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Triple helix formation: binding avidity of acridine-conjugated AG motif third strands containing natural, modified and surrogate bases opposed to pyrimidine interruptions in a polypurine target

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ABSTRACT

A critical issue for the general application of triple-helix-forming oligonucleotides (TFOs) as modulators of gene expression is the dramatically reduced binding of short TFOs to targets that contain one or two pyrimidines within an otherwise homopurine sequence. Such targets are often found in gene regulatory regions, which represent desirable sites for triple helix formation. Using intercalator-conjugated AG motif TFOs, we compared the efficacy and base selectivity of 13 different bases or base surrogates in opposition to pyrimidines and purines substituted into selected positions within a paradigm 15-base polypurine target sequence. We found that substitutions closer to the intercalator end of the TFO (positions 4–6) had a more deleterious effect on the dissociation constant (K_d) than those farther away (position 11). Opposite T residues at position 11, 3-nitropyrrole or cytosine in the TFO provided adequate binding avidity for useful triplex formation (K_ds of 55 and 110 nM, respectively). However, 3-nitropyrrole was more base selective than cytosine, binding to T ≥4 times better than to A, G or C. None of the TFOs tested showed avid binding when C residues were in position 11, although the 3-nitropyrrole-containing TFO bound with a K_d of 200 nM, significantly better than the other designs. Molecular modeling showed that the 3-nitropyrrole·T:A triad is isomorphous with the A·A:T triad, and suggests novel parameters for evaluating new base triad designs.

INTRODUCTION

Gene-specific regulation of cell functions has become a realistic goal with the availability of agents that can sequence specifically target DNA or RNA and modulate gene expression (1–4). Triplex-forming oligonucleotides (TFOs) bind in the major groove of duplex DNA by virtue of Hoogsteen-type hydrogen bonds between the bases of the third strand and the polypurine strand of the duplex, forming T·A:T and C+·G:C triads in the case of parallel binding pyrimidine motif TFOs or A·A:T and G·G:C triads for the antiparallel binding purine motif TFOs. In favorable cases, strong triple helix binding has been shown to prevent RNA transcription both *in vitro* and *in vivo* (5–14). However, the use of TFOs as gene-regulating agents cannot have widespread application until a number of problems are solved. One particularly vexing problem is the limited number of homopurine sequences at appropriate locations within a gene whose expression one may wish to modulate. Using currently available TFO motifs, pyrimidine interruptions within polypurine target sequences greatly reduce the binding avidity of a short oligonucleotide (15–17), even when triplex formation is stabilized by conjugation of the oligo to an intercalating agent (18). Achieving high avidity binding to sites containing even a single pyrimidine would substantially increase the number of targetable sites. For example, the HIV (Bru strain) proviral genome contains five homopurine targets 15 bases long, while there are 20 target sites of the same length containing one pyrimidine. Similarly, there is only one 17-base homopurine site in the proviral genome, while there are seven sites of that length containing a single pyrimidine interruption.

Numerous efforts have been made to address this problem (2,19), but no direct comparisons between several different designs have been published. To investigate TFO binding to sites containing a pyrimidine, we modified the sequence of a 15-base homopurine target at selected sites. We then measured the equilibrium dissociation constants of intercalator (acridine) conjugated AG motif TFOs having various bases or base surrogates opposite those sites in order to determine both the avidity and selectivity of binding. Our studies showed that for

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these TFOs, the position of the pyrimidine in the target relative to the acridine intercalation site had a strong influence on binding, and that for certain substitutions not too close to the intercalation site, adequate binding avidity for potential biological applications can be achieved. Additionally, the molecular modeling studies of base triads reported here offer new insights into parameters which may be diagnostic for triplex formation.

MATERIALS AND METHODS

Oligonucleotide synthesis

Unconjugated oligonucleotides, intercalator conjugated oligonucleotides, and duplexes were prepared and purified as previously described (18,20,21). All oligonucleotides except those containing 3-nitropyrrole were synthesized on an Applied Biosystems DNA synthesizer. Phosphoramidites of the following were purchased from Glen Research (Sterling, VA): purine (P), 2-amino-6-methoxyaminopurine (K), isoguanine (iG), O4-methylthymine (mT), carboxythymine (cT), 5-nitroindole (N), 3-nitropyrrole (M) and chloroacridine (J). Uni-Link Aminomodifier phosphoamidite (S) was purchased from Clontech (Palo Alto, CA). 6,9-Diamino-2-methoxyacridine (DAMA) was conjugated to the 5′ end of the oligos as previously described (20). All TFOs except those containing 3-nitropyrrole had an aminopropyl protecting group on the 3′ end (22) (3′amino-modifier C3 CPG, Glen Research), and all were purified by HPLC (20).

Synthesis of 3-nitropyrrole-containing TFOs

The 3-nitropyrrole-containing oligonucleotides were synthesized on a Biosearch Model 8700 DNA synthesizer according to the following method which was developed to allow the use of small amounts of scarce phosphoramidites. Automated oligonucleotide synthesis was carried out according to the manufacturer's recommendations to the point at which the scarce phosphoramidite [3-nitropyrrole phosphoramidite (M)] was to be introduced. After detritylation, the column was dried on the synthesizer with a stream of dry He. Meanwhile, 10 µmol (for a 1 µmol oligo synthesis) of the phosphoramidite was weighed into a conical vial equipped with a septum screw cap and the vial was purged with dry He. While the He was still gently flowing, one end of the column was attached to the exhaust needle and the other end was fitted with a disposable tuberculin syringe (Fig. 1A) so that contamination with atmospheric moisture was minimized. The syringe/column/needle assembly (a, b and c in Fig. 1A) was transferred onto a septum-sealed 10 ml serum vial containing 0.45 M tetrazole in anhydrous acetonitrile under dry He. Enough of this activator solution was drawn into the assembly to slightly overfill the synthesis column. The assembly was transferred back to the phosphoramidite vial and the activator injected. After dissolution, the activated phosphoramidite solution was drawn back and forth between the vial and column for ~5 min. The column was then returned to the synthesizer and the remaining synthetic steps were carried out. As shown by measurement of the OD of the trityl groups removed during the synthesis (shown in Fig. 1B for the synthesis of AGM6), the coupling yield was comparable to that obtained from the fully automated steps.

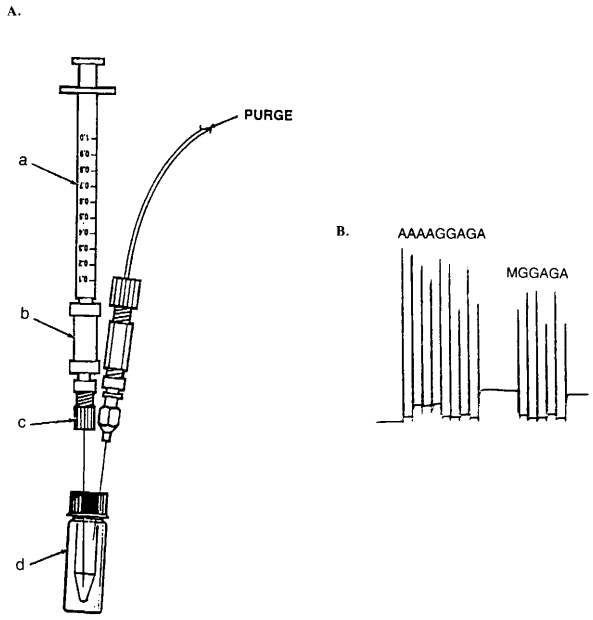

Figure 1. Semi-manual oligonucleotide synthesis. (**A**) Reaction assembly. a, inert syringe; b, synthesis column; c, exhaust/transfer needle, a 1/4 28 hub-22 gauge needle (Aldrich) fitted to a 1/4 28 male luer adapter (Upchurch) counterbored at the threaded end to 0.125 inches in diameter × ~0.125 inches deep; d, phosphoramidite vial, Wheaton 986294 (1 ml v-vial with septum screw cap) or Wheaton 986214 (1 ml serum cap v-vial). (**B**) OD of trityl groups removed during oligonucleotide synthesis.

TFO binding assays

Band-shift assays (20,21) and footprinting assays (18) were performed as previously described (and see legend to Fig. 4). Oligonucleotides were initially screened in band-shifts and avidly binding oligonucleotides were retested with extensive dilution curves. Footprinting assays were used to confirm the dissociation constants for the most avidly binding oligonucleotides. Plasmids containing duplexes with T in the positions shown in Figure 2 and plasmids containing duplexes with A or C instead of T were prepared as described (18) by cloning into the *Hin*cII site of the pUC19 vector. Plasmids were purified by CsCl/ethidium bromide banding, and the inserts were verified by Maxam and Gilbert sequencing (23). The *Hin*dIII–*Sst*I restriction fragment was isolated, labeled, and then used for footprinting experiments as described (18). Footprinting dissociation constants were determined by video densitometry of autoradiographs (IMAGE for Macintosh, Wayne Rasband, NCI, Bethesda, MD) (18,20,21). The individual lanes were standardized by dividing the intensities of the protected bands within the oligopurine tract by the intensities of three unprotected bands, and then plotted as a function of the concentration of the oligonucleotide.

RESULTS

Figure 2A shows the structure of the DNA intercalator (DAMA), and Figure 2B shows the sequence of the paradigm target duplex and TFO. All of the TFOs used for these experiments were conjugated to DAMA through a substituted urea-type linker (19) because the greater binding affinity afforded by this derivitization

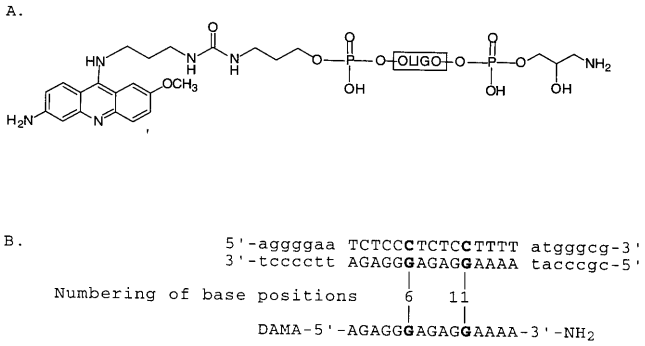

Figure 2. Intercalator structure, oligonucleotide and duplex targets. (**A**) The chemical structure of DAMA and the urea linker attachment to the oligonucleotides are shown. (**B**) The sequences of the unchanged duplex target and oligonucleotide are shown. The DAMA-conjugated AG15 oligonucleotide (AG15C) is shown in antiparallel orientation to the purine-rich binding strand of the target. The positions of substitutions in the target sequence and oligonucleotide for investigation of binding specificity are indicated by the numbers.

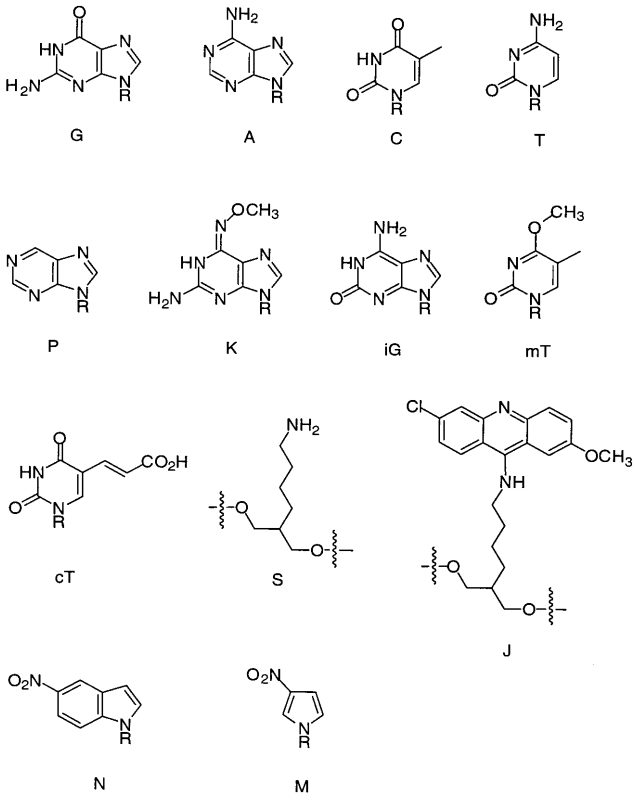

Figure 3. Structures of bases and base surrogates used in the binding studies. G, guanine; A, adenine; C, cytosine; T, thymine; P, purine; K, 2-amino-6-methoxyaminopurine; iG, isoguanine; mT, *O*-4-methylthymine; cT, carboxythymine; S, amino spacer; J, chloroacridine spacer; N, 5-nitroindole; M, 3-nitropyrrole (R, 2-deoxyribose).

would more realistically indicate therapeutic potential. The 3′-amino terminator, which prevents degradation of oligos *in vivo* (24) does not have an effect on binding avidity (18). It was not used on the 3-nitropyrrole-containing oligos as a matter of synthetic convenience. Substitutions in the binding strand were located at either the 6 or 11 position relative to the intercalator end of the acridine (Fig. 2B). These positions were chosen both in order to test the effect of distance from the intercalator and because removal of the stronger G to G Hoogsteen pairing versus A to A should provide a greater triplex destabilization and therefore a better opportunity for measuring surrogate-dependent effects.

Previous studies have demonstrated little a priori predictability for the binding avidity and selectivity of triple helix interactions with novel base surrogates (25–28), and therefore we tested a variety of base substitutions and modifications (Fig. 3) against all four natural bases in the target strand. At the same time, we developed a convenient method whereby oligonucleotides could be produced in excellent yield from scarce phosphoramidites. In this method, the phosphoramidite and activator were mixed and added to the synthesis column off the synthesizer. After coupling, the column was returned to the machine, and automated synthesis continued. The 3-nitropyrrole-containing TFOs were produced in this way. 5′-Amino modifiers (Glen Research), 5′-chemical phosphorylation reagent (Clontech), dC and dT amino modifiers (Biogenex and Glen Research, respectively) have also been successfully coupled using this method (B.W., unpublished).

Binding of TFOs containing substitutions at position 11

The apparent equilibrium dissociation constants (K_ds) of the TFOs containing substitutions at position 11 were measured using the band-shift assay (29) (Table 1). The two most avidly binding oligos, which contained 3-nitropyrrole (M11) (30) or cytosine (C11) (31) opposite a T in the binding strand of the target duplex (Fig. 2B) were further characterized by quantitative dimethylsulfate (DMS) footprinting experiments (18,32) (Fig. 4). Quantitation of the autoradiographs by densitometry showed that the dissociation constants for these TFOs were 55 and 110 nM, respectively. These numbers are in good agreement with those obtained by the band-shift assay (40 and 125 nM, respectively). Binding of the 3-nitropyrrole-substituted TFO M11 to duplexes with bases other than T in position 11 of the binding strand was considerably less strong, resulting in a selective specificity of triplex formation for this oligonucleotide. Table 1 shows that the 3-nitropyrrole preference for T at position 11 is 4-fold over C and 6-fold over either purine base. Cytosine at the same place in the TFO had a very low preference for T over the purines (2-fold over G; 3-fold over A), but a slightly greater one for C (9-fold). As shown in Table 1, none of the substitutions that have been reported to bind well opposite T [i.e., guanine (G11) (33), purine (P11) (34), acridine (J11) (35) and an amino spacer (S11) (36)], achieved a K_d <200 nM. The TFO containing the purine base (P11) was the only other one showing a preference for binding to T interruptions, but the K_d was relatively high (300 nM), and the specificity was low (only a factor of 2–3 over the other bases). Although the preference for T shown by 3-nitropyrrole is substantially less than in the case of the natural bases (e.g., G binding to G in the duplex at position 11 has a minimum preference of almost 50-fold over T), the specificity shown by 3-nitropyrrole could contribute in a positive way to the overall binding specificity of a TFO.

Table 1. Apparent dissociation constants for TFO binding to a target duplex containing various bases at the 11 position (K_ds in nM)

Substitution in TFO	Base in target duplex			
	G(native)	T	C	A
Guanine (AG15)	5	240	2500	500
Adenine (A11)	300	700	1000	60
Cytosine (C11)	200	110[a]	1000	300
Thymine (T11)	1000	3000	7000	1000
Purine (P11)	400	300	800	600
2-Amino-6-methoxyaminopurine (K11)	ND	ND	ND	ND
Isoguanine (iG15)	ND	ND	ND	ND
CarboxyT (cT11)	600	500	600	100
O4-methylT (mT11)	5000	>10 000	>10 000	>10 000
Amino spacer (S11)	1000	2000	6000	10 000
Acridine (J11)	200	700	1000	500
5-Nitroindole (N11)	500	700	700	200
3-Nitropyrrole (M11)	340	55a	200	350

[a]Derived from DMS footprinting.

Table 2. Apparent dissociation constants for TFO binding to a target duplex containing various bases at the 6 position (K_ds in nM)

Substitution in TFO	Base in target duplex			
	G(native)	T	C	A
Guanine (AG15)	5	3000	11 000	18 000
Adenine (A6)	1000	>10 000	>10 000	100
Cytosine (C6)	ND	ND	ND	ND
Thymine (T6)	>30 000	>30 000	>30 000	15 000
Purine (P6)	10 000	15 000	15 000	10 000
2-Amino-6-methoxyaminopurine (K6)	40	>10 000	>10 000	500
Isoguanine (iG6)	20	6000	>10 000	>10 000
CarboxyT(cT6)	>10 000	>10 000	5000	2000
O4-methylT (mT6)	5000	>10 000	>10 000	>10 000
Amino spacer (S6)	10 000	30 000	>50 000	>50 000
Acridine (J6)	1000	ND	ND	ND
5-Nitroindole (N6)	>10 000	>10 000	>10 000	>10 000
3-Nitropyrrole (M6)	>>10 000	15 000	>30 000	>>10 000

Binding of TFOs containing substitutions at other positions

We also tested TFOs containing bases and base surrogates in the 6 position. As shown in Table 2, none was able to achieve submicromolar dissociation constants with duplexes containing either T or C in the binding strand opposite that site. 3-Nitropyrrole still exhibited a preference for T, but with a K_d of 5 μM. If the T was located closer to the intercalation end of the TFO, e.g. at position 4 or 5, the binding of TFOs containing 3-nitropyrrole at those positions was almost undetectable (data not shown). A 3-nitropyrrole double substitution opposite Ts in positions 11 and 12 was able to bind with only a 3 μM K_d, markedly increased from the single substitution K_d of 55 nM. The TFO with C at the 6 position (C6) was not tested due to an anomalous secondary structure feature which caused it to migrate faster on electrophoresis than similar oligos. The TFOs with isoG and K at the 6 position (iG6 and K6) bound nearly as well as the TFO with the canonical G opposite G in the duplex and not at all well against the other bases, and so the TFOs with those substitutions in the 11 position were not tested (Table 1). It is of interest to note that replacing a G with an A, even though a 'perfect' triple helix can still be formed, raised the K_d by a factor of 12 in the case of the 11 position (Table 1), and a factor of 20 for position 6 (Table 2), reconfirming the importance of the number and placement of G residues for high binding affinity of TFOs.

Molecular modeling

In order to determine why 3-nitropyrrole showed a preferential binding affinity for T, models were created for the A·A:T, G·G:C, M·T:A, M·C:G, M·G:C and M·A:T triads using the software program Chem3D (CambridgeSoft Corporation). The Watson–Crick base pairs were fixed using the parameters C1′–C1′ and λ as defined in Figure 5 (37,38). The A·A:T and G·G:C triads were created by placing the third base in optimal hydrogen bonding position relative to the Watson–Crick base pair as illustrated in the figure for A·A:T. Additional parameters that uniquely define the third base position were defined as λ2′, λ3 and $C^2{}_{1'}$–$C^3{}_{1'}$ as shown in the diagram. The triads containing 3-nitropyrrole in the third strand position were then modeled to optimize van der Waals interaction with the Watson–Crick base pair. Hydrogen bonding to 3-nitropyrrole was not considered. As

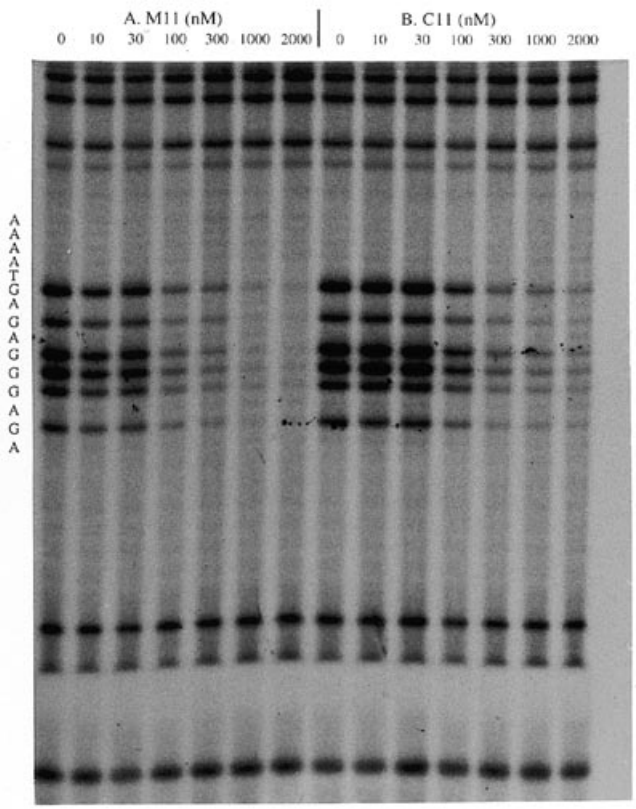

Figure 4. M11 and C11 K_ds by footprinting. The plasmid duplex fragment labeled with ^{32}P on the strand harboring the oligopurine binding target was incubated with increasing concentrations of the indicated TFO in TM buffer (pH 7.4, 10 mM $MgCl_2$). After a brief exposure (3 min) to 0.5% DMS, the reaction was stopped, and the DNA was recovered and treated with hot piperidine to cleave it at sites of DMS methylation. The DNA was again recovered, then dissolved in formamide and run out on a sequencing gel. The sequence of the relevant fragment area is listed on the left, and the concentration of the TFO in each sample is shown above the lanes.

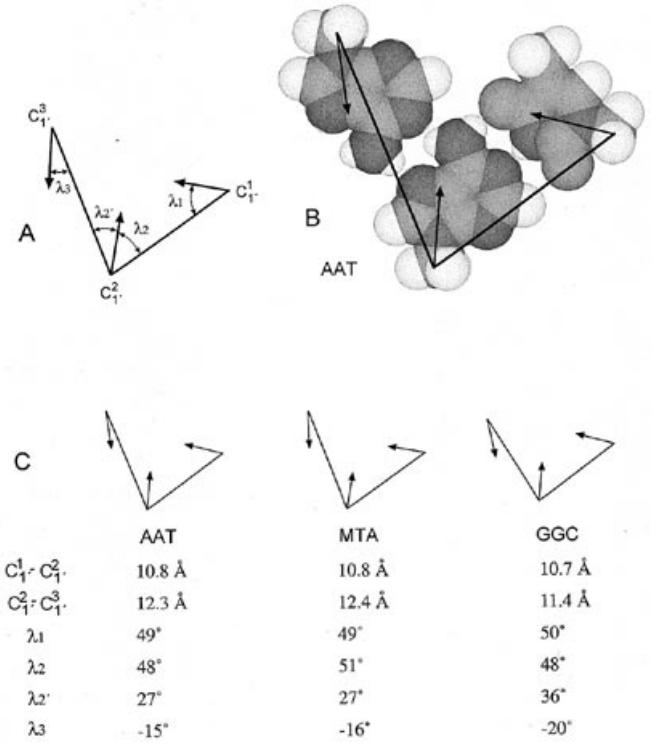

Figure 5. Base triads modeled in Chem3D (CambridgeSoft Corporation). (**A**) The space filling models are shown with a methyl group in place of $C_{1'}$. The parameters $C^1{}_{1'}$–$C^2{}_{1'}$, $C^2{}_{1'}$–$C^3{}_{1'}$, λ_1, λ_2, $\lambda_{2'}$, λ_3 are defined as depicted on the triad parameter map. (**B**) The triad parameter map is shown superimposed on a space filling model of the A · A:T triad. (**C**) The three triad parameter maps of the A · A:T, M · T:A (M, 3-nitropyrrole), and G · G:C triads as obtained from space filling models. The parameters measured from the models are included in the table below the parameter maps.

shown in Figures 5 and 6, the M · T:A triad is isomorphous with the A · A:T but not the G · G:C triad. The M · T:A triad maintains the geometry of the A · A:T triad as judged from the close match between the parameters λ2′, λ3 and $C^2{}_{1'}$–$C^3{}_{1'}$. To achieve van der Waals interaction with A:T or G:C base pairs would require a significantly smaller $C^2{}_{1'}$–$C^3{}_1$ distance. For example, if the angles λ2′ and λ3 were maintained then this distance would have to be decreased from 12.3 to 9.8 Å for the M · A:T triad and from 11.4 to 10.2 Å for the M · G:C triad in order to maintain van der Waals contact.

DISCUSSION

To realize the full potential of triplex formation for gene regulation, it is essential to be able to incorporate natural or modified bases or base surrogates into a TFO so that the presence of pyrimidine mismatches in the target polypurine strand will be tolerated and not substantially degrade the binding avidity and specificity necessary for biological activity. Much work has been done using G as the base best suited for binding to T interruptions, especially concerning TFOs with G and T residues opposite G and A in the target strand (22,39,40) and some of these TFOs have exhibited unusually strong binding (41). This may be due to the exact sequence and/or length of the target and TFO, or perhaps due to the presence of long strings of G residues (42,43). The sequence which we and others have studied is found in the promoter region of the IL2Rα gene (6,18,21,44). It has only three contiguous Gs, with seven Gs and eight As overall, and the K_d of the unconjugated TFO is 2.5 μM as compared to 5 nM for the conjugated version (21). We find that single pyrimidine interruptions in the target strand of the duplex, especially those near the intercalator end of the conjugated TFO, reduce binding avidity drastically (Tables 1 and 2). It had been shown previously, both by modeling and experimental studies, that cytosine was the best natural base for binding to a T:A base pair which was located centrally in a long target duplex (31,45) and our data confirm that result for the 11 position of our target sequence (K_d of 110 nM for C11 versus 240 for G11, 700 for A11 and 3000 for T11). However, we find that that among the candidates we tested, the best binding to T was provided by the 3-nitropyrrole-containing TFO, M11 (K_d of 55 nM), and a preference for T 4–6-fold over the other bases. This is sufficient binding avidity and specificity to warrant future investigations using 3-nitropyrrole opposite T interruptions for intervention of gene expression by triple helix binding. The 3-nitropyrrole was also the best compound for binding to C of the designs tested, and in some cases of C interruptions it could prove useful.

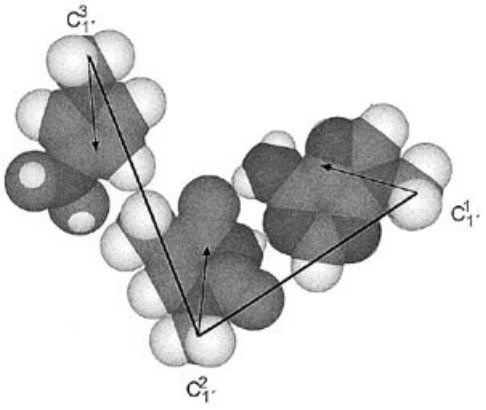

Figure 6. Space filling model of the base triad M · T:A (M, 3-nitropyrrole). The model is shown with a methyl group in place of $C_{1'}$.

3-Nitropyrrole has been shown to base pair non-discriminately with all four natural bases in Watson–Crick duplexes (46). When incorporated into a pyrimidine motif third strand and tested at low pH, it was found in one report to destabilize the triple helix (47) and in another to discriminate G:C from C:G, A:T and T:A in the presence of a triplex-specific ligand (48). In the present instance, we utilized a purine motif third strand because of its greater binding avidity at physiological pH (21). The ability of 3-nitropyrrole to pair specifically with T in the triplex context M · T:A in these TFOs provides insight into the geometry requirements for the design of new base triad motifs. For nucleic acid duplexes, the parameters C1′–C1′ and λ (Fig. 5) provide the best indication of fit. C1′–C1′ is the interstrand distance between the two glycosidic C1′ carbons of a given base pair and the angle λ is defined by C1′–C1′–N1 (pyrimidine) and C1′–C1′–N9 (purine) (37,38). For normal A:T and C:G base pairs in a B-DNA duplex the value of C1′–C1′ is typically in the range of 10.7–11.1 Å, while λ may range from ~46 to 54°. For a given base pair the two values of λ are usually within a few degrees of one another. For example the values 11.1 Å, 50° and 51° have been measured for a T:A base pair. In comparison, the parameters for base mispairs which destabilize the double helix fall well outside of this range. Typical values for a T · G mismatch are 10.3 Å, 69° and 42°; for a C:A mismatch, 10.3 Å, 68° and 46°; G:A mismatch (*anti–anti*), 12.5 Å, 53° and 52°; and for a G:A mismatch (*anti–syn*), 10.7 Å, 58° and 40°. It follows that for optimum stability one might expect it to be necessary for the third strand of a nucleic acid triplex to maintain a restricted range of parameters. As illustrated in Figure 5, the additional parameters that uniquely define the third base position are λ2′ and λ3 and $C^2{}_{1'}$–$C^3{}_{1'}$. The model supports previous observations that the A · A:T and G · G:C triads are not strictly isomorphous (49). The difference in position of the third A in the A · A:T triad and G in the G · G:C triad is about equivalent to the difference in position that one sees between a normal A:T base pair and a G:T mismatch in duplex DNA. If that is the case, why are triplexes based on G · G:C and A · A:T triads stable? First of all, they are not that stable; the third-strand association constant is considerably less than the association constant for melting of the Watson–Crick duplex. Second, duplexes containing missteps (adjacent base pairs with differing C1′–C1′ and λ parameters; for example, G:T mismatches interspersed with A:T base pairs) are usually less stable than duplexes containing only perfectly stepped natural base pairs, but still may be sufficiently stable to maintain duplex structure at normal temperatures. For example, the duplex formed by the DNA sequence d(CGCGATATTGCG) containing two G:T mismatches (equivalent to 4 missteps out of 11 steps) melts at 51.5°C, in comparison to the natural self-complementary sequence d(CGCGATATCGCG) which melts at 68.5°C (50). Although the difference in parameters between A · A:T and G · G:C triads is not sufficiently great to impair the formation of stable triplexes, substitution of a third base pair which does not fit within this parameter range can be significantly destabilizing. The parameters for the A · A:T and M · T:A triads are virtually identical if the 3-nitropyrrole occupies a position in which the hydrophobic C5 methyl is in van der Waals contact with the C4-H and NO_2 group of the nitropyrrole. Recent results indicated that 3-nitropyrrole greatly prefers to pair opposite itself rather than a hydrogen bonding base (51), and hydrogen bonding studies by NMR showed that 3-nitropyrrole exhibits virtually no hydrogen bonding association with the natural bases. As discussed in the Results section, models show that if A, C or G was placed opposite 3-nitropyrrole in the triad, there would have to be a significant shift in base position to achieve van der Waals contact. Furthermore, A, C and G give only hydrophilic contacts in the region that would be adjacent to the 3-nitropyrrole. Based on these results, it appears that the parameters λ2′ and λ3 and $C^2{}_{1'}$–$C^3{}_{1'}$ may be useful for evaluating new base triad designs.

Future biological applications of triplex forming oligonucleotides will require high avidity, high specificity compounds which discriminate effectively at low concentrations *in vivo*. A number of studies examining binding of either purine or pyrimidine motif TFOs containing only natural bases opposite pyrimidine interruptions in a target site found relatively low avidity binding (15,16,52). Since conjugation of an intercalator to an AG motif TFO can dramatically enhance binding avidity, giving dissociation constants in the low nanomolar range, we and others have examined the binding of such conjugates to 'perfect' polypurine targets (18,53). In addition, we have previously found that GA motif TFOs bind more strongly than GT (antiparallel) or TC (parallel) motif ones (21) to such targets. However, expansion of the repertoire of useful triplex target sites to all or most genes will require the deployment of novel moieties opposite pyrimidines contained within a target sequence so as to permit the highest avidity and specificity of binding. We find that although the position of the pyrimidine is crucial to triplex binding with intercalator conjugated TFOs, and this may limit the choice of targets somewhat, the use of the base surrogate 3-nitropyrrole against a T insertion should allow sufficient binding avidity and specificity for biological applications. In conclusion, the data presented in this paper indicate that even when the target is a mixed sequence, a high degree of specific binding is achievable with TFOs, in particular short ones conjugated to a strong intercalating agent.

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