Asymmetrical di–aromatic guanidinium/2–aminoimidazolinium derivatives: synthesis and DNA affinity

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Abstract

In this paper we report the synthesis of three families of new amidine–based aromatic derivatives as potential DNA minor groove binding agents for the treatment of cancer. The preparation of *mono*–guanidine, *mono*–2–aminoimidazoline and asymmetric diphenyl guanidine/2–aminoimidazoline derivatives (compounds **1a,b,c** to **8a,b,c**) is presented. The affinity of these substrates and of a family of *mono*– and *bis*–isoureas (previously prepared in Rozas' laboratory) for DNA was evaluated by means of DNA thermal denaturation measurements. In particular, compounds **2c**, **5c**, **6c**, **7c**, and **8c** were found to bind strongly both to natural DNA and to Adenine–Thymine oligonucleotides, showing a preference for the Adenine–Thymine base pairs sequences.

Abbreviations: HB, hydrogen bond; ΔT_m , increment in DNA denaturation temperature; AT, Adenine–Thymine pairs; MES, 2–(*N*–morpholino)ethanesulfonic acid; P/D, ratio between base pairs and ligand (drug);

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Introduction

The DNA minor-groove is the interaction site for many replicative and repair enzymes and transcription control proteins. It also provides an opportunity to design drugs that are capable of selective binding and hence the potential to interfere with these processes. Minor-groove binding agents typically have long and planar structures that allow them to adopt a crescent shape that fits into the minor-groove forming close contacts in the deep, narrow space formed between the two DNA strands. These DNA/minor-groove binder complexes are typically stabilized by hydrogen bonds (HBs), van der Waals contacts and/or ionic interactions.¹ Binding is typically driven by hydrophobic interactions and if the molecule is positively charged this is usually accompanied by the liberation of a large number of water molecules. The positively charged natural products distamycin and netropsin were among the first examples of molecules that specifically target the minor-groove. Since their discovery, many drugs that target the minor-groove have been developed. They are highly selective and they compromise the fundamental biochemistry of a cell through inhibition of the actions of DNA-dependent enzymes, for example by direct inhibition of transcription.^{2,3,4}

Sophisticated synthesis has yielded oligoamides capable of highly specific binding;⁵ further development has also witnessed the emergence of hairpins polyamides to latch around the groove.⁶ While many different minor-groove binders have been synthesised and tested as anticancer agents, their severe toxicity has prevented any therapeutic use. However, some dicationic aromatic diamidines such as furamidine (Figure 1),⁷ have shown promising antiproliferative activity against different tumour cell-lines. The furamidine derivative furimidazoline is one particularly good example.⁸ It is worth noting that fluorescence microscopy indicates the rapid nuclear accumulation of some furamidines in tumour cells.⁹ These findings together open new possibilities for developing novel aromatic dicationic derivatives with enhanced interaction and selectivity within the DNA minor-groove and possibly with more selective toxicity.

We have previously prepared a large number of symmetric diaromatic *bis*-guanidinium and *bis*-2-aminoimidazolinium derivatives, which are structurally related to furamidine, with potential to treat sleeping sickness (Figure 1).¹⁰ These compounds showed good affinity for DNA as indicated by the increment of the melting temperature (ΔT_m) values obtained in thermal denaturation measurements. The results encouraged us to develop new ligands that might more efficiently bind to DNA.

Figure 1. Structure of furamidine (left) and general structure of the related compounds previously reported by Rozas' group¹⁰ that have shown good affinity towards DNA.

Hence, we have further developed this set by preparing three new additional families (Figure 2), comprising a series of diaromatic *mono*-guanidinium and *mono*-2-aminoimidazolinium derivatives (family I: compounds **1g/h** to **8g/h**), a series of diaromatic *mono*- and *bis*-isouroniums (family II: compounds **9i/j** to **11i/j**) and a series of asymmetric diaromatic guanidinium/2-aminoimidazolinium dications (family III: compounds **1c** to **8c**). Consideration of the properties of each of these families has allowed us to explore the requirements needed for improved binding to the DNA minor-groove. All of the compounds described here contain the basic furamidine scaffold which has been modified by systematically changing both the terminal cationic arms and the bridging furan moiety.

Figure 2. Structure of the three families studied

The cationic groups may bind to DNA in two ways, firstly through direct H-bonds through the NH groups and secondly through water-mediated interactions with the bases. These two mechanisms of binding mean that different interactions, depending on the nature of the cation, can exist. In this work the amidinium cations have been exchanged with guanidinium, 2-aminoimidazolinium and isouronium cations. In these cases, we sought to enhance HB contacts in the minor groove by increasing the number of HB donors (NH groups in the guanidinium and 2-aminoimidazolinium cations) or introducing an additional HB acceptor (an O atom in the isouronium cation). Furthermore, these are more articulated cations than amidinium. The structure of the supporting molecular scaffold or linker, is very important if we wish to capitalize on these multiple binding interactions. The optimum linker will allow for a better orientation of the positive charges within the groove and introduce the curvature or geometry best suited to fitting. However, it is now known that curvature is not essential to fit into the minor groove, since Boykin *et al.* have shown that linear molecules are also capable of strongly binding to the minor-groove.¹¹ Work by Nguyen *et al.* showed that in the linear molecule CPG-40215A, the *N,N'*-diaminoguanidine bridging group served as a ‘see-saw’ hinge facilitating a variety of interactions with the bases through motions that position the terminal amidine cations in a range of binding modes.¹²

Moreover, to investigate the optimal linking scaffold we have exchanged the furan moiety of furamide, for a number of smaller functional groups (O, S, NH, CO, CH₂, CH₂CH₂ or NHCONH). We hypothesized that such a substitution should afford a two-fold advantage by firstly facilitating the interaction with DNA through HBs (as donors or acceptors) and secondly, as our cations are larger than in the furamide series, making this central group smaller should ensure optimization of the distance between the cationic moieties (guanidinium and 2-aminoimidazolinium) for DNA binding.

To assess the binding to DNA we have performed thermal denaturation experiments in natural DNA (salmon sperm, 68% Adenine-Thymine base pair –AT- content). Furthermore, since binding to the minor groove requires the presence of a run of five AT base pairs binding experiments were also conducted in the presence of double stranded AT homopolymers [poly(dA•dT)₂ and poly(dA)•poly(dT)] to assess their potential selectivity for the minor groove .

Results and Discussion

Chemistry

On the basis of our previous experience and taking into account the good results obtained by our group^{10,13,14} the guanidinylation and 2-aminoimidazolinylation of the present target molecules was performed following our established methodology. This consists of the reaction of a deactivated aromatic amine with *N,N'*-bis(*tert*-butoxycarbonyl)thiourea (Boc protected guanidine precursor) or *N,N'*-di(*tert*-butoxycarbonyl)imidazoline-2-thione¹³ (Boc protected 2-aminoimidazoline precursor) assisted by mercury (II) chloride in the presence of an excess of triethylamine. Deprotection of the Boc-intermediates of the first stage and further treatment with Amberlyte resin in water leads to the hydrochloride salts of the target molecules.^{10,13,14,15} Preparation of the corresponding *mono*-isouronium (**9i**, **10i**, **11i**, Table 1) and *bis*-isouronium derivatives (**9j**, **10j**, **11j**, Table 2) has been reported by our group in a

previous article¹⁶ using a similar synthetic approach to the one just described starting from the corresponding aromatic alcohols.

Synthesis of asymmetric guanidine/2-aminoimidazoline derivatives (Family III)

All our starting materials are symmetric and, hence, the two amino moieties have the same reactivity. As we intend to introduce different groups at both ends of the molecule, an excess of the diamines was required to avoid di-functionalization. Thus, three equivalents of each of the corresponding diamines were treated with just one equivalent of *N,N'*-bis(*tert*-butoxycarbonyl)thiourea, one equivalent of mercury (II) chloride and an excess of triethylamine to afford the desired *mono*-functionalized Boc-protected guanidines (**1a–8a**) as main products with yields ranging from 29 to 82% (see Scheme 1). Working under these conditions, less than 5% of the di-functionalized products was observed.

Scheme 1

In the second stage of the synthesis, one equivalent of each of the previous intermediates was reacted with one equivalent of *N,N'*-di(*tert*-butoxycarbonyl)imidazoline-2-thione, a slight excess of mercury (II) chloride and an excess of triethylamine to afford the

asymmetric Boc-protected derivatives (**1b–8b**) in moderate to good yields. Standard deprotection of the Boc groups with an excess of trifluoroacetic acid in dichloromethane followed by treatment with Amberlyte resin in water led to the hydrochloride salts of the asymmetric guanidine- and 2-aminoimidazoline containing products (**1c–8c**).

Synthesis of amino-guanidinium and amino-2-aminoimidazolinium derivatives (Family I)

Regarding the synthesis of the *mono*-guanidine and *mono*-2-aminoimidazoline containing derivatives, in the first step we decided to protect one of the amino groups of the starting amines with Boc (see Scheme 2) to increase the solubility of the intermediates and allow a faster development of the column chromatography.

Following the same strategy as mentioned above, three equivalents of each of the starting diamines were treated with one equivalent of Boc anhydride. The *mono*-Boc-protected amines (**1d–6d**) were obtained as the main products in good yields.

The urea derivative **8d** was prepared following a different route (see Scheme 2). Thus, reaction of 4-nitrobenzoisocyanate with *mono*-Boc-protected 4-aminoaniline yielded the corresponding Boc protected 4-[3-(4-nitrophenyl)ureido]aniline (**12**), which upon hydrogenation produced derivative **8d**. However, the carbonyl group proved to be too deactivating and compound **7d** (and subsequently **7e**, **7f** and **7h**) could not be prepared. All these compounds are new, except for **1d**¹⁷ and **3d**¹⁸ that have been already described.

In the second stage, the *mono*-Boc-protected amines were treated with one equivalent of *N,N'*-bis(*tert*-butoxycarbonyl)thiourea or *N,N'*-di(*tert*-butoxycarbonyl)imidazoline-2-thione, a slight excess of mercury (II) chloride and triethylamine. The guanidine precursors (**1e–6e** and **8e**) were purified by column chromatography in silica gel, whereas for the 2-aminoimidazoline ones (**1f–6f** and **8f**), neutral alumina was used instead. Finally, deprotection of the Boc groups afforded the final *mono*-amidinium-like products after three

stages in moderate to good overall yields. It should be mentioned that the amino–guanidine derivatives **1g–8g** could also be obtained after Boc deprotection of **1a–8a**. In fact, this was the only possible route to prepare compound **7g** and proved to be a shorter and more efficient methodology, producing higher overall yields.

Scheme 2

All the diamines used as starting materials are commercially available either from Aldrich or Fluka, except for the 1,4-(*bis*-4-aminophenyl)piperazine, whose synthesis has been described previously.¹⁹ It is also worth mentioning that, the unreacted starting diamines could be recovered from the column eluting with a more polar solvent system.

Biophysical Results: Thermal Denaturation Assays

Thermal Denaturation experiments are easy to perform and provide quick and reliable information on the binding of small molecules to DNA. These assays, for example in unspecific salmon sperm DNA can be used as a general screening for the suitability of the compounds studied as DNA binders. Afterwards, the preference for specific base sequences can be explored.

Denaturation studies of families I–III in the presence of natural DNA

The interaction of all the molecules with DNA was firstly examined by performing thermal denaturation experiments using a mixed sequence salmon sperm DNA with a 32 % GC content. The melting temperature of salmon sperm DNA ($T_{m(SS)}$) was measured in the presence of the different families of molecules.

The results found for some of the *mono*–amidinium–like derivatives (guanidinium, 2–aminoimidazolinium and isouronium derivatives from families I and II) are presented in Table 1. The *mono*–amidinium–like compounds tested were found to have a slight stabilizing interaction with natural DNA, as indicated by the moderate increase in melting temperature, with $\Delta T_{m(SS)}$ values of around 3 °C being obtained.

Table 1.– Results obtained from the thermal melting experiments showing the change in melting temperature in the presence of the *mono*–amidine–like cations (**1g,h**–**6g,h**, **7g**, **8g,h**, **9i**–**11i**) to salmon sperm DNA ($\Delta T_{m(SS)}$, °C)^a

X		$\Delta T_{m(SS)}, ^\circ\text{C}$	$\Delta T_{m(SS)}, ^\circ\text{C}$	$\Delta T_{m(SS)}, ^\circ\text{C}$	
CH ₂	1g	3	1h	3	9i 2
CH ₂ CH ₂	2g	0	2h	0	
O	3g	2	3h	0	10i 4
S	4g	2	4h	0	11i 2
NH	5g	2	5h	2	
Piperazine	6g	2	6h	1	
CO	7g	0			
NHCONH	8g	2	8h	4	

^a Melting temperature in phosphate buffer (10 mM) is 69 °C.

Progressing from the monocationic species to the dicationic symmetric *bis*-isouronium derivatives (**9j** – **11j**) resulted in moderately greater changes (Table 2). For these compounds, the O linker (**10j**) was found to have the greatest effect on the melting temperature resulting in an increase of 6 °C in the T_m value.

More pronounced changes were observed when the measurements were repeated for the asymmetric dicationic compounds of family III (**1c** – **8c**). These compounds were found to significantly increase the T_m by a range of 6 and 12 °C indicating an enhanced stabilization of the duplex structure that is attributed to improved binding.

Comparison of the $\Delta T_{m(SS)}$ values obtained for the symmetric *bis*-isouronium (**9j**-**11j**) and the asymmetric dicationic (**1c**, **3c**, **4c**) possessing the CH₂, O or S linkers, revealed very little difference in the melting temperature between the compounds with different cations.

Table 2.— Results obtained in the thermal melting experiments showing the change in melting temperature of DNA in the presence of the *bis*-isouronium derivatives (**9j**-**11j**) and asymmetric diphenyl dicationic compounds (**1c**-**8c**).

X	$\Delta T_{m(SS)}$ (°C)		$\Delta T_{m(AT)}$ (°C)	Discretion $\Delta T_{m(AT)} - \Delta T_{m(SS)}$ (°C)	$\Delta T_{m(A)(T)}$ (°C)	Sequence Specificity
	Salmon sperm DNA ^a	Poly(dA•dT) ₂ DNA ^b	Poly(dA)•poly(dT) DNA ^c			
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	Cmp		Family II
CH ₂	9j	4	—	—	—	—
O	10j	6	—	—	—	—
S	11j	5	—	—	—	—
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	Cmp		Family III
CH ₂	1c	6	6	0	17.2	None
CH ₂ CH ₂	2c	8	16	8	29	AT
O	3c	7	10	3	25.3	AT
S	4c	7	9	2	23	AT
NH	5c	12	17.5	5.5	35.1	AT
Piperazine	6c	9	15	6	31.1	AT
CO	7c	8	15	7	32	AT
NHCONH	8c	11.5	14	2.5	30	AT
Netropsin		—	39	—	—	—

^aDNA melting temperature in phosphate buffer (10 mM) is 69 °C.

^bP/D = 10. Poly(dA•dT)₂ melting temperature in phosphate buffer (10 mM) is 47 °C. Experiments performed in TCD.

^cP/D = 3. Poly(dA)•poly(dT) melting temperature in MES buffer (10 mM) is 43 °C. Experiments performed by WD Wilson and co. in Georgia State University.

When we compare the melting results of the molecules with the methylene (**1c**; see thermal melting experiment results in Figure 3) and ethylene (**2c**) bridges in the presence of natural DNA, we observe that the ethylene linker gave a slightly better melting result, which is indicative of improved binding. A possible reason for this is that the extra CH₂ increases hydrophobicity thereby improving the hydrophobic interactions. Another possible conclusion for this is that the more flexible ethylene linker provides another point of contact with the DNA allowing for a better binding.

A comparable change was also found in the case of the bulky piperazine linker (**6c**), which yielded intermediate stabilization of DNA. This is interesting given the linear and bulky nature of the piperazine linker, and thus, the combination of both features could play a role in its entry and fitting into the minor groove. The greatest change was observed for the NH (**5c**; see thermal melting experiment results in Figure 3) and NHCONH (**8c**) species (~ 12 °C). Interestingly, both of these compounds are capable of strong hydrogen bonding interactions. The strong binding of compound **8c** to salmon sperm DNA (Table 2) could possibly be explained because it has a urea linker that could provide enough freedom of rotation allowing a good induced fit inside the DNA minor groove. However, it should be noted that the changes do not seem to reflect the difference in the length of the linker in the case of **5c** and **8c**.

Figure 3.— Graph showing the ΔT_m for **1c** ($\Delta T_m = 6$ °C, red) and **5c** ($\Delta T_m = 12$ °C, green) in salmon sperm DNA (blue)

Denaturation studies of family III in the presence of poly(dA•dT)₂ and poly(dA)•poly(dT)

The experiments carried out on the asymmetric series in the presence of the salmon sperm DNA revealed that, considering the linkers, the order of affinity is: $\text{--NH--} \approx \text{--NHCONH--} > (\text{--Piperazine--}, \text{--CO--}, \text{--CH}_2\text{CH}_2\text{--}, \text{--O--}, \text{--S--}, \text{--CH}_2\text{--})$. In light of the promising results found for family III, the experiments were repeated in the presence of poly(dA•dT)₂ using similar experimental conditions. Here, the absence of the G•C base pair reduces the width of the groove allowing for a tighter fit, with minor groove complexes typically forming with a run of four A•T base pairs. In almost all experiments, a greater change in melting temperature was found in the presence of the asymmetric dications (**2c–8c**) in the AT oligonucleotides. This result was taken as a strong indication of a preference for the minor groove binding site afforded in homopolymer DNA and indicates sequence selectivity for the AT domains of DNA (Table 2). The exception was found for the CH₂ species (**1c**) which was found to stabilize the homopolymer to the same extent as natural DNA.

However, in the case of the ethylene linker (**2c**) the melting temperature showed a two-fold increment compared to salmon sperm DNA. This would imply that while **2c** binds to AT rich domains, which are characterized by a narrow minor groove, **1c** is unlikely to bind in the same fashion as the increased availability of binding sites in the AT homopolymer is not accompanied by increased binding affinity. Thus, it may be concluded that the restricted degree of rotation conferred by the $\text{--CH}_2\text{--}$ bridge does not favour insertion into the narrower minor groove present in the AT DNA and the molecule most probably binds more effectively through an alternative mode. However, the higher degree of rotation due to the extra CH₂ in **2c**, allows the molecule to wrap around the minor groove better than **1c**.

Next, we considered the influence of the other bridging groups in the selective binding between the different DNA systems. By comparing molecules **1c**, **3c**, **4c** and **5c** (linker: $\text{--CH}_2\text{--}$, --O-- , --S-- and --NH-- respectively), it was noted that compound **5c** had the highest melting temperature in both salmon sperm DNA and poly(dA•dT)₂. This was

followed by molecules **3c** and **4c** that have the same melting temperature in salmon sperm DNA but slightly different in poly(dA•dT)₂ DNA (by 1 °C). As noted previously, the smallest change was observed for compound **1c**. These results seem to indicate the importance of hydrogen bonding with a possible HB donation to the O2 of thymine being a potential dominant factor. The fact that **3c** and **4c** gave better melting results in comparison to **1c** illustrates further the order of binding since the oxygen and sulphur linkers could be HB acceptors, but the methylene bridge is a known weak HB donor.

However, if we consider the relative sensitivity of the melting temperature (ΔT^{rel}) defined to the DNA type ($\Delta T_{m(AT)} - \Delta T_{m(SS)}$; see Table 2), two distinct groupings emerge. The first group contains the compounds **2c** (–CH₂CH₂–), **5c** (–NH–), **6c** (–piperazine–) and **7c** (–CO–) and shows a ΔT^{rel} change of the order of 6 °C. The second grouping shows significantly less sensitivity to the two forms of DNA, **1c** (–CH₂–), **8c** (–NHCONH–), **4c** (–S–) and **3c** (–O–), with the ether linker showing the greatest difference of 3 °C.

When comparing the results for molecules **1c**, **5c** and **7c** (linkers: –CH₂–, –NH– and –CO– respectively), the primary difference is the HB characteristics of the corresponding linkers, with the methylene bridge being a weak HB donor, the –NH– a stronger HB donor and the carbonyl group a HB acceptor. The order of binding to DNA and AT oligonucleotides, from best to worst, was found to be: strong HB donor (NH) > HB acceptor (CO) > weak HB donor (CH₂). This indicates that, in the synthesis of future molecules, incorporating more HB donors would be advantageous. The geometry around the bridging linker must also be considered as it is likely to play an important role. Here, there are two important factors. Firstly, the steric nature of the geometry (tetrahedral –CH₂– vs trigonal planar –CO– and trigonal pyramidal –NH–) will influence the approach to and binding in the pocket. Secondly, the rotational freedom conferred by the bridging moiety would be expected to influence the binding. There is no freedom of rotation in **7c** (trigonal planar) whereas there

is freedom of rotation around the linker for molecules **1c** (tetrahedral linker) and **5c** (trigonal pyramidal linker). It is interesting to note that compound **6c** not only shows a medium affinity towards natural DNA but also to poly(dA•dT)₂. This linker is longer than the rest of the groups considered and the relatively good results obtained could be justified by the large size and linear characteristics of the piperazine ring. The ΔT_m of netropsin (a well known minor groove binder) was also measured in this homopolymer for the sake of comparison. Even though this compound shows a two-fold binding strength compared to the asymmetric dications, this can be explained by the large number of HB donor and acceptor groups present in this molecule.

AT sequence selectivity between different 5-base-pair binding sites has previously been reported for the different binding modes (1:1 versus 2:1) of the classic minor groove binder distamycin.²⁰ In the study by Chen *et al.*, a preference for the ATATA over AAAAA was observed. In addition, small molecules can bind in a 2:1 and a 1:1 fashion in the minor-groove and this seems to strongly depend on the sequence. It has been found by Vesnaver and co.²¹ that the narrowest groove is that of five base pairs of AT and Netropsin prefers 1:1 binding in such system. For this reason, we also considered the interesting results already published by us in collaboration with W.D. Wilson (Georgia State University, USA) on the thermal denaturation of the symmetric *bis*-guanidines and *bis*-2-aminoimidazolines,⁸ and pursued further collaboration with this group using those reported conditions with the asymmetric dications and the poly(dA)•poly(dT) oligonucleotide (Table 2). It was felt that these experiments would help to confirm the selectivity of the asymmetric dications towards the AT sequences.

The most significant differences in conditions were on the one hand, the higher loading of the drug to DNA, with a ratio between base pairs and ligand (P/D) of 3 rather than that of 10 used above and, on the other hand, the buffer used, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), as opposed to 10 mM phosphate buffer. Due to these

differences, the results obtained cannot be quantitatively compared to the $\text{poly(dA}\cdot\text{dT)}_2$ results. However, the trend observed is similar and a good correlation was found between both sets of results as shown in Figure 4. Comparing the results previously published with Wilson's group on the thermal denaturation of the symmetric *bis*-guanidines and *bis*-2-aminoimidazolines,¹⁰ and those found now using the same Wilson's conditions (Table 2), it is interesting to observe how the ΔT_m values obtained for the asymmetric compounds are almost an average of those measured for the *bis*-guanidines (smaller) and those obtained for the *bis*-2-aminoimidazolines (larger). This would imply a similar binding mode in both AT DNAs and, most probably linked to a 1:1 mode as would be expected given the *bis*-cationic nature of the compounds under study.

Figure 4. Correlation found between the ΔT_m $\text{poly(dA}\cdot\text{dT)}_2$ vs. ΔT_m $\text{poly(dA)}\cdot\text{poly(dT)}$

From these studies, it can be concluded that hydrophobic interactions, hydrogen bonding, electrostatic interactions and molecular conformation are all important factors in DNA minor groove binding for the asymmetric dications. More importantly, these show strong binding to DNA showing AT-sequence specificity.

Conclusions

We have prepared three different families of *mono*- and *bis*-amidinium-based derivatives, in order to explore their potential as DNA minor-groove binders. First, with respect to family I, the *mono*-guanidinium and *mono*-2-aminoimidazolinium derivatives have been prepared following the synthetic pathways previously described by us.^{10,13,14} Second, regarding family II, the preparation of the asymmetric guanidinium/2-aminoimidazolinium di-cations has been performed from the corresponding *mono*-guanidinium derivatives and subsequent introduction of the 2-aminoimidazoline group. Third, regarding family III, the preparation of the *mono*- and *bis*-isouronium derivatives has been reported in a previous paper from our group.¹⁶

The ability of these compounds to bind to DNA has been evaluated by thermal denaturation experiments not only with mixed sequence DNA (salmon sperm), which comprises all four bases, but also with poly(dA•dT)₂ and poly(dA)•poly(dT) oligonucleotides. Despite showing cationic groups at both ends of the di-aromatic moieties, *mono*-amidinium-like derivatives (ammonium and guanidinium or 2-aminoimidazolinium) and *bis*-isouroniums (two isouronium cations) have shown to poorly/moderately bind to DNA. Nevertheless, in general, asymmetric *bis*-amidinium-based derivatives displayed a good affinity towards DNA and a preference for the AT sequences. In particular, compounds **2c**, **5c**, **6c**, **7c** and **8c** strongly bind to both unspecific and AT oligonucleotides.

Taking into consideration these promising results, we believe that these asymmetric compounds deserve more investigation as DNA targeting agents and thus, more biophysical studies are ongoing and will be reported soon.

Experimental

Chemistry

All the commercial chemicals were obtained from Sigma–Aldrich or Fluka and were used without further purification. Deuterated solvents for NMR use were purchased from Apollo. Dry solvents were prepared using standard procedures, according to Vogel, with distillation prior to use. Chromatographic columns were run using Silica gel 60 (230–400 mesh ASTM) or Aluminium Oxide (activated, Neutral Brockman I STD grade 150 mesh). Solvents for synthesis purposes were used at GPR grade. Analytical TLC was performed using Merck Kieselgel 60 F₂₅₄ silica gel plates or Polygram Alox N/UV₂₅₄ aluminium oxide plates. Visualisation was by UV light (254 nm). NMR spectra were recorded in a Bruker DPX–400 Avance spectrometer, operating at 400.13 MHz and 600.1 MHz for ¹H–NMR and 100.6 MHz and 150.9 MHz for ¹³C–NMR. Shifts are referenced to the internal solvent signals. NMR data were processed using Bruker Win–NMR 5.0 software. Electrospray mass spectra were recorded on a Mass Lynx NT V 3.4 on a Waters 600 controller connected to a 996 photodiode array detector with methanol, water or ethanol as carrier solvents. Melting points were determined using an Electrothermal IA9000 digital melting point apparatus and are uncorrected. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrometer equipped with a Gateway 2000 4DX2–66 workstation and on a Perkin Elmer Spectrum One FT–IR Spectrometer equipped with Universal ATR sampling accessory. Elemental analyses (C, H, N) of the target compounds, which were performed at the Microanalysis Laboratory (School of Chemistry and Chemical Biology, University College Dublin), are within ±0.4% of the calculated values confirming ≥ 95% purity. Fractional moles of water frequently found in amidinium-like salts could not be prevented despite 24–48 h of drying in vacuum.

General method for the synthesis of *mono*-functionalized Boc-protected guanidines:

Method 1 [Scheme 1, conditions (A)].

Over a solution containing an excess (9.0 mmol) of each of the starting diamines, 3.0 mmol of *N,N'*-di(*tert*-butoxycarbonyl)thiourea and 1.3 mL (9.3 mmol) of TEA in DCM (5 mL) at 0 °C, 3.3 mmol of HgCl₂ were added. The resulting mixture was stirred at 0 °C for 1 h and for the appropriate duration at room temperature. Then, the reaction was diluted with EtOAc and filtered through a pad of Celite. The filter cake was rinsed with EtOAc. The organic phase was washed with water (2 x 30 mL), washed with brine (1 x 30 mL), dried over anhydrous Na₂SO₄ and concentrated under vacuum to give a residue that was purified by silica gel column chromatography, eluting with the corresponding hexane:EtOAc mixture to yield the desired compound as a solid. Less than 5% of the disubstituted substrate was observed.

General procedure for the synthesis of Boc-protected asymmetric guanidine/2-aminoimidazoline, Boc-protected amino/2-aminoimidazolidine, and Boc-protected amino/guanidine derivatives: Method 2 [Scheme 1, conditions (B); Scheme 2, conditions (B) and (C)].

Each of the corresponding amines was treated in DCM at 0 °C with 1.1 equivalents of mercury (II) chloride, 1.0 equivalent of *N,N'*-di(*tert*-butoxycarbonyl)imidazolidine-2-thione (for the 2-aminoimidazoline precursors) or *N,N'*-di(*tert*-butoxycarbonyl)thiourea (for the guanidine precursors) and 3.1 equivalents of TEA. The resulting mixture was stirred at 0 °C for 1 hour and for the appropriate duration at room temperature. Then, the reaction mixture was diluted with EtOAc and filtered through a pad of Celite to get rid of the mercury sulfide formed. The filter cake was rinsed with EtOAc. The organic phase was washed with water (2 x 30 mL), washed with brine (1 x 30 mL), dried over anhydrous Na₂SO₄ and concentrated under vacuum to give a residue that was purified by silica gel (guanidine precursors) or

neutral alumina column flash chromatography (2-aminoimidazoline precursors), eluting with the appropriate hexane:EtOAc mixture.

General procedure for the synthesis of *mono*-Boc-protected 4,4'-diphenyl diamines:

Method 3. Compounds 1d to 6d [Scheme 2, conditions (A)].

Over a solution containing an excess (30.0 mmol) of each of the corresponding diamines in DCM (200 mL), 10.0 mmol of TEA and 10.0 mmol of di-*tert*-butyl dicarbonate (Boc₂O) were added at 0 °C. The resulting mixture was stirred at 0 °C for 1 h and 16 h more at room temperature. Then, the reaction was concentrated under vacuum to give a residue that was purified by silica gel column chromatography, eluting with the appropriate hexane:EtOAc mixture. The *mono*-Boc-protected compound was obtained as a solid. The excess of unreacted starting material diamine was recovered from the column. Less than 10% formation of the di-Boc-protected compound was observed.

General procedure for the synthesis of the hydrochloride salts: Method 4.

Each of the corresponding Boc-protected precursors (0.5 mmol) was treated with 15 mL of a 50% solution of trifluoroacetic acid in DCM for 3 h. After that time, the solvent was eliminated under vacuum to generate the trifluoroacetate salt. This salt was dissolved in 20 mL of water and treated for 24 h with IRA400 Amberlyte resin in its Cl⁻ form. Then, the resin was removed by filtration and the aqueous solution washed with DCM (2 x 10 mL). Evaporation of the water afforded the pure hydrochloride salt. Absence of the trifluoroacetate salt was checked by ¹⁹F NMR.

Dihydrochloride salt of *N*-{4-[4-(imidazolidin-2-ylideneamino)benzyl]phenyl} guanidine (1c): Method 4.

White solid (96%); mp 78–80 °C; ¹H NMR (D₂O) δ 3.58 (s, 4H, CH₂), 3.76 (s, 2H, PhCH₂Ph), 6.98 (d, 2H, *J* = 8.0 Hz, Ar.), 7.01 (d, 2H, *J* = 8.0 Hz, Ar.), 7.15 (d, 2H, *J* = 8.0 Hz, Ar.), 7.16 (d, 2H, *J* = 8.0 Hz, Ar.); ¹³C NMR (D₂O) δ 39.6 (PhCH₂Ph), 42.2 (CH₂ Im), 123.3, 125.1, 129.6, 129.7, 131.5, 132.6, 139.7, 140.4 (Ar.), 155.5, 157.6 (CN); HRMS (ESI⁺) *m/z* 309.1728 calcd [M + H]⁺; found 309.1732. Anal. (C₁₇H₂₂Cl₂N₆·1.5H₂O) C, H, N.

Dihydrochloride salt of *N*-(4-{2-[4-(imidazolidin-2-ylideneamino)phenyl]ethyl} phenyl)guanidine (2c): Method 4.

White solid (94%); mp decomposes over 215 °C; ¹H NMR (D₂O) δ 2.84 (s, 4H, CH₂), 3.67 (s, 4H, CH₂ Im), 7.03–7.13 (m, 4H, Ar.), 7.15–7.25 (m, 4H, Ar.); ¹³C NMR (D₂O) δ 35.4, 35.5 (2CH₂), 42.2 (CH₂ Im), 123.3, 125.1, 129.4, 129.5, 131.3, 132.3, 140.3, 140.9 (Ar.), 155.6, 157.8 (CN); HRMS (ESI⁺) *m/z* 323.1984 calcd. [M + H]⁺; found 323.1988. Anal. (C₁₈H₂₄Cl₂N₆·0.8H₂O) C, H, N.

Dihydrochloride salt of *N*-{4-[4-(imidazolidin-2-ylideneamino)phenoxy]phenyl} guanidine (3c): Method 4.

Yellowish solid (94%); mp 48–50 °C; ¹H NMR (D₂O) δ 3.70 (s, 4H, CH₂ Im), 6.97–7.06 (m, 4H, Ar.), 7.17–7.26 (m, 4H, Ar.); ¹³C NMR (D₂O) δ 42.3 (CH₂), 119.5, 119.6, 125.8, 127.4, 129.0, 130.1, 154.8, 155.4 (Ar.), 155.8, 158.1 (CN); HRMS (ESI⁺) *m/z* 311.1594 calcd. [M + H]⁺; found 311.1595. Anal. (C₁₆H₂₀Cl₂N₆O·2.8H₂O) C, H, N.

Dihydrochloride salt of *N*-{4-[4-(imidazolidin-2-ylideneamino)phenylsulfanyl]phenyl}guanidine (4c): Method 4.

Yellowish solid (95%); mp 94–96 °C; ^1H NMR (D_2O) δ 3.67 (s, 4H, CH_2), 7.05–7.12 (m, 4H, Ar.), 7.19–7.27 (m, 4H, Ar.); ^{13}C NMR (D_2O) δ 42.2 (CH_2), 123.7, 125.5, 131.5, 132.0, 132.4, 132.9, 133.6, 134.1 (Ar.), 155.3, 157.4 (CN); HRMS (ESI^+) m/z 327.1293 calcd. $[\text{M} + \text{H}]^+$; found 327.1295. Anal. ($\text{C}_{16}\text{H}_{20}\text{Cl}_2\text{N}_6\text{S} \cdot 1.8\text{H}_2\text{O}$) C, H, N.

Dihydrochloride salt of *N*-{4-[4-(imidazolidin-2-ylideneamino)phenylamino]phenyl}guanidine (5c): Method 4.

Green solid (94%); mp 66–68 °C; ^1H NMR (D_2O) δ 3.60 (s, 4H, CH_2), 6.95–7.09 (m, 8H, Ar.); ^{13}C NMR (D_2O) δ 42.2 (CH_2), 117.8, 118.0, 125.2, 126.0, 126.9, 127.3, 141.3, 142.0 (Ar.), 155.9, 158.1 (CN); HRMS (ESI^+) m/z 310.1722 calcd. $[\text{M} + \text{H}]^+$; found 310.1724. Anal. ($\text{C}_{16}\text{H}_{21}\text{Cl}_2\text{N}_7 \cdot 1.7\text{H}_2\text{O}$) C, H, N.

Tetrahydrochloride salt of *N*-(4-{4-[4-(imidazolidin-2-ylideneamino)phenyl]piperazin-1-yl}phenyl)guanidine (6c): Method 4.

Brownish solid (93%); mp 168–170 °C; ^1H NMR (D_2O) δ 3.62 (s, 4H, CH_2 Im), 3.71 (s, 8H, CH_2 Pip), 7.23–7.31 (m, 4H, Ar.), 7.37 (d, 2H, $J = 9.0$ Hz, Ar.), 7.41 (d, 2H, $J = 8.5$ Hz, Ar.); ^{13}C NMR (D_2O) δ 42.3 (CH_2 Im), 50.2, 50.9 (CH_2 Pip), 120.0, 120.3, 125.0, 126.6, 131.4, 133.0, 141.9, 143.1 (Ar.), 155.6, 157.8 (CN); HRMS (ESI^+) m/z 379.2359 calcd. $[\text{M} + \text{H}]^+$; found 379.2362. Anal. ($\text{C}_{20}\text{H}_{30}\text{Cl}_4\text{N}_8 \cdot 1.0\text{H}_2\text{O}$) C, H, N.

Dihydrochloride salt of *N*-{4-[4-(imidazolidin-2-ylideneamino)benzoyl]phenyl} guanidine (7c): Method 4.

White solid (94%); mp 99–101 °C; ^1H NMR (D_2O) δ 3.80 (s, 4H, CH_2), 7.38 (d, 2H, $J = 8.5$ Hz, Ar.), 7.42 (d, 2H, $J = 8.5$ Hz, Ar.), 7.78–7.84 (m, 4H, Ar.); ^{13}C NMR (D_2O) δ 42.3 (CH_2), 121.8, 123.5, 131.5, 131.6, 133.7, 134.2, 138.9, 139.5 (Ar.), 155.3, 157.5 (CN), 197.5 (CO); HRMS (ESI^+) m/z 323.1620 calcd. $[\text{M} + \text{H}]^+$; found 323.1619. Anal. ($\text{C}_{17}\text{H}_{20}\text{Cl}_2\text{N}_6\text{O} \cdot 2.0\text{H}_2\text{O}$) C, H, N.

Dihydrochloride salt of 1-(4-guanidinophenyl)-3-[4-(imidazolidin-2-ylideneamino)phenyl]urea (8c): Method 4.

White solid (94%); mp decomposes over 200 °C; ^1H NMR (D_2O) δ 3.57 (s, 4H, CH_2), 6.98 (d, 2H, $J = 8.5$ Hz, Ar.), 7.03 (d, 2H, $J = 8.5$ Hz, Ar.), 7.16 (d, 2H, $J = 8.5$ Hz, Ar.), 7.20 (d, 2H, $J = 8.5$ Hz, Ar.); ^{13}C NMR (D_2O) δ 42.1 (CH_2), 120.0, 120.1, 123.4, 125.4, 128.3, 129.3, 136.1, 136.8 (Ar.), 153.4, 155.4, 157.3 (CO, CN); HRMS (ESI^+) m/z 353.1838 calcd. $[\text{M} + \text{H}]^+$; found 353.1832. Anal. ($\text{C}_{17}\text{H}_{22}\text{Cl}_2\text{N}_8\text{O} \cdot 2.0\text{H}_2\text{O}$) C, H, N.

DNA Binding Assays

DNA salmon sperm and poly(dA•dT)₂ experiments

Thermal melting experiments were conducted with a Varian Cary 300 Bio spectrophotometer equipped with a 6x6 multicell temperature-controlled block. Temperature was monitored with a thermistor inserted into a 1-mL quartz curvet containing the same volume of water as in the sample cells. Absorbance changes at 260 nm were monitored from a range of 20 °C to 90 °C with a heating rate of 1 °C/min and a data collection rate of five points per °C. The salmon sperm DNA was purchased from Sigma Aldrich (extinction coefficient $\epsilon_{260} = 6600 \text{ cm}^{-1}\text{M}^{-1}$ base). A quartz cell with a 1-cm path length was filled with a 1-mL solution of

DNA polymer or DNA–compound complex. The DNA polymer (150 μ M base) and the compound solution (15 μ M) were prepared in a phosphate buffer [0.01 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$], adjusted to pH 7) so that a compound to DNA base ratio of 0.1 was obtained. The thermal melting temperatures of the duplex or duplex–compound complex obtained from the first derivative of the melting curves are reported.

Poly(dA)•poly(dT) experiments

Thermal melting experiments were conducted with a Cary 300 Bio UV–Visible spectrophotometer (Varian, Inc.) in 1–cm quartz cells. The solutions were prepared in MES buffer containing 10 mM 2–(N–morpholino)ethanesulfonic acid, 0.1 M NaCl, 1 mM EDTA, pH 6.25. The DNA polymer poly(dA)–poly(dT) was purchased from Amersham Biosciences (NJ, USA) and used with an extinction coefficient $\epsilon_{260} = 6000 \text{ cm}^{-1} \text{ M}^{-1}$ base, room temperature, MES buffer. The samples were mixed with ratio of one compound per two base pairs and scanned up and down a temperature range of 25 $^{\circ}\text{C}$ – 95 $^{\circ}\text{C}$ with a heating/cooling rate of 0.5 $^{\circ}\text{C}/\text{min}$. Temperature was recorded with a temperature probe inserted into a cuvet filled with water. The procedure was repeated twice to generate four thermal melting curves for each sample. One strong transition was observed for every curve. The data were processed with a software package included with the instrument.

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Supporting Information Available

Preparation, ^1H NMR, ^{13}C NMR and MS data of all new Boc-protected derivatives (**1a–8a**, **1b–8b**, **1d–6d**, **8d**, **1e–6e**, **8e**, **1f–6f**, **8f**, **12**) and all *mono*-guanidines and *mono*-2-aminoimidazolines (**1g–8g**, **1h–6h**, **8h**) prepared; a table containing the combustion analysis data of the new target compounds; and the ^1H and ^{13}C NMR spectra for the final compounds (**1c–8c**) is presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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