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***N*-Naphthoyl-substituted indole thio-barbituric acid analogs inhibit the helicase activity of the hepatitis C virus NS3**

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Abstract

The hepatitis C virus (HCV) represents a substantial threat to human health worldwide. The virus expresses a dual-function protein, NS3 having both protease and RNA helicase activities that are essential for productive viral replication and sustained infections. While viral protease and polymerase inhibitors have shown great successes in treating chronic HCV infections, drugs that specifically target the helicase activity have not advanced. A robust and quantitative 96-well plate-based fluorescent DNA unwinding assay was used to screen a class of indole thio-barbituric acid (ITBA) analogs using the full-length, recombinant HCV NS3, and identified three naphthoyl-containing analogs that efficiently inhibited NS3 helicase activity in a dose-dependent manner, with observed IC₅₀ values of 21–24 μM. Standard gel electrophoresis helicase assays using radiolabeled duplex DNA and RNA NS3 substrates confirmed the inhibition of NS3 unwinding activity. Subsequent anisotropy measurements demonstrated that the candidate compounds did not disrupt NS3 binding to nucleic acids. Additionally, the rate of ATP hydrolysis and the protease activity were also not affected by the inhibitors. Thus, these results indicate that the three ITBA analogs containing *N*-naphthoyl moieties are the foundation of a potential series of small molecules capable of inhibiting NS3 activity via a novel interaction with the helicase domain that prevents the productive unwinding of nucleic acid substrates, and may represent the basis for a new class of therapeutic agents with the potential to aid in the treatment and eradication of hepatitis C virus.

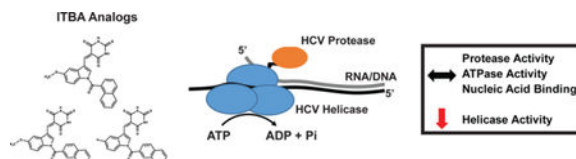
Graphical Abstract

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Keywords

helicase; Hepatitis C virus; nonstructural protein 3; thio-barbituric acid.

Hepatitis C virus (HCV) is a world-wide health concern as it affects an estimated 71 million individuals¹ with at least 2.7 million people in the US living with chronic viral infection.² Untreated, HCV can lead to hepatic fibrosis, cirrhosis and hepatocellular carcinoma.³ Powerful new therapies have been developed to combat viral infection, but with continued heavy use and the error-prone nature of viral replication⁴, resistant viruses will continue to appear in the population. Without a viable vaccine, low cost new drugs specifically targeting critical viral components are needed to alleviate the health risks to infected individuals, and provide opportunities to continue the therapeutic successes into the future.

The HCV genome encodes several non-structural enzymes and accessory proteins as well as structural coat and envelope proteins for virion assembly and productive infections. Translation of the genome results in a single polyprotein that is proteolytically processed by host peptidases and a virally encoded protease into functional proteins.⁵ In addition, replication requires the helicase activity of NS3 to unwind secondary structures in the nascent transcript, and NS5B, a functional RNA-dependent polymerase.⁶ Interestingly, NS3 is a dual-function viral protein with an N-terminal protease domain involved in viral processing, and a C-terminal helicase domain that is classified as a member of the superfamily 2 (SF2) DExH/D-box helicases.^{7, 8} NS3 has an affinity for single-stranded RNA and DNA, and is able to unidirectionally translocate in the 3' to 5' direction along the nucleic acid strand to displace nucleic acid binding proteins⁹ that may impede NS5B polymerase progression as well as unwinding secondary structures and duplex RNA that form in the large viral genome.

Recent therapeutic successes with compounds specifically targeting the HCV RNA polymerase (NS5B), RNA binding protein (NS5A) and N-terminal protease (NS3-protease), either as single drug regimens or in a combinational cocktail,^{10, 11} demonstrate that severely disabling the viral replication complex without toxic damage to hepatocyte function leads to viral eradication in the patient. Despite its essential function during HCV replication, however, few successes have been reported targeting the NS3 helicase activity, perhaps owing to the conserved structure of NS3 helicase domain and the essential nature of endogenous helicase activity in a multitude of cellular processes. Effective direct-acting antiviral agents specifically inhibiting the NS3 helicase activity without significant off-target effects have been particularly difficult to identify.

Penthala *et al*¹² described the rapid synthesis scheme for this series of *N*-aroyl-substituted indole thio-barbituric acid (ITBA) analogs (Figure 1A) by refluxing a selection of *N*-

aroylindole-3-carboxaldehyde precursor molecules with 2-thiobarbituric acid in methanol. The resulting 5((1-*aroyl*-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione analogs exhibited low micromolar potency as anti-neoplastic agents against a large library of cancer cells¹². Subsequently, Coggins *et al*¹³ and Zafar *et al*¹⁴ used the ITBA library in search of inhibitors of the stress-response human DNA polymerase η . Their structure-activity data suggested that the ITBA analogs, particularly those containing an indolic *N*-naphthoyl substituent, bound with micromolar affinities in and around the nucleic acid binding site, and inhibited the polymerase with selectivity over other cellular polymerases¹³. The encouraging results from these studies with a nucleic acid binding protein led us to use the ITBA library in search of compounds that would specifically interact with the helicase domain of HCV NS3 and provide the basis for a new class of HCV therapeutic compounds.

Initially, we employed a real-time FAM-based quantitative assay as a first-pass screen of the ITBA library to determine the extent of compound-mediated inhibition of helicase catalyzed DNA unwinding (See Supplemental Data and Figure S1). In the presence of saturating amounts of ATP and Mg^{2+} , recombinant full-length NS3 efficiently unwound the duplex DNA substrate as measured by the increase in FAM fluorescence. Of the twenty-one ITBA analogs screened, three (Analog **14**, **16** and **19** (Figure 1B)) inhibited NS3 helicase activity at 30 μ M concentration (Figure 2), and were selected for further study. Interestingly, all three analogs contained an *N*-1-naphthoyl or *N*-2-naphthoyl substituent consistent with enhanced DNA polymerase η inhibition through suspected interactions with nucleic acid binding motifs.¹⁴ The interaction of the ITBA analogs with NS3 also seem to favor the 5-methoxyindole moiety (analog **16** and **19**), although analog **14** had no 5-substituent in the indole ring, yet exhibited significant inhibition of helicase activity. However, the presence of a 5-chloroindole moiety (analog **15** and **18**) exhibited poor inhibition, suggesting an important role for the 5-indole substituent in NS3 binding. Using the fluorescent DNA unwinding assay, we also obtained an IC_{50} value in the range of 21–24 μ M for the ITBA analogs (Table 1) suggesting that analogs **14**, **16** and **19** were reasonably potent NS3 helicase inhibitors worthy of further study.

To investigate the effects of analogs **14**, **16** and **19** on the DNA unwinding activity of NS3 more rigorously, we utilized a polyacrylamide gel-based unwinding assay in the presence of 25 μ M of the inhibitors. Employing a radiolabeled duplex DNA substrate in the presence of excess ATP, enzyme activity was measured as the amount of the substrate that was unwound by the NS3 helicase at the designated time points, plotted and fit to a single exponential (Figure 3). The resulting reduction in the rate of duplex DNA unwinding closely agreed with our real-time assay indicating that the presence of the inhibitor reduced NS3 DNA unwinding activity. These results provide strong evidence that the ITBA analogs form specific interactions with the enzyme to reduce the ability of NS3 to unwind DNA substrates. Thus, using recombinant HCV NS3, we were able to screen multiple potential inhibitory compounds using a 96-well plate real-time assay to identify potential candidate compounds as NS3 helicase inhibitors and confirm their activity with additional helicase activity measurements.

Since RNA is the natural substrate for NS3 during viral replication, we also investigated RNA duplex unwinding in the same polyacrylamide-based unwinding assay utilizing a 38 bp duplex RNA (Figure 4). Our RNA substrate was susceptible to degradation resulting in a faster migrating RNA duplex species that melted upon heating (Figure 4, **compare the first lane to the last lane**). Since NS3 does not bind and unwind duplex RNA lacking a single-stranded 3' tail,²³ we considered only the loss of dsRNA and the appearance of the ssRNA in our evaluation of the RNA helicase activity. Curiously, the ITBA analogs had less of an effect on the rate of RNA unwinding than DNA unwinding, perhaps owing to the different length of the nucleic acid duplex substrates. Although the rate of product formation was similar under the conditions of our experiments, we observed a 50% reduction in the quantity of NS3-mediated product with all three compounds supporting the hypothesis that the binding of the inhibitors to the full-length NS3 protein has a significant and specific effect on both the DNA- and RNA-dependent helicase activities.

Multiple reports have described HCV NS3 helicase inhibitors that disrupt or modify the nucleic acid binding region of the protein to alter the viral replicative capacity.^{15–19} To determine if the mode of inhibition includes the disruption of protein-nucleic acid interactions, we used fluorescence polarization to measure the anisotropy of the NS3-DNA complex to determine the equilibrium dissociation constant (K_D) in the presence of analogs **14**, **16** and **19**. Our results indicated that the K_D for the NS3 binding to ssDNA was unaffected by 25 μM of the ITBA analogs (Figure 5) indicating that the ITBA analogs do not interfere with nucleic acid binding to the helicase.

The interaction of the NS3 protease with the helicase domain is important for NS3 activity,²⁰ therefore we measured the effect of the ITBA analogs on the protease activity of recombinant NS3-4A, an isolate with full protease activity using an EDANS/DABCYL fluorescent peptide substrate. While 10 μM telepravir, a well-characterized NS3 protease inhibitor,¹⁰ reduced the protease activity by 85%, the ITBA analogs **14**, **16** and **19** had no effect (Figure 6) on the protease activity of the protein. Thus the inhibition of NS3 helicase activity does not involve interference with the active site of the protease or its proteolytic activity, *per se*.

Finally, since NS3 utilizes the energy from the hydrolysis of ATP to unwind nucleic acid duplexes, the RNA-stimulated ATPase activities of NS3 at sub-saturating and saturating concentrations of ATP were measured using a coupled spectrophotometric assay. The maximal rate of ATP hydrolysis with excess polyU RNA (75 μM nt) and ATP (5 mM) was unaffected by 25 μM of ITBA analogs **14**, **16** or **19**, unlike the inhibition of the helicase activity with similar ATP concentrations (Figure 7). At sub-saturating ATP concentrations, the presence of the inhibitors had a small effect on the ATPase rate and the calculated K_M indicating that at low concentrations of ATP, the ITBA analogs may affect the association of the ATP in the active site. Based on the conditions in our nucleic acid unwinding assays, we contend that the observed increase in the K_M would have a minor contribution to the inhibition of the helicase activity. Taken together, our results suggest that the ITBA analogs have a unique binding site affecting NS3 activity that does not interfere with nucleic acid binding, protease activity or ATPase activity.

In conclusion, we evaluated a series of 5((1-aryl-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione analogs as HCV NS3 helicase activity inhibitors using a rapid plate-based fluorescent assay, and confirmed the ability of analogs **14**, **16** and **19** to inhibit the nucleic acid unwinding ability of NS3 with an IC₅₀ of approximately 21 μM without directly affecting nucleic acid binding, ATP hydrolysis or the protease activity. These small compounds are unique, since their apparent mode of action does not seem to involve the disruption of nucleic acid binding as reported for other helicase inhibitors.^{15–19} Full-length NS3 containing both the helicase and the protease domains has been shown to require conformational changes^{21, 22} in order to efficiently unwind duplex RNA. The unique binding properties of the naphthoyl moiety in the ITBA analogs **14**, **16**, and **19** to NS3 may involve a specific disruption of the conformational changes leading to greatly reduced helicase activity. The discovery of these three candidate molecules that inhibit viral helicase activity via a novel mechanism is an exciting start for this new class of compounds. Further refinements in the drug-likeness, water solubility and efficacy of these compounds will allow for a strong foundation to build the next-generation of HCV NS3 therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

NS3	nonstructural protein 3
HCV	hepatitis C virus
ITBA	indole thio-barbituric acid
ssDNA	single-stranded DNA
dsDNA	Double-stranded DNA
DMSO	dimethylsulfoxide
FAM	6-carboxyfluorescein

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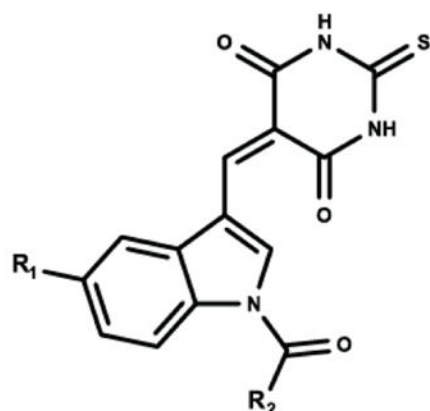
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Highlights

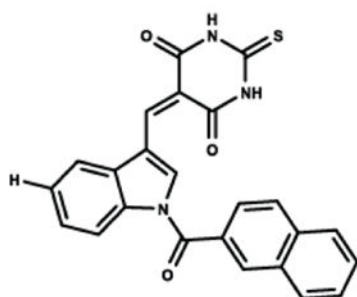
- Hepatitis C Virus NS3 helicase activity is inhibited by *N*-naphthoyl-containing indole thiobarbituric acid analogs
- Both RNA and DNA-dependent unwinding activities of NS3 helicase are inhibited.
- Nucleic acid binding, protease and ATPase activities are not inhibited by the compounds.

A.

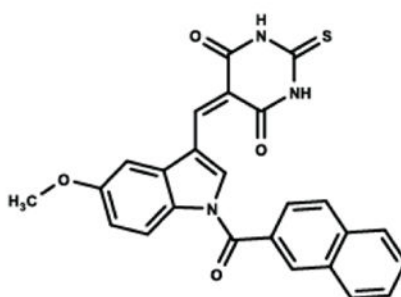


Compound	PubChem CID	R ₁	R ₂
1	71561621	H	C ₆ H ₅
2	71561891	H	2-Br-C ₆ H ₄
3	71561892	Cl	2-Br-C ₆ H ₄
4	71562016	OCH ₃	2-Br-C ₆ H ₄
5	71561622	Cl	C ₆ H ₅
6	71561624	OCH ₃	C ₆ H ₅
7	71562015	Br	2-Br-C ₆ H ₄
8	71561623	Br	C ₆ H ₅
9	71561759	H	4-OCH ₃ -C ₆ H ₄
10	71561760	Cl	4-OCH ₃ -C ₆ H ₄
11	71561761	Br	4-OCH ₃ -C ₆ H ₄
12	71561762	OCH ₃	4-OCH ₃ -C ₆ H ₄
13	71561625	H	4-F-C ₆ H ₄
14	71562140	H	1-naphthyl
15	71562141	Cl	1-naphthyl
16	71562142	OCH ₃	1-naphthyl
17	71562017	H	2-naphthyl
18	71562018	Cl	2-naphthyl
19	71562020	OCH ₃	2-naphthyl
20	71561887	H	4-CN-C ₆ H ₄
21	71561889	H	4-CO-OCH ₃ -C ₆ H ₄

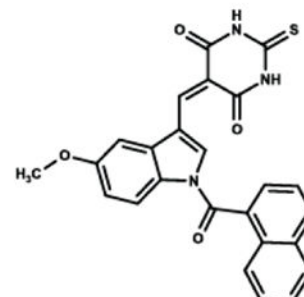
B.



14



16



19

Figure 1.

Small library of indole thio-barbituric acid (ITBA) analogs for screening as HCV NS3 inhibitors. A) Chemical scaffold, PubChem CID numbers and substituent groups used to construct the library. B) The structural formulas of three N-naphthoyl-substituted indole thio-barbituric acid analogs identified in this study.

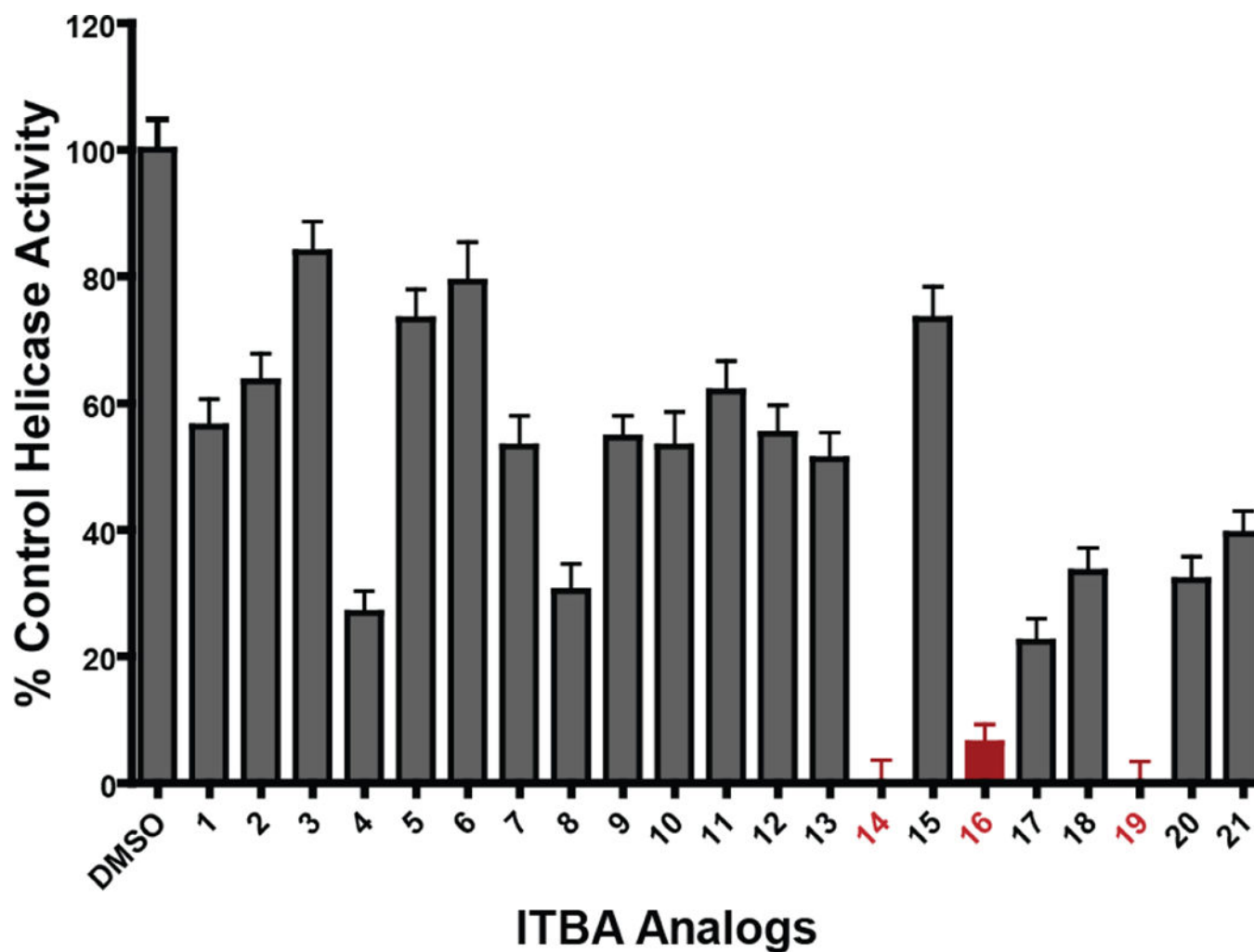


Figure 2. Inhibition of recombinant NS3 helicase activity using fluorescent substrates in the presence of 30 μM of the ITBA analogs solubilized in 5% DMSO. The DNA unwinding activity of recombinant full-length NS3 was measured using a fluorescently labeled duplex-containing DNA substrate in the presence of ATP and Mg^{2+} . The NS3 unwinding rates in the presence of the inhibitors were normalized to the DMSO-containing control samples. Red bars indicate the candidate inhibitors selected for further study. Bars represent the mean \pm standard deviations of triplicate measurements.

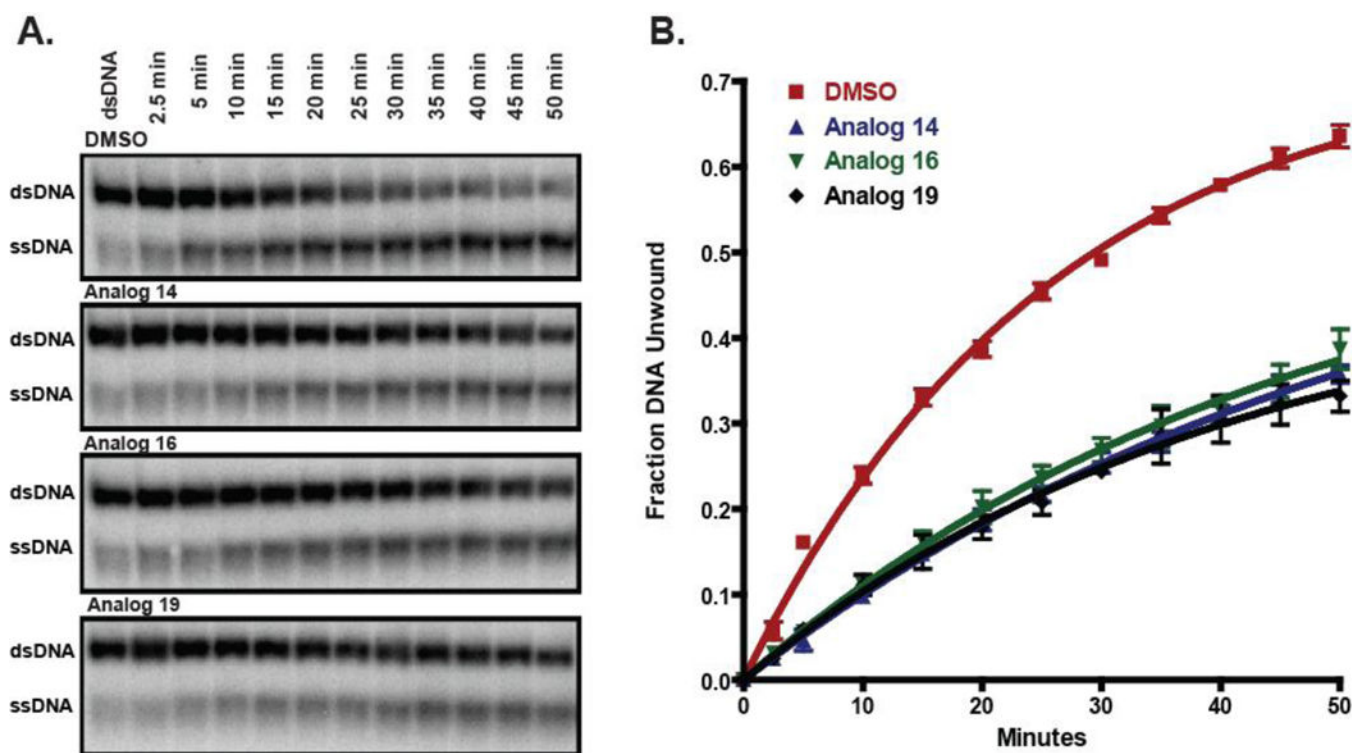


Figure 3.

ITBA analogs inhibit in vitro NS3 DNA unwinding activity. A) Unwinding of 2 nM of radiolabeled duplex DNA by 100 nM of recombinant NS3 in the presence of 25 μ M of the indicated ITBA analogs. Representative gels from triplicate measurements are shown. B) Quantitation of the radioactivity for time points of triplicate experiments were averaged and fit to a single exponential to calculate the following DNA unwinding rate constants: DMSO control (red squares): $0.039 \pm 0.002 \text{ min}^{-1}$, Analog 14 (blue triangles): $0.018 \pm 0.001 \text{ min}^{-1}$, Analog 16 (green inverted triangles): $0.021 \pm 0.002 \text{ min}^{-1}$, and Analog 19 (black diamonds): $0.024 \pm 0.002 \text{ min}^{-1}$.

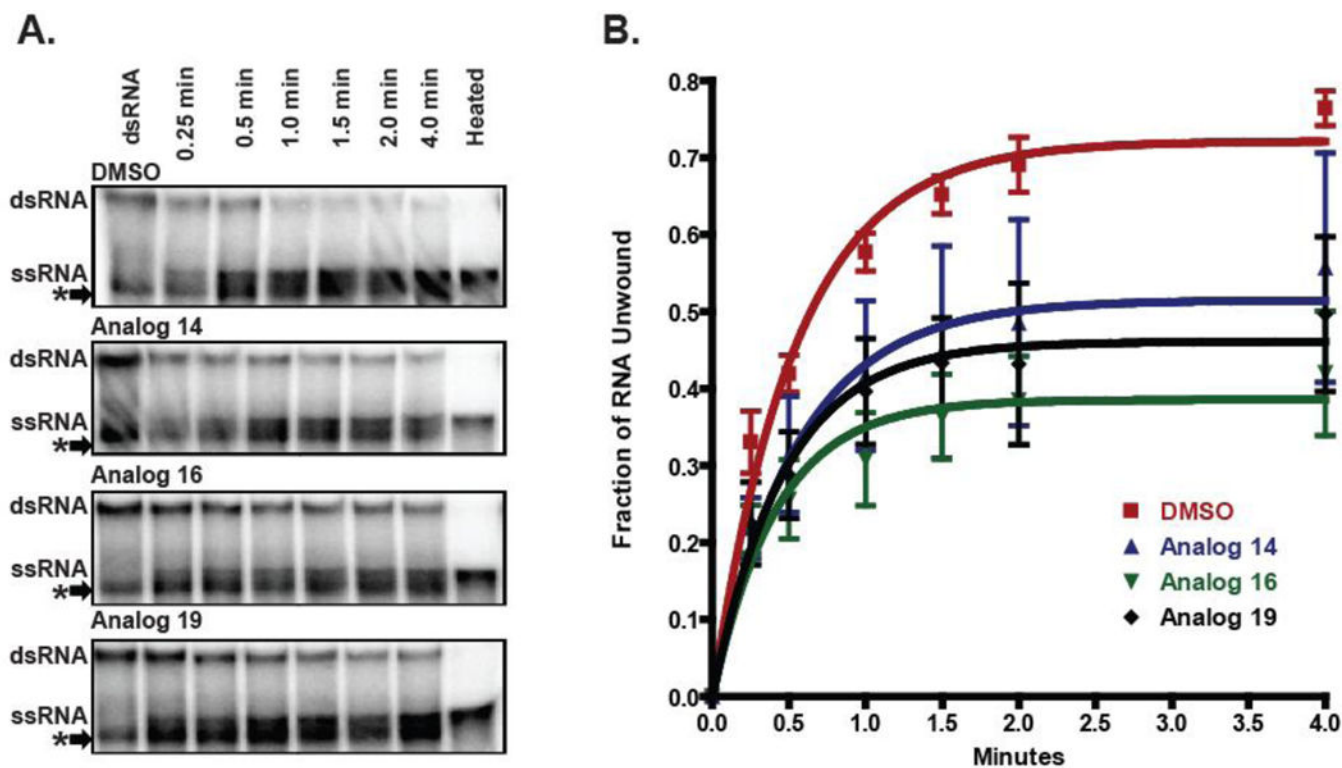


Figure 4:
ITBA analogs inhibit in vitro NS3 RNA unwinding activity. A) Unwinding of 2 nM of radiolabeled duplex RNA by 500 nM of recombinant NS3 in the presence of 25 μ M of the indicated ITBA analogs. A contaminating duplex RNA species indicated by an asterisk (*) was disregarded in the evaluation of the helicase activity (see text and Supplemental data). Representative gels from triplicate measurements. B) Quantitation of the radioactivity for time points of triplicate experiments were averaged and fit to a single exponential to calculate the following RNA unwinding rate constants: DMSO control (red squares): $1.84 \pm 0.19 \text{ min}^{-1}$, Analog 14 (blue triangles): $1.81 \pm 0.77 \text{ min}^{-1}$, Analog 16 (green inverted triangles): $2.38 \pm 0.72 \text{ min}^{-1}$, and Analog 19 (black diamonds): $2.14 \pm 0.69 \text{ min}^{-1}$.

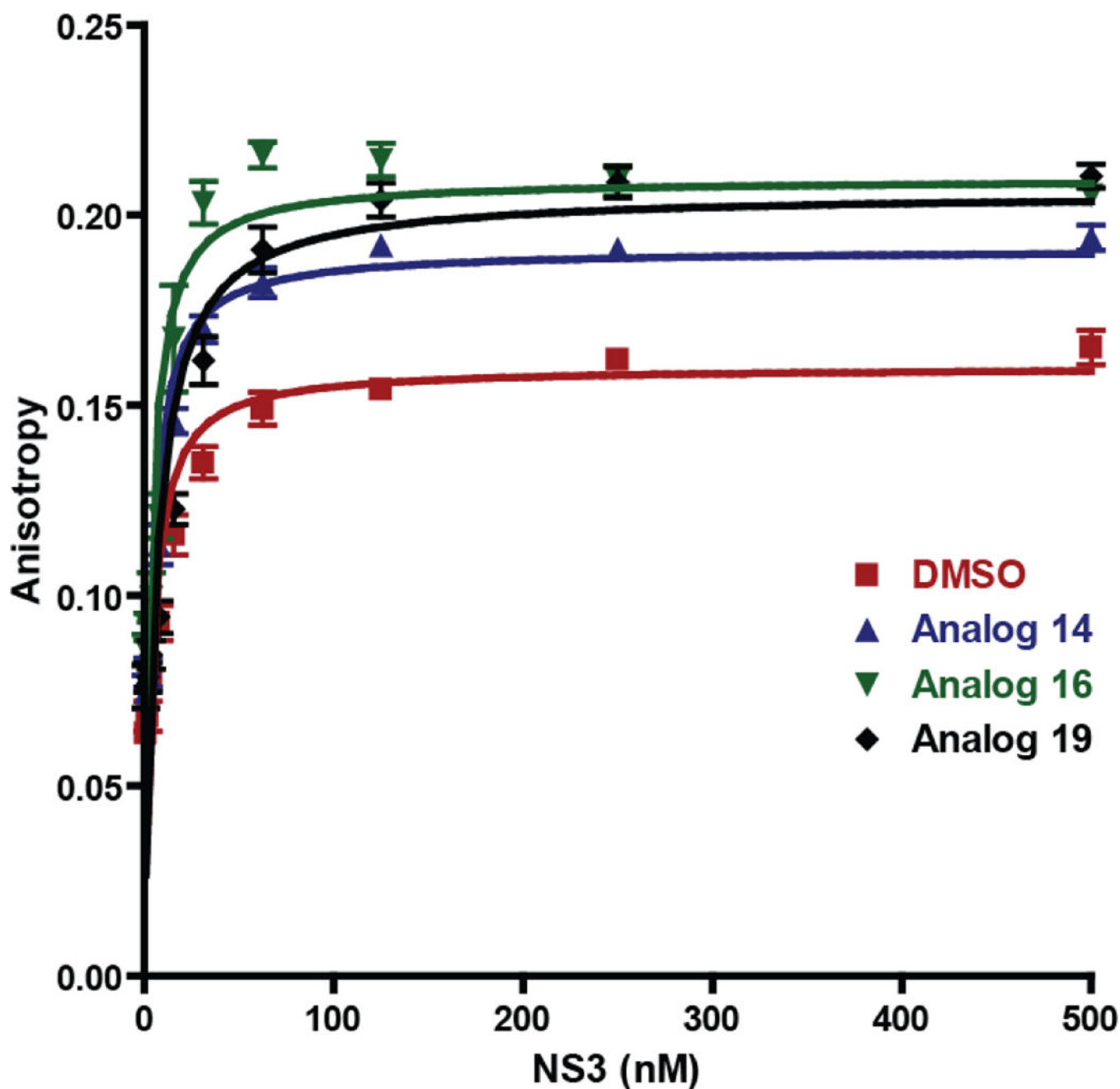


Figure 5: ITBA analogs do not alter the interaction of HCV NS3 with nucleic acids. Binding of increasing concentrations of full-length NS3 to fluorescein-labelled T₂₀ oligomer was measured by fluorescence polarization in the presence of 1 nM DNA and 25 μ M of the indicated ITBA analogs. The anisotropy of the NS3-ssDNA complex was calculated and fit to a quadratic function to obtain the protein-ssDNA dissociation constants: DMSO control (red squares): $K_D = 3.3 \pm 0.2$ nM, Analog 14 (blue triangles): $K_D = 3.1 \pm 0.2$ nM, Analog 16 (green inverted triangles): $K_D = 2.8 \pm 0.4$ nM, and Analog 19 (black diamonds): $K_D = 5.7 \pm 0.6$ nM.

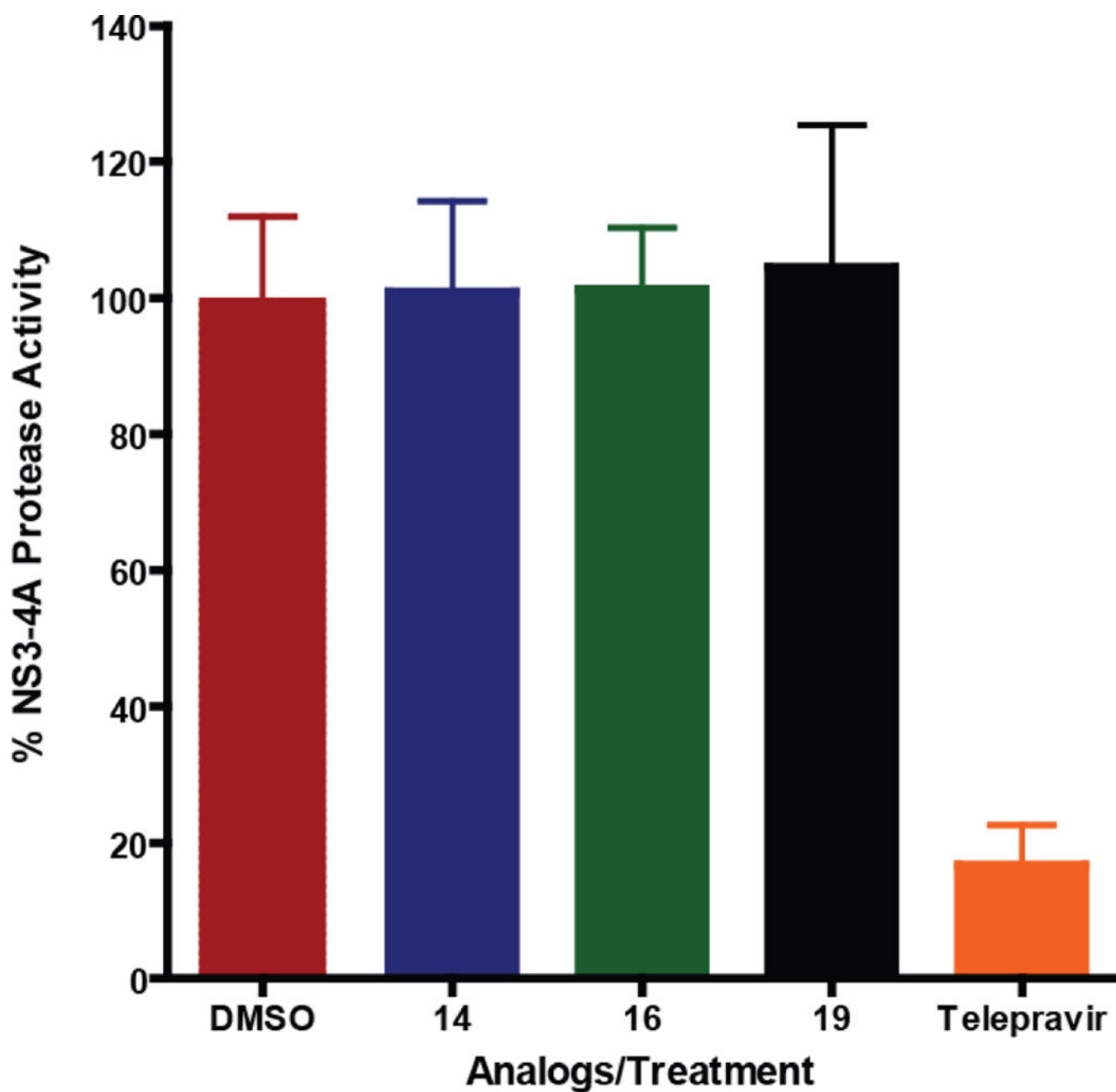


Figure 6:

ITBA analogs do not inhibit the protease activity of HCV NS3-4A. The protease activity of NS3-4A was measured with the internally quenched substrate Ac-Asp-Glu-Asp-EDANS-Glu-Glu-Abu-L-Lactoyl-Ser-Lys DABCYL-NH₂ that fluoresces after cleavage by NS3-4A. ITBA analogs were examined at 25 μ M in the reactions. The NS3-4A inhibitor Telepravir (10 μ M) was used as the positive control reducing protease activity by 85%. Bars represent the mean \pm standard deviation of normalized protease activities from triplicate measurements.

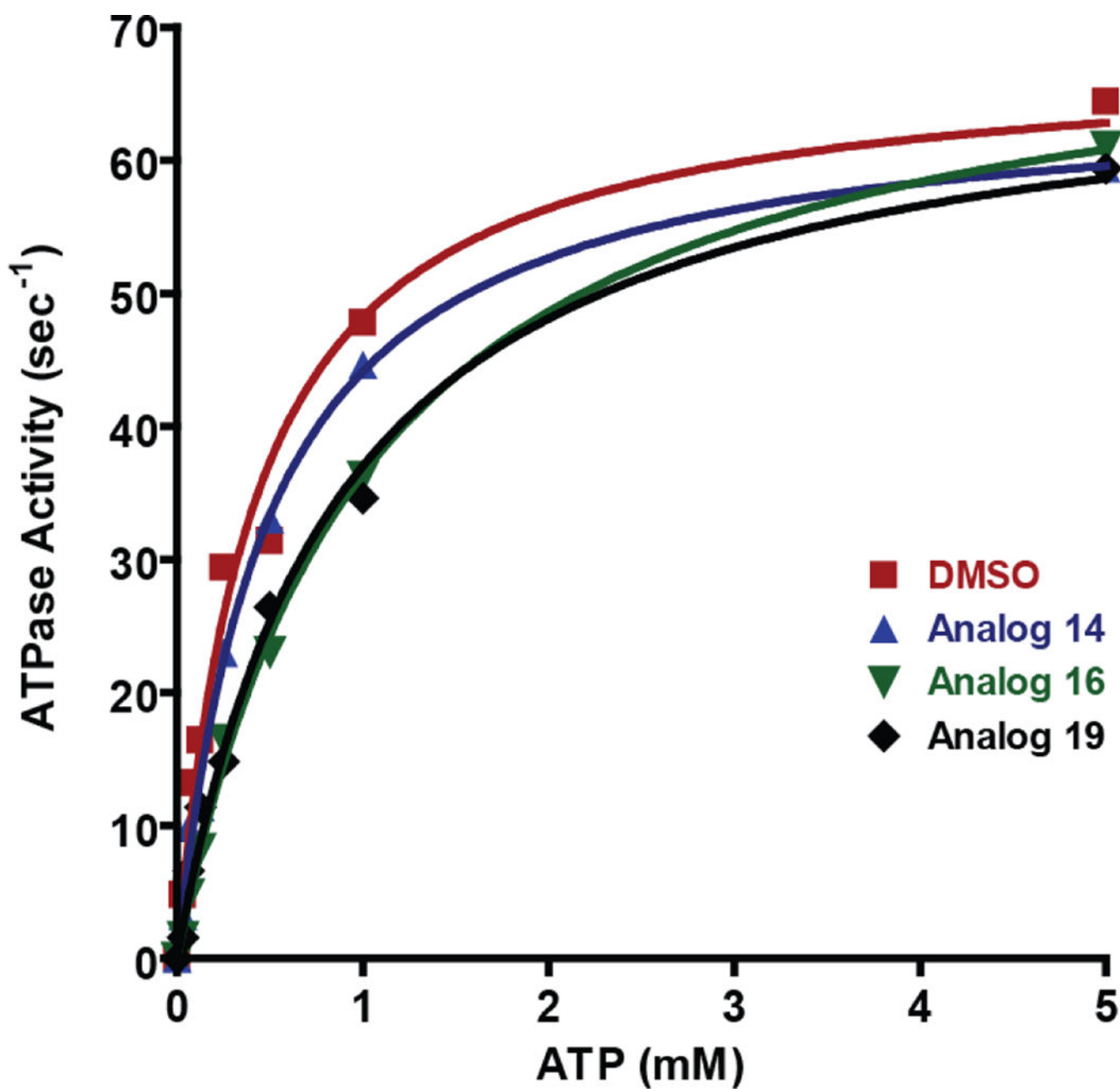


Figure 7:

ITBA analogs do not alter the HCV NS3 RNA-stimulated ATPase activity. ATPase activity of 50 nM full-length NS3 was measured in the presence of saturating amounts of RNA (75 μ M (nucleotides) polyU) and 25 μ M of the indicated ITBA analogs. The ATP concentration was varied from 0–5 mM, and the kinetic constants k_{cat} and K_M calculated using the Michaelis-Menton equation: DMSO control (red squares): $K_M = 0.41$ mM, $k_{cat} = 68.0$ s⁻¹, Analog 14 (blue triangles): $K_M = 0.48$ mM, $k_{cat} = 65.3$ s⁻¹, Analog 16 (green inverted triangles): $K_M = 1.00$ mM, $k_{cat} = 73.0$ s⁻¹ and Analog 19 (black diamonds): $K_M = 0.86$ mM, $k_{cat} = 68.7$ s⁻¹.

Table 1:The IC₅₀ for NS3 DNA Unwinding Activity for candidate ITBA analogs

Helicase Activity	
	IC₅₀ (μM)
DMSO	-
14	21.6 ± 1.9
16	21.4 ± 2.4
19	23.5 ± 1.8

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