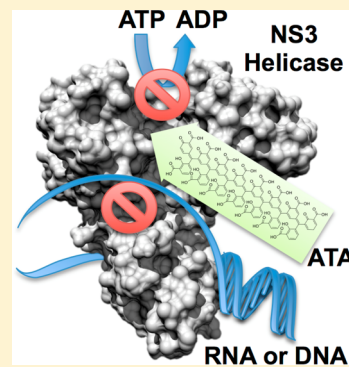


Aurintricarboxylic Acid Modulates the Affinity of Hepatitis C Virus NS3 Helicase for Both Nucleic Acid and ATP[‡]

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ABSTRACT: Aurintricarboxylic acid (ATA) is a potent inhibitor of many enzymes needed for cell and virus replication, such as polymerases, helicases, nucleases, and topoisomerases. This study examines how ATA interacts with the helicase encoded by the hepatitis C virus (HCV) and reveals that ATA interferes with both nucleic acid and ATP binding to the enzyme. We show that ATA directly binds HCV helicase to prevent the enzyme from interacting with nucleic acids and to modulate the affinity of HCV helicase for ATP, the fuel for helicase action. Amino acid substitutions in the helicase DNA binding cleft or its ATP binding site alter the ability of ATA to disrupt helicase-DNA interactions. These data, along with molecular modeling results, support the notion that an ATA polymer binds between Arg467 and Glu493 to prevent the helicase from binding either ATP or nucleic acids. We also characterize how ATA affects the kinetics of helicase-catalyzed ATP hydrolysis, and thermodynamic parameters describing the direct interaction between HCV helicase and ATA using microcalorimetry. The thermodynamics of ATA binding to HCV helicase reveal that ATA binding does not mimic nucleic acid binding in that ATA binding is driven by a smaller enthalpy change and an increase in entropy.



The red dye aurintricarboxylic acid (ATA, PubChem¹ CID 2259) is a triphenylmethane that inhibits most enzymes that synthesize, degrade, or manipulate DNA and RNA.^{2,3} The molecular basis for the sensitivity of nucleic acid enzymes to submicromolar ATA concentrations likely results from the ability of ATA to form polymers in solution that resemble nucleic acids (Figure 1).^{4,5} ATA polymers bind RNA⁶ and also bind to nucleic acid binding clefts on nucleases and similar proteins.^{3,4,7–10}

ATA is part of Sigma's Library of Pharmacologically Active Compounds (LOPAC), and consequently, ATA has been observed to inhibit a wide variety of proteins. Our interest in ATA stems from our recent discovery that ATA inhibits the helicase encoded by the hepatitis C virus (HCV),¹¹ but the list of ATA targets includes over 100 listed in PubChem Bioassay.¹² This list includes other viral enzymes,¹³ Bloom's syndrome helicase (BLM) (Pubchem AID 2364), and the human RECQ1 protein (AID 2549). ATA also inhibits other ATP-fueled proteins like topoisomerase II,¹⁴ protein kinases in the JAK/STAT pathway, and other kinases needed in metabolism.^{15–18}

HCV infects almost 150 million people today, causing liver cancer, cirrhosis, and liver failure. HCV is a single-stranded, positive sense RNA virus with a genome encoding a single multifunctional polyprotein. Host and viral proteases cleave the HCV polyprotein into 10 structural and nonstructural proteins. Besides inhibiting HCV helicase, ATA also inhibits the HCV RNA-dependent RNA polymerase, which is formed by nonstructural protein 5B (NS5B).¹⁰ The HCV helicase is part of the multifunctional nonstructural protein 3 (NS3), which is also a protease when NS3 binds its essential protease cofactor, nonstructural protein 4A (NS4A). HCV helicase unwinds

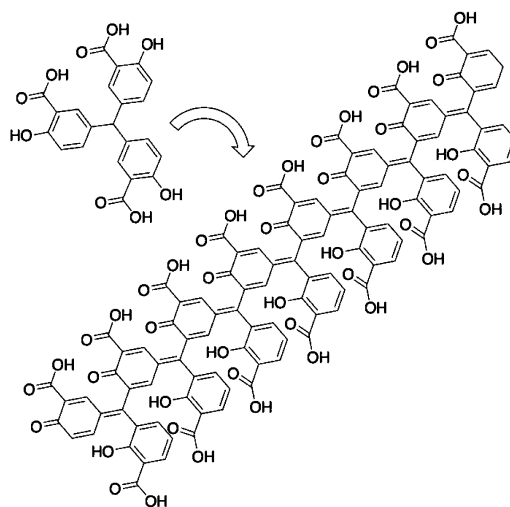


Figure 1. Aurintricarboxylic acid (ATA). Chemical structure of an ATA monomer and an idealized ATA polymer formed in aqueous solution.⁵

duplex RNA and DNA, even though only duplex RNA intermediates are known to be involved in HCV replication.¹⁹ Two recently approved antiviral drugs (boceprevir²⁰ and telaprevir²¹) inhibit the protease function of the NS3/NS4A complex, but relatively few potent NS3 helicase inhibitors have

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been reported, and no HCV helicase inhibitors have entered clinical trials.²² HCV and related viruses encode the only proteins in which protease and helicase active sites are covalently linked in the same polypeptide. The NS3 N-terminal domain is the protease, and the NS3 C-terminal domain is the helicase. Each NS3 domain influences the activity of the other,^{23,24} but it is still not clear exactly how the NS3 helicase and protease are coordinated during HCV replication. Only a few compounds are known that inhibit both the NS3/NS4A protease and helicase functions, and ATA is not one of these.²⁵

Because HCV helicase is a motor protein fueled by ATP hydrolysis, helicase inhibitors can act by preventing ATP binding, ATP hydrolysis, or RNA binding or by decreasing the ability of NS3 to move along nucleic acids or the ability of NS3 to separate a duplex. Mukherjee et al. showed that ATA causes NS3 to dissociate from single-stranded DNA, an effect that might be due to ATA binding to the NS3 nucleic acid binding site, to an allosteric site, or to both.¹¹ The helicase portion of NS3 (i.e., NS3h) has three domains, two of which resemble conserved motor domains shared by all helicases and related proteins. ATP binds between the motor domains, and one strand of RNA (or DNA) binds the cleft that separates the motor domains from a novel helical domain not seen in other helicase structures.²⁶ ATP binding between the NS3h motor domains causes the ATP binding cleft to close so that NS3h binds RNA more weakly and can slide toward the 5' end of RNA like a Brownian motor.²⁷ Because compounds that mimic ATP, like nonhydrolyzable nucleotide analogs, also cause NS3h to release DNA,^{28,29} it is possible that ATA might cause NS3h to release DNA by binding to the ATP-binding site rather than the RNA-binding site. Such a hypothesis that ATA binds NS3h in place of ATP is supported by the observation that a similar triphenylmethane dye called blue HT inhibits HCV helicase by binding in place of ATP (PDB file 2ZJO).³⁰ The goal of this study is therefore to determine if ATA interacts with HCV helicase like DNA or if ATA interacts with NS3h at the ATP binding site, like blue HT.

Here we present evidence that ATA directly binds NS3h and that ATA influences the binding of both nucleic acid and ATP to NS3h. These data can be interpreted as further evidence of communication between the ATP and nucleic acid binding sites on NS3h or as evidence that ATA inhibits HCV helicase by interacting with both the ATP and nucleic acid binding sites. We performed assays monitoring NS3-catalyzed ATP hydrolysis in the presence and absence of ATA, and at various concentrations of DNA, along with direct binding assays with ATA, wild type NS3h, and NS3h harboring amino acid substitutions known to affect either ATP binding or DNA binding to NS3.

EXPERIMENTAL PROCEDURES

Materials. ATA (catalog #A1895, lot #051M0200 V) was from Sigma (St. Louis, MO). The truncated C-terminally His-tagged NS3 proteins lacking the protease domain (NS3h) were purified as described before: NS3h (wild type),³¹ NS3h_D290N, NS3h_E291Q,³² NS3h_H369K, and NS3h_E493Q.³³ NS3h R467E was generated for this study using the Quik-Change site directed mutagenesis kit (Agilent technologies) to alter the p24NS3h_1b(con1) plasmid using the oligonucleotides 5'-GCG GCG AGG CAG GAC TGG TGA GGG CAG GAT GGG CAT TTA C-3' and 5'-GTA AAT GCC CAT CCT GCC CTC ACC AGT CCT GCCTCG CCG C-3'. The R467E protein was expressed and purified as

described for the wild type enzyme.³¹ Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA).

Electrophoretic Mobility Shift Assay. Binding assays containing 25 mM MOPS, pH 7.5, 1.25 mM MgCl₂, 10 nM Cy5-dT15 (5'-/SCy5/-TTT TTT TTT TTT TTT-3'), and 30 nM NS3h were incubated for 5 min at room temperature. Following the addition of indicated concentrations of ATA, the binding reactions were incubated another 5 min at 23 °C. A 15% polyacrylamide Tris-borate-EDTA (TBE) gel was prerun at 4 °C for 30 min at 100 V. Ten microliters of each sample was loaded onto the gel. The gel was run for 5 min at 200 V to allow samples to enter the gel, then they were run for 60 min at 100 V at 4 °C. The gel was scanned on a BioRad Molecular Imager FX phosphorimager.

Fluorescence Polarization (FP)-Based DNA Binding Assay. Binding assays were performed as described by Mukherjee et al.¹¹ in low volume 384-well microplates (Greiner Bio-One, Kremsmünster, Austria, catalog #784076-25). Reactions were assembled in 19 μ L, then 1 μ L of DMSO or ATA dissolved in DMSO was added, such that the final concentrations in each assay was 5 nM Cy5-dT15, 25 mM MOPS, pH 7.5, 1.25 mM MgCl₂, 0.0025 mg/mL BSA, 0.005% (v/v) Tween 20, 0.025 mM DTT, 5% DMSO (v/v), and the indicated amounts of NS3h or ATA. Polarization was monitored with a TECAN Infinite M1000 PRO multimode microplate reader by exciting at 635 nm (5 nm bandwidth) and measuring total fluorescence intensity, parallel and perpendicular polarized light at 667 nm (20 nm bandwidth). *G*-factors were calculated from wells with Cy5-dT15 alone.

To calculate the concentration of NS3h needed to bind 50% of the oligonucleotide (*EC*₅₀) and to calculate the amount of ATA needed to displace 50% of the protein (*IC*₅₀), polarization values were fitted to standard dose response equations using GraphPad Prism (version 6). The *K*_i of ATA was calculated for each compound using the Cheng-Prusoff relationship³⁴ (i.e., eq 1) by assuming ATA competes with the DNA oligonucleotide to bind NS3h.

$$K_i = IC_{50} / ([Protein] / EC_{50} + 1) \quad (1)$$

In eq 1, *IC*₅₀ is the concentration of ATA that reduces polarization by 50%, *EC*₅₀ is the concentration of NS3h needed to increase polarization by 50%, and [Protein] is the NS3h concentration used to measure *IC*₅₀.

ATP Hydrolysis (ATPase) Assays. NS3h-catalyzed ATP hydrolysis was monitored using two different colorimetric assays that monitor inorganic phosphate released from ATP. Assay 1, which has a larger dynamic range but is less sensitive, was used when ATP concentrations were greater than 1 mM (Figure 4), and assay 2 was used when ATA concentrations were less than 1 mM (Figure 5). ATA did not interfere with either assay, as evidenced by the fact that up to 100 μ M ATA did not affect the absorbance of inorganic phosphate controls in either assay.

Assay 1. Assays were assembled in 27 μ L in clear 96-well microtiter plates (Corning Inc., Catalog #9017) and initiated by adding 3 μ L of ATP such that the final reactions contained 15 nM NS3h, 1 mM ATP, 25 mM MOPS, pH 6.5, 1.25 mM MgCl₂, 5% DMSO, 50 μ g/mL BSA, 0.01% Tween 20, and the indicated concentrations of ATA and oligonucleotide dT20 (5'-TTT TTT TTT TTT TTT TTT TT). After 15 min at 23 °C, reactions were terminated by adding 200 μ L of a solution containing 0.034% (w/v) malachite green, 1 N HCl, 1%

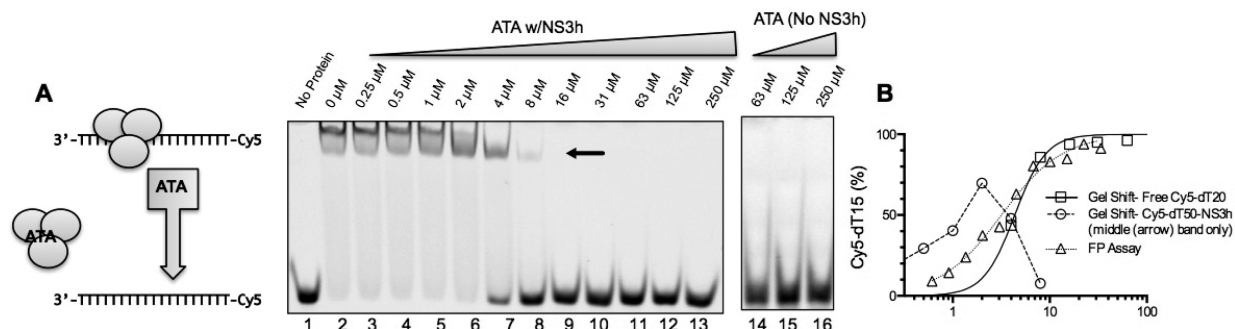


Figure 2. ATA displacing DNA from the HCV helicase. (A) Effect of ATA on the electrophoretic mobility of an oligonucleotide (Cy5-dT15) in the presence and absence of NS3h. The diagram on the left shows the likely band identity. Samples containing Cy5-dT15 alone (lane 1) or Cy5-dT15, NS3h, and indicated concentrations of ATA were separated on a nondenaturing 15% polyacrylamide gel. The gel on the right (lanes 14–16) shows the electrophoretic mobility of Cy5-dT15 in the absence of NS3h, but in the presence of the highest concentrations of ATA analyzed. (B) Comparison of the effect of ATA on Cy5-dT15-NS3h fluorescence polarization as monitored with a FP-based DNA binding assay (triangles)¹¹ and with the amount of free Cy5-dT15 present in the lowest band in the gel shift assay (squares). The relative amount of the middle band (arrow) of NS3h-shifted complex is also shown (circles).

ammonium molybdate, and 0.025% Tween 20, followed within 10 s by addition of 25 μ L of 35% (w/v) sodium citrate. After 20 min at 23 $^{\circ}$ C, an absorbance at 630 nm was read in a Varioskan multimodal plate reader (ThermoFisher, Inc.). Phosphate released was determined from a standard curve after subtracting A_{630} values obtained in a reaction lacking NS3h.

Concentration of ATP cleaved in 15 min (ν) and total enzyme concentration (E_t) were used to calculate specific activities (ν/E_t), which were globally fit to eqs 2–4 using GraphPad Prism (version 6.0).

$$\frac{\nu}{E_t} = k_{\text{fast}} \frac{ED - EI}{E_t} \quad (2)$$

$$ED = \frac{(E_t + D_t + K_{\text{DNA}}) - \sqrt{(E_t + D_t + K_{\text{DNA}})^2 - 4D_tE_t}}{2} \quad (3)$$

$$EI = \frac{ED I^n}{K_i^n + I^n} \quad (4)$$

In eq 2–4, E_t is NS3h concentration, D_t is dT20 concentration, I is ATA concentration, K_i is the apparent dissociation constant for NS3h and ATA, n is the Hill coefficient, ED is the NS3h-DNA complex, EI is the NS3h-ATA complex, and k_{fast} is the turnover rate of DNA-stimulated NS3h-catalyzed ATP hydrolysis.

Assay 2. Assays were assembled in 45 μ L in clear 96-well microtiter plates (Corning Inc., Catalog #9017) and initiated by adding 5 μ L of a 10x solution containing ATP and MgCl_2 at equimolar concentrations such that the final concentrations were 15 nM NS3h, 25 mM MOPS, pH 6.5, 1.25 mM (excess) MgCl_2 , 5% DMSO, 50 μ g/mL BSA, 0.01% Tween 20, and the indicated concentrations of MgATP^{2-} and ATA. After incubation for 30 min at 37 $^{\circ}$ C, reactions were terminated by adding 100 μ L of BioMol Green reagent (Enzo Life Sciences). After 60 min at 23 $^{\circ}$ C, an absorbance at 620 nm was read. The amount of phosphate released was determined from a standard curve after subtracting A_{620} values obtained in a reaction lacking NS3h.

Concentration of ATP cleaved in 30 min (ν) and total enzyme concentration (E_t) were used to calculate specific

activities (ν/E_t), which were globally fit to eq 5 using GraphPad Prism (version 6.0).

$$\frac{\nu}{E_t} = \frac{k_{\text{slow}} S}{K_m \left(1 + \frac{I}{K_i} \right) + S} \quad (5)$$

In eq 4, E_t is NS3h concentration, I is ATA concentration, K_i is the apparent dissociation constant for NS3h and ATA, and k_{slow} is the turnover rate of NS3h-catalyzed ATP hydrolysis in the absence of DNA.

Differential Scanning Calorimetry (DSC). Experiments were performed in a Nano-DSC (TA Instruments). Wild type NS3h was diluted to 10 μ M in a solution containing 25 mM MOPS, pH 7.0, 1.25 mM MgCl_2 , 5% DMSO, 50 μ g/mL BSA, and 0.01% Tween 20. In experiments containing DNA, dT20 was included also at 10 μ M. ATA was added at the concentrations indicated, ranging from 10 μ M to 5 mM. Heating was performed in the range 20–100 $^{\circ}$ C at a rate of 1 $^{\circ}$ C/min. In each experiment, a duplicate heating was conducted on the same sample to serve as a baseline for data analysis. This second heat of the sample could be used because the components of the buffer were thermally stable and the protein did not refold when heated to 100 $^{\circ}$ C. The second heat scan also worked well as the baseline because of the similar thermal history of the sample and reference scan. Data was analyzed with NanoAnalyze software using the two-state scaled model (version 2.3.6, TA Instruments, New Castle, DE).

Binding by Isothermal Titration Calorimetry (ITC). Binding of ATA to NS3h was monitored using a Nano-ITC (TA Instruments, New Castle, DE). NS3h was dialyzed overnight into a solution containing 25 mM MOPS, pH 7.0, 1.25 mM MgCl_2 , and 0.01% Tween 20. NS3h concentrations were determined after dialysis by absorbance at 280 nm using a molar extinction coefficient (ϵ_{280}) of 52 360 $\text{M}^{-1} \text{cm}^{-1}$. Enzyme was diluted in dialysis buffer to a final concentration of 20 μ M for titrations with ATA and 15 μ M for titrations with dT20. ATA was dissolved in the same dialysis buffer to 250 μ M (or dT20 at 100 μ M) and added to 174 μ L of dialyzed NS3h in 16 incremental 3 μ L injections at 300 s intervals at 5 $^{\circ}$ C. Data from the first injection was excluded due to pre-equilibration mixing between the contents of cell and syringe at the syringe tip. Peak areas were integrated, normalized, and then fitted using the independent sites model by nonlinear regression

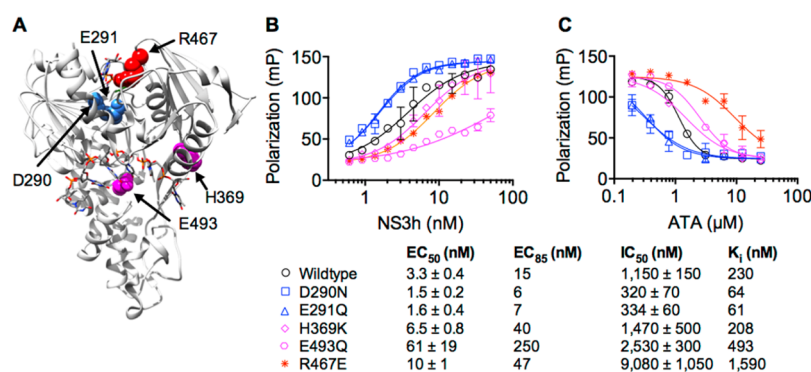


Figure 3. Effect of amino acid substitutions in the NS3h ATP and DNA binding clefts on the interaction of ATA with NS3h. (A) Locations of amino acids of interest mapped on PDB 3KQL,²⁹ where DNA and an ATP analog are also shown as sticks. D290 and E291 (blue) line one side of the ATP binding cleft of domain 1, R467 (red) on domain 2 completes the ATP-binding site, and H369 and E493 (magenta) line the DNA binding cleft. (B) Cy5-dT15 titrations with wild type NS3 or its site-directed mutants. Points are averages of two replicates with error bars showing standard deviations. All assays were performed with 5 nM Cy5-dT15. Data are fitted to a four parameter dose–response equation with the indicated EC₅₀ values, which approximate the dissociation constant describing the affinity of NS3h for DNA. (C) Ability of ATA to displace NS3h and each of its site-directed mutants from Cy5-dT15. Displacement assays were performed using the concentration of each NS3h protein that yielded 85% of the maximum observed change in polarization (EC₈₅), which was calculated from the curve fits using GraphPad Prism. Data are fitted to standard dose–response equations with the IC₅₀ values shown. K_i values were calculated for the concentration of DNA in each assay (shown) using eq 1. Errors are 95% confidence intervals of the curve fits.

using NanoAnalyze (version 2.3.6, TA Instruments, New Castle, DE).

Molecular Modeling. The ATA heptamer (Figure 1) was docked into PDB file 2ZJO³⁰ that was stripped of its ligand blue HT. Using UCSF Chimera 1.6.2,³⁵ water molecules and counterions were removed from the PDB file, histidine protonation states were calculated, and incomplete side chains were automatically filled. A 3D conformation of the ATA heptamer from Figure 1 was generated using Open Babel GUI, saved as a PDB file, and positioned using a rigid body orienting code in UCSF DOCK 6.5.³⁶ During modeling, the ligand-binding site of the protein was constrained to be the largest cluster of spheres surrounding the molecular surface of the protein as generated by UCSF DOCK's *sphgen* module. No additional constraints were made on the resolution of the binding site.

RESULTS

ATA Displaces DNA from NS3h. Mukherjee et al.¹¹ recently reported that ATA is an HCV helicase inhibitor by using a polarization-based assay that monitors the ability of NS3h to bind a Cy5-labeled DNA oligonucleotide. They found that ATA decreases the polarization of NS3h to cause a polarization shift of a Cy5-labeled oligonucleotide, with an IC₅₀ value of 1.4 μM. They also found that ATA inhibited the ability of HCV helicase to unwind DNA and RNA, with IC₅₀ values of 0.6 ± 0.1 μM and 0.4 ± 0.2 μM, respectively.

The displacement of DNA from NS3h might result from ATA interacting with either the oligonucleotide or NS3h. If ATA binds to DNA, it might cause a shift in the mobility of a DNA oligonucleotide in a gel, as is seen when an oligonucleotide binds its complement. To test this hypothesis, we analyzed the effect of ATA on the electrophoretic mobility of an NS3h–oligonucleotide complex using a gel-shift assay. When increasing concentrations of ATA (lanes 3–12, Figure 2A) were applied to a solution containing NS3h (30 nM) and a Cy5-labeled oligonucleotide (Cy5-dT15, 10 nM) (lane 2, Figure 2A), ATA caused the nucleotide to migrate more rapidly. At saturating ATA concentrations, the labeled oligonucleotide migrated to the same location where the

oligonucleotide migrated in the absence of NS3h (lane 1, Figure 2A). The concentration of ATA needed to shift 50% of the oligonucleotide to the position where free DNA migrates in the gel was 4 μM (lane 7, Figure 2A), but lower concentrations of ATA caused the oligonucleotide to migrate slightly faster than it does in the absence of ATA. The lower concentration was more comparable to the concentration of ATA needed to decrease Cy5-dT15 polarization by 50% (circles, Figure 2B). One explanation might be due to the fact that NS3h forms oligomers on DNA. The middle band in lane 2 (arrow, Figure 2A) might represent an NS3h monomer bound to DNA, and the more intense upper band might represent an NS3h oligomer bound to DNA. High concentrations of ATA did not appear to alter the mobility of Cy5-dT15 either in the presence of NS3h (lanes 3–13) or in the absence of NS3h (lanes 14–16).

Amino Acid Substitutions in Both the NS3h ATP and DNA Binding Sites Alter Relative Sensitivity of NS3h to ATA. To better localize the site where ATA interacts with NS3h, we examined the interaction of ATA with NS3h harboring amino acid substitutions that were previously demonstrated to disrupt the interaction of either ATP or DNA with NS3h (Figure 3A). Three substitutions were in the ATP binding cleft, and two were in the DNA binding cleft. Asp290 binds the magnesium ion needed to bridge ATP to NS3h,²⁹ and a D290N substitution causes magnesium to bind NS3h 500 times more weakly.³² Glu291 is the catalytic base needed to activate the water that attacks the gamma phosphate of ATP upon hydrolysis,²⁹ and an E291Q mutation leads to a protein that cleaves ATP about 1,000 times more slowly.³² Arg467 acts as an “arginine finger” to stabilize the transition state. The R467E protein generated here retains the ability to bind DNA (Figure 3B) but does not hydrolyze ATP or unwind DNA substrates (data not shown). Glu493 and His369 both contact DNA when it is bound to NS3h,^{26,29} and both E493Q and H369K substitutions cause NS3h to bind DNA less tightly under optimal conditions.³³

We used the above FP-based DNA binding assay¹¹ to investigate the ability of ATA to displace DNA bound to each of the above proteins (Figure 3B). The FP-based binding assay

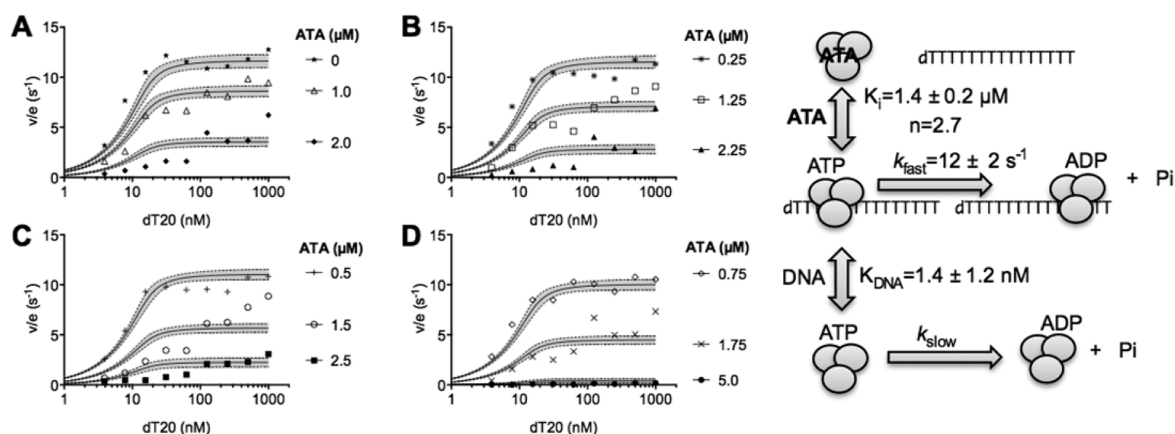


Figure 4. Effect of ATA on the ability of DNA to stimulate NS3h-catalyzed ATP hydrolysis. Specific activities of NS3h (mol ATP hydrolyzed/(s · mol NS3h)) are observed in assays with 1 mM ATP and with indicated concentrations of ATA and the oligonucleotide dT20. Data shown in panels A–D are fitted to eqs 2–4 by nonlinear regression, with indicated parameters. Uncertainties are 95% confidence intervals (dotted lines, gray shading) from the nonlinear regression.

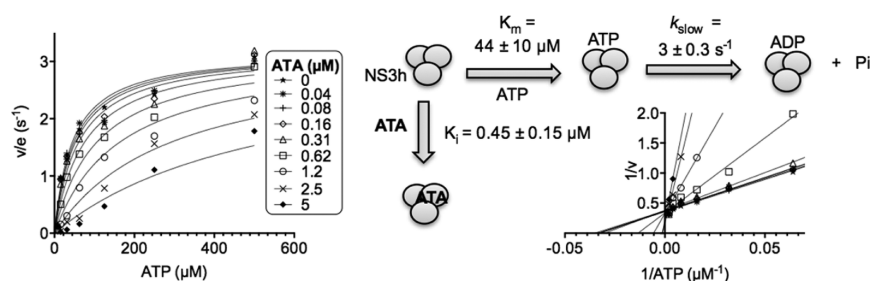


Figure 5. Effect of ATA on helicase-catalyzed ATP hydrolysis in the absence of nucleic acids. Specific activities are observed at indicated ATP and ATA concentrations in the absence of DNA. Data are fitted to eq 5 by nonlinear regression with indicated parameters. Uncertainties are 95% confidence intervals from the nonlinear regression. (Inset) Data on a Lineweaver–Burk plot with lines fitted by linear regression.

confirmed earlier observations that the substitutions in the DNA binding site cause NS3h to bind DNA more weakly and that the substitutions in the ATP binding site result in tighter DNA binding (Figure 3B).^{33,37} Less ATA was needed to displace DNA bound to D290N or E291Q than was needed to displace DNA from wild type NS3, and more ATA was needed to displace DNA from H369K, E493Q, and R467E (Figure 3C). Because the displacement assays were all performed at different NS3h concentrations (i.e., the EC_{85} values from Figure 3B), data were normalized using the Cheng–Prusoff relationship (eq 1) to calculate a relative K_i for ATA with each protein.³⁴ The resulting K_i 's were similar for both wild type and the H369K protein, but the K_i for ATA was notably lower for the D290N and E291Q proteins and higher for both E493Q and R467E.

ATA Affects the Affinity of NS3h for DNA. If a compound binds NS3h in the place of nucleic acids, it should also prevent RNA or DNA from stimulating NS3h-catalyzed ATP hydrolysis. NS3h hydrolyzes ATP both in the presence and absence of DNA (or RNA), but nucleic acids stimulate rates of NS3h-catalyzed ATP hydrolysis by 10–100 fold (Figure 4) depending on the nucleic acid sequence and base composition. Polypyrimidines, such as the oligonucleotide dT20 (5'-TTT TTT TTT TTT TTT TT-3'), are some of the most efficient stimulators of NS3h-catalyzed ATP hydrolysis.^{38,39} If a compound only binds NS3h in place of DNA, then inhibition of NS3h-catalyzed ATP hydrolysis should be observed at low levels of dT20, but high levels of dT20 should be able to compete with the inhibitor to still activate ATP hydrolysis. To

test if ATA binds only the NS3h DNA binding site, we therefore examined initial rates of NS3h-catalyzed ATP hydrolysis at various concentrations of dT20 and ATA (Figure 4). Data did not fit a model that assumes that dT20 and ATA compete for the same binding site on NS3h, as has been previously observed with NS3h inhibitors that simply mimic DNA.³¹ Instead, data fit a model that assumes ATA binds NS3h to produce a complex incapable of hydrolyzing ATP even when excess DNA is present (eq 2–4). The model (Figure 4) assumes that dT20 binds NS3h with a dissociation constant (K_{DNA}) of 1.4 ± 1.2 nM, that ATA binds NS3h with a dissociation constant (K_i) of 1.4 ± 0.2 μM, and that in the absence of ATA, NS3h hydrolyzes ATP at a turnover rate (K_{fast}) of 11.6 ± 2 s⁻¹ (uncertainties represent the 95% confidence intervals of the nonlinear regression).

ATA Affects the Affinity of NS3h for ATP. Because all of the above ATPase assays were performed with 1 mM ATP, a concentration that is over 10 times the K_m of ATP in this reaction,³² they do not reveal whether or not ATA might influence the ability of NS3h to bind ATP. To address this issue and examine how ATA affects the ability of NS3h to hydrolyze ATP in the absence of nucleic acids, we used a more sensitive assay (Assay 2 in Experimental Procedures) to monitor the effect of ATA on NS3h-catalyzed ATP hydrolysis even in the absence of DNA, and this inhibition was more pronounced at low ATP concentrations than at higher ATP concentrations (Figure 5). Data fit a model that assumes that ATA acts as a competitive inhibitor (eq 4), with a K_i (ATA) of

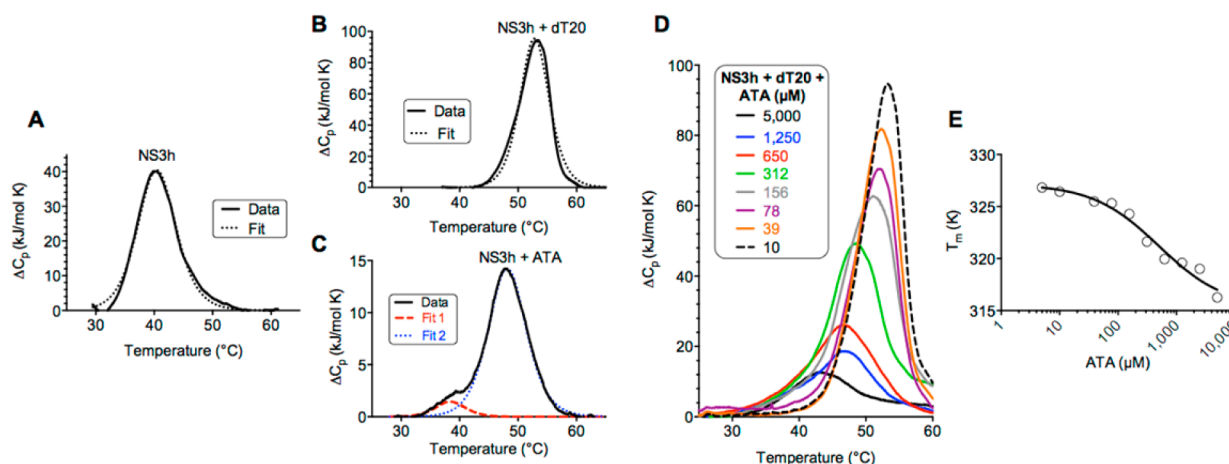


Figure 6. Interaction of NS3h, DNA, and ATA as monitored by DSC. (A) NS3h (10 μM) was heated from 20 to 100 $^{\circ}\text{C}$ at a rate of 1 $^{\circ}\text{C}/\text{min}$. Baseline corrected data were fitted to a scaled van 't Hoff two-state model (dotted line). (B) NS3h (10 μM) and dT20 (10 μM) were heated and analyzed as described in panel A. (C) NS3h (10 μM) and 300 μM ATA were heated as described in panel A and deconvoluted data fitted to two scaled van 't Hoff two-state models (dotted lines). (D) NS3h (10 μM) and dT20 (10 μM) with indicated concentrations of ATA were heated as described in panel A. Only baseline corrected raw data are shown in panel D. (E) Melting temperatures observed at each ATA concentration for data shown in panel D fitted to a dose response equation.

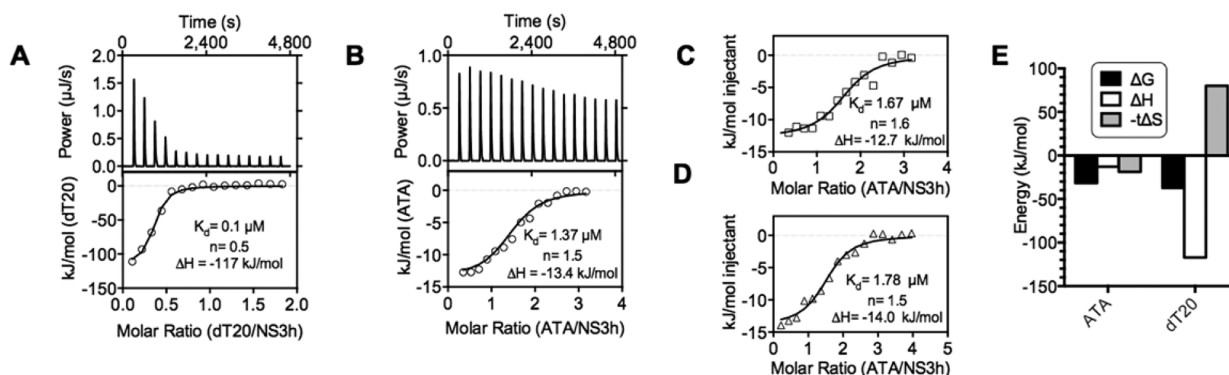


Figure 7. Interaction of NS3h with DNA, or ATA, as monitored by ITC. (A) NS3h (15 μM) was titrated with dT20 (100 μM) at 25 $^{\circ}\text{C}$, and heat released monitored with time (top panel). Resulting heats of injection were fitted by nonlinear regression with indicated parameters (lower panel). (B) NS3h (20 μM) was titrated with ATA (250 μM) at 5 $^{\circ}\text{C}$, and heat released monitored with time (top panel). Note: In panels A and B, data are plotted with exothermic events in the positive direction and the first data points removed for curve fits. (C) and (D) Repeat titrations of NS3h with ATA under the same conditions as in panel B. (E) Comparison of the thermodynamic parameters (adjusted to 25 $^{\circ}\text{C}$) describing the interaction of ATA or dT20 with NS3h.

$0.45 \pm 0.15 \mu\text{M}$, a K_m (ATP) of $44 \pm 10 \mu\text{M}$, and a turnover rate (k_{slow}) of $3 \pm 0.3 \text{ s}^{-1}$ (Figure 5). Competitive inhibition was also clear when reciprocal data were fit by linear regression on a Lineweaver–Burk plot (Figure 5 inset).

ATA Binds NS3h in the Absence of DNA or ATP. The above data suggest that ATA binds NS3h with a dissociation constant in the low micromolar range, but they do not directly show an ATA–NS3h interaction. Therefore, we next examined the direct interaction of ATA with NS3h in the absence of any helicase substrates using microcalorimetry.

Differential scanning calorimetry (DSC) was first used to examine the ATA–NS3h interaction. The goal of the DSC experiments were to understand if ATA forms a stable complex with NS3h. ATA increased the unfolding temperature of NS3h (compare Figure 6 panels A and C), but not to the same extent as a natural NS3h ligand like the oligonucleotide dT20 (compare Figure 6 panels A and B). In the presence of DNA, the unfolding temperature of NS3h increased by 10 $^{\circ}\text{C}$, and this change was accompanied by a 300 kJ/mol increase in the enthalpy of unfolding ($\Delta\Delta H$) (Figure 6B). When ATA was

added to NS3h, the unfolding temperature of NS3h increased by 8 $^{\circ}\text{C}$, but ATA caused a decrease in the enthalpy of unfolding for NS3h ($\Delta\Delta H = -218 \text{ kJ/mol}$, Figure 6B). DSC data obtained from unfolding the NS3–dT20 complex fit a scaled van 't Hoff two-state model, which assumes that only one species melts in a single event (Figure 6B). However, data obtained with the NS3h–ATA complex fit a model that assumes two species are present and that both undergo a two-state transition (Figure 6C). The first species had the same T_m as free NS3h (red, line Figure 6C), suggesting that not all NS3h was bound to ATA under these conditions. When ATA was added to a preassembled NS3h–dT20 complex (10 μM each), ATA lowered the magnitude and T_m of the observed melting event, lowering the observed melting temperature in a concentration-dependent manner, most likely by replacing bound DNA (Figure 6D). The T_m decreased $11 \pm 3 \text{ }^{\circ}\text{C}$, and about 500 μM ATA was needed to decrease the T_m by 50% of this maximum change (Figure 6E).

Isothermal titration calorimetry (ITC) also revealed that ATA interacts with NS3h in the absence of DNA or ATP

(Figure 7). For comparison, we first performed a similar ITC experiment with NS3h and the oligonucleotide dT20 (Figure 7A). ATA titrations generated notably smaller heats (Figure 7B–D), but the areas of these heats still fitted a standard n -site (noncooperative) binding model. For these experiments, 20 μ M NS3h was titrated with aliquots of a 250 μ M ATA solution. On the basis of the dissociation constant determined, the “ c value” (i.e., the product of the receptor concentration and the binding constant for each titration with ATA) was ~ 18 , which is well within the accepted range for ITC experiments.⁴⁰ Determining the end point of the titration with ATA was challenging because even though both ATA and NS3h were in exactly the same buffer, there was still a clear heat of dilution remaining after NS3h was saturated with ATA, which was about twice that observed with DNA. Nevertheless, we were confident that end points were reached because similar heats were obtained in two additional titrations performed under the same conditions (Figure 7C,D) and because the initial heats observed in each titration were almost 20 kJ greater than the baseline. Thus, for model fitting, baseline heats were adjusted to zero on the basis of the average of the heats of the last three injections.

NS3h bound dT20 more tightly than ATA, with two NS3h protomers assembling on the oligonucleotide. More heat was released upon DNA binding to NS3h, however, and DNA binding was accompanied by a decrease in entropy (Figure 7E). ATA bound NS3h differently, with 1.5 ATA monomers binding per NS3h with a dissociation constant of $1.3 \pm 0.2 \mu$ M and a ΔH of -13.4 ± 0.6 kJ/mol (Figure 5B). The K_d determined using ITC was remarkably similar to most of the IC_{50} 's and K_i 's determined above. A K_d of 1.3 μ M corresponds to a ΔG of -30.9 ± 0.3 kJ/mol (at 5 $^{\circ}$ C), and considering the fact that the change in enthalpy upon binding was only -13.4 kJ/mol, binding of ATA to NS3h appeared to be mainly driven by a positive change in entropy ($\Delta S = 63$ J/(mol K); at 5 $^{\circ}$ C, $-T\Delta S = -17.5$ kJ/mol).

DISCUSSION

Some of the data presented above could be interpreted to support the hypothesis that ATA binds NS3 helicase at the site normally occupied by RNA (or DNA), as has been observed with a variety of other nucleic acid enzymes.^{2,7} However, several key observations do not fit this simple explanation. First, NS3h harboring amino acid substitutions in the ATP binding alter the ability of ATA to displace helicase-bound DNA (Figure 3). Second, ATA modulates ATP binding in the absence of DNA (Figure 4). Third, ATA modulates the ability of DNA to stimulate ATP hydrolysis in a cooperative manner, rather than as a linear competitive inhibitor of activation (Figure 5).^{28,32,41} Last, the thermodynamic parameters describing the interaction of ATA do not resemble those describing the interaction of DNA with NS3h (Figures 6 and 7).

To explain all our observations, we envision a model where an ATA polymer interacts with both the NS3h RNA-binding site, and the NS3h ATP-binding site, so that ATA causes a conformational change that prevents the protein from interacting with either ATP or nucleic acids (Figure 8). If ATA binds in place of ATP, it could cause an abnormal rotation of the second motor domain (i.e., domain 2), as has been observed to occur when the similar triphenylmethane blue HT (a.k.a. methyl blue, CID 455837) binds NS3h.¹⁰ Domain 2 of NS3h is lined with positively charged amino acids, one of which

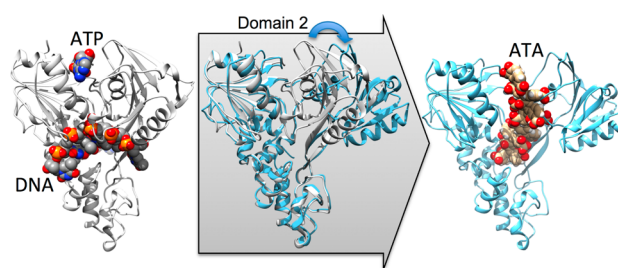


Figure 8. Molecular model depicting how ATA might inhibit HCV helicase. The left structure shows a ternary complex of NS3h, DNA, and an ATP analog (PDB file 3KQL²⁹) with the protein depicted as ribbons and the ligands as space-filled models. The far right structure shows an ATA heptamer positioned in PDB file 2ZJO³⁰ using UCSF Dock 6.5.³⁶ The middle panel shows how NS3h domain 2 might rotate to accommodate ATA.

is Arg467,^{42,43} which acts as an arginine finger during ATP hydrolysis.²⁹ On the basis of our site-directed mutagenesis data, we propose that ATA polymers bind near Arg467 in the second NS3h motor domain and Glu493 in the DNA binding cleft so that ATA might simultaneously interfere with the ability of NS3h to bind ATP and RNA (or DNA).

To test this hypothesis, we used UCSF Dock 6.5³⁶ to examine the interaction of an ATA heptamer with PDB file 2ZJO³⁰ after the blue HT ligand was removed. Remarkably, the ATA polymer docked as predicted, with one end of ATA within 3 Å of Glu493 in the DNA binding cleft and the other end within 3 Å of Arg467 (Figure 8 right panel). A comparison of the docked ATA model with PDB file 2ZJO²⁹ (Figure 8, left panel) reveals the conformational change that we propose releases DNA from the enzyme (Figure 8, middle). Of note, altering either R467 or E493 causes the protein to be less responsive to ATA (Figure 3). The observation that proteins harboring substitutions on the domain 1 side of the ATP-binding site are more responsive to ATA (Figure 3) might be because such mutations cause the protein to assume an open conformation more frequently.

Our data lend less support to the idea that ATA inhibits helicases because ATA binds nucleic acid substrates or because ATA sequesters the metal ion cofactors needed for helicase action. The interaction of ATA with aluminum is a standard assay used to detect metal ions in water, and others have shown that ATA binds RNA.⁶ However, ATA does not shift the electrophoretic mobility of our DNA oligonucleotides (Figure 2), and metal ion chelation seems unlikely because all assays were performed with a vast excess of divalent metal cofactors. One other explanation for the ability of ATA to inhibit NS3 helicase action is that ATA might disrupt the formation of NS3h oligomers that are needed to efficiently unwind DNA and RNA.^{44,45} Monitoring how a small molecule disrupts NS3h oligomers is challenging. However, if the multiple bands seen on native gels with NS3h and DNA (Figure 2) represent different oligomeric forms of NS3h, low levels of ATA appear to disrupt the NS3h oligomer, and this disruption might contribute to the potent ATA inhibition seen in helicase assays.

All studies here were performed with recombinant NS3 protein that was truncated to remove its protease domain (a.k.a. NS3h). Some studies have been repeated with full-length NS3 in both the presence and absence of NS4A, and we have not noted any differences in the sensitivity of these more biologically authentic helicase constructs to ATA. Likewise, we have no evidence that ATA also inhibits the protease

function of NS3. Nevertheless, the NS3 protease domain, NS4A, or other HCV or host proteins might influence how ATA interacts with NS3 in the cell.

On a final note, results here might inspire the development of more specific ATA derivatives as possible HCV antiviral agents. However, synthesis of more active analogs will likely be challenging because without a polymeric tail, triphenylmethanes, like blue HT and its optimized derivatives, are all relatively poor NS3h inhibitors, with IC₅₀ values between 10 μ M and 40 μ M.^{10,46} It might be worthwhile to start such a study by first examining the effect on HCV of the collection of ATA derivatives, which were previously synthesized to inhibit the reverse transcriptase encoded by the human immunodeficiency virus (HIV).^{5,47} ATA derivatives might be particularly effective HCV antivirals because ATA inhibits both NS3h and the HCV NS5B RNA-dependent RNA polymerase.¹⁰

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Notes

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ABBREVIATIONS

ATA, aurintricarboxylic acid; HCV, hepatitis C virus; NS3h, nonstructural protein 3 helicase domain

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