Chemical synthesis, DNA incorporation and biological study of a new photocleavable 2′-deoxyadenosine mimic

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ABSTRACT

The phototriggered cleavage of chemical bonds has found numerous applications in biology, particularly in the field of gene sequencing through photo-induced DNA strand scission. However, only a small number of modified nucleosides that are able to cleave DNA at selected positions have been reported in the literature. Herein, we show that a new photoactivable deoxyadenosine analogue, 3-nitro-3-deaza-2′-deoxyadenosine (d(3-NiA)), was able to induce DNA backbone breakage upon irradiation (λ>320 nm). The d(3-NiA) nucleoside was chemically incorporated at desired positions into 40-mer oligonucleotides as a phosphoramidite monomer and subsequent hybridization studies confirmed that the resulting modified duplexes display a behaviour that is close to that of the related natural sequence. Enzymatic action of the Klenow fragment exonuclease free revealed the preferential incorporation of dAMP opposite the 3-NiA base. On the other hand, incorporation of the analogous 3-NiA triphosphate to a primer revealed high enzyme efficiency and selectivity for insertion opposite thymine. Furthermore, only the enzymatically synthesized base pair 3-NiA:T was a substrate for further extension by the enzyme. All the hybridization and enzymatic data indicate that this new photoactivable 3-NiA triphosphate can be considered as a photochemically cleavable dATP analogue.

INTRODUCTION

Selective DNA fragmentation is undeniably a useful technique for the structural and dynamic analysis of nucleic acids. For instance, DNA sequencing using microarrays, which require preliminary DNA fragmentation into oligomers of 20–40 nt in length, is a very promising technology in the field of medical diagnosis. DNA strand scission can involve either native restriction enzymes, synthetic nucleases (e.g. metallic complexes) (1–5), or photocleaving agents (6). The advantages of photocleaving agents are numerous: the cleavage reactions can be performed in reagent-free medium, they allow the spatial and temporal control of the reactions, and reaction by-products are easily removed. If many DNA-photocleaving agents have been developed so far, only a very few of them are nucleoside analogues that can be incorporated within DNA strands (7–10). These reported nucleoside analogues typically rely on the photochemistry of the O-nitrobenzyl group, which is attached at one C-position of the sugar backbone (11–16). Unfortunately, they often behave as universal nucleobases and then are not specifically recognized by enzymes (17–19). Thus, there is a significant incentive to develop new photoactive nucleoside derivatives that display only minor structural modifications in comparison with their natural analogues in order to maintain their specific recognition properties.

Previously, we reported the synthesis of 7-nitro-indole nucleoside (d(7-Ni)) (20,21) (Figure 1). Photochemical and cleavable properties of DNA strands incorporating d(7-Ni) have been examined (22). Upon irradiation in aerated solution, a chemical rearrangement involving the nitro group occurs, affording the corresponding deoxyribonolactone. Subsequently, mild alkaline or thermal treatment leads to DNA strand cleavage through a β-elimination reaction (Figure 2). DNA polymerase recognition of d(7-Ni) incorporated either in a DNA matrix or to a primer as its corresponding triphosphate has also been studied (23). It was shown that the modified base was not specifically recognized by the enzyme.

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We recently designed and synthesized a second-generation photocleavable nucleoside, 3-nitro-3-deaza-2'-deoxyadenosine (d(3-NiA)), which conserves the base-pairing ability of the natural adenine and was therefore expected to be specifically recognized by polymerase enzymes as an adenine (Figure 1). Previously, we described the synthesis of d(3-NiA) and its incorporation in oligonucleotides as a phosphoramidite monomer (24,25). Photocleavage of DNA containing the modified 3-NiA base was shown to be quantitative and rapid, both in single- and double-strand DNA (22).

In the present paper, we report the thermodynamic properties of oligonucleotide duplexes that incorporate d(3-NiA) in the middle of their sequences. The enzymatic recognition by a DNA polymerase Klenow fragment exonuclease free of the 3-NiA unnatural base has also been studied. The synthesis of the analogous d(3-NiA)TP triphosphate as well as its enzymatic incorporation into DNA are described.

MATERIALS AND METHODS

Materials

All reagents were of the highest quality commercially available. Oligonucleotides were labelled using [γ-32P]-ATP (specific activity 3000 Ci/mmol) (PerkinElmer) and T4 polynucleotide kinase purchased from MBI fermentas. ATP (specific activity 3000 Ci/mmol) (PerkinElmer) was added. For incorporation of the modified nucleoside d(3-NiA), standard coupling time was extended for 10 min and quantity of capping reagent was multiplied by five. After automated synthesis the oligomers were deprotected on column, cleaved from support by conc. aqueous ammonia 30% for 1 h at room temperature, then deprotected by heating at 55°C for 24 h.

These oligomers were purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7-M urea. The concentration of oligodeoxynucleotides was determined by UV. Oligonucleotide sequences used for experiments have been reported in Table 1.

Melting temperature experiments

Experiments were performed on 11-bp DNA duplexes containing d(3-NiA) paired with dG, dC, dA or dT. Experimentally, the modified oligonucleotides were mixed with their complementary strands in buffer consisting of 10 mM sodium phosphate/1 mM EDTA/100 mM NaCl adjusted to pH 7. UV absorption spectra (at 260 nm) and melting experiments were recorded using a CARY 400 Scan UV-Visible spectrophotometer (Varian) equipped with a Peltier thermoelectric Cary temperature controller (Varian). Before each melting experiment, samples were heated at 80°C for 5 min and cooled slowly to ensure that the oligonucleotides were in the duplex state. The absorbance was monitored at 260 nm from 5°C to 80°C at a heating rate of 0.3°C per minute. Data were recorded every 1 min. Melting temperature (Tm) has been determined from the derivative method using the “Cary Win UV-bio” (version 2) application software. Error in the determination of Tm has been estimated as ±0.6°C (average of three independent measurements).

Synthesis

1-[3'-O-acetyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxy-β-D-erythro-pentofuranosyl]-7-nitro-4-(2,4,6-trimethylphenox)-1H-imidazo[4,5-c]pyridine: The compound 2 (200 mg, 0.28 mmol) was co-evaporated twice with dry pyridine for 1 μmol DNA synthesis were modified since double amounts of phosphoramidites were used for coupling steps and after this step also double amount of capping reagent was added. For incorporation of the modified nucleoside d(3-NiA), standard coupling time was extended for 10 min and quantity of capping reagent was multiplied by five. After automated synthesis the oligomers were detitylated on column, cleaved from support by conc. aqueous ammonia 30% for 1 h at room temperature, then deprotected by heating at 55°C for 24 h.

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imidazo[4,5-c]pentofuranosyl]-7-nitro-1-deoxy-2-erythro-pentofuranosyl]-7-nitro-1-deoxy-2-erythro-pentofuranosyl]-7-nitro-hydroimidazo[4,5-c]pyridine (3): The 5′-O-DMT-nucleoside (200 mg; 0.26 mmol) was dissolved in dichloromethane (10 ml) and methanol (200 μl) was added. To this solution, trichloroacetic acid (400 μl, 50% solution in dichloromethane) was added and the mixture was stirred for 45 min at room temperature. The solution was then washed with NaHCO3 saturated aqueous solution (8 ml) and dried with MgSO4. After evaporation of solvents, the oily residue was purified by flash chromatography (elution with AcOEt/cyclohexane 70/30) to afford the compound 3 (97 mg, 82%). Rf (AcOEt/cyclohexane 1/1): 0.25. 1H NMR (300 MHz, CDCl3): δ = 8.82 (s, 1 H), 8.75 (s, 1 H), 6.95 (s, 2 H), 6.82 (t, J = 6.2 Hz, 1 H), 5.45 (m, 1 H), 4.23 (m, 1 H), 3.97 (m, 2 H), 2.88 (m, 1 H), 2.77 (m, 1 H), 2.67 (m, 1 H), 2.32 (s, 3 H), 2.14 (s, 3 H), 2.10 (s, 6 H). 13C NMR (75 MHz, CDCl3): δ = 170.8, 158.7, 147.6, 144.3, 140.9, 135.8, 132.2, 130.6, 130.3, 130.0, 129.7, 89.4, 86.2, 76.7, 61.9, 41.2, 210 (2C), 16.5. MS (DCI, NH3/isobutane): 457.0 [M + H]+. Anal. Calcd for C22H24N4O7 (456.5): C 57.50, H 5.30, N 11.86.

5′-Triphosphate of 4-amino-1-[2′-deoxy-β-D-erythro-pentofuranosyl]-7-nitro-hydroimidazo[4,5-c]pyridine (5): The 3′-O-acetyl nucleoside 3 (30 mg, 66 μmol) was dried by co-evaporation with dry pyridine (twice) and then dissolved under argon in dry pyridine (66 μl) and dry dioxyane (198 μl). To this solution, 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one 4 (86 μl of 1 M solution freshly prepared in dry dioxyane, 86 μmol) was added and stirred at room temperature under argon for 10 min. Then tributylammonium pyrophosphate (212 μl of 0.5 M solution in dry DMF, 106 μmol) and tributylamine (86 μl, 352 μmol) were added simultaneously and stirred for 10 min. The reaction mixture was then treated with iodine (1.3 ml of 1% solution in aqueous pyridine solution: pyridine/water = 98/2 v/v, 192 μl) for 15 min and then the excess of iodine was decomposed by addition of sodium hydrogen sulphite aqueous solution (0.5%). After stirring for 10 min, solvents were removed in vacuo; then the residue was dissolved in 1 ml of water. The crude product was purified by reverse-phase silica gel chromatography (LiChroprep RP 18 silica gel, 40–63 μm, elution with 0–80% MeOH/H2O). To combined fractions containing 5, NaHCO3 aqueous solution (0.2 M, 500 ml) was added and solvents were removed by evaporation in vacuo to afford 41 mg (47%) of 5. Rf (propan-1-ol/H2O/NH3 11/2/7): 0.65. 1H NMR (300 MHz, D2O): δ = 9.01 (s, 1 H), 8.70 (s, 1 H), 7.13 (s, 2 H), 6.93 (t, J = 6.3 Hz, 1 H), 5.58 (m, 1 H), 4.60 (m, 1 H), 4.37 (m, 2 H), 2.92 (m, 2 H), 2.37 (m, 3 H), 2.23 (s, 3 H), 2.10 (s, 6 H). 31P NMR (75 MHz, D2O): δ = −21.74 (t, Pβ), −10.91 (d, Pβ), −5.80 (d, Pγ). MS (ESI): 784.9 [M–3H + + 4Na+]+, 762.9 [M – 2H + + 3Na+]+. UV 84 μM in H2O: λmax (ε) = 322 (4265).

5′-Triphosphate of 3-deaza-3-nitro-2-deoxyadenosine (I): The triphosphate 5 (4.5 μmol) was dissolved in water (200 μl) and concentrated NH4OH (30%) solution (1.2 ml) was added and the solution was heated at 60°C for 2 h. After evaporation of solvents, the residue was purified by reverse-phase silica gel chromatography (LiChroprep RP 18 silica gel, 40–63 μm, elution with H2O) to afford 1 (3.46 μmol, 77%). Rf (propan-1-ol/H2O/NH3 11/2/7): 0.46. 1H NMR (500 MHz, D2O): δ = 8.76 (s, 1 H), 8.70 (s, 1 H), 6.88 (m, 1 H), 5.58 (m, 1 H), 4.77 (m, 1 H), 4.29 (m, 3 H), 2.70 (m, 2 H). 31P NMR (121 MHz, D2O): δ = −22.18 (t, Pβ), −10.98 (d, Pβ), −7.31 (d, Pγ).
Sequence 13) template (100 nM) hybridized to 50 (X = d(3-NiA), sequence 8) or unmodified (X = A, template).

5 min, then cooling to room temperature over 2 h.

500 (mM each) for 20 min at 30°C in a final volume of 10 μL. Reactions were quenched by adding 30 μl of formamide loading buffer (95% formamide, 20 mM EDTA pH 8, 0.1% bromophenol blue, 0.1% xylene cyanol) and heating at 70°C for 5 min. Aliquots (2 μl) were loaded on a 20% denaturing PAGE.

Primer extension by KF exo- opposite d(3-NiA) present in template. Reactions were conducted either on modified (X = d(3-NiA), sequence 8) or unmodified (X = A, sequence 13) template (100 nM) hybridized to 5’-end labelled primer (sequence 9, 50 nM). Primer extension reaction catalysed by KF exo- (0.1 U) was carried out in buffer hybridization in presence of single dNTP (20 μM) or all four dNTPs (20 μM each) for 20 min at 30°C and analysed by 20% denaturing PAGE.

Preparation of the 3-deaza-3-nitro-2’-deoxyadenosine triphosphate 1

The 3-deaza-3-nitro-2’-deoxyadenosine triphosphate 1 was prepared from the trimethylphenoxy precursor nucleoside 2. The 5’-DMT nucleoside 2 (see Figure 3) was first converted into the 3’-protected nucleoside 3, which was then directly converted to the triphosphate 5 using the Ludwig–Eckstein method (26). Thus, the compound 3 was treated successively (1) with 2-chloro-4H-1,3,2-benzo-dioxaphosphorin-4-one (4), (2) with tributylammonium pyrophosphate and (3) with iodine-aqueous pyridine. Purification of the crude mixture was carried out by reverse-phase chromatography. The triphosphate 5 was obtained with a 47% overall yield and characterized by 1H and 31P NMR and by ES-MS spectrometry. Finally the aroylhemostidin was replaced by NH2 and the 3’-O-acetyl group was deprotected to afford the triphosphate 1 when 5 was treated with aqueous NH3 at 60°C for 2 h. The triphosphate 1 was obtained with 77% yield and characterized by 1H and 31P NMR and by electrospray mass ES-MS spectrometry.

Hybridization properties

We evaluate the ability of the 3-nitro-3-deaza-2’,deoxyadenosine to pair with another base into a DNA duplex. Thermodynamic parameters were determined for a series of 11-mer duplexes containing the modified d(3-NiA) nucleoside in the middle of the sequence (sequence 1) and facing one of the four natural bases (sequences 2–5). For comparison, stability of duplexes containing the natural base pairs A:T or G:C (sequence 6: sequence 3) and sequence 7: sequence 5, respectively) or the mismatched bases A:C (sequence 6: sequence 5) were determined. The sigmoidal curves γ = f(T) obtained from the standard UV melting curves for the different duplexes exhibited melting cooperativity and were analysed to derive the Tm values and the Van’t Hoff transition enthalpy ΔH, entropy ΔS and free energy ΔG°. The resulting data listed in Table 2 showed higher stability for the 3-NiA:T unnatural base pair duplex (Tm = 59°C) compared to the other duplexes in which 3-NiA was opposite to C, G or A (Tm = 46°C, 53°C and 49°C, respectively). Very similar Tm values were determined respectively for the matched duplexes A:T and 3-NiA:T (Tm = 59 and 60°C, respectively) and for the mismatched duplexes A:C and 3-NiA:C (Tm = 46°C for both). The stabilization energies determined respectively for the matched duplexes A:T and 3-NiA:T were also similar (ΔS° = 231 and 236 cal/mol/K, ΔH° = 85 and 86 kcal/mol, respectively and ΔG° = 16 kcal/mol for both). On the contrary, the decrease of the Tm (ΔTm = 8°C) was measured when the natural base pair C:G was replaced by the unnatural pair 3-NiA:G. This corresponds to a loss of free energy of 4 kcal/mol. All these results indicate that the 3-NiA unnatural base behaves like an adenine.
Experiments were run in triplicate. See ‘Materials and Methods’ section for details.

Kinetic parameters for insertion of dNMP opposite d(3-NiA). Duplex containing the 3-NiA nucleobase was evaluated as a substrate for the exonuclease-deficient Klenow fragment of *Escherichia coli* DNA polymerase I (KF exo-). Studies were investigated using a 40-mer modified template containing the unnatural bases 3-NiA in the middle of the sequence (sequence 8) hybridized to the 18-mer primer (sequence 9). For comparison, a template containing the natural base A (sequence 13) hybridized to the same 18-mer primer was also examined. The resulting duplexes were subjected to primer extension in the presence of one single dNTP (Figure 4). DNA synthesis on template containing the natural base (sequence 13) led to the expected incorporation of dTMP opposite A (data not shown). When using the 3-NiA containing strand as a template (sequence 8), dTMP was incorporated as expected opposite 3-NiA. dAMP and guanosine monophosphate (GMP) were also incorporated opposite the unnatural base with quite different efficiency. Insertion of dCMP could not be detected in these conditions (Figure 4, lane 3).

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### Table 2. Melting temperatures and thermodynamics parameters of 11-mer duplexes containing natural or unnatural base pairs (X:Y) in the middle of the sequence

| Duplex          | X       | Y       | Tm (°C) | ΔS° (cal/mol/K) | ΔH° (kcal/mol) | ΔG° (kcal/mol) |
|-----------------|---------|---------|---------|----------------|----------------|----------------|
| 3’ CGCACXCACGC 5’ | Unnatural base pair | 3-NiA | A       | 49             | 85             | 36             | 10             |
| 5’ GCGTGYGTCGC 3’ |          |         |         |                |                |                |
| 3-NiA A         |         | 5’ GCGTGYGTCGC 3’ | 3-NiA | T       | 60             | 236            | 86             | 16             |
| 3-NiA G         |         | 5’ GCGTGYGTCGC 3’ | 3-NiA | C       | 53             | 182            | 67             | 13             |
| 3-NiA C         |         | 5’ GCGTGYGTCGC 3’ | 3-NiA | G       | 46             | 154            | 57             | 11             |
| Natural base pair | A       | T       | 59             | 231            | 85             | 16             |
|                 | G       | C       | 61             | 240            | 89             | 17             |
| Mismatch        | A       | C       | 46             | 185            | 67             | 12             |

Experiments were run in triplicate. See ‘Materials and Methods’ section for details.

### Figure 4. Single nucleotide incorporation opposite 3-NiA. Reactions were performed on 40-mer templates containing the unnatural base: 3-NiA, sequence 8 (lanes 1–5; 100 nM) primed with 32P-labeled 18-mer (sequence 9, 50 nM) in the presence of one single dNTP (20 μM) and KF exo- (0.1 U). Reactions were incubated for 20 min at 30°C before processing as described in ‘Materials and Methods’ section.
opposite the modified base. The accumulation of this intermediate suggests that a pause occurs after the first incorporation.

Incorporation of d(3-NiA) triphosphate opposite the four natural bases A, C, G or T. Efficiency of insertion of the triphosphate of d(3-NiA) against each natural base in the template has been measured. Experiments were run using the 19-mer primer (sequence 17) successively hybridized to the four templates (sequences 10, 14–16) varying by the nature of the base N at the initiation site of the polymerization. The kinetic parameters for insertion of the unnatural 3-NiA base opposite the different natural bases (Table 4). The triphosphate of d(3-NiA) was preferentially incorporated opposite to thymine in the template (kcat/Km = 40.6 × 10^2 min^{-1} mM^{-1}). The kcat/Km value was 6.3 times, 52 times and 110 times, respectively, higher than measured for incorporation opposite natural bases A, C and G. Furthermore, incorporation of d(3-NiA)TP opposite natural thymine was 16 times higher than incorporation of natural dATP opposite thymine (kcat/Km = 2.53 × 10^2 min^{-1} mM^{-1}).

Extension of the primer terminated by d(3-NiA). The ability of KF exo- to extend a primer terminated by the unnatural 3-NiA base opposite the different natural bases N in the template was studied (Figure 6). Reactions were conducted on the 18-mer primer (sequence 9) annealed to the 40-mer templates in which base N19 was successively a thymine (sequence 10), an adenine (sequence 13), a cytosine (sequence 11) or a guanine (sequence 12). In a first step, primer extension was run in the presence of the triphosphate d(3-NiA)TP (Figure 6, lanes 2, 7, 12 and 17). Incorporation of one d(3-NiA) nucleoside was observed for all duplexes, permitting to obtain all possible unnatural base pairs 3-NiA: N. In a second step polymerization was continued on these extended primers in the presence either of dATP (the next complementary base in the template) (Figure 6, lanes 3, 8, 13 and 18) or in the presence of the four dNTPs (Figure 6, lanes 4, 9, 14 and 19). No extension could be observed for the duplexes in which d(3-NiA) was facing adenine, cytosine or guanine, neither in the presence of dATP (Figure 6, lanes 8, 13 and 18) nor in the presence of the mixed dNTPs (Figure 6, lanes 9, 14

Table 3. Steady-state kinetic parameters (kcat/Km) for incorporation by KF exo- of single nucleotides opposite the natural or unnatural bases present in the template (X = A or 3-NiA, respectively)

| X     | dYTP   | kcat (min^{-1}) | Km (μM) | kcat/Km (μM^{-1} min^{-1}) × 10^{-2} |
|-------|--------|-----------------|---------|-------------------------------------|
| 3-NiA | dATP   | 5.86±0.08       | 19±2    | 30.70                               |
| 3-NiA | dCTP   | 1.10±0.01       | 235±2   | 0.47                                |
| 3-NiA | dGTP   | 3.26±0.07       | 52±5    | 6.16                                |
| 3-NiA | dTTP   | 0.62±0.01       | 38±4    | 1.62                                |
| 3-NiA | d(3-NiATP) | 2.53±0.05         | 21±4    | 11.87                               |

Natural triphosphate
A | dTTP | 1.92±0.02 | 60±6 | 3.2 |

Table 4. Efficiency of KF exo- incorporation of unnatural triphosphate (d(3-NiA)TP) opposite natural bases N in the template

| N     | kcat (min^{-1}) | Km (μM) | kcat/Km (μM^{-1} min^{-1}) × 10^{-2} |
|-------|-----------------|---------|-------------------------------------|
| A     | d(3-NiA)TP      | 4.3±0.1 | 67±7                               | 6.4 |
| T     | d(3-NiA)TP      | 7.7±0.1 | 19±2                               | 40.6|
| G     | d(3-NiA)TP      | 0.136±0.004 | 36±4                        | 0.37|
| C     | d(3-NiA)TP      | 0.47±0.01 | 61±5                          | 0.77|

Natural triphosphate
T | dATP | 2.37±0.04 | 93±6 | 2.53 |
and 19). Only the 3-NiA:T base pair was substrate for extension of the modified primer. In this case, formation of a full-length product was observed in the presence of the four natural triphosphates (Figure 6, lane 4). Incorporation of the modified monophosphate (d(3-NiA)MP) at the 3′ primer terminus has been proved for all extension reactions. Indeed, primers extended by one or two d(3-NiA)MP [products of lanes 7, 12, and 17 for one incorporation and lane 2 for extension by two d(3-NiA)MP: Figure 6] were first irradiated at λ > 320 nm for 60 min, in order to convert the modified monophosphate into deoxyribonolactone. Secondly, cleavage at the lactone site by piperidine treatment produced the initial 18-mer primer (Figure 6, lanes 5, 10, 15 and 20).

**Figure 6.** Chain extension reaction catalysed by KF exo- in the presence of the modified triphosphate d(3-NiA)TP. Experiments were performed with different natural templates (100 nM) varying by the nature of the base N19 (N = T, sequence 3; N = C, sequence 4; N = G, sequence 5; N = A, sequence 6) hybridized to 18-mer primer, sequence 8 for 20 min at 30°C (lanes 2, 7, 12 and 17). In the second step, reactions were carried out using KF exo- (0.1 U) in the presence of dATP (lanes 3, 8, 13, 18) or in the presence of a mixture of the four dNTPs (lanes 4, 9, 14, 19) for 20 min at 30°C. One part of products of reactions corresponding to lanes 3, 8, 13 and 18 were illuminated at λ > 320 nm for 20 min and then treated with NaOH 1 M for 20 min at 70°C (lanes 5, 10, 15 and 20).

**Figure 7.** Effect of the downstream template base N20 on chain extension reaction catalysed by KF exo-. (i) 3-NiA Incorporation opposite T in the template. (ii) Incorporation of the complementary correct nucleotide opposite varying nucleotide in the template. Experiments were performed with different templates (100 nM) varying by the nature of the base N20 (N = T, sequence 10; N = A, sequence 14; N = C, sequence 15; N = G, sequence 16) hybridized to 18-mer primer (sequence 2, 50 nM). Addition of one d(3-NiA) to the primer was carried out using KF exo- (0.1 U) in presence of d(3-NiA)TP (500 μM) for 5 min at 30°C (lanes 2, 6, 10 and 14). The second step of reaction was performed using KF exo- (0.1 U) in the presence of one single dNTP (lanes 3, 7, 11 and 15) or in the presence of the mixture of the four dNTPs (lanes 4, 8, 12 and 16) for 20 min at 30°C.

Influence of the downstream base on the extension of a primer terminated by the unnatural 3-NiA base. In this experiment, we investigated the influence of the downstream template base (N20) on the extension of the primer terminated by the 3-NiA unnatural base. Experiments were conducted on the same 18-mer primer (sequence 9) hybridized to different 40-mer templates (sequences 10, 14–16) varying only by the nature of base N20 (Figure 7). The primers were first elongated by one 3-NiA base as described in the previous experiment (Figure 7, lanes 2, 6, 10 and 14). It should be noted that the 18-mer primer annealed to template in which N20 was a guanine was only partially converted in the conditions used. In a second step, elongation was continued either in the presence of the triphosphate complementary to the next base in the template (Figure 7, dATP for T20: lane 3, dTTP for A20: lane 7, dGTP for C20: lane 11, dCTP for G20: lane 15) or in the presence of the four dNTPs (Figure 7, lanes 4, 8, 12 and 16). All the templates allowed elongation of the primer by one 3-NiA. For each duplex, primers terminated by 3-NiA were extended in the presence of a unique triphosphate (Figure 7, lanes 3, 7, 11 and 15). However, primer extension in the presence of dCTP only led to small amounts of reaction product. Incorporation of three guanines was observed for the primer annealed to template in which N20 was a cytosine.

**Figure 7.** Effect of the downstream template base N20 on chain extension reaction catalysed by KF exo-.
DISCUSSION

In this article, we first studied the hybridization properties of DNA duplexes containing the 3-NiA unnatural base opposite each native base. Comparison of the thermodynamic parameters determined for the unnatural 3-NiA: N (N = A, C, G or T) base-pair duplexes and for the natural matched or mismatched base-pair duplexes clearly showed that 3-NiA behaves like the deoxyadenosine nucleoside.

We then investigated recognition by KF exo- of the unnatural nucleobase 3-NiA in the DNA template. The incorporation frequency opposite the unnatural base followed the order: dAMP > dGMP > dTMP > dCMP. It appears that d(3-NiA) was recognized by KF exo- rather like a pyrimidine since insertion of purines dAMP or dGMP opposite the unnatural base was favoured over dTMP and dCMP. The small preference for dAMP incorporation suggests that similar to non-informative lesions, such as abasic sites (2'-deoxyribose, dR or 2'-deoxyribonolactone, dL), 3-NiA might obey the ‘A-rule’. Indeed, when the modified nucleosides are unable to fit within the polymerase active site, the enzyme inserts in most cases an adenine opposite the non-instructional nucleobase (27). Although 3-NiA is very similar to the natural adenine, we assume that the presence of the nitro group could disturb the polymerase binding and subsequently prevents its conformational change, which is necessary for the matched nucleotide insertion. Consequently, 3-NiA cannot be considered as a true photocleavable deoxyadenosine mimic. In addition, these primer extension experiments showed that the presence of d(3-NiA) in the template led to a decrease of the polymerase activity, although full-length products were obtained.

We also examined the incorporation of the d(3-NiA) triphosphate opposite each native base in the template. The measured kinetic parameters were significantly different between the four natural bases. Indeed, the modified d(3-NiA) triphosphate was preferentially inserted opposite thymine. The insertion frequency opposite each natural base followed the order T >> A > C ~ G. If we refer to other studies in which the fidelity of KF exo- has been checked (28), it appeared that d(3-NiA)TP is inserted with a selectivity that is nearly as high as that observed for dATP. In addition, the d(3-NiA) triphosphate was also efficiently inserted opposite itself in the template. This observation is in good agreement with a previous study from Kool, who reported that hydrophobic aromatic nucleosides are preferentially incorporated opposite themselves (29).

Extension of primers terminated by the unnatural base 3-NiA has been also monitored. We found that 3-NiA behaves as chain terminator when facing adenine, guanine or cytosine in the template. When facing thymine, the 3-NiA primer was elongated by one adenine in the presence of dAMP and full-length elongation occurred in the presence of the four native base triphosphates, indicating that only the terminal 3-NiA:T pair could be recognized by KF exo-. This elongation was not dependent on the nature of the base downstream to thymine in the template.

Several models have been proposed in order to explain how most DNA polymerases maintain an incredible degree of substrate fidelity during DNA synthesis. Replication fidelity has historically been interpreted with respect to the formation of proper hydrogen bonds between the nascent base pairs. According to this model, the incoming dNTP directly pairs opposite to its complementary base via direct hydrogen bonding, the enzyme catalysing the formation of the phosphodiester bond only if the two nucleobases are aligned correctly. However, several studies also indicate that hydrogen bonding should be not the unique factor involved in nucleotide selection by polymerase during DNA synthesis. It was proposed that solvation, base stacking and steric (size and shape) factors should play a role in the selection process as well. In particular, Berdis et al. (30,31) highlighted the significant contribution of the stacking interactions between the π electrons-rich residues of both aromatic amino acids in the catalytic site of the enzyme and the incoming nucleobase. These non-covalent interactions were shown to facilitate the nucleotide binding and the conformational change step preceding the phosphoryl transfer during DNA synthesis, both steps being the most significant contributors towards catalysis fidelity. A complete kinetic scheme describing the polymerization of dNTP by the Klenow fragment of DNA polymerase I has been established by Kuchta (32).

With regards to the results obtained by Berdis, Kool and Kuchta, we suggest the hypothetic mechanism described explains the efficiency and selectivity of the d(3-NiA)TP incorporation by KF exo- opposite thymine in the template. The nucleobase moiety of the non-natural triphosphate d(3-NiA)TP contains an extended π-electron surface area (NO2 group) with reference to the natural dATP. This enhanced π electron surface area could be responsible of higher binding stabilization of the incoming nucleobase through π–π stacking interactions with the aromatic amino acids in the dNTP-binding site of the enzyme. Upon binding of d(3-NiA)TP, the polymerase/DNA complex then undergoes a conformational change to further align the incoming non-natural triphosphate into a favourable geometrical arrangement for subsequent phosphoryl transfer. Additionally, the hydrogen bonds formation in the nascent base pair, similar to which existing in the natural T:A Watson–Crick base pair, could facilitate this arrangement and consequently play a significant role in the selective incorporation of d(3-NiA)TP by KF exo- opposite thymine in the template. In this model, we propose that shape complementary, hydrogen bonding and an extended π-stacking all contribute to the efficiency and the selectivity of the d(3-NiA)TP insertion by KF exo- opposite thymine in the template.

In conclusion, the 3-NiA nucleoside was designed by analogy with the previously reported 7-nitroindole nucleoside to be a photocleavable precursor of the highly labile deoxyribonolactone lesion. In contrast with 7-Ni, the 3-NiA nucleoside is potentially able to establish two hydrogen bonds with thymine and was thus expected to be specifically recognized by enzymes as an adenine. Enzymatic experiments involving the 3-NiA nucleotide incorporated in a template indicated that the modified base was not recognized as an adenine. However, the
selective recognition by KF exo- of the d(3-NiA)TP triphosphate as a dATP analogue has been demonstrated. Compared to the first-generation 7-Ni nucleoside, which was a universal nucleoside analogue, the 3-NiA was recognized by the DNA polymerase as an adenosine. In addition, hybridization properties of oligonucleotides that incorporate (3-NiA) are very similar to the corresponding native biopolymers. Further work also showed that d(3-NiA) is actually a highly interesting motif to generate DNA backbone cleavage at pre-selected positions. From these results, we propose that the d(3-Ni-A)TP may be a useful new fragmentation tool for DNA sequencing.

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