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Oligonucleotides Bearing 5-Formyl-2'-*O*-methyluridine: Preference in Binding Affinity to the NF-κB $(p50)_2$ Homo- and p50/p65 Heterodimers

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Abstract: 5-Formyl-2'-*O*-methyluridine was incorporated into the various positions of oligonucleotide 26-mers containing the NF-κB binding sequence. Some of them showed binding selectivity toward the homo- and heterodimers of subunits of NF-κB.

The Rel/NF-κB family of proteins including Rel, Rel A, Rel B, p50 and p52 is a group of transcription factors which share a highly conserved domain of approximately 300 amino acids termed the "rel homology domain" (RHD).[1] Through the RHD, they form either a homodimer or a heterodimer and bind to a decameric sequence motif (κB site), which is present in the enhancers of the genes involved in immune and inflammatory responses. Recently X-ray crystallography disclosed the structure of NF-κB p50 bound to DNA, in which the recognition loop of the protein reaches into the DNA and interacts with bases.[2] On the other hand, γ irradiation to DNA causes oxidation of a thymidine 5-methyl to a formyl group, which would be able to react with a proximal ε-amino group of a lysine residue of histone to form DNA-protein crosslink.[3] We were interested in investigating interaction between NF-κB and modified oligonucleotides, in which thymidine was replaced by a 5-formyluridine derivative inside or outside of the κB site to create a new type of NF-κB inhibitors.

The precursor of the 5-formyl-2'-*O*-methyluridine, 5-(1,2-diacetoxy)ethyl-2'-*O*-methyluridine[4] (U*) being an appropriate form for a DNA synthesizer, was synthesized in a gram scale from O^2,2'-cyclouridine **1** (Scheme 1). Selective 2'-*O*-methylation was accomplished by the ISIS method in 89% yield,[5] and the following iodination of the 5-position with CAN-I_2 in AcOH gave **3** in 74% yield.[6] The 3',5'-diol was protected by TBDMS groups to afford **4** in quantitative yield. Stille coupling with tributyl(vinyl)tin[7] using $Pd(CH_3CN)_2Cl_2$ as a catalyst followed by dihydroxylation of the resulting vinyl group using *cat.* OsO_4/NMO gave **6** in 77% yield in two steps. Acetylation of the vicinal diol and selective deprotection of 3',5'-dihydroxyl groups afforded **8** in 96% yield. Dimethoxytritylation (89%) of the 5'-hydroxyl group to yield **9**[8] followed by 3'-*O*-phosphitylation[9] (90%) gave the nucleoside phosphoramidite unit **10** ready to incorporate into the 26-mer including the κB site, *i.e.* 5'-GGGACTTTCC-3' or its complementary sequence of 3'-CCCTGAAAGG-5' (Scheme 2).

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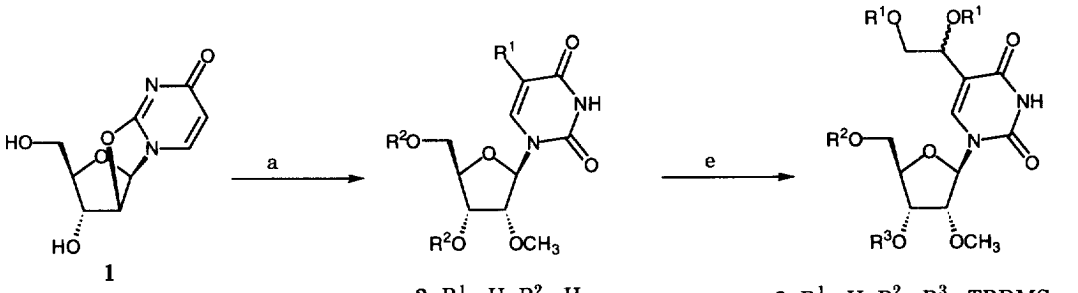

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b: **2**: R^1= H, R^2= H
b, c: **3**: R^1= I, R^2= H
c: **4**: R^1= I, R^2= TBDMS
d: **5**: R^1= vinyl, R^2= TBDMS
11: R^1= CHO, R^2= H

f: **6**: R^1= H, R^2= R^3= TBDMS
f, g: **7**: R^1= Ac, R^2= R^3= TBDMS
g, h: **8**: R^1= Ac, R^2= R^3= H
h, i: **9**: R^1= Ac, R^2= DMT, R^3= H
i: **10**: R^1= Ac, R^2= DMT, R^3= P(N-*i*Pr$_2$)OCH$_2$CH$_2$CN

Scheme 1. *Reagents and conditions*: a) see ref. 5. b) I_2 (0.6 eq), CAN (0.5 eq), AcOH, 80 °C. c) TBDMSCl (3.5 eq), imidazole (5 eq), DMF. d) 5 mol% $Pd(CH_3CN)_2Cl_2$, $Bu_3SnCH{=}CH_2$, CH_3CN, 80 °C. e) *cat.* OsO_4, NMO (2.5 eq), acetone-H_2O-^{t}BuOH (4:1:1). f) Ac_2O (4 eq), py. g) TBAF (2 eq), AcOH (2 eq), THF. h) DMTCl (1.5 eq), py. i) $[(iPr)_2N]_2POCH_2CH_2CN$ (1.8 eq), DCI (0.7 eq), CH_3CN-CH_2Cl_2 (1:10).

A. *a* 5'-A GCT TCA GAG **GGG ACT TTC** CGA GAG G-3'
b 3'-AGT CTC **CCC TGA AAG** GCT CTC CAG CT-5'

B. ① 3'-AGU* CTC **CCC TGA AAG** GCT CTC CAG CT-5'
② 3'-AGT CU*C **CCC TGA AAG** GCT CTC CAG CT-5'
③ 3'-AGT CTC **CCC U*GA AAG** GCT CTC CAG CT-5'
④ 3'-AGT CTC **CCC TGA AAG** GCU* CTC CAG CT-5'
⑤ 3'-AGT CTC **CCC TGA AAG** GCT CU*C CAG CT-5'
⑥ 5'-A GCT TCA GAG **GGG ACU* TTC** CGA GAG G-3'
⑦ 5'-A GCT TCA GAG **GGG ACT U*TC** CGA GAG G-3'
⑧ 5'-A GCT TCA GAG **GGG ACT TU*C** CGA GAG G-3'

Scheme 2. A: Nucleotide sequence of the duplex 26-mer. NF-κB recognizes the 5'-GGGACTTTCC-3' sequence. B: Eight modified 26-mers synthesized are shown, in which T was replaced by 5-(1,2-diacetoxy)ethyl-2'-*O*-methyluridine (U*) one by one inside or outside of the κB site, described in the bold letters.

After ammonolysis, the 26-mers were purified by HPLC and treated with $NaIO_4$ to create the 5-formyl at the U* position.[4] Enzymatic hydrolysis and HPLC analysis proved the existence of the 5-formyl-2'-*O*-methyluridine unit in the strands.[10] It is worthy to note that only aldehyde **11**[11] shows UV absorption up to 350 nm, and the detection of the minor component **11** (1/26) was effective using both wavelengths of 260 nm (like normal nucleosides) and 304 nm (specific to **11**).

Each 26-mer was annealed with its natural complementary strand (four bases overhanged each other), respectively, for example modified strand ① with natural strand *a* in Scheme 2. Ability of these eight double strands to inhibit the interaction of the p50 homodimer or the p50/p65 heterodimer with oligonucleotides containing the κB site (duplex A shown in Scheme 2) was tested in electrophoretic mobility shift assays (Figure 1). The complex formations between

the two dimers and a ^{32}P end-labeled duplex A were completely inhibited nonselectively by the 20 times amount of the cold duplex A (lane 9). Although modifications at the four base pair-outside of the κB site did not significantly affect the binding with the proteins (lanes 1 and 5), replacements at the close position and inside of the κB site allowed the probe-protein bindings: these duplexes do not bind the κB site efficiently (lanes 2, 3, 7, and 8). Interestingly, however, duplexes consist of ④ and ⑥ showed selective competitive inhibitory activity, namely the former has preference in binding affinity toward the p50/p65 heterodimer and the latter possesses the selectivity to the p50 homodimer (lanes 4 and 6). According to the X-ray work of Verdine *et al*,[2b] the position of the 5-formyl group in ⑥ seems to be within the reach of the ε-amino group of Lys 244 which is the key residue of the recognition loop of the p50 homodimer. Since the p50/p65 heterodimer acts as an activator of transcription whereas the p50 homodimer acts as a suppresser, the modified duplexes could affect the immune and inflammatory systems *in vivo* in a distinct manner.

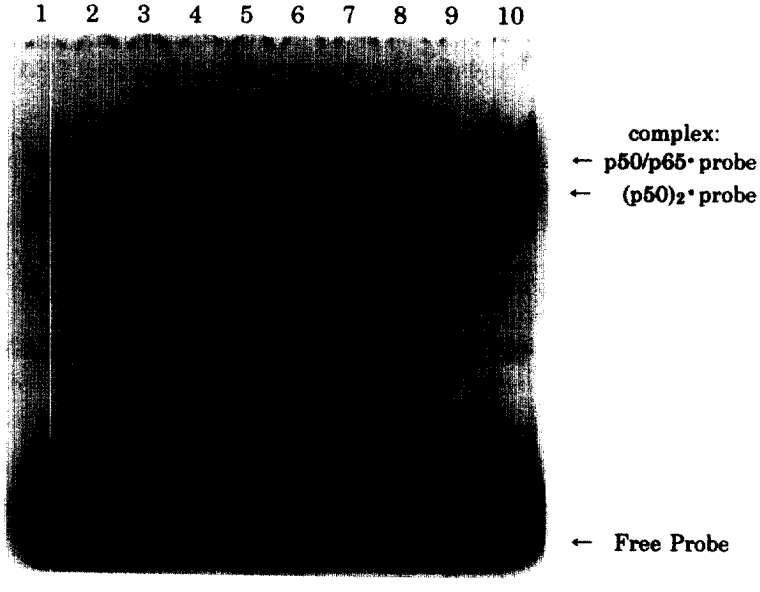

Figure 1. Selective binding affinity of various modified duplexes to the p50 homodimer and the p50/p65 heterodimer of NF-κB. Electrophoretic mobility shift assays were performed as described[12] using nuclear extract prepared from Jurkal T cells stimulated with TNF-α (10 ng/mL, 20 min). The 5'-^{32}P-labeled probe (duplex A, 1 ng) was incubated with the nuclear extract (5 μg protein) in binding buffer [15 mM Tris•HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT, 7.5% (w/v) glycerol, 0.3% (w/v) NP-40, 1 μg/μL BSA] in the presence or absence of each duplex (20 ng) at 25 °C for 20 min. DNA-protein complexes were analyzed on 4% nondenaturing polyacrylamide gels. Lanes 1-8: in the presence of duplex ①-⑧, respectively; Lane 9: in the presence of duplex A (20 ng); Lane 10: no competitor.

The 5-methyl group of T exists in the major groove of DNA and modification at C5 of 2'-deoxyuridine and its incorporation into oligomers is a useful design target for the study of DNA-protein interaction.[13] It would be considered that the formyl group, the oxidation form of the methyl in T, is not an essential factor to raise the selectivity. The sugar pucker of ribonucleoside, even 2'-*O*-methylated one like **11**, keeps the 3'-*endo* conformation in RNA, while 2'-deoxyribonucleoside prefers the 2'-*endo* puckering mode which is maintained in oligomer or

polymer to form B-DNA.[14] This conformational difference in **11** and/or the bulkiness of the 2'-methoxyl group would affect the protein binding affinity. It has been reported that NF-κB makes a large number of contacts with DNA, and it may account for the fine selectivity.[15]

The possibility of introducing 5-formyl-2'-deoxyuridine into the κB-sites is currently under investigation to compare the binding affinity and to realize making crosslink between the formyl group and an ε-amino group of a certain lysine residue in NF-κB.

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8. The two diastereomers of **9** were separated by HPLC on a Shim-Pack Prep-SIL (H) column (2×25 cm, eluted with 25% hexane in EtOAc at a flow rate of 10 mL/min). Anal. Calcd for $C_{37}H_{40}N_2O_{12}\cdot 2/3H_2O$: C, 62.00; H, 5.81; N, 3.91. For the isomer with retention time 15.1 min; Found: C, 61.89; H, 5.89; N, 3.68. For the other isomer, 16.5 min; Found: C, 61.73; H, 5.87; N, 3.65.
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10. The oligo was treated with phosphodiesterase and alkaline phosphatase in 50 mM phosphate buffer (pH 7.2) at 37 °C for 5 h. HPLC analysis on a PRODIGY ODS column (4.6× 150 mm, 40 °C, eluted with 0-10% CH_3CN in 50 mM HCO_2NH_4, linear gradient over 20 min, at a flow rate of 1.0 mL/min) showed the peak of 5-formyl-2'-*O*-methyluridine[11] with the retention time of 16.3 min (the detector 1; 260 nm) and 16.4 min (the detector 2; 304 nm). There is a time lag (0.1 min) between the two detectors 1 and 2.
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