Specific Binding of the DNA Repair Enzyme AlkA to a Pyrrolidine-Based Inhibitor

Orlando D. Schärer, Jean-Yves Ortholand, A. Ganesan, Khosro Ezaz-Nikpay, and Gregory L. Verdine*

Department of Chemistry, Harvard University Cambridge, Massachusetts 02138

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In the cell, DNA is continuously subjected to damage resulting from spontaneous hydrolysis, oxidation, deamination, alkylation, and errors in replication.[1] To counter the toxic and mutagenic lesions thus produced, DNA repair enzymes are expressed in all known organisms. Whereas DNA repair was formerly viewed as an independent housekeeping function of cells, recent evidence has linked DNA repair to other essential biological processes, including progression through the cell cycle[2] and gene transcription.[3] Moreover, it is now firmly established that defects in DNA repair contribute directly to carcinogenesis.[4] Despite widespread interest in the molecular basis for recognition and repair of DNA, no high-resolution structure of a DNA repair enzyme bound specifically to its target DNA has been solved.[5] In part, this situation arises from the necessarily fleeting nature of enzyme–substrate complexes, which makes them difficult to observe. To overcome this problem, we have initiated an effort toward the design and synthesis of molecules that bind DNA repair proteins to form stable, long-lived complexes suitable for structural analysis. Here we report potent inhibition of the *Escherichia coli* 3-methyladenine DNA glycosylase II (AlkA) by a pyrrolidine-containing oligonucleotide, **1**.

AlkA catalyzes the removal of 3-methyladenine and 7-methylguanine, as well as several other minor DNA lesions, through glycosidic bond hydrolysis (Figure 1).[6] An especially interesting structural problem is posed by the fact that this 31 kDa protein can recognize such a diversity of substrates, irrespective of whether the modified site is in the major or minor groove.[7] Although the reaction mechanism employed by AlkA is not known in detail,[8] evidence in related systems suggests that it proceeds through one or more transition states in which substantial positive charge is accumulated at O-1′ and C-1′ (Figure 2).[9,10]

Naturally occurring compounds bearing a positively charged nitrogen in the place of the endocyclic oxygen are known to exhibit potent inhibition of glycosyl transfer enzymes, presumably by mimicking the developing positive charge on the ring during the normal catalytic process (Figure 2).[9] With regard to the present study, a key finding was the inhibition of nucleoside hydrolase by the substituted pyrrolidine 1,4-dideoxy-1,4-iminoribitol.[10] To test the application of this approach toward the inhibition of AlkA, we synthesized a phosphoramidite derivative that allows the incorporation of a pyrrolidine residue into DNA site specifically and examined the interaction of **1**-containing oligonucleotides with AlkA.

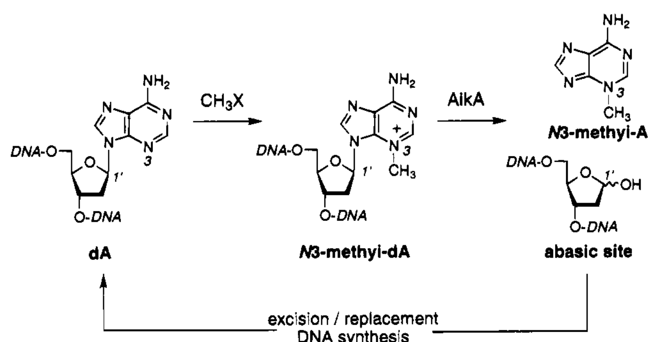

Figure 1. Production and repair of N3-methyladenine residues in DNA. Alkylation of an adenine residue by an exogenous or endogenous methylating agent (CH_3X) generates N3-methyl-dA, which undergoes glycosidic bond cleavage, catalyzed by AlkA. The abasic site thus generated is excised by additional enzymes in the overall repair pathway.

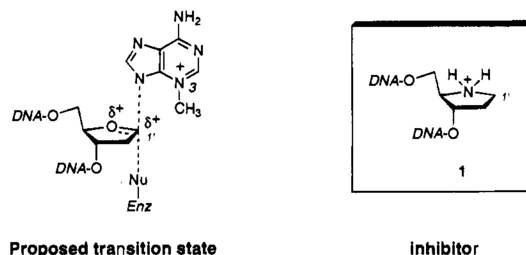

Figure 2. Analogy between the proposed transition state for enzyme-mediated glycosidic bond cleavage and inhibitor **1**.

The core structure of **1**, the natural product (2*R*,3*S*)-2-hydroxymethyl-3-hydroxypyrrolidine, has been synthesized previously.[11] Our synthesis of **1**[12] (Scheme 1) begins with the known homoallylic alcohol **2**,[13] which contains both stereocenters of the final product in the correct configuration. Deprotection of the acetal functionality of **2** afforded diol **3**. Treatment with 4 equiv of TBDMS-triflate followed by 1.0 equiv of *tert*-butlylammonium fluoride yielded the protected diol, with concomitant cleavage of the *t*-Boc group.[14] The free amine was reprotected as the Fmoc carbamate,[15] which upon ozonolysis and reduction gave alcohol **5**. Mesylation of the alcohol followed by thermal cyclization resulted in formation of the pyrrolidine ring, and cleavage of the silyl groups afforded the Fmoc-protected pyrrolidine **6**. Tritylation of the primary

* To whom correspondence should be addressed.

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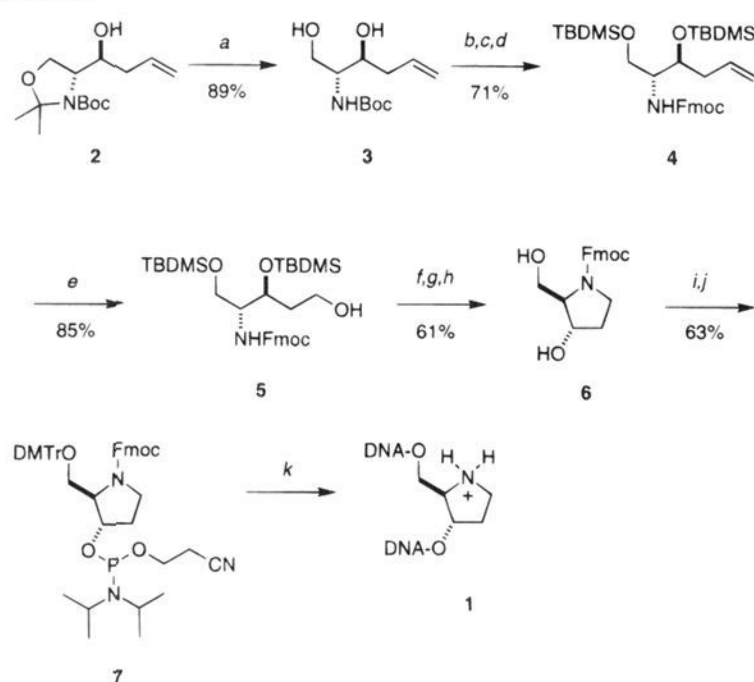
Scheme 1[a]



[a] Reagents and conditions: (a) catalytic TsOH, MeOH, 89%; (b) TBMDSOTf, 2,6-lutidine, CH_2Cl_2; (c) 1.0 equiv of TBAF, THF, 87% (2 steps); (d) FmocCl, Et_3N, CH_2Cl_2, 81%; (e) O_3, MeOH, −78 °C; Me_2S, then $NaBH_4$, −78 °C → room temperature, 85%; (f) MsCl, Et_3N, CH_2Cl_2, 83%; (g) iPr_2NEt, ClC_2H_4Cl, reflux, 74%; (h) 1% HCl, EtOH, 98%; (i) DMTrCl, DMAP, Et_3N, CH_2Cl_2, 74%; (j) $iPr_2NP(Cl)OC_2H_4CN$, iPr_2NEt, CH_2Cl_2, 85%; (k) solid phase DNA synthesis.

alcohol and phosphitylation using standard methodology yielded the phosphoramidite **7**.[16]

To study the interaction of the pyrrolidine-containing DNA with AlkA, we employed **7** in the solid phase synthesis of a 25-mer oligunucleotide containing a central pyrrolidine residue (5′-GGA TAG TGT CCA **1** GTT ACT CGA AGC-3′). Following deprotection and purification by standard methods,[16] the 25-mer was subjected to nucleoside composition analysis, which revealed the presence of the desired pyrrolidine residue, together with a small amount (~10%) of a byproduct.[17] The **1**-containing oligonucleotide was 5′-^{32}P-end labeled and annealed to a complementary 25-mer containing a thymine residue opposite the pyrrolidine (5′-GCT TCG AGT AAC **T** TGG ACA CTA TCC-3′).

The binding of AlkA to this singly modified duplex 25-mer was analyzed by the electrophoretic mobility shift assay (EMSA),[18] which detects the difference in size between protein-bound and free DNA. Titration of the **1**-containing duplex with increasing concentrations of AlkA resulted in the appearance of a band with the retarded mobility characteristic of a protein−DNA complex. This band was resistant to competition by a 100-fold excess of an unmodified duplex 25-mer but was completely abolished in the presence of a 100-fold excess of unlabeled **1**-containing duplex 25-mer. These results demonstrate that the protein specifically recognizes the presence of the pyrrolidine moiety in DNA. Further EMSA experiments under K_d conditions ([DNA] < K_d) revealed that AlkA binds the **1**-containing duplex with a K_d of 16 ± 3 pM.[19] As a design test, we analyzed in parallel the binding of AlkA to a 25-mer containing a tetrahydrofuran analog[20] in the place of the pyrrolidine. In contrast to the exceedingly tight binding of AlkA to the charged pyrrolidine, the uncharged tetrahydrofuran exhibited little or no specific binding to AlkA (Figure 3); we

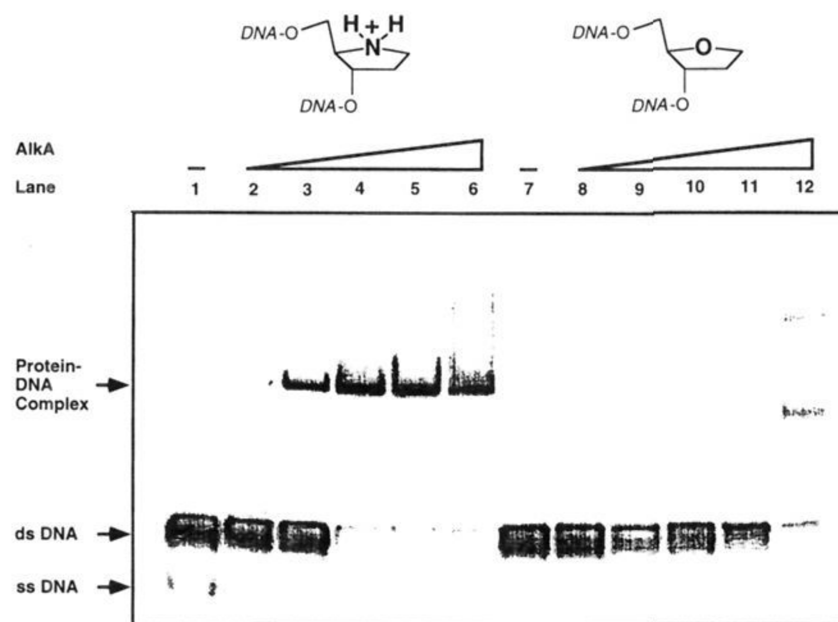


Figure 3. EMSA assay to detect binding of pyrrolidine (**1**)-containing DNA (lanes 1−6) and tetrahydrofuran-containing DNA (lanes 7−12) to various concentrations of AlkA. Oligonucleotide concentration, 0.01 nM. AlkA concentrations: lanes 1 and 7, 0 nM; lanes 2 and 8, 0.02 nM; lanes 3 and 9, 0.2 nM; lanes 4 and 10, 2 nM; lanes 5 and 11, 20 nM; lanes 6 and 12, 200 nM.

estimate the difference in K_d values between these two to be at least 10 000-fold (5.6 kcal/mol). Thus, AlkA exhibits exquisite selectivity for the presence of a basic nitrogen in the inhibitor. To test the ability of **1**-containing DNA to inhibit glycosidic bond cleavage of normal substrate by AlkA, we incubated the enzyme with [^{3}H]methylated calf thymus DNA in the presence of various amounts of **1**-containing DNA.[6a] When present in slight molar excess over AlkA, the **1**-containing duplex inhibited the release of [^{3}H]methylated bases from DNA by >90% (supplementary material). Addition of the same amount of unmodified DNA had no effect on the enzyme-catalyzed reaction. Thus, **1**-containing DNA clearly functions as an inhibitor of AlkA.

Here we have demonstrated specific binding of a DNA glycosylase enzyme to a designed pyrrolidine-containing inhibitor. This binding interaction is unusually strong, exceeding that observed even for the majority of sequence-specific protein−DNA complexes. These studies open the way for high-resolution structural analysis of AlkA bound specifically to DNA, which should provide much-needed insight into the molecular basis for substrate recognition and catalysis.[21]

In preliminary studies, we have found that **1**-containing DNA binds a number of glycosylases other than AlkA, including those derived from humans.[22] Future studies will aim to build in specificity for a particular glycosylase through appropriate attachment of a base moiety to the pyrrolidine framework.

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Supplementary Material Available: Synthetic procedures and ^{1}H-NMR, ^{13}C-NMR, IR, and HRMS data for all new compounds; procedures for enzyme purification, activity and EMSA assays; representative example of assays (14 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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