Effect of Intercalator Substituent and Nucleotide Sequence on the Stability of DNA- and RNA- Naphthalimide Complexes

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ABSTRACT

DNA intercalators are commonly used as anti-cancer and anti-tumor agents. As a result, it is imperative to understand how changes in intercalator structure affect binding affinity to DNA. Amonafide and mitonafide, two naphthalimide derivatives that are active against HeLa and KB cells *in vitro*, were previously shown to intercalate into DNA. Here, a systematic study was undertaken to change the 3-substituent on the aromatic intercalator 1,8-naphthalimide to determine how 11 different functional groups with a variety of physical and electronic properties affect binding of the naphthalimide to DNA and RNA duplexes of different sequence compositions and lengths. Wavelength scans, NMR titrations, and circular dichroism were used to investigate the binding mode of 1,8-naphthalimide derivatives to short synthetic DNA. Optical melting experiments were used to measure the change in melting temperature of the DNA and RNA duplexes due to intercalation, which ranged from 0 to 19.4 °C. Thermal stabilities were affected by changing the substituent, and several patterns and idiosyncrasies were identified. By systematically varying the 3-substituent, the binding strength of the same derivative to various DNA and RNA duplexes was compared. The binding strength of different derivatives to the same DNA and RNA sequences was also compared. The results of these comparisons shed light on the complexities of site specificity and binding strength in DNA-intercalator complexes. For example, the consequences of adding a 5'-TpG-3' or 5'-GpT-3' step to a duplex is dependent on the sequence composition of the duplex. When added to a poly-AT duplex, naphthalimide binding was enhanced by 5.6-11.5 °C, but when added to a poly-GC duplex, naphthalimide binding was diminished by 3.2-6.9 °C.

INTRODUCTION

DNA is the molecule in all cellular forms of life that contains the genetic information for biological development. DNA can be a major drug target because of its vital role in the cell. Pharmaceuticals designed to target DNA may be used to interfere with replication, transcription, or gene expression, all of which are key steps in the cell growth and division process. There is a need to understand the structural features of an intercalator that control the specificity and strength of its binding properties, which may help improve site specificity and selectivity.^{1,2} The ability to identify structural elements of a drug responsible for the specificity of binding to DNA or RNA may improve the screening process for new drugs.

Intercalation is one general way in which organic molecules can interact with DNA (groove binding being the other). Intercalators are generally planar aromatic compounds that can bind covalently or non-covalently between the aromatic base pairs of DNA or RNA.^{3,4} More specifically, intercalators can insert themselves either perpendicular or parallel with respect to the long axis of the base pair.⁵ The substituents on an aromatic intercalator dictate how much the compound intercalates into the DNA. Partial insertion of an intercalator can occur when bulky substituents are introduced onto the flat aromatic molecule. The DNA- or RNA-intercalator complex is stabilized by π - π , hydrophobic, and polar interactions as well as electrostatic forces.^{4,6} There are a number of factors that play a role in determining sequence selectivity of intercalator binding to DNA, such as size, shape, and substituents. Numerous experiments have shown that simple intercalators typically bind to 5'-pyrimidine-purine-3' steps,^{4,7-9} with a preference for 5'-CpG-3' steps.⁴

An intercalation event will cause an increase in helical rise between base pairs and an unwinding event where the molecule inserts itself. The classical model of intercalation proposes

that each intercalated molecule will lengthen the duplex by 3.4 Å,¹⁰ thereby increasing the helical rise between adjacent base pairs to about 6.8 Å.^{10,11} In order to accommodate this space increase and unwinding, the sugar-phosphate torsional angles and sugar pucker must change.^{6,11,12} The structure of the intercalating molecule greatly impacts the way in which the intercalation event affects the overall duplex. For example, ethidium bromide reduces the helical twist of B-form DNA from 36° to 10°, and proflavin unwinds the duplex by approximately 17°.¹³⁻¹⁶ Although intercalation does not lead to a disruption of Watson-Crick base pairs, it can induce significant structural perturbations.^{11,12,17} Additionally, when an intercalator is bound, the base pair step adjacent to the intercalator is now structurally hindered and thus prevents another intercalation event from occurring at the adjacent step, commonly known as the neighbor exclusion principle.^{4,18,19}

During replication or transcription, these changes in the duplex structure may result in alterations to the information content.¹³ The consequences of these alterations could cause a complete halt in replication or could induce a mutation, which can be lethal or non-lethal.¹³ If lethal, these base mutations will eventually lead to apoptosis.¹³

Previously, Braña and co-workers published the synthesis and cytotoxicity of the first series of naphthalimides which combined several elements of other known intercalators into a single molecule with biological activity against HeLa and KB cells *in vitro*.^{20,21} From these studies, two compounds were found to be the most active, amonafide and mitonafide, which differ at the 3-position, with a -NH₂ or a -NO₂ group, respectively (Figure 1). Additionally, studies confirmed that these compounds constitute a new class of intercalative topoisomerase II active drugs.²² Due to limited activity against solid tumor tissues, mitonafide was terminated in

phase II clinical trials while amonafide was terminated in phase III clinical trials due to its excessive toxicity.²³

Figure 1. Structure of 1,8-naphthalimide. The derivatives studied here are as follows: X= -H (**1**), -NO₂ (mitonafide) (**2**), -NH₂ (amonafide) (**3**), -Cl (**4**), -Br (**5**), -I (**6**), -OH (**7**), -NHCH(CH₃)₂ (**8**), -NHCOCH₃ (**9**), -Ph (**10**), and -CH₃ (**11**).

Using absorbance spectra and viscosity studies with calf thymus DNA, Waring et al. have shown that mitonafide does intercalate into DNA.²⁴ Bailly et al. have shown via DNase I footprinting experiments that amonafide and mitonafide have a sequence preference for alternating purine-pyrimidine steps, particularly 5'-TpG-3' and 5'-GpT-3' steps.¹⁷ These primary binding sites go against the typical 5'-CpG-3' sequence preference of simple intercalators.⁴

There are currently no three-dimensional structures of the naphthalimides studied herein complexed with DNA or RNA; therefore, it is difficult to predict where the substituents are positioned and how the naphthalimide and the nucleic acid base pairs interact. However, there is a solution structure of a bisnaphthalimide intercalator complexed with 5'-d(ATGCAT)₂-3' solved via NMR.²⁵ The solved structure shows the compound binding to the two 5'-TpG-3'

steps. The linker connecting the two naphthalimide cores of the bisnaphthalimide may be influencing how the bisnaphthalimide is binding to the DNA. However, this structure may still provide some insight into how our naphthalimides are binding.

In this study, 11 previously synthesized naphthalimide derivatives²⁶⁻³¹ (Figure 1) were individually mixed with short DNA and RNA duplexes of varying sequence composition and length to form DNA- and RNA-naphthalimide complexes. These complexes were then thermodynamically characterized; the binding of the same derivative to various DNA and RNA duplexes and the binding of different derivatives to the same DNA and RNA duplex were compared. Since short oligonucleotide duplexes were used, the idiosyncrasies of naphthalimide binding were investigated. By varying the DNA or RNA duplex base composition, it was determined that G-C pairs are necessary but not sufficient for measurable binding; a 5'-TpG-3' step results in more stabilization than a 5'-GpT-3' step, and the consequence of adding a 5'-TpG-3' or 5'-GpT-3' step to a duplex is dependent upon the sequence composition of the duplex.

METHODS AND MATERIALS

PREPARATION OF NUCLEIC ACIDS. All oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). Oligonucleotides were obtained resulting in the following duplex sequences: 5'-d(ATATATATATATAT)₂-3' (**A**), 5'-d(ATATATGATATA)-3'/5'-(TATATCATATAT)-3' (**B**), 5'-d(ATATAGTATATA)-3'/5'-(TATATACTATAT)-3' (**C**), 5'-d(GTAATATTAC)₂-3' (**D**), 5'-d(GCGCGCGC)₂-3' (**E**), 5'-d(GTCCGCGGAC)₂-3' (**F**), 5'-d(GTCCGTCCGAC)-3'/5'-(GTCCGACGGAC)-3' (**G**), 5'-r(GCGCGCGC)₂-3' (**H**), 5'-r(GUCCGCGGAC)₂-3' (**I**), and 5'-d(CGATCG)₂-3' (**J**). The purification of **A-I** followed standard procedures previously described,³² while **J** was purified on a Shimadzu LC-10 system using a Waters μ Bondapak C-18 column (9 x 300 mm). The mobile phase was 10 mM

ammonium acetate (pH 7.0) with a flow rate of 1 mL/min. The elution was performed over a linear gradient from 0.1 to 20% acetonitrile over 20 min.

PREPARATION OF NAPHTHALIMIDE DERIVATIVES. Naphthalimide derivatives were selected to encompass a range of electron donating and withdrawing properties, molecular size or steric effects, and other physical properties as outlined in Hardebeck et al.²⁶ Naphthalimide derivatives were synthesized as previously described.²⁶⁻³¹ Stock solutions were prepared by reconstituting dried compounds in DMSO (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mM. Compound numbers are defined in Figure 1.

UV-VIS WAVELENGTH SCANS. Absorption spectra were collected using a Beckman-Coulter DU800 spectrophotometer. The spectra were collected from 800 to 200 nm. The effect of intercalation on the absorbance spectra of these naphthalimide compounds was measured by adding varying aliquots of DNA to a solution containing the naphthalimide compound of interest (250 μ M in 10 mM NaH₂PO₄, 1 mM Na₂EDTA, pH 7.0) in a quartz cuvette. A new sample was prepared at each duplex base pair (bp) concentration.

NUCLEAR MAGNETIC RESONANCE. Single strands were annealed in 80 mM NaCl, 3 mM NaH₂PO₄, 7 mM NaHPO₄, and 0.5 mM Na₂EDTA (pH 7.0) to give duplex concentrations of ~1 mM. For the nonexchangeable proton experiments, D₂O exchange was performed with lyophilization from 99.96% D₂O (Sigma-Aldrich, St. Louis, MO). The samples were then redissolved in ~250 μ L of 100% D₂O (Acros Organics, Hampton, NH). Experiments on exchangeable protons were recorded in 90:10 H₂O:D₂O. The appropriate amount of stock compound was added to give the correct duplex:intercalator ratio.

All spectra were acquired on a Bruker Avance 400 MHz spectrometer equipped with a broadband observe probe. One-dimensional (1D) imino proton spectra for the DNA duplex

without intercalator were acquired using a jump-and-return pulse sequence³³ optimized for water suppression, and the spectra of the DNA:intercalator complexes were acquired with a WET pulse sequence. The WET pulse sequence was used to suppress the water and DMSO solvent peaks.³⁴ 1D data were acquired over a temperature range of 0-40 °C. NMR data were processed using TOPSPIN (Bruker Inc., Billerica, MA). Spectra were referenced to HDO at the appropriate temperature.

CIRCULAR DICHROISM. Single strands were annealed in 10 mM NaH₂PO₄ and 1 mM Na₂EDTA (pH 7.0) to give a duplex concentration of 50 μM. The effect of intercalation on the CD spectra of these naphthalimide compounds was measured by adding the appropriate amount of stock compound to give the following duplex:intercalator molar ratios: 0:1 and 0.5:1 for most samples and 0:1, 0.5:1, 1:1, 1.5:1, and 2:1 for one sample. The CD spectra of the complexes were collected on two separate Jasco J815 spectropolarimeters (Japan Spectroscopic Co., LTD, Tokyo, Japan) at 10 °C. Data from 350 to 230 nm were signal averaged over four scans with a sensitivity of 10 mdeg and a resolution of 1 nm (scan speed = 50 nm/min).

OPTICAL MELTING EXPERIMENTS. An aliquot was removed from the stock naphthalimide solution and diluted to a total volume of 1 mL with MilliQ water (Millipore System, Bedford, MA). This solution was then used to dissolve 50 μM dried dsDNA or dsRNA in 10 mM NaH₂PO₄ and 1 mM Na₂EDTA (pH 7.0). Buffer conditions were chosen to mimic that of previous work on naphthalimides done by Remers and co-workers.³⁵ An appropriate amount of each compound in the same buffer was added to give the correct 1:1 duplex:intercalator molar ratio.

Optical melting experiments were performed on the complexes described above and monitored at 260 nm for DNA and 280 nm for RNA using a heating rate of 1 °C/min from ~10 to

90 °C on a Beckman-Coulter DU800 spectrophotometer with a Beckman-Coulter high performance temperature controller. The absorbance of the intercalator at these wavelengths was minimal in comparison to the DNA or RNA.

DETERMINATION OF THERMODYNAMIC PARAMETERS FOR DUPLEXES AND COMPLEXES. The resulting melt curves were analyzed using *Meltwin*³⁶ and were fit to a two-state model. The model assumes linear sloping baselines and temperature-independent ΔH° and ΔS° values.^{36,37} Each individual melt curve was analyzed to give ΔG°_{37} , ΔS° , and ΔH° values. The average ΔS° and ΔH° values from all of the melts were used to calculate the T_M at 1×10^{-4} M total strand concentration. The ΔT_M values upon intercalation were found by subtracting the values for the naked duplex from the values obtained for the complex; it was assumed that nucleic acid-intercalator mixtures with melts resulting in ΔT_M values < 1 °C did not form complexes.

RESULTS AND DISCUSSION

In this study, the substituent at the 3-position on the aromatic core of 1,8-naphthalimide was systematically altered, and the substituent's effects on binding to DNA and RNA duplexes were analyzed. The 11 substituents were selected for various reasons as described in the Methods and Materials section. Altering the substituent at the 3-position does in fact change the binding properties of 1,8-naphthalimide to short chemically synthesized DNA and RNA duplexes, as described below.

Simple intercalators typically bind to 5'-pyrimidine-purine-3' steps,^{4,7-9} with a preference for 5'-CpG-3' steps⁴ while amonafide and mitonafide have been shown to bind to 5'-TpG-3' or 5'-GpT-3' steps.¹⁷ Various DNA and RNA duplexes (see Methods and Materials section for full sequences of each duplex) used in this study contain a wide range of sequence compositions

(summarized in Table S1). For example, duplex **A** is a poly-AT sequence and has five 5'-YpR-3' steps, no 5'-CpG-3' steps, and no 5'-GpT-3' or 5'-TpG-3' steps. Duplex **B** is a poly-AT sequence with a single G-C pair in the middle of the duplex. It has five 5'-YpR-3' steps, no 5'-CpG-3' or 5'-GpT-3' steps, and one 5'-TpG-3' step. Duplex **C** is also a poly-AT sequence with a G-C pair in the middle of the duplex. It has five 5'-YpR-3' steps, no 5'-CpG-3' or 5'-TpG-3' steps, and one 5'-GpT-3' step. Duplex **D** is an A-T rich sequence with G-C pairs at the duplex termini. This sequence has three 5'-YpR-3' steps, one 5'-GpT-3' step, and no 5'-CpG-3' or 5'-TpG-3' steps. Duplex **E** is a poly-GC duplex and has three 5'-YpR-3' steps, three 5'-CpG-3' steps, and no 5'-GpT-3' or 5'-TpG-3' steps. Duplex **F** is mostly G-C pairs with A-T pairs at the penultimate positions. This sequence has two 5'-YpR-3' steps, two 5'-CpG-3' steps, one 5'-GpT-3' step, and no 5'-TpG-3' step. Duplex **G** is mostly G-C pairs with an A-T pair in the middle of the duplex and at the penultimate positions. Duplex **G** has two 5'-YpR-3' steps, two 5'-GpT-3' steps, and no 5'-TpG-3' steps. Duplex **J** is mostly G-C pairs with two A-T pairs in the middle. This sequence has two 5'-YpR-3' steps, two 5'-CpG-3' steps, and no 5'-GpT-3' or 5'-TpG-3' steps and was only used for NMR studies. In order to compare binding strengths gained via intercalation between DNA and RNA, the corresponding RNA duplexes **H** and **I** were obtained for DNA duplexes **E** and **F**, respectively.

CONFIRMATION OF INTERCALATION. A previous study by Waring et al.²⁴ reported that amonafide (compound **2**) binds to nucleic acids by intercalation. A few experiments (UV-vis wavelength scans, NMR titrations, and circular dichroism) on select complexes were performed in the current investigation to confirm that the 1,8-naphthalimide derivatives studied here were also binding via intercalation.

UV-vis wavelength scans of all individual compounds (**1-11**) mixed with each individual duplex (**A-I**) were performed to confirm that these naphthalimide derivatives intercalate into DNA. The complexation results in a slight red shift and broadening of the absorbance maximum of the compound, both typical of an intercalation event³⁸⁻⁴¹ and similar to that seen previously for the same compound when complexed with calf thymus DNA.²⁴ The scan for compound **2** mixed with duplex **F** can be seen in Figure S1. All other complexes displayed similar results (data not shown).

Broadening and shifting of DNA proton resonances via NMR upon addition of ligand are also indicative of DNA intercalation.^{42,43} NMR titrations were performed using compounds **2** (data not shown) and **3** (Figure S2) with duplex **J** and resulted in broadening and shifting of resonances in the amino region of the proton spectra. The stacking of the naphthalene moiety between the base pairs results in the upfield shift and broadening of the DNA protons as seen here, which is consistent with previous work with other DNA intercalators.⁴⁴⁻⁴⁶

Circular dichroism can be used to investigate the binding of compounds to DNA.^{47,48} When a compound binds to DNA via intercalation, an induced circular dichroism (ICD) is observed. This ICD is caused either by the geometric change in the drug or the coupling between its electronic transitions and those of the duplex.⁴⁸ Five compounds were titrated into five different duplexes which resulted in ICDs around 280 nm, suggesting binding via intercalation. The CD spectra for compound **2** with duplex **F** can be seen in Figure S3.

Based on previous experiments with naphthalimides²⁴ and the representative data from UV-vis wavelength scans, NMR titrations, and CD spectroscopy for select complexes in the current investigation, it was assumed that when the naphthalimide derivatives studied here bind to DNA or RNA, they are binding via intercalation.

OPTICAL MELTING EXPERIMENTS. The stability of a DNA or RNA duplex can be experimentally measured using optical melting experiments. The absorbance versus temperature plot results in a sigmoidal curve from which the melting temperature (T_M) can be determined. When the same duplex is complexed with an intercalator, another sigmoidal curve can be obtained, and the T_M will be shifted to a higher temperature in comparison to the non-complexed DNA.¹⁰ The difference between the melting temperature of naked DNA and of the complex (ΔT_M) can be attributed to the stability gained by intercalation.^{6,10,49} ΔT_M values equate well to binding strength, and an increase in intercalator concentration leads to an increase in stability.^{10,49} The ΔT_M values for all 11 naphthalimide derivatives, obtained with seven DNA duplexes and two RNA duplexes at a 1:1 molar ratio, are listed in Table 1. A plot of the melt curves for all compounds complexed with duplex **F** is shown in Figure S4. Similar melt curves were obtained with all other DNA and RNA duplexes reported here.

In general, when electron-withdrawing substituents were placed at the 3-position on the naphthalimide, the average ΔT_M values for those compounds were higher for all sequences studied here. For the electron-withdrawing substituents (compounds **2**, **4**, **5**, and **6**), the average ΔT_M is 6.5 °C; for the electron donating substituents (compounds **3**, **7**, **8**, **9**, **10**, and **11**) the average ΔT_M is 4.8 °C. For compound **1**, the average ΔT_M is 8.9 °C. When electron-withdrawing substituents are added to the naphthalene ring, it becomes more electron deficient and is therefore able to interact more favorably with the electron rich aromatic bases in DNA and RNA.⁵⁰

Table 1. ΔT_M^a values (°C) for naphthalimides complexed with various DNA and RNA duplexes

Compound	Duplexes									Average ^d
	A	B^b	C^b	D	E^c	F	G^b	H	I	
1	N/B ^e	19.4	11.6	8.1	8.7	5.6	N/B	N/B	N/B	8.9
2	N/B	17.2	11.7	12.6	14.3	13.3	5.0	1.8	2.2	12.4
3	N/B	14.6	10.1	11.6	16.9	5.1	N/B	1.1	1.0	9.7
4	N/B	7.0	N/B	4.5	6.3	1.1	N/B	N/B	N/B	3.2
5	N/B	13.9	10.8	6.3	9.5	6.0	N/B	1.6	2.0	7.8
6	N/B	8.4	1.7	4.4	2.5	N/B	N/B	N/B	N/B	2.8
7	N/B	14.7	5.7	4.4	1.6	1.6	2.2	N/B	N/B	5.0
8	N/B	16.4	N/B	7.3	7.9	N/B	2.1	N/B	1.1	5.6
9	N/B	8.8	4.7	8.6	4.6	5.6	N/B	N/B	N/B	5.4
10	N/B	1.3	5.8	N/B	N/B	N/B	N/B	N/B	N/B	1.2
11	N/B	5.1	N/B	N/B	3.2	2.7	N/B	N/B	N/B	1.8
Average	N/B	11.5	5.6	6.2	6.9	3.7	N/B	N/B	N/B	

^aValues were determined by subtracting the values for the naked duplex from the values obtained for the complex. ^bThe sequence was melted with its complementary sequence. ^cValues for duplex **E** were taken from ref. 26. ^dAverage values for each compound when complexed with DNA duplexes **B-G**. Note that DNA duplex **A** and RNA duplexes **H** and **I** were not included in this average. N/B entries were treated as 0 for the calculation of the average. ^eN/B= no binding.

Same naphthalimide, different duplex. The same naphthalimide derivative binds to a variety of DNA duplex compositions and gives a wide range of ΔT_M values (Table 1). At least three intercalators bind to six DNA duplexes, duplexes **B-G**, with a minimum of 1 °C of stabilization. However, they do not have the same degree of duplex stabilization (Table 1). When comparing the same naphthalimide derivative between sequences, it does not exhibit the same binding strength to each sequence. For example, compound **1** stabilizes different DNA duplexes from 0 to 19.4 °C, the largest range seen for the compounds investigated. Compound **1**

stabilizes duplex **B** with a ΔT_M of 19.4 °C, the highest seen for any duplex. Compound **2** exhibits the highest average stabilization gained by intercalation to DNA duplexes (duplexes **B-G**) with an average ΔT_M of 12.4 °C (Table 1). While there are compounds that significantly stabilize DNA, there are also compounds that do not. For example, compounds **10** and **11** exhibit weak stabilizing interactions to DNA duplexes (duplexes **B-G**), with an average ΔT_M of 1.2 and 1.8 °C, respectively.

There are several possible explanations for why a naphthalimide binds differently to different DNA duplexes. One explanation could be that there are different primary binding sites for each duplex. For example, compound **3** could be binding to a different sequence step in duplex **E** than in duplex **F**; the naphthalimide derivative could be binding to the 5'-GpC-3' step in duplex **E** but to a 5'-GpT-3' step in duplex **F**; therefore, differences in binding strength would be observed. Rigorous footprinting experiments would need to be completed in order to determine if substituents change the naphthalimide's primary binding site.

A second possibility for why a naphthalimide binds differently to different DNA duplexes is that the naphthalimide compounds could be binding to the same binding step in every duplex but inducing duplex unwinding to different extents in each duplex. When an intercalator binds, the DNA will unwind and lengthen, but the amount of helical twist is determined by a balance between destabilizing and stabilizing interactions between the intercalator and the bases. For example, the sequence composition could play a role in determining how much unwinding takes place. Some sequences may be able to better accommodate the intercalator than other sequences, or certain sequences may be better able to balance the destabilizing versus stabilizing interactions, as mentioned above.

Same duplex, different compounds. The data in Table 1 reveal the compound that is most stabilizing for each of the DNA or RNA sequences. None of the compounds studied bind to the poly-AT sequence, duplex **A**. For duplexes **B** and **C**, compounds **1** and **2** are the most stabilizing with ΔT_M values of 19.4 and 11.7 °C, respectively (Table 1). For duplexes **D** and **E**, compounds **2** and **3** are the most stabilizing with ΔT_M values of 12.6 and 16.9 °C, respectively (Table 1). For duplex **F**, compounds **2** and **5** are the most stabilizing with ΔT_M values of 13.3 and 6.0 °C, respectively. For duplex **G**, compounds **2** and **7** are the most stabilizing with ΔT_M values of 5.0 and 2.2 °C, respectively. It is interesting to note that the most stabilizing compound varies with duplex. Compound **2** is the most stabilizing to duplexes **C**, **D**, **F**, and **G**. Compound **1** is the most stabilizing to duplex **B**, and compound **3** is the most stabilizing to duplex **E**. The ΔT_M values for duplex **B** range from 1.3 to 19.4 °C, the largest range observed. Duplex **G** has a much smaller range of ΔT_M values from 0 to 5.0 °C.

RNA equivalents of all DNA duplexes were tested but only two, duplexes **H** and **I**, exhibited two-state and reproducible melt curves. RNA duplexes **H** and **I** are the equivalent of DNA duplexes **E** and **F**. These two RNA duplexes were used to obtain binding strengths for the same set of compounds, and the ΔT_M values are listed in Table 1. Only 3 out of the 11 compounds investigated bind to duplex **H**. Similarly, there are only four compounds that bind to duplex **I**. Compound **2** has the largest stabilizing effect towards RNA duplexes, duplexes **H** and **I**, yielding a ΔT_M of 1.8 and 2.2 °C, respectively. Duplex **H** has a ΔT_M range from 0 to 1.8 °C. Similarly, duplex **I** has a ΔT_M range from 0 to 2.2 °C.

There are several possible explanations for why different intercalators bind differently to the various sequences. First, changing the substituents on the naphthalene ring could alter the position of the entire compound within the base step, thereby changing the interactions between

the compound and the nucleic acid bases. This change in orientation would alter the stacking interactions between the naphthalene moiety and the aromatic base pairs resulting in different binding strengths. Since these stacking interactions are responsible for stabilizing the DNA-intercalator complex,^{5,11} intercalators tend to maximize these interactions. Therefore, intercalators with structural elements that increase van der Waals contacts tend to have a higher binding strength.^{49,51} A systematic structural study utilizing NMR, x-ray crystallography, or molecular modeling would need to be employed to determine the specific orientation of these naphthalimide compounds with various substituents.

Second, if the substituent is able to be in a coplanar orientation with the ring system, then it could potentially increase the stacking ability of the molecule and exhibit an increased binding strength. For example, compound **2**, with a nitro group at the 3-position, has an average ΔT_M of 12.4 °C, yet, compound **11**, with a methyl group at the 3-position, has an average ΔT_M of 1.8 °C. The ability of the nitro group to be coplanar with the ring system and the inability of the methyl group to be coplanar with the ring system might explain this difference in stability. Similarly, compound **10**, with a phenyl group at the 3-position, may adopt a conformation with the phenyl substituent twisted with respect to the naphthalimide aromatic core. If so, this could partially explain its relatively low average ΔT_M (1.2 °C).

Third, the overall size of the substituent could be playing a role in the compound's binding strength. Compound **6**, with a large iodo substituent at the 3-position, has a low average ΔT_M (2.8 °C). Similarly, compound **10**, with a large phenyl substituent at the 3-position, also has a low average ΔT_M (1.2 °C). When comparing the large and bulky substituents, compounds **6** and **10**, to a small substituent, compound **1**, there is a large difference in average ΔT_M values. The large and bulky phenyl and iodo groups could be preventing intercalation, therefore resulting

in low average ΔT_M values. Without a systematic structural study, with naphthalimides or other intercalators, it is difficult to estimate a size range of substituents that allow partial intercalation or completely inhibit intercalation.

DNA vs RNA. When comparing the number of compounds that bind to DNA versus RNA, there is a significant difference. There are only three compounds that bind to RNA duplex **H**, whereas there are 10 compounds that bind to the corresponding DNA duplex **E**. Similarly, there are only four compounds that bind to RNA duplex **I**; however, there are nine compounds that bind to its DNA equivalent duplex **F**. When comparing the compounds that bind to both DNA and RNA, a difference between ΔT_M values can be seen. Compound **2** stabilizes RNA duplex **H** by only 1.8 °C but stabilizes DNA duplex **E** by 14.3 °C. Additionally, compound **5** exhibits a similar trend by stabilizing duplexes **H** and **E** by 1.6 and 9.5 °C, respectively. Compound **2** stabilizes RNA duplex **I** by 2.2 °C whereas it stabilizes DNA duplex **F** by 13.3 °C.

Intercalation into DNA duplexes appears to be more stabilizing than intercalation into RNA duplexes. These differences in stabilization energies could be a result of the differences in helical parameters between RNA and DNA.⁵² Helical rise, twist, sugar pucker, and the 2'-OH group on the ribose sugar in RNA could cause steric interference and reduced π - π interactions.¹¹ Additionally, the difference in binding affinity between DNA and RNA could be a result of the major type of stacking interactions involved, in DNA there is mostly intra-strand stacking whereas in RNA there is mostly inter-strand stacking. .¹¹

Sequence Specificity. By using short chemically synthesized oligonucleotides of known composition, as opposed to calf thymus or herring sperm DNA, this study revealed some of the finer complexities of site specificity. Some general trends for naphthalimide binding to DNA and RNA are observed based on sequence composition, but sequence specificity is convoluted.

It can be seen that there is no observed binding to duplex **A**, a poly-AT duplex (Table 1). When G-C pairs are introduced into the middle of a poly-AT duplex, duplexes **B** and **C**, or at the termini of a poly-AT duplex, duplex **D**, binding is observed. The average ΔT_M for duplex **A** is 0 °C but increases to 11.5 and 5.6 °C for duplexes **B** and **C**, respectively. Duplexes **B** and **C** both have five 5'-YpR-3' steps, but duplex **B** has one 5'-TpG-3' step while duplex **C** has one 5'-GpT-3' step. The higher average ΔT_M for duplex **B** suggests that the naphthalimide compounds have a more favorable interaction with a 5'-TpG-3' step than a 5'-GpT-3' step. Similarly, when G-C pairs are added to the termini of a poly-AT duplex, as in duplex **D**, the average ΔT_M increases from 0 °C for a poly-AT duplex to 6.2 °C for duplex **D** (Table 1). Duplex **D** has two 5'-GpT-3' steps, but because both are at the helix termini, an unstable terminal G-C pair could result upon intercalation. This may explain why duplex **D**, with two terminal 5'-GpT-3' steps, is only slightly more stabilized upon intercalation than duplex **C**, with one internal 5'-GpT-3' step.

The data for the A-T rich duplexes suggest that a G-C pair is required for binding and that 5'-TpG-3' steps are preferred over 5'-GpT-3' steps, with internal 5'-GpT-3' steps being more stabilizing than terminal 5'-GpT-3' steps. The data for the G-C rich sequences (Table 1), however, are contrary to these trends. Duplex **E**, a poly-GC duplex, has an average ΔT_M of 6.9 °C, suggesting that a 5'-GpT-3' or 5'-TpG-3' step is not required for binding. Interestingly, when two penultimate A-T pairs (resulting in two terminal 5'-GpT-3' steps) were added to a poly-GC duplex, duplex **F**, the average ΔT_M (3.7 °C) decreased from that observed for the poly-GC duplex **E**. When an internal 5'-GpT-3' step (duplex **G**) was added to a poly-GC duplex with terminal 5'-GpT-3' steps, binding was significantly reduced.

Taking the data for the A-T rich and G-C rich sequences together, the complicated nature of the sequence specificity becomes apparent. For example, a G-C pair is required but not

sufficient for binding. Although a G-C pair is required for binding, a G-C rich duplex is not necessarily stabilized more than an A-T rich duplex upon intercalation. A single internal or two terminal 5'-GpT-3' steps are sufficient to result in binding to an A-T rich duplex but not sufficient for binding to a G-C rich duplex and can even decrease binding.

The consequence of adding a 5'-TpG-3' or 5'-GpT-3' step to a duplex appears to be dependent upon the sequence composition of the duplex. For example, when added to a poly-AT duplex, naphthalimide binding was enhanced by 5.6-11.5 °C, but when added to a poly-GC duplex, naphthalimide binding was diminished by 3.2-6.9 °C. Clearly, binding of naphthalimides to DNA or RNA is not dependent solely on the A-U or G-C content, the number of C-G steps, or the number of 5'-GpT-3' or 5'-TpG-3' steps.

Further characterization of naphthalimide binding, perhaps by footprinting, molecular modeling, NMR, or x-ray crystallography, may help understand why naphthalimides stabilize different duplexes more than others, why different naphthalimides bind differently to the same sequence, why there is a difference between naphthalimide binding to DNA and RNA, and why the sequence specificity appears to be so complex.

ASSOCIATED CONTENT

Notes

The authors declare no competing financial interest.

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Graphical Abstract