except for ATP for two minutes, then initiated by injecting in 1/10 volume of ATP such that the final concentration of all components was as noted above. Conditions were as described above except that 5% v/v DMSO was present in each assay. Initial reaction velocities were calculated by fitting first-order decay equation to data obtained after ATP addition and calculating an initial velocity from the resulting amplitude and rate constant. The concentration at which a compound causes a 50% reduction in reaction velocity (IC_{50}) was calculated by fitting compound concentration to initial velocity using eq 3:

$$\nu_i = \nu_0 / \left(1 + ([I]/IC_{50})^h\right) \quad (3)$$

where ν_0 is the velocity observed in DMSO-only controls-inhibition, h is the Hillslope coefficient, and [I] is the concentration of test compound.

DNA Binding Assays. Fluorescent intercalation displacement (FID) assays[18] were used to measure the ability of a compound to bind the MBHA substrate. The concentration at which half the ethidium bromide is displaced (EC_{50}), was determined using the different conditions as above to more closely mimic the conditions of a standard helicase assay. Each 100 μL reaction contained 25 mM MOPS pH 6.5, 0.16 μM MBHA DNA substrate (lacking Cy5 and IBQ-RQ modifications), 2 μM ethidium bromide, and various concentrations of test compound. Fluorescence of ethidium bromide was monitored using excitation and emission wavelengths of 545 and 595 nm, respectively, on a Cary Eclipse fluorescence spectrophotometer in white 96-well plates. The amount of ethidium bromide-DNA complex fluorescence was used to estimate the ability of compound to bind DNA, and therefore displace the fluorescent intercalator (ethidium bromide).

$$\text{binding} = (1 - ((Fc - F(+))/(F(-) - F(+)))) \times 100 \quad (4)$$

In eq 4, Fc is the fluorescence in the presence of the compound, $F(-)$ is the average DMSO-only negative controls, and $F(+)$ is the average positive controls (100 μM berenil). EC_{50} values were from a normalized concentration−response curve.

A modified FID assay in which ethidium bromide was replaced with SYBR Green I (Invitrogen) was used to estimate a compound's affinity for the MBHA substrate. Reactions were performed as described above except that the DNA substrate was present at 0.32 μM, ethidium bromide was absent, and SYBR Green was present at (0.68 μM). Data were normalized as described above and fit to concentration−response equation using GraphPad Prism software. Titrations with each compound were performed in triplicate, and EC_{50} values from three independent titrations are reported ± standard deviations. Average percent bound at 100 μM is reported for compounds that did not decrease the fluorescence of SYBR Green stained DNA more than 50% at the highest concentration tested.

ATP Hydrolysis Assays. A modified "malachite green" assay was used to measure ATP hydrolysis.[37] The 50 μL reactions contained 25 mM MOPS pH 6.5, 5 mM $MgCl_2$, 2 mM ATP, 5 μg/mL BSA, 0.01% (v/v) Tween20, 0.05 mM DTT, and poly(U) RNA (Saint Louis, MO) as indicated. Reactions were initiated by adding NS3h_1b(con1). Reactions were incubated for 15 min at 37 °C then stopped by mixing 40 μL of the reaction into 200 μL of the malachite green reagent (3 volumes 0.045% (w/v) malachite green, 1 volume 4.2% ammonium molybdate in 4N HCl, 0.05 volume of 20% Tween 20). Then 25 μL of 34% sodium citrate was quickly added to each reaction and color allowed to develop for 15 min. Absorbance at 630 nm was proportional to the concentration of phosphate produced; free phosphate produced was measured against a standard curve.

HCV Subgenomic Replicon Assay. An HCV *Renilla* luciferase (HCV RLuc) reporter construct was used to measure the effect of each compound on cellular HCV RNA levels. The replicon was a generous gift from Seng-Lai Tan. In HCV RLuc, the HCV internal ribosome entry site (IRES) drives the translation of the neomycin and *Renilla* luciferase genes, while the HCV nonstructural proteins (NS3 to NS5B) are translated from the encephalomyocarditis virus IRES.[29] The plasmid DNA was cleaved with *Sca* I, purified by phenol/chloroform extraction followed by ethanol precipitation, and used as template for RNA transcription using MEGAscript T7 RNA transcription kit (Ambion, Austin, TX). The RNA transcripts were treated with 2 U DNase I (Ambion) at 37 °C for 30 min, purified by acid phenol/chloroform extraction, followed by isopropyl alcohol precipitation, and suspended in diethylpyrocarbonate-treated water. RNA concentration was determined by spectrophotometry by measuring the A_{260}. RNA integrity and size was checked on 1% agarose gel. Transcribed RNA was stored in aliquots at −80 °C until needed.

Huh-7.5 cells RNA were transfected with HCV RNA by electroporation. Briefly, subconfluent Huh7.5 cells were trypsinized, suspended in complete growth medium, and centrifuged at 1000*g* for 5 min at 4 °C. The cell pellets were then washed twice with ice-cold phosphate-buffered saline (PBS) and suspended at 1.75×10^7 cells/mL in ice-cold PBS. Replicon RNA (5 μg) was mixed with 0.4 mL of cell suspension and transferred to 2 mm gap width electroporation cuvette (Eppendorf AG, Germany) and pulsed with five times for 99 μs at 820 V over 1.1 s intervals using the ECM 830 electroporator instrument (BTX Havard Apparatus, Holliston, MA). After 5 min recovery period at room temperature, cells were transferred to 10 mL complete growth medium and seeded into 10 cm diameter cell culture dishes. Twenty-four hours after transfection, the medium was replaced with fresh complete DMEM supplemented with 1 mg/mL Geneticin (Invitrogen) and the medium was replaced every three to four days with fresh medium containing 1 mg/mL Geneticin. Geneticin-resistant colonies were selected for a period of two weeks and expanded in the presence of 250 μg/mL Geneticin.

HCV RLuc replicon cells were seeded at a density of 10×10^3 cells per well in 96-well plates and incubated for 4−5 h to allow the cells to attach to the plate. The compounds dissolved in dimethyl sulfoxide (DMSO) were added at a final concentration of 10 μM (DMSO solvent final concentration was 0.5%), and the cells were incubated for 72 h at 37 °C under 5% CO_2 atmosphere. The effects of compounds on HCV replication were then assessed by measuring the *Renilla* luciferase activity in compound-treated versus DMSO-treated cells. At the end of the incubation period, the medium was aspirated and the cells were washed with 1× PBS. The *Renilla* luciferase reporter gene assay was performed using the *Renilla* luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the cells were lysed by addition of 50 μL of 1× *Renilla* luciferase lysis buffer followed by two cycles of freeze/thaw. The luciferase activity content of the lysate was measured with a FLUOstar Omega microplate reader instrument (BMG Labtech, Germany) after injecting 50 μL of luciferase substrate and reading for 5 s.

Cell Viability Assay. To assess compound toxicity toward Huh-7.5 cells, cells were plated and treated as above and cell viability was assessed using the CellTiter-Glo luminescent cell viability kit (Promega) following the manufacturer's instructions. Briefly, at the end of a 72 h incubation period, the medium was aspirated and the cells were washed with 1× PBS, and then an equal volume of growth medium and CellTiter-Glo reagent was added and the lysis was initiated by mixing on an orbital shaker. The plate was incubated at 23 °C for 30 min, and the luciferase activity was measured for 1 s using the FLUOstar Omega microplate reader (BMG Labtech).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC purity analysis and screening results of the NCI Mechanistic Set. Sample helicase assays with various concentrations of each compound. General protocols for the PK assays, experimental details for the isolation of primuline and thioflavine S components, the synthesis of compounds **5**−**36**, and compound characterization data, including HPLC purity

and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants U54 HG005031, R03 MH085690-01, and R01 AI088001 and a Research Growth Initiative Award from the UWM Research Foundation. We thank Dmitriy Minond, Peter Hodder, Jill Ferguson, and Katharine Emery of the Scripps Research Institute, Florida, for help with assay development and for uploading the screening results into PubChem BioAssay. We are also grateful to Doreen Badheka, Yoji High, William Shadrick, and John J. Hernandez for their help with various enzymatic assays, and to Seng-Lai Tan for providing the HCV replicon. Finally, we thank Arianna Mangravita-Novo, Michael Vicchiarelli, and Layton H. Smith of the Sanford–Burnham Medical Research Institute at Lake Nona for providing the solubility and PK profiling measurements.

ABBREVIATIONS USED

HCV, hepatitis C virus; DAA, direct acting antiviral; NS3, nonstructural protein 3; MBHA, molecular beacon-based helicase assay; FID, fluorescent intercalator displacement; PK, pharmacokinetics

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