Title: Evaluation of the Base-pairing Properties of 5-(5-Indolylethynyl) and 5-(5-Indolyl)-2'-deoxyuridine Modified Triplex and Duplex

Authors: Maha Fatthalla, Ph.D; Erik Pedersen

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Abstract: Indole was conjugated to deoxyuridine either directly or via ethynyl linkage to target faulty single strand, double strand or protein prior transcription or translation processes, provided that having high selectivity and binding affinity. One of the successful oligonucleotide therapies is antisense strategy which targets single-stranded RNA (mRNA). Hitherto, four antisense drugs have been approved by the Food and Drug Administration (FDA) namely fomivirsen for treating cytomegalovirus retinitis, migomersen for homozygous familial hypercholesterolemia, fomivirsen for treating cytomegalovirus retinitis, and nusinersen for spinal muscular atrophy.

Targeting double-stranded RNA possessing a purine-rich strand is another strategy which is called antigene strategy. Targeting double-stranded RNA possessing a purine-rich strand is another strategy which is called antigene strategy. The thermal denaturation experiments showed higher triplex stabilization for 5-(5-indolyethyl)-2'-deoxyuridine over 5-(5-indolyl)-2'-deoxyuridine as the triple bond allows twisting to put the indole into a proper position within the triplex encouraging better π-π stacking.

Introduction

Oligonucleotide therapy (ONT) serves as a new class of molecular medicines to target faulty single strand, double strand or protein prior transcription or translation processes, provided that having high selectivity and binding affinity. One of the successful oligonucleotide therapies is antisense strategy which targets single-stranded RNA (mRNA). Hitherto, four antisense drugs have been approved by the Food and Drug Administration (FDA) namely fomivirsen for treating cytomegalovirus retinitis, migomersen for homozygous familial hypercholesterolemia, fomivirsen for treating cytomegalovirus retinitis, and nusinersen for spinal muscular atrophy.

Targeting double-stranded DNA possessing a purine-rich strand is another strategy which is called antigene strategy. In this strategy, triplex-forming oligonucleotide (TFO) binds through sequence-specific Hoogsteen base pairing to its targeted oligopurine of the DNA double helix. Two Hoogsteen H-bonds are formed thanks to the presence of donor and acceptor groups in the major grooves establishing two types of triplex motifs; parallel or antiparallel triplex relative to the targeted oligopurine strand. The formed triplex helical structure represents an ideal tool to target transcription start-sites and consequently blocks gene expression which would lead to gene therapy.

Effective TFO should overcome nuclease degradation, resist the high repulsion to its negative charge, and survive at physiological pH as cytosine should be protonated at N-3 to form the second Hoogsteen H-bond with N-7 in Guanine. The procedure could be improved by inserting additional nucleotide, called intercalator, having polyaromatic residue in order to increase the π-π stacking ability to its targeted duplex. More research is needed to fully apply this approach in therapy, since more than sixty years have passed since triplex discovery and no approved drug yet exists.

Indole is an important reactive structural compound whose derivatives are widely distributed in nature and its synthetic molecules have diverse biological activities. Moreover, indole was incorporated into DNA as an artificial alkyne base modification at specific sites of DNA double helix. Indole represents a very promising charge trap in DNA charge-transport studies due to its low oxidation potential and the characteristic transient absorption of the corresponding radical. Recently, new classes of bisindoles have been synthesized as small molecules to interact with G-quadruplex DNA structures, and some examples were stacked efficiently to the top 5'G-tetrad.

Palladium assisted reactions are excellent to functionalize aromatic compounds. Sonogashira cross-coupling is one of these reactions which add alkyne terminal to aromatic systems. Moreover, bi-aryl compounds can be synthesized via Suzuki cross-coupling which utilizes organoboron moieties as nucleophile and aryl halide. Both reactions have been extensively used to synthesize conjugated natural product analogues which have made a revolution in medicinal chemistry and pharmaceutical industry.

Several groups were interested in incorporating indole as a base modification of DNA, LNA or acyclic-nucleoside to test its ability to stabilize the corresponding duplexes or triplexes through N-1, C-2 or C-3 of indole. In addition, we previously reported the enhanced intercalation ability due to phenanthrene and pyrene modified indoles to stabilize parallel DNA triplexes when inserted as a bulge through N-1 or C-3 of indole. We were curious to see the effect of having free N-1 and C-3 indole and therefore we synthesized two modified deoxyuridine nucleosides connected through C-5 of indole. The modified nucleotides dUind and dUfimod (Fig. 1) have been incorporated in the middle of tetradecamer pyrimidinyl strands in order to test the stability of the formed triplexes in addition to DNA and RNA duplexes.

![Fig 1. Structure of nucleotides dUind and dUfimod](image-url)
Results and Discussion

Synthesis and properties of phosphoramidite building blocks

Sonogashira cross-coupling reaction has been used to synthesize ethynyl containing nucleoside 6 (dU\textsuperscript{315}). The strategy entails two palladium catalyzed reactions: the first step was coupling of 5-iodoindole (1) with trimethylsilylacetylene (TMSA) followed by protodesilylation using NaOH/Methanol to produce the desired 5-ethyl-1H-indole (3) in 82% yield in addition to its dimer 1,4-di(1H-indol-5-yl)buta-1,3-dyne (4) in 9% yield (Scheme1). The second step utilized 5-ethyl-1H-indole (3) and the commercially available 5-ido-2'-deoxyuridine (5, dU). On the other hand, nucleoside 11 (dU\textsuperscript{315}) has been synthesized using Suzuki reaction. The strategy involves borylation of 5-bromoindole (9) followed by transmetalation with 5-ido-2'-deoxyuridine (5) as dU/Pd(0) complex to afford 5-(5-iodo-2'-deoxyuridine (11, dU\textsuperscript{115}) (Scheme1). Suzuki coupling gave the desired product in addition to deoxyuridine (dU) as a by-product which can be easily removed by washing with water. Borylation of aryl bromide is normally carried out by the addition of trimethyl borate B(O\textsubscript{3})\textsubscript{3} to the aryl bromide/DBU mixture at -78°C. However, it is found better in terms of yield and feasibility to neutralize the acid N-H of the bromoindole with KH before adding KHDBU to ensure the formation of 5-indolyboronic acid (10). Moreover, the produced boronic acid was purified by flash chromatography to remove the unreacted 5-bromoindole 9 affording 10 as white solid (75%) which was visualized on TLC by a curcumin staining solution.

Purification by column or preparative thin layer chromatography of the nucleosides 6 and 11 from the starting nucleoside dU was very laborious due to their very similar Rf values even though many different eluting systems were attempted. However, using about 2.5-3 fold of 5-ethyl-1H-indole 3 and 5-indolyboronic acid (10), ensured elevated conversion to indolyltetynucleoside 6 (dU\textsuperscript{315}) and indolyl nucleoside 11 (dU\textsuperscript{115}), respectively, leading to more easy purification of the products. DMT-protected compounds were prepared by protecting the primary hydroxyl group of diols 6 and 11 using 4,4’-dimethoxytrityl chloride (DMT-Cl) in anhydrous pyridine and Et\textsubscript{3}N. The crude products were washed twice with NaHCO\textsubscript{3} solution to remove the produced HCl salts prior to column chromatography purification using 1% Et\textsubscript{3}N with the suitable eluting systems. However, HNMR data of the purified products showed contamination with Et\textsubscript{3}N/HCl salt. Therefore, subsequent washing with DCM/NaHCO\textsubscript{3} mixture was done and it showed its effectiveness to remove the salt. We think the washing step was important to avoid interference with the tetrazolide activator in the following phosthylation step. A complicating factor could also be the free NH in the indole containing nucleosides which could also be the reason the low yields of the pure DMT protected compounds 7 and 12 being 30 % and 31 % yield, respectively. Phosphoramidites 8 and 13 have been synthesized by overnight phosphitylation of the secondary hydroxyl group using 2-cyanoethyN,N,N’-tetraisopropyl phosphorodiamidite in the presence of disopropyl ammonium tetrazolide as activator (Scheme1). As can be seen from \textsuperscript{13}C NMR, the produced phosphoramidites are a diastereomeric mixture\textsuperscript{24} due to the chiral phosphorus atom\textsuperscript{25} resulting in epimer formation.\textsuperscript{26} The produced phosphoramidites 8 and 13 were incorporated into oligonucleotides using an automated DNA synthesizer.

Synthesis and characterization of oligonucleotides

In order to test the stability of triplex formation, the modified monomers dU\textsuperscript{315} and dU\textsuperscript{115} were inserted in the middle of 14-mer poly pyrimidine strands. The standard phosphoramidite protocol was used to synthesize ON3 containing dU\textsuperscript{315} with extended coupling time to 25 min to increase the coupling efficiency. MALDI-TOF-MS analysis of the crude oligonucleotide showed a peak for the correct oligo mass in addition to another peak higher by 126 mass unit which couldn’t be explained as accompanying salts. This mass corresponds to iodination of dU\textsuperscript{115} during the oxidation step using iodine oxidizer solution [THF/water/pyridine/iodine, 66/12/22/0.6 (v/v/v/v)]. The oxidizer was used primarily to convert phosphate to phosphate group resulting in a cyanoethyl-protected phosphate backbone. Under the aforementioned conditions, we believe that only C-3 iodine could be iodinated due to its high electron density, making it highly prone to electrophilic substitution by iodine.\textsuperscript{27-32} HPLC purification resulted in two oligomers ON3 and ON4 containing dU\textsuperscript{315} and its iodinated monomer dU\textsuperscript{115}, respectively. The new oligos have very close retention time 15.16 and 14.99 min, respectively, and approximately equal yield (Table 1, figure S1-S4 in the Supporting Information). Based on our experience with indole containing oligonucleotides and the best of our knowledge, this is the first time to have iodination reaction of iodine during automated DNA synthesis as we suspect that happened because of N-1 and C-3 of iodine being unsubstituted.\textsuperscript{18,19} In addition, the high yield of iodinated oligo is due to the repeated oxidation process of the following nucleotides. On the other hand, this observation could be used for further post-synthesis oligonucleotide modifications. To avoid the iodinated product, the oligonucleotide synthesis protocol was modified to use the non-aqueous CSO oxidizer [(1S)-(+)-(10-camphorsulfonyl) oxaziridine] which has been useful in the oligonucleotide synthesis of iodine sensitive monomers like 7-deaza-2'-deoxyguanosine.\textsuperscript{33} The protocol was programmed to 5 min wait step in oxidation process which shows better yield than 3 min proposed by the manufacturer. The yield of ON3 was improved (Table 1) and MALDI-TOF-MS analysis of the crude oligonucleotide didn’t show any additional peaks corresponding to ON4, which confirms that ON3 is the sole oligonucleotide. ON5 having monomer dU\textsuperscript{115} was directly synthesized using CSO oxidizer and its MALDI-TOF-MS spectrum showed the desired oligo mass identical with the calculated values and the yield was comparable to unmodified oligo.

The properties of the modified oligonucleotides (ON3-5) were characterized by its ability to form stable triplex and/or duplex derived from thermal denaturation experiments. The melting temperature (Tm) was determined as the first derivative of melting curve which measured at λ = 260 nm. Two types of parallel triplexes have been tested.

### Table 1: MALDI-MS and oligonucleotide yield of synthesized ONs (1.0 μmol scale)

<table>
<thead>
<tr>
<th>Entry</th>
<th>TFO</th>
<th>CCC</th>
<th>TTX</th>
<th>TTT</th>
<th>TTT</th>
<th>Found m/z</th>
<th>Calcd. m/z</th>
<th>ON yield (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON3</td>
<td>X</td>
<td>dU\textsuperscript{115}</td>
<td>4549.03</td>
<td>4549.8</td>
<td>70.6</td>
<td>0.0</td>
<td>161.8</td>
<td></td>
</tr>
<tr>
<td>ON4</td>
<td>X</td>
<td>dU\textsuperscript{115}</td>
<td>4674.36</td>
<td>4675.8</td>
<td>61.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>ON5</td>
<td>X</td>
<td>dU\textsuperscript{115}</td>
<td>4527.19</td>
<td>4525.8</td>
<td>na\textsuperscript{[b]}</td>
<td>264.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Yield calculated on the basis of measured optical density (OD) in 1 mL of water
[b] not synthesized
Scheme 1. Reagents and conditions: (i) CuI, Pd(PPh₃)₄, TMSA, ACN, Et₃N; (ii) MeOH, NaOH, 82% for 3 and 9% for 4; (iii) CuI, Pd(PPh₃)₄, DMF, Et₃N, 79%; (iv) DMT-Cl, pyridine, Et₃N 30% for 7 and 31% for 12; (v) N,N-Diisopropylammonium tetrazolide, 2-cyanoethyl N,N,N,N´-tetraisopropylphosphoramidite, DCM (in case of dU≡ind, 60%) and ACN (in case of dUind, 79%); (vi) DNA synthesizer using (a) I₂ oxidizer; (b) CSO oxidizer; (vii) KH, t-BuLi, B(OMe)₃, THF, 75%; (viii) 5, CsF, Pd(PPh₃)₄, DMF, H₂O, 62%.
Two types of triplexes were investigated, the first type carried the modified deoxyuridine in the TFO as a bulge to its complementary duplex (ON3/D1, ON4/D1 and ON5/D1), while the second, TFO, had the modified deoxyuridine as a substituent to deoxouridine (dT) in ON2 to form triplexes upon mixing with duplex (ON3/D2, ON4/D2 or ON5/D2). The data showed reduced stability of the parallel triplexes (ON3/D1, ON4/D1 and ON5/D1) compared to the corresponding wild-type (ON1/D1) by 9.0-14.5°C at different pHs (Table 2). Interestingly, ON3/D1 and ON4/D1 showed differences in melting temperature despite the presence of large iodine atom in ON4. Most likely, the indole ring system, with or without the iodine atom, is outside the triple helical structure when inserted as a bulge. This is opposite to an inserted pyrene intercalator which stabilizes the triplex.[34] On the other hand, when ON3 and ON4 were hybridized with D2 having T.A base pair to complement dU^ind or dU^ind, the formed triplex motifs (ON3/D2 and ON4/D2) were discriminated and destabilized by 4.5°C for ON3/D2 and 16.5°C for ON4/D2 at pH 6.0 when compared to wild-type ON2/D2 (Table 2). The loss of some stability is likely as T.A is slightly less thermally stable than T.A as stated previously by Mergny and coworkers.[35] It is believed that the iodine atom in dU^ind inhibits the anticipated π-π stacking due to its residing in the major groove of the ON4/D2 triplex which may explain the low melting temperature compared to ON3/D2. Also, truncation of the triple bond had a destabilizing effect on the triplex stability as ON5/D2 showed 3.0°C lower melting temperature when compared to ON3/D2.

The ability of the modified oligos ON3-5 for duplex formation was also studied using two complementary DNA and RNA sequences ON6 and ON7, respectively (Table 3). By using sodium cacodylate buffer adjusted to pH 6.0, the DNA duplexes with ON3 and ON5 were destabilized by 2.0 and 3.0°C, respectively, while ON4 destabilized the formed duplex by 7.5°C compared to the unmodified duplex ON2/ON6.

Hybridization of the modified oligos ON3 comprising dU^ind and ON5 comprising dU^ind with RNA sequence ON7 using medium salt phosphate buffer at pH 7.0 resulted in only a slight destabilization (1.0 - 1.5°C) when compared to wild-type duplex ON2/ON7. On the other hand, ON4/ON7 which had dU^ind is destabilized by 5.0°C compared to wild-type duplex ON2/ON7. Previously, pyrene [36,37] and naphthalene [38] modified deoxyuridines have been synthesized to be used as fluorescence probes, however, their duplexes showed no additive thermal stability.

Examining Tables 2 and 3, one finds better thermal stability of triplex or duplex formation due to ON3 when compared to ON5 which might be attributed to the flexibility of dU^ind having the triple bond linker which may allow planarity of the indole and uracil ring systems which is less likely to happen in the truncated structure dU^ind.

### Conclusion

Two new indole containing nucleotides dU^ind and dU^ind have been synthesized in order to add functionality to deoxyuridine at its 5-position with 5-indolyl and 5-ethyl indole. The main purpose of the modifications is to test their ability to improve the electrostatic properties to their targeted duplexes and stabilize the corresponding parallel triplexes through free N-H. The modified dU^ind monomer upon oligonucleotide synthesis reacts with iodine oxidizing solution to produce another oligonucleotide having dU^ind which showed great destabilization compared to dU^ind. To the best of our knowledge, it is the first time to address this iodination problem during automated DNA synthesis when working with indole modified nucleobases. We attribute the iodination of indole during standard oligonucleotide synthesis to the high reactivity of indole toward electrophilic substitution reactions especially when N-1 and C-3 were
unsubstituted. Changing the oxidizer from iodine into CSO oxidizer showed its effectiveness to afford the desired oligo in higher yields. dU\textsuperscript{ind} and dU\textsuperscript{ind} were able to form stable triplexes without any substantial sharing from N-H. Modified indole DNA/RNA duplexes showed comparable stability to the corresponding wild-type duplexes while the DNA/DNA duplexes were slightly less stable. Oligos containing dU\textsuperscript{ind} were able to stabilize their triplexes better than the truncated monomer dU\textsuperscript{ind}, when substituting dT in the wild-type triplex. While inserting dU\textsuperscript{ind} and dU\textsuperscript{ind} as bulge distorted the formed triplexes.

Supporting information summary

SI includes all experimental procedures and purification of the synthesised compounds and oligonucleotides as well. Further, it contains reversed-phase HPLC profile, MALDI-TOF-MS analysis, and ONs melting temperature profiles. NMR data are also included.

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Keywords: CSO oxidizer, deoxyuridine, indole, nucleobase iodination, parallel triplex


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Free (NH)-indole containing nucleosides were synthesized and linked to deoxyuridine either directly or via ethynyl linkage. The free NH accelerates unprecedented iodination reaction of indole during DNA synthesis using iodine oxidizer; the problem was solved by using CSO-oxidizer. The ability to form stable triplexes and duplexes was evaluated to show the enhanced ability of ethynyl nucleoside to stabilize parallel triplexes, antiparallel RNA and DNA duplexes compared to truncated nucleoside. Moreover, a great destabilization was observed due to the iodinated nucleoside.