DNA Damage

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Sequence-Specific Nucleic Acid Damage Induced by Peptide Nucleic Acid Conjugates That Can Be Enzyme-Activated**

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Sequence-specific targeting and cleavage of nucleic acids (in DNA or RNA) can be achieved using oligo-2′-deoxyribonucleotides (ODN) or peptide nucleic acids (PNA) covalently attached to a reactive chemical.[1] These conjugates combine the ability to recognize (through the ODN or the PNA) and react (through the chemical) with a selected sequence, which thus can be either redox cleaved or hydrolyzed. However, the great majority of the chemical reactants used in this context to date are non-natural compounds (such as, transition-metal complexes, polyamines, photosensitizers) and, as a consequence, such systems in general require an activating mechanism (e.g., light, strong reductants) unavailable within the cell. An attractive solution to this problem might be provided by ODN conjugates carrying a chemical moiety serving to recruit a cellular enzyme and directing the action of that enzyme towards selective nucleic acid cleavage. Such a chemical can be a substrate or an inhibitor of the enzyme. To our knowledge, there is only one example of such a conjugate that can be "enzyme activated" illustrating this strategy.[2] It is provided by studies of ODN-camptothecin (or rebeccamycin) conjugates in which the chemical moieties are selective inhibitors of Topoisomerase I.[2] In that case the chemical group has two roles: 1) by recruiting Topoisomerase I, it directs its action to the selected sequence; 2) by blocking the religation step, it ensures the persistence of DNA breaks. There is no reported example of a PNA conjugate used in this application. Herein we report a PNA–flavin conjugate **1** (Figure 1) in which the flavin moiety can recruit an NAD(P)H:flavin oxidoreductase (flavin reductase) allowing the flavin–enzyme combination to activate molecular oxygen in the presence of NADH and to selectively damage a complementary ODN in vitro.

PNAs, which are non-ionic ODN analogues with a non-natural polyamide backbone, have many advantages over ODN: 1) they are resistant to nucleases and proteases; 2) they display a very high affinity for complementary DNA and RNA and can bind to single- and double-stranded DNA targets.[3] Flavin reductases are ubiquitous enzymes, present in all living organisms, which catalyze the reduction of free

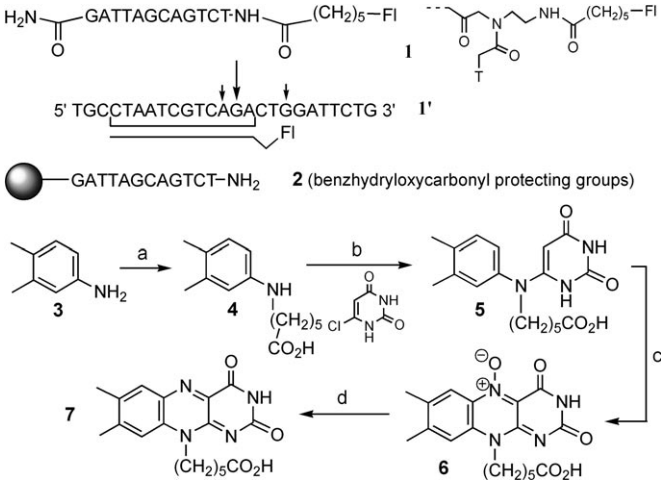

Figure 1. Structures of the compounds reported herein. Flavin **7** was prepared in 4 steps: a) 6-chlorohexanoic acid, TEA, EtOH, reflux (53%); b) dioxane/H_2O (1:1), pH 7.5, reflux (71%); c) $NaNO_2$, acetic acid (81%); d) DTT, dioxane/EtOH (1:1), reflux (90%). The arrows on top of **1′** indicate the sites of oxidation. The bridge marks the sequence in **1′** that is complementary to the PNA sequence in **1**. The orientation of **1** when hybridized to **1′** is also indicated. Fl indicates the isoalloxazine ring system of the flavin. The carboxylic acid side chain on N^{10} in **7** is a highly modified version of the normal flavin ribityl chain. TEA = triethylamine, DDT = 4,4′-dichlorodiphenyl-1,1,1-trichloroethane.

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flavins (riboflavin, flavin mononucleotide (FMN), or flavin adenine dinucleotide (FAD)) by reduced pyridine nucleotides (NAD(P)H). Reduced flavins react with oxygen efficiently thus generating radical and oxidizing species (hydroxyl radicals and H_2O_2) potentially reactive towards DNA.[4] They also efficiently transfer electrons to ferric ions.[5] In a recent study, flavin reductase was shown to promote oxidative DNA damage in *E. coli* cells through reduction of intracellular free iron by enzymatically reduced flavins.[6] This observation is the basis for studying the PNA–flavin/flavin reductase combination for selectively destroying a DNA target.

There is only one reported synthesis of flavin-tethered PNA.[7,8] In that case the synthetic procedure allows a given nucleic base to be replaced at various positions along the PNA chain by the isoalloxazine ring system of the flavin which attaches through nitrogen atoms, either N^3 or N^{10}.[8] The novel compounds reported herein, with N^{10} of the isoalloxazine ring system linked to the N terminal of the PNA through a highly modified version of the ribityl chain of riboflavin, are shown in Figure 1.

The PNA–flavin conjugate **1** was synthesized (see Supporting Information) by coupling the carboxylic acid function of **7** to the N terminal end of the protected 12-mer PNA **2** attached to the solid support of synthesis using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling agent (Figure 1). After release and deprotection using trifluoroacetic acid (TFA)/*m*-cresol, the resulting conjugate **1** was purified by HPLC on C_{18} reversed phase and characterized by mass spectrometry and UV/Vis spectroscopy.

Strong binding of compound **1** to the 25-mer ODN **1′** containing a complementary sequence (Figure 1) and **1**:**1′** duplex formation were confirmed by melting assays (4 μM, 0.1 M NaCl, $T_m = 61\,°C$).

Flavin reductase activity is generally assayed from the oxidation of NAD(P)H and spectrophotometrically monitored at 340 nm during aerobic incubation with the enzyme and a flavin substrate. The enzyme used herein as a model is the flavin reductase from *E. coli* named Fre. We have previously shown that Fre has very broad substrate specificity since it accepts riboflavin, FMN, or FAD as a substrate.[9] This indicates that the ribityl chain of a flavin plays almost no role in its recognition by Fre. The crystal structure of the Fre–riboflavin complex has been determined and indeed shows that the protein/substrate interaction almost exclusively involves the isoalloxazine ring system and not the ribityl chain located at the surface.[10] Riboflavin analogues with modified lateral chains at N^{10} are excellent substrates.[9] The flexibility is such that ODN–flavin conjugates, with the isoalloxazine ring system attached to the 5′-end of the ODN are also substrates.[11] Using increasing concentrations of **1** as the unique flavin component in the assay mixture and 200 μM NADH at pH 7.8 and 25 °C, we observed a saturation behavior for the enzyme activity. The data could be fitted with a V_m value of 18000 nmol oxidized NADH/min mg^{-1} protein and a K_m value for **1** of 3 μM (data not shown), which are in the same order-of-magnitude range as the values obtained with FAD ($V_m = 26000$, $K_m = 1$ μM) and riboflavin ($V_m = 70000$, $K_m = 1.5$ μM), thus showing that **1** is well recognized as a substrate by Fre.

The most remarkable result is that flavin reductase retains as much as approximately 25% activity when **1** (10 μM) is hybridized to **1′** (1 equiv) at 25 °C (below the T_m value). This activity remained unchanged upon further addition of **1′**. The **1**:**1′** complex is thus a substrate of Fre and this is in marked contrast with comparable ODN–flavin conjugates which were shown to be unable to promote NADH oxidation in the presence of small excesses of a complementary ODN.[11] Further studies are required to understand the molecular basis for such a drastic difference.

The above result thus made PNA–flavin conjugates good candidates as DNA (or RNA) damaging agents that can be enzyme activated. This potential was evaluated in an in vitro system in which **1** was incubated with a 2-fold excess of **1′** (to ensure full complexation) in the presence of NADH (5 mM), various concentrations of $FeSO_4$ and Fre (1 μM) in 50 mM Tris buffer (tris(hydroxmethyl)aminomethane) pH 7.8. The reaction was carried out in the dark to avoid photoreactions of the flavin moiety and the reaction mixture was analyzed by gel electrophoresis after 30 min incubation and piperidine treatment (see Supporting Information). The results are shown in Figure 2. They demonstrate an interesting selectivity of the

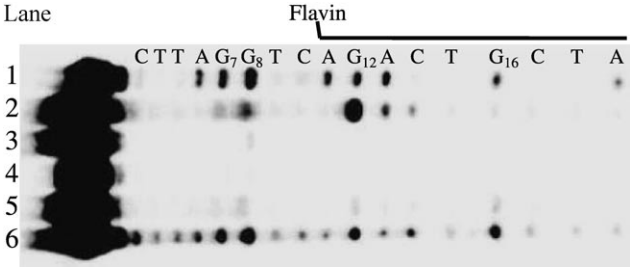

Figure 2. Phosphoimager picture of a 15% polyacrylamide/7 M urea gel showing the cleavage products of the 25 base-pair target **1′** ^{32}P-labeled at the 5′-end. Lane 1: Sequencing products (G + A) from **1′** in formic acid; lane 2: **1**:**1′** duplex (4 μM with **1′** in 2-fold excess) incubated in the dark at 37 °C, pH 7.8 (50 mM Tris buffer) for 30 min in the presence of NADH (5 mM), flavin reductase (Fre; 1 μM), $FeSO_4$ (40 μM); lane 3: as in lane 2 but without NADH and Fre; lane 4: as in lane 2 but without any iron; lane 5: as in lane 2 with the addition of SOD (4 μM) and catalase (0.5 μM); lane 6: as in lane 2 but with **1′** (8 μM) and riboflavin (4 μM) instead of the **1**:**1′** duplex; see text for details. The orientation of the complementary chain of the PNA–flavin **1** is also shown.

reaction promoted by the Fre/**1** combination. Indeed, cleavage occurred mainly at the duplex junction where the flavin is located. The major product resulted from the oxidation of the closest guanine residue (G12) within the PNA binding site and a minor one was observed at the closest guanine (G8) of the GG sequence adjacent to the duplex-simplex junction (lane 2). This result is consistent with a flavin-dependent production of reactive species and a short diffusion of the latter. The cleavage was both enzyme- and iron-dependent (lanes 3 and 4, respectively) and was inhibited by addition of superoxide dismutase (SOD) and catalase (lane 5) or by exclusion of oxygen. This result suggests that the reaction proceeds by: 1) reduction of the isoalloxazine ring system of

the **1**:**1**′ complex by NADH, catalyzed by Fre; 2) reduction of O_2 by the reduced flavin, generating H_2O_2 in close proximity of the target, 3) oxidation of adjacent reactive bases by OH· radicals (Fenton reaction). The cleavage yield was iron-concentration dependent but cleavage could be observed with no addition of iron as a result of the presence of iron impurities in the buffer. Accordingly, no cleavage could be observed after treating the buffer with chelating resins (Chelex) to remove these impurities (lane 4). When riboflavin was used in place of **1**, the selectivity was lost and almost all bases of the target were oxidized, with guanines (in particular GG doublets) being, as expected, the most reactive sites (lane 6). Furthermore, the efficiency of the reaction was significant with a 20% yield based on the target obtained after only 30 min reaction.

In conclusion, we report herein the first PNA-based artificial agent that can be enzyme activated for nucleic acid oxidation. The PNA–flavin **1** displays two interesting properties: 1) in complex with a complementary strand, it is recognized as a substrate by a flavin reductase; 2) under enzyme-activity conditions, specific and efficient oxidation of the complementary strand at reactive guanines adjacent to the flavin moiety occurs. Thus **1** combines DNA damaging activity, arising from its flavin moiety, and selectivity, arising from its PNA moiety.

Future experiments will aim at finding whether such PNA–flavin compounds might function in vivo by recruiting cellular flavin reductases to direct their action against a target sequence. Flavin reductases are ubiquitous enzymes found in all living organisms and have been shown to promote intracellular Fenton reactions and DNA damaging in vivo.[6] Thus the flavin- and iron-dependent DNA damage reaction studied herein occurs in vivo and the question is whether we can make it sequence-selective with PNA-flavins as shown herein in vitro. A limitation might be the very poor cellular uptake of PNAs in general and thus specific transfection strategies might be required.[12] On the other hand, since riboflavin conjugation has been shown to facilitate protein entry into human cells in culture[13] and PNA conjugation to lipophilic groups results in improved cell uptake,[12] conjugates such as **1** could have better cell-penetrating properties than the corresponding flavin-free PNA. This is under investigation.

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[1] P. B. Arimondo, A. S. Boutorine, J.-C. François, *Recent Res. Dev. Bioconjugate Chem.* **2002**, *1*, 29–53; A. S. Boutorine, P. B. Arimondo, *Nucleic Acids Mol. Biol.* **2004**, *18*, 243–268.
[2] M. Matteucci, K.-Y. Lin, T. Huang, R. Wagner, D. Sternbach, M. Mehrotra, J. M. Besterman, *J. Am. Chem. Soc.* **1997**, *119*, 6939–6940; P. B. Arimondo, G. S. Laco, C. J. Thomas, L. Halby, D. Pez, P. Schmitt, A. Boutorine, T. Garestier, Y. Pommier, S. M. Hecht, J.-S. Sun, C. Bailly, *Biochemistry* **2005**, *44*, 4171–4180.
[3] P. Wittung, S. K. Kim, O. Buchardt, P. Nielsen, B. Nordèn, *Nucleic Acids Res.* **1994**, *22*, 5371–5377.
[4] P. Gaudu, D. Touati, V. Nivière, M. Fontecave, *J. Biol. Chem.* **1994**, *269*, 8182–8188.
[5] M. Fontecave, J. Covès, J.-L. Pierre, *Biometals* **1994**, *7*, 3–8.
[6] A. N. Woodmansee, J. A. Imlay, *J. Biol. Chem.* **2002**, *277*, 34055–34066.
[7] H. Ikeda, K. Yoshida, M. Ozeki, I. Saito, *Tetrahedron Lett.* **2001**, *42*, 2529–2531.
[8] M. K. Cichon, C. H. Haas, F. Grolle, A. Mees, T. Carell, *J. Am. Chem. Soc.* **2002**, *124*, 13984–13985.
[9] F. Fieschi, V. Nivière, C. Frier, J.-L. Décout, M. Fontecave, *J. Biol. Chem.* **1995**, *270*, 30392–30400.
[10] M. Ingelman, S. Ramaswamy, V. Nivière, M. Fontecave, H. Eklund, *Biochemistry* **1999**, *38*, 7040–7049.
[11] P. Simon, C. Dueymes, M. Fontecave, J.-L. Décout, *Angew. Chem.* **2005**, *117*, 2824–2827; *Angew. Chem. Int. Ed.* **2005**, *44*, 2764–2767.
[12] U. Koppelhus, P. E. Nielsen, *Adv. Drug Delivery Rev.* **2003**, *55*, 267–280.
[13] S. R. Holladay, Z.-F. Yang, M. D. Kennedy, C. P. Leamon, R. J. Lee, M. Jayamani, T. Mason, P. S. Low, *Biochim. Biophys. Acta* **1999**, *1426*, 195–204.

