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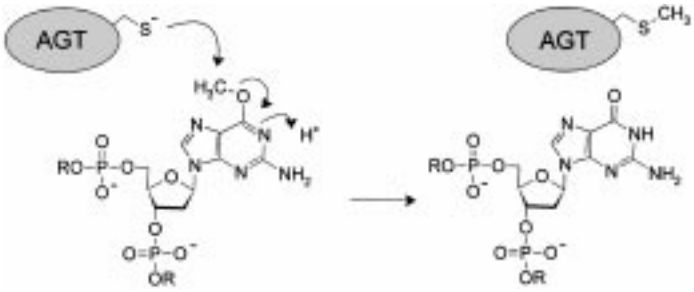

Scheme 1. *Schematic representation of the DNA repair reaction catalyzed by AGT (see text for details).*

Synthesis and Applications of Chemical Probes for Human *O*^{6}-Alkylguanine-DNA Alkyltransferase

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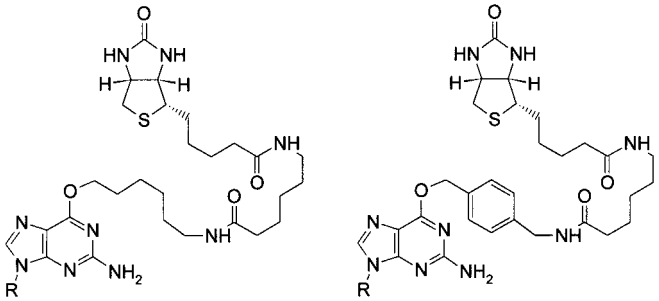
KEYWORDS:

O^6-alkylguanine-DNA alkyltransferase · DNA repair · nucleobases · phage display · transferases

The design and synthesis of chemical probes to study and to elucidate complex biological problems is becoming an increasingly important field in chemistry. We are interested in the repair of *O*^{6}-alkylated guanines in DNA, a DNA lesion that results from alkylation by *S*-adenosylmethionine or exogenous toxins and which has been shown to be highly mutagenic and carcinogenic.[1] The DNA repair protein *O*^{6}-alkylguanine-DNA alkyltransferase (AGT; EC 2.1.1.63) reverses this alkylation by transferring the alkyl group to a reactive cysteine residue in the protein, leading to repaired DNA and an irreversibly alkylated protein (Scheme 1).[2] The expression level of human AGT (hAGT) in tumor cells is also crucial for their sensitivity to chemotherapeutic agents that alkylate DNA, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide.[2] Consequently, hAGT has become a target in cancer chemotherapy, as its inhibition would increase the efficiency of currently used DNA-alkylating drugs.[3] A simple and reliable assay to measure the activity of hAGT in cell extracts would be of great importance for research on the role of hAGT in the chemotherapy of tumors, as currently used assays rely on radioactively labeled substrates and a subsequent HPLC separation. We describe here the synthesis of oligonucleotides containing *O*^{6}-alkylated guanine derivatives of the type **1** and **2** that serve as affinity labels for hAGT and their use in a highly specific assay for this alkyltransferase. In addition, we introduce a novel system for the directed molecular evolution of hAGT,[4] which relies on the display of active hAGT on phage λ and on oligonucleotides containing *O*^{6}-alkylated guanine derivatives of the type **1** and **2**.



1 **2**

To incorporate nucleosides of the type **1** and **2** into oligonucleotides, the corresponding phosphoramidites **3** and **4** were synthesized starting from the readily available nucleoside **5** (Figure 1 A, B).[5] The phosphoramidites **3** and **4** were then incorporated into oligonucleotides with coupling yields of about 93 % (Figure 1). After synthesis and complete deprotection of the oligonucleotides, the amino group of the *O*^{6}-alkylguanine moiety was biotinylated with *N*-(+)-biotinyl-6-aminocaproic acid *N*-succinimidyl ester.[5] The incorporation of **1** and **2**, their complete deprotection and biotinylation as well as the base composition of **7** and **8** were verified by digesting the oligonucleotides with snake venom phosphodiesterase and calf intestinal alkaline phosphatase followed by HPLC analysis.[6] The nucleosides **1** and **2** were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI) of the corresponding HPLC peak eluates.[5]

To demonstrate the use of the *O*^{6}-alkylated oligonucleotides **7** and **8** (Figure 1 C) as sensitive probes for active hAGT, the double-stranded oligonucleotides were incubated with recombinant hAGT[5, 7] and the reaction mixture transferred into streptavidin-coated microtiter plate wells, leading to immobilization of biotinylated hAGT. After washing of the wells, immobilized hAGT was detected by an enzyme-linked immunosorbent assay (ELISA) based on an anti-hAGT antibody and an

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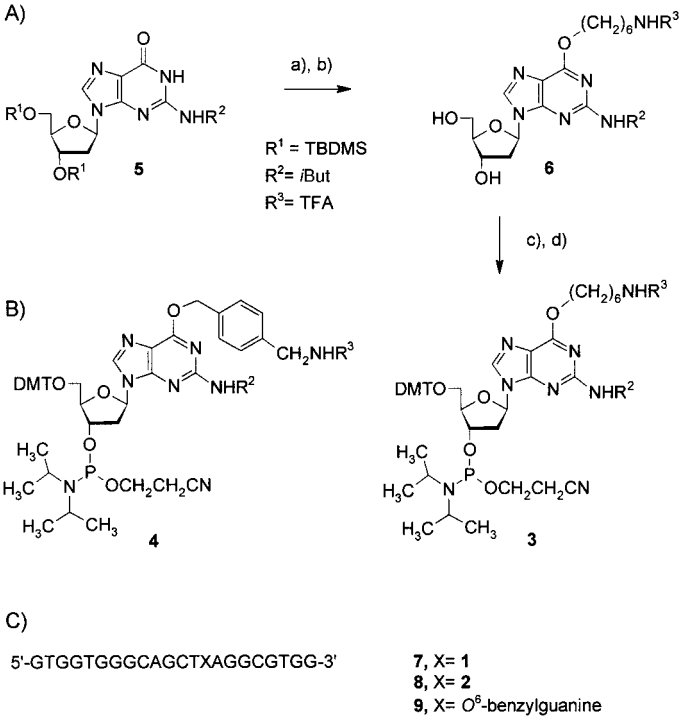

Figure 1. *A) Synthesis of the phosphoramidite **3**: a) 1.5 equiv diethyl azodicarboxylate, 1.5 equiv triphenylphosphine, 1.5 equiv 6-(trifluoroacetylamino)-1-hexanol, THF, 25 °C, 69%; b) pyridine/HF, 25 °C, 74%; c) 1.21 equiv 4,4′-dimethoxytriphenylmethyl chloride, pyridine, 25 °C, 96%; d) 0.5 equiv diisopropylammonium tetrazole, 1.2 equiv 2-cyanoethyl-N,N,N′,N′-tetraisopropylphosphordiamidite, CH_2Cl_2, 25 °C, 85%. B) Structure of phosphoramidite **4**, which was synthesized by using the same reaction sequence as for **3**. C) Sequence of the oligonucleotides **7** – **9**. Assays and selections were performed with the corresponding double-stranded oligonucleotides, in which C was used as the complementary base to X. DMT = 4,4′-Dimethoxytriphenylmethyl; iBut = isobutyryl; TBDMS = tert-butyldimethylsilyl; TFA = trifluoroacetyl.*

anti-mouse antibody – peroxidase conjugate as a secondary antibody (Figure 2).[5] Varying the hAGT concentration at a fixed concentration of the double-stranded oligonucleotide showed that the ELISA signal depends linearly on the concentration of hAGT. To compare the reactivity of the biotinylated substrates to

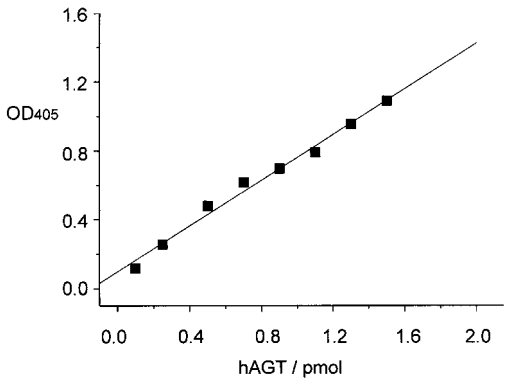

Figure 2. *ELISA signal (optical density at 405 nm, OD_{405}) after incubation of hAGT (0.1 – 1.5 pmol) with double-stranded **7** (1 pmol) and subsequent capture in streptavidin-coated microtiter plates.[7] For the measurement of the ELISA signal, wells were incubated with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; 1 mg mL^{-1}) and H_2O_2 (3 mM) at pH 4 for 25 min and the OD_{405} was measured.*

that of known substrates of hAGT, we performed competition experiments (Figure 3) in which double-stranded **7** and double-stranded **8** were mixed with varying concentrations of a double-

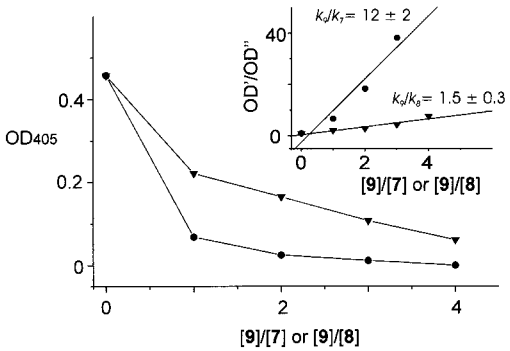

Figure 3. *ELISA signal (OD_{405}) as a function of the ratios [**9**]/[**7**] (●) and [**9**]/[**8**] (▼). Inset: Ratio of OD′/OD″ as a function of the ratios [**9**]/[**7**] (●) and [**9**]/[**8**] (▼), where OD′ is OD_{405} in the absence of **9** and OD″ is OD_{405} at a given ratio of either [**9**]/[**7**] (●) or [**9**]/[**8**] (▼).*

stranded oligonucleotide **9** containing O^6-benzyl-2′-deoxyguanosine (see Figure 1 C). In the competition experiments, reaction of hAGT with **9** instead of **7** or **8** would lead to a decrease in the ELISA signal. O^6-Benzyl-2′-deoxyguanosine incorporated into oligonucleotides has been shown to be the preferred substrate of hAGT.[8] Assuming that the DNA repair of double-stranded oligonucleotides **7** – **9** under these conditions can be described by the single rate constants k_7, k_8, or k_9,[9] the ratio of the ELISA signals (measured as optical density, OD) in the absence of **9**, that is OD′, and at various ratios of [**9**]/[**7**] and [**9**]/[**8**], that is, OD″, can be expressed by Equations (1) and (2) (see Figure 3).

$$OD'/OD'' = 1 + k_9[\mathbf{9}]/k_7[\mathbf{7}] \quad (1)$$

$$OD'/OD'' = 1 + k_9[\mathbf{9}]/k_8[\mathbf{8}] \quad (2)$$

The obtained ratio for k_9/k_8 of 1.5 indicates that substitution of the O^6-benzyl group in the 4-position does not significantly affect the activity of **8** as a substrate of hAGT compared to **9**. A comparison of the values for k_9/k_8 (1.5) and k_9/k_7 (12) shows that **8** is preferentially repaired over **7**.

These experiments demonstrate the utility of the synthesized affinity labels to quantitatively detect active hAGT with high sensitivity, as about 100 fmol of the alkyltransferase can be reliably detected. We therefore expect that these probes will become important tools in those areas of research where the detection and quantification of active hAGT is required, an example being the research on the role of hAGT in tumor resistance and sensitivity to DNA-alkylating drugs.[10] The competition experiments also show how these affinity labels can be used to study questions regarding substrate and sequence specificity of hAGT.[9]

Another envisioned application of the synthesized probes was their use in the directed molecular evolution of hAGT by using phage λ display (Figure 4), allowing for the use of evolutionary techniques to address mechanistic questions as well as to

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Table 1. *In vitro selections of λhAGT out of mixtures of λhAGT and λfooDc at different ratios of λhAGT/λfooDc.*[a]

λhAGT input [pfu]	λhAGT output [pfu]	λfooDc input [pfu]	λfooDc output [pfu]	Enrichment factor
2×10^6	171	1.2×10^{10}	38	2.7×10^4
2×10^6	91	1.2×10^9	4	1.3×10^4
1.3×10^5	11	1.2×10^9	4	2.5×10^4
9×10^3	4	1.2×10^9	8	6.6×10^4

[a] 10 pmol of double-stranded **7** were used in the selections. The phage titer was measured by using *E. coli* XL-1Blue as a host strain and the enrichment factor was determined by blue/white screening.[13, 14] pfu = plaque-forming units.

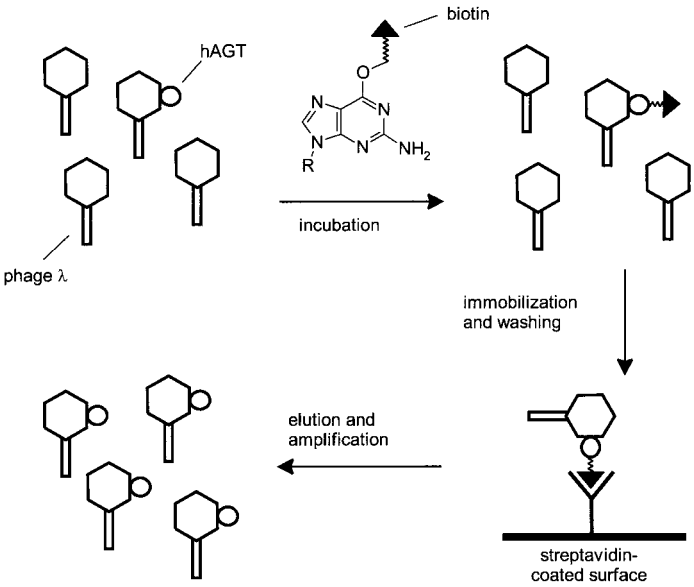

Figure 4. *General scheme for the in vitro selections of phage λ displaying active hAGT out of mixtures of phage λ displaying no or inactive hAGT.*

generate novel mutants for practical applications.[4, 11] A more traditional in vivo selection scheme for the directed molecular evolution of hAGT, which relies on the growth of bacteria in the presence of high levels of DNA-alkylating agents to select for bacteria expressing active hAGT, has already been introduced.[2, 12] The display of active hAGT on the surface of phage λ and the use of these phages in in vitro selections would complement this approach, as it allows for a much higher variability and control of the selection conditions. Phage λ was chosen over the much more utilized filamentous phages, as its lytic life cycle should facilitate the display of cytosolic proteins with unpaired cysteine residues such as hAGT.[13] For the display of hAGT on phage λ, we used the vector λfooDc.[13] λfooDc allows for the fusion of foreign proteins to the C terminus of the major head protein D (gpD). In this vector, the gene coding for gpD is followed by an amber stop codon, a recognition sequence for the protease collagenase, a long linker consisting of proline and threonine repeats and the *lacZ′* gene. The collagenase sequence allows for the cleavage of the displayed hAGT from the phage surface and thus the elution of phage λ in in vitro selections (Figure 4). The hAGT gene was cloned into the linker region of λfooDc and the resulting vector λhAGT packaged in vitro into λ particles.[5] *Escherichia coli* strain Q358, which possesses a strong suppressor activity for the amber stop codon,[13] was used for amplification. The expression of hAGT as a gpD fusion protein on the surface of the phage was confirmed by Western blotting.[5] In order to demonstrate that hAGT is indeed actively displayed on the surface of phage λ as well as to demonstrate the feasibility of our selection system, we performed model in vitro selections by mixing predetermined ratios of λhAGT and λfooDc phages. The phage mixtures were incubated with the double-stranded oligonucleotide **7**, biotinylated phages were captured on streptavidin-coated microtiter plates and, after vigorous washing of the wells, eluted by collagenase cleavage (Figure 4, Table 1).[5] The observed enrichment factors were above 10^4 per round of selection. This clearly demonstrates the display of active hAGT on the surface of phage λ and the feasibility of our selection scheme for the directed molecular evolution of hAGT.

This work was supported by the Swiss National Science Foundation (grants 3100-055666.98/1 and 21-56420.99) and by the Deutsche Forschungsgemeinschaft. The authors would like to thank P. G. Schultz and G. von Kiedrowski for helpful discussions and support, M. Wüstefeld for help with the synthesis of the oligonucleotides, I. Maruyama for the vector λfooDc and the E. coli *strain Q358, and K. Bender for the plasmid pQE9-hAGT.*

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