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Synthesis of oligonucleotides carrying fluorescently labelled O⁶-alkylguanine for measuring hAGT activity

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ABSTRACT. O⁶-alkylguanine-DNA-alkyltransferase (hAGT) activity provides resistance to cancer chemotherapeutic agents and its inhibition enhances chemotherapy. We herein present the development of a novel fluorescence assay for the detection of hAGT activity. We designed a dsDNA sequence containing a fluorophore-quencher pair, where the fluorophore was attached to an O⁶-benzylguanine. This precursor was synthesized using the Mitsunobu reaction to introduce the benzyl group. The alkyl-fluorophore group is transferred to the active site during the dealkylation, producing an increase in fluorescence which is correlated to hAGT activity. This assay can be used for the evaluation of potential inhibitors of hAGT in a straightforward manner.

Keywords: Fluorescence, DNA repair, Oligonucleotide synthesis, Mitsunobu, O⁶-alkylguanine-DNA alkyltransferase

Alkylating agents such as carmustine or temozolamide are chemotherapeutic anticancer drugs that produce their cytotoxic effect by generating adducts at multiple sites in DNA.¹ The most relevant adduct in terms of mutagenesis and carcinogenesis is the alkylation of the O⁶ position of guanines.²⁻⁹ In particular, the alkylating agent 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) initially attacks at the O⁶ guanine position, causing its cross-link with the opposite cytosine, blocking DNA replication and producing G₂/M arrest.^{10, 11} In addition to the well-known side effects and limitations of chemotherapeutic agents, these substances also show acquired tumour resistance problems. The DNA-repair protein human O⁶-alkylguanine DNA alkyltransferase (hAGT or MGMT) is responsible for removing alkyl adducts from the O⁶ position of guanines, thereby blocking the cytotoxic effects of the alkylating agents and making a crucial contribution to the resistance mechanism.¹²⁻¹⁴ It is well established that tumoral cells show greater expression of this protein, which explains their low sensitivity to chemotherapeutic

drugs in a large number of cancers.¹⁵⁻¹⁷ On the contrary, the promoter methylation of hAGT has been associated with patients' longer survival.¹⁸⁻²² Therefore, pharmacological inhibition of hAGT has the potential to enhance the cytotoxicity of a diverse range of anticancer agents.²³

hAGT is a DNA-binding protein that contains a highly conserved internal cysteine, which acts as the acceptor site for alkyl groups. It behaves like a suicidal non-enzyme, inactivating itself since the S-alkylcysteine formed is not regenerated.^{24, 25} For this reason, intense research effort has been devoted to the identification of small molecules capable of inhibiting hAGT activity and significantly enhancing the cytotoxic effect of BCNU in prostate, breast, colon and lung tumour cells.²³

Given the potential relevance of hAGT as a prognostic marker of cancer and as a therapeutic target, several methods are available to characterize its activity. Moreover, they are also able to evaluate the capacity of small molecules for inhibiting hAGT. Most of these methods involve radioactivity assays, while others are based on multiple-step enzymatic reactions²⁶⁻³⁰ or *in vivo* hAGT labeling.³¹⁻³⁴ However, the first methods require the use of rigorous safety procedures and the others are discontinuous and time-consuming due to the necessity of multiple steps. In addition, validation of inhibitors for hAGT activity *in vivo* needs to consider the cellular uptake of these compounds as well as their toxicity *per se*. Recently, two methods based on the conformational change of an intramolecular G-quadruplex were developed by our group.^{35, 36}

In this paper we describe the development of a one-step FRET assay which improves the efficiency of our previous methods because it increases the repair rate of hAGT by using double-stranded DNA, the natural substrate of the protein. In addition, the detection of a fluorescence increase that is proportional to the repair rate of hAGT represents a low-cost, straightforward and rapid method.

For this purpose, we designed a double-stranded DNA sequence containing a fluorophore-quencher pair (Scheme 1). The fluorophore was post-synthetically and covalently attached to a modified O⁶-benzylguanine (^FdG) and the quencher was introduced in a very close position of the complementary strand of the duplex (^QdU-complementary or ^QdU-mismatch), quenching the fluorescence. The fluorophore was transferred together with the benzyl group to hAGT's active site when the protein repaired the DNA, restoring the guanine. The removal of the fluorophore brought it apart from the quencher, producing a significant increase in fluorescence which allowed to measure the repair reaction of hAGT.

Scheme 1. **A.** Chemical structure of the O⁶-benzylguanine with the fluorophore covalently attached to the alkyl group. **B.** Chemical structure of the quencher group (Dabcyl), covalently attached to a 2'-deoxyuridine. **C.** Schematic representation of the fluorescence assay. 1) Annealing of the two strands containing a fluorophore and a quencher group, with the correspondent extinction of fluorescence. 2) hAGT repair activity over the benzylguanine, dragging the fluorescein group and significantly increasing fluorescence.

The ^{Bz}dG oligonucleotide was prepared using the appropriate modified 2'-deoxyguanosine phosphoramidite (**7**) in the automated DNA synthesizer using standard protocols³⁷.

Derivative **7** was prepared from 2'-deoxyguanosine and the protected N-(4-(hydroxymethyl)benzyl)-trifluoroacetamide (**1**) (Scheme 2). Its incorporation at the O⁶ position of the guanine was performed through a Mitsunobu reaction. The guanine amino function was protected with a dimethylformamidine protecting group. Finally, the protection and functionalization of the two hydroxyl groups were carried out under standard conditions.

The O⁶-benzyl-2'-deoxyguanosine-containing oligonucleotide and its complementary strands with a quencher group (dabcyl, Q) either at the complementary position or at one nucleotide shift were synthesized, purified by HPLC and characterized by MALDI-TOF (see Supplementary Data). Subsequently, the fluorescein label (F) was attached to the O⁶-benzyl-2'-deoxyguanosine amino group by the fluorescein isothiocyanate (FITC) reaction.³⁸ The DNA duplexes, one complementary and the other containing a mismatch due to the positioning of the dU-quencher phosphoramidite pairing with the modified ^FdG, were prepared from the fluorophore-quencher pair of oligonucleotides under proper annealing conditions (Table 1). We explored to different positions of the quencher group in the complementary sequences to ensure the maximal quenching of fluorescence and to minimize steric effects and complementary discrepancies. In the case of the first duplex the placement of the quencher in the complementary base of the modified guanine provokes a mismatch in the sequence (G:dU) instead of a (G:C) pair. The second duplex contained the quencher group at one nucleotide shift with respect to the benzyl-2'-deoxyguanosine in the opposite sequence to maintain

complementarity, expecting that the fluorescence extinction would remain efficient and intending to minimize the steric impediments for the repair action of hAGT.

Scheme 2. A) Chemical synthesis of the O⁶-benzyl-2'-deoxyguanosine phosphoramidite. a) TEA, Et₃NFA in DCM, 0°C to r.t. o/n b) Ac₂O, DMAP, TEA in DMF, r.t. 4h; c) Compound 1, PPh₃, DEAD in Dioxane, r.t. o/n; d) MeOH:NH₃ (1:1), 55°C 4h; e) (CH₃O)₂CH-N(CH₃)₂ in DMF; f) DMT-Cl, DIEA in Pyr, r.t. o/n; g) DIEA, CNEOP(Cl)N(iPr)₂ in DCM, r.t.

Table 1. Sequences of the two different duplexes used in this work.

Name	Sequence
Oligo- ^F dG: Complementary- ^Q dU	5'-ATC TTC TC ^F G ATT CA-3' 3'-TAG AAG AGC ^Q dUAA GT-5'
Oligo- ^F dG:Mismatch- ^Q dU	5'-ATC TTC TC ^F G ATT CA-3' 3'-TAG AAC AG ^Q dU TAA GT-5'

First, to see the possible effect of these two bulky groups in the duplex formation, its thermal stability was studied at 260 and 485 nm and compared to each other (see Table S2 at Supplementary Data). In general, we observed destabilization of the duplex at 260 nm in the presence of the bulky substituent in the O⁶ position of the central guanine, compared to the controls. However, both duplexes are stable at 30°C. The duplex structure of both pairs were studied by CD spectra at 25°C confirming that the fluorescein-benzyl substitution did not affect

the overall secondary structure of the DNA (See Figure S1, SD). In order to be able to use these two duplexes in a fluorescence-quenching assay, we studied how the stability of the duplexes affects the emission of fluorescence by monitoring the emitted fluorescence at excitation wavelength of 485 nm (See Figure S2, SD). Significant differences in the melting temperature were observed due to the separation of the fluorophore and the quencher and a great increase in the fluorescent signal was detected. In the case of the mismatched sequence, this increase in fluorescence is produced at 26°C while in the fully complementary duplex, the quencher effect persisted until 28°C, allowing us to use it at the lower enzymatic temperature range (25°C) for hAGT activity.

Figure 1. HPLC profile of the repair reaction of the ^FdG double stranded sequence in the absence of the quencher group in the complementary strand. The HPLC analysis was performed at 37 °C.

Efficient reaction with hAGT requires the ^FdG modification to correctly accommodate into the active site. This brings the CH₂ which is attached at the O⁶ position in closer proximity to the thiol group of Cys145, making the reaction possible. To study the capacity of the fluorescein-benzyl group to enter the active site of hAGT without affecting the hAGT activity, the reaction was analyzed by HPLC. For this purpose, the full-length hAGT was over-expressed and purified as previously described.^{35, 39} Increasing concentrations of the protein were incubated with an excess of the double-stranded ^FdG sequence for 90 min at 30°C and analyzed using HPLC. The reaction was performed in substoichiometric conditions due to the fact that high amount of DNA was required to detect the peak corresponding to the repaired sequence. Figure 1 shows one of the HPLC profiles of the reaction's final products of hAGT at 37°C. The appearance of a peak with a shorter retention time corresponds to the repaired sequence, formed by the removal of the fluorescein-benzyl group. Even if the reaction was not quantitative due to

the lower amount of hAGT used in the experiment, the repair of the alkylguanine indicated that hAGT has the capacity to accommodate bulky groups, including a fluorophore, in the active site without upsetting its repair activity. This study was performed in the absence and presence of the quencher in the complementary strand with similar results.

We then tested the effectiveness of our proposed fluorescence method to evaluate the repair activity of hAGT. We performed the hAGT reaction using a 10 nM concentration of the fluorescently-dabcyl labelled duplex for 20 min, using different hAGT concentrations. We determined 10 nM to be the minimum amount of double-stranded DNA required to achieve a detectable and reliable difference in intensity compared to the background. As expected, the fluorophore-quencher pair gave low background fluorescence, because of the quenching effect produced by the proximity of these two groups in the base paired sequence. The presence of hAGT produced a remarkable increase in fluorescence intensity caused by the transfer of the fluorescein-benzyl group to its active site. The increase rate in fluorescence intensity correlated directly with the amount of hAGT in the reaction mixture (Figure 2). Moreover, the inactive mutant hAGT-C145S did not exhibit any decrease in fluorescence due to its inability to repair the alkylated DNA (see Supplementary data).

In summary, we have described a novel route for the synthesis of an O⁶-benzyl-2'-deoxyguanosine precursor which incorporated to an oligonucleotide sequence, can become a fluorescently labelled substrate for measuring hAGT repair activity. Annealing with a complementary quencher strand produces a duplex with low basal fluorescence. Using this labelled oligonucleotide, we have developed a new *in vitro* assay for the quantification of the DNA repair activity of hAGT and the identification of potential inhibitors as chemotherapy enhancers. The novelty of this method compared to existing ones²⁶⁻³³ is the single-step and real-time measurement of the reaction *in vitro*, representing a rapid and straightforward method that reduces cost, time and effort. In contrast with our previous developed methods to quantify hAGT activity,^{35, 36} this system uses a double-stranded oligonucleotide, which is the natural substrate for the hAGT activity, improving efficiency and reliability due to its easy quantification of the fluorescence increase upon activity. We believe that this new method will facilitate the search for new and more potent inhibitors which enhance the effect of chemotherapeutic drugs. However, the thermal stability of the oligonucleotides should be improved to ensure their stability at physiological temperature. Work in this direction is currently underway.

Figure 2. hAGT activity assay. A) Increase of fluorescence with respect to the background fluorescence of the oligonucleotide during 20 minutes. B) Total increase of fluorescence after 20 minutes of reaction with different concentrations of hAGT

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Supplementary data

Experimental data, NMR traces for all novel compounds, HPLC assays and enzymatic reactions can be found in the supplementary data.

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Supplementary Data

Synthesis of oligonucleotides carrying fluorescently labelled O⁶-alkylguanine for measuring hAGT activity.

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1. Material and methods

1.1 Abbreviations:

Ac: acetyl, Ac₂O: acetic anhydride, ACN: acetonitrile, AcOEt: ethyl acetate, anh: anhydrous, Ar: aromatic, BCNU: bis-chloroethylnitrosourea, Bz: benzyl, Bzl: benzoyl, CPG: controlled pore glass, Dabcyl: dimethylamino-azobenzoic acid, DCM: dichloromethane, DEAD: diethyl azodicarboxylate, DIEA: *N,N*-diisopropylethylamine, DMAP: *N,N*-dimethylaminopyridine, DMF: *N,N*-dimethylformamide, dmf: dimethylaminomethylidene, DMT: 4,4'-dimethoxytrityl, DMT-Cl: 4,4'-dimethoxytrityl chloride, DMSO: dimethylsulfoxide, DTT: dithiothreitol, EtTFA: ethyl trifluoroacetate, F: fluorescein, ^FG: fluorescein-benzylguanosine, FITC: Fluorescein isothiocyanate, FRET: fluorescence resonance energy transfer, hAGT: human O⁶-alkylguanine-DNA alkyltransferase, LCAA: long chain amino alkyl, MALDI: matrix-assisted laser desorption/ionization, MeOH: methanol, PPh₃: triphenylphosphine, Pyr: pyridine, ^QdU: (N-4'-carboxy-4-(dimethylamino)-azobenzene)-aminohexyl-3-acrylamido]-2'-deoxyuridine, RP-HPLC: reverse phase high pressure liquid chromatography, TCA: trichloroacetic acid, TEA: triethylamine, TEAA: triethylammonium acetate, THAP: trihydroxyacetophenone monohydrate, THF: tetrahydrofuran, TLC: thin-layer chromatography, TOF: time of flight, UV: ultraviolet.

1.2 Chemicals

Reagents for oligonucleotide synthesis including phosphoramidite monomers of dA^{Bzl}, dC^{Bzl}, dG^{dmf} and T, the 5'-deblocking solution (3% TCA in DCM), activator solution (0.4 M 1H-tetrazole in ACN), CAP A solution (Ac₂O /Pyr/THF), oxidizing solution (0.02 M iodine in THF/Pyr/water (7:2:1), supports and LCAA-CPG were purchased from Applied Biosystems (PEBiosystems Hispania S.A., Spain) and Link Technologies Ltd.(Scotland) and used as received. Sephadex G-25 (NAP-10) columns were purchased from GE Healthcare, USA. The 96-well Optical reaction plates were purchased to Nunc, USA. HiTrapTM FF column were purchased at GE Healthcare. FITC (isomer I) and the rest of chemicals were purchased from Aldrich, Sigma, or Fluka (Sigma-Aldrich Quimica S.A., Spain), and used without further purification. Anhydrous solvents and deuterated solvents (CDCl₃, CD₃OD and DMSO-*d*₆) were obtained from reputable sources and used as received. Thin-layer chromatography (TLC) was carried out on aluminium-backed Silica-Gel 60 F₂₅₄ plates. Column chromatography was performed using Silica Gel (60 Å, 230 x 400 mesh).

1.3. Instrumentation

Oligonucleotide sequences were synthesized on an Applied Biosystems 3400 DNA Synthesizer (Applied Biosystems, USA). RP-HPLC purifications were performed on a Waters chromatography system using Nucleosil semi-preparative 120 C₁₈ (250x4 mm) columns. Analytical RP-HPLC was performed using a XBridge OST C18 2.5 µm column and a Nucleosil Analytical column 120 C18 (250x4mm). Oligonucleotides were quantified by UV absorption at 260 nm with a Jasco V650 spectrophotometer. Mass spectra were recorded on a MALDI *Perseptive* Voyager DETM RP time-of-flight (TOF) spectrometer (Applied Biosystems, USA) equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/mL in ACN/ water 1:1) and ammonium citrate (50 mg/mL in water). NMR spectra were measured on Varian Mercury-400. Chemical shifts are

given in parts per million (ppm); *J* values are given in hertz (Hz). All spectra were internally referenced to the appropriate residual undeuterated solvent.

UV analyses were performed using a Jasco V-650 instrument equipped with a thermoregulated cell holder. Set temperature was controlled with an 89090A Agilent Peltier device and Hellma quartz cuvettes were used (1000 μ L volume). CD spectra were recorded on a JASCO spectropolarimeter J-810. Fluorometric measurements were performed on a JASCO FP6200 spectrofluorometer at 25°C and with a Multi-Detection Microplate Reader SpectraMax M5 (Molecular devices BioNova Scientific, Sunnyvale, USA).

2. Chemical synthesis of the O⁶-benzyl-2'-deoxiguanosine phosphoramidite precursor

2.1. Preparation of N-(4-(hydroxymethyl)benzyl)-trifluoroacetamide (1)

4-aminomethylbenzylalcohol (500 mg, 2.89 mmol) was dissolved in 3 ml DCM. TEA (3.0 eq, 1.169 ml, 8.4 mmol) was added dropwise and kept under argon atmosphere. Afterwards, 400 μ l (1.2 eq, 3.36 mmol) of EtTFA were added dropwise and the mixture was stirred in argon atmosphere at room temperature for 4 hr. The reaction was followed up by TLC (DCM:MeOH 5%). The reaction mixture dissolved in DCM and was washed with brine and the organic phase dried with MgSO₄. The organic phase was filtered and evaporated. The crude of the reaction was purified by chromatography (silica gel), eluted with a gradient of 0-5% MeOH in DCM. Yield (1, 500 mg, 76%, white solid). TLC R_f=0.36 (DCM:MeOH 95:5), MS (EI 70 eV) m/z calcd for C₁₀H₁₄F₃N₂O₂ (M⁺ + NH₄⁺) 251.1002 found 251.1002.

¹H-NMR (400 MHz, CDCl₃) δ [ppm] 4.52 (d, 2H *J*=5.8 Hz, Bz-CH₂NH), 4.70 (s, 2H, Bz-CH₂O), 6.5 (s, 1H, OH), 7.25 (d, 2H *J* = 8 Hz, Bz-H_{arom}), 7.37 (d, 2H *J* = 8 Hz, Bz-H_{arom}).

¹³C NMR (100 MHz, CDCl₃) δ [ppm] 43.64 (Bz-CH₂NH), 64.79 (Bz-CH₂O), 115.70 (q, *J* = 287.9 Hz, C_qF₃), 127.55 (2C_{arom}), 128.18 (2C_{arom}), 135.16 (C_{q-arom}), 141.0 (C_{q-arom}), 157.09 (q, *J* = 157.19 Hz, COCF₃).

¹⁹F NMR (376 MHz, CDCl₃) δ [ppm] -75.84 (COCF₃).

2.2. Preparation of 3',5'-O-diacetyl-2'-deoxyguanosine (2)

2'-Deoxyguanosine (1 g, 3.78 mmols, 1 eq.) was dried by coevaporation with anhydrous DMF and dissolved in 20 ml of anhydrous DMF. Then, 90 mg of DMAP (0.74 mmols, 0.2 eq.) were dissolved in 2 ml of anhydrous DMF and added dropwise. Next, 2.6 mL of TEA (18.5 mmols, 5.0 eq.) and 1.05 ml of Ac₂O (11.1 mmols, 3.0 eq.) were added and the mixture dissolved completely. The solution was left to react for 4 hours at r.t. Afterwards cold DCM was added to precipitate the desired compound. The mixture was vacuum-filtered and the desired compound dried. Yield (2, 1.1 g, 91.6%, white solid). MS (EI 70 eV) m/z calcd for C₁₄H₁₈N₅O₆ (M⁺ + H⁺) 352.12 found 352.12

¹H-NMR (400 MHz, MeOD) δ [ppm] 1.93 (s, 3H, CH₃O), 2.01 (s, 3H, CH₃O), 2.47 (m, 1 H, 2'), 2.91 (m, 1H, 2''), 4.2 (m, 1 H, 4'), 4.26 (m, 2H, 5', 5''), 5.34 (m, 1H, 3'), 6.15 (m, 1H, 1'), 7.76 (s, 1H, 8).

2.3. Preparation of O⁶-(N-4-((hydroxymethyl)benzyl)-trifluoroacetylmiide)-3',5'-O-diacetyl-2'-deoxyguanosine (3)

3',5'-O-diacetyl-deoxyguanosine (**2**, 620 mg, 1.79 mmol) was dried 3 times by evaporation of DMF. PPh₃ (1.42 g; 5.4 mmol, 3 eq) and the protected phenol (**1**, 500 mg, 2.15 mmol, 1.2 eq) was added and left under vacuum for 2 h. Then, the mixture was dissolved in anhydrous dioxane (10.0 ml) and maintained under an argon atmosphere. Finally, DEAD (860 µl; 5.4 mmol 3 eq) was added dropwise. The reaction mixture was stirred for 4 hours at room temperature and the progress of the reaction was monitored by TLC (DCM:MeOH 9:1). The orange reaction was concentrated under vacuum to obtain a viscous oil. The oil was purified by flash chromatography using a gradient of MeOH in DCM to yield a white solid (750 mg, 75%). TLC R_f=0.42 (DCM:MeOH 9:1), MS (EI 70 eV) m/z calcd for C₂₄H₂₆F₃N₆O₇ (M⁺ + H⁺) 567.18 found 567.181 [M⁺].

¹H-NMR (400 MHz, CDCl₃) δ [ppm] 2.08 (s, 3H, CH₃CO), 2.12 (s, 3H, CH₃CO), 2.52 (m, 1H, 2'), 2.98 (m, 1H, 2''), 4.33 (m, 2H, 5', 5''), 4.52 (m, 1H, 4'), 4.90 (s, 2H, Bz-CH₂NH), 5.43 (m, 1H, 3'), 5.55 (s, 2H, Bz-CH₂O), 6.28 (dd, 1H, J = 8 Hz, 1'), 7.27 (d, 2H, J = 8 Hz, Bz-H_{arom}), 7.49 (d, 2H, J = 8 Hz, Bz-H_{arom}), 7.74 (s, 1H, 8).

¹³C NMR (100 MHz, CDCl₃) δ [ppm] 20.76 (CH₃CO), 20.93(CH₃CO), 36.69 (C-2'), 43.60 (Bz-CH₂NH), 63.79 (C-5'), 67.51 (Bz-CH₂O), 74.61 (C-3'), 82.30 (C-1'), 84.44 (C-4'), 115.85 (q, J = 287 Hz, CF₃), 116.11 (C-5), 128.06 (2C_{arom}), 128.77 (2C_{arom}), 135.74 (C-8), 136.49 (C_{q-arom}), 137.56 (C_{q-arom}), 153.53 (C-4), 156.63-157.74 (q, J = 37 Hz COCF₃), 159.15 (C-2), 160.87 (C-6), 170.27 (CO), 170.61 (CO).

¹⁹F NMR (376 MHz, CDCl₃) δ [ppm] -75.76 (COCF₃).

2.4. Preparation of the deprotected O⁶-(N-4-((hydroxymethyl)benzyl)-trifluoroacetylmiide)-2'-deoxyguanosine (4)

Compound (**3**) was dissolved in 3 ml of MeOH and transferred to a polypropylene conical tube (50 ml) with a stirring bar. Ammonium hydroxide (20%) 3 mL was added and the mixture was stirred during 2 hours. The reaction was followed up by TLC. The solvent was evaporated and the crude was purified by flash chromatography (DCM:MeOH 95:5 to 90:10), yielding the expected compound **4** (112 mg, 46 %, solid). TLC R_f=0.18 (DCM:MeOH 95:5), MS (EI 70 eV) m/z calcd for C₂₀H₂₂F₃N₆O₅ (M⁺ + H⁺) 483.1598 found 483.1599 [M⁺].

¹H-NMR (400 MHz, MeOD) δ [ppm] 2.31 (m, 1H, 2'), 2.75 (m, 1H, 2''), 3.73 (dd J = 12 Hz, J = 3, 1H, 5'), 3.83 (dd J = 12 Hz, J = 3, 1H 5''), 4.03 (m, 1H, 4'), 4.43 (s, 2H, Bz-CH₂NH₂), 4.54 (m, 1H, 3'), 5.48 (s, 2H, Bz-CH₂O), 6.28 (dd, 1H J = 8 Hz, J = 6 Hz, 1'), 7.27 (d, 2H J = 8 Hz, Bz-H_{arom}), 7.45 (d, 2H J = 8 Hz, Bz-H_{arom}), 8.01 (s, 1H, 8).

¹³C NMR (100 MHz, MeOD) δ [ppm] 39.73 (C-2'), 42.58 (Bz-CH₂NH), 62.32 (C-5'), 67.25 (Bz-CH₂O), 71.73 (C-3'), 85.39 (C-1'), 88.24 (C-4'), 114.55 (C-5), 116.049 (q J = 286.7 Hz, CF₃), 127.4 (2C_{arom}), 128.31 (2C_{arom}), 135.91 (C-8), 137.01 (C_{q-arom}), 138.65 (C_{q-arom}), 152.87 (C-4), 157.54 (q, J = 37 Hz COCF₃), 159.9 (C-2), 160.66 (C-6).

^{19}F -NMR (376 MHz, MeOD): δ [ppm] -77.20 (CF_3)

2.5. Preparation of the N^2 -(N' , N' -dimethylamino)methylen)- O^6 -(N -4-((hydroxymethyl)benzyl)-trifluoroacetamide)-2'-deoxyguanosine (**5**)

The product (**4**, 0.23 mmol) was dried by evaporation with anhydrous DMF and dissolved in 2 ml of DMF under argon. Dmf protecting group 125 μL (4 eq, 0.93 mmol) was added and the mixture was stirred during 4 hours. The reaction was followed by TLC. The solvent was evaporated and crude was purified by flash chromatography over silica gel yielding the expected compound **5** (112 mg, 46 %, solid). TLC R_f =0.28 (DCM:MeOH 90:10), MS (EI 70 eV) m/z for $\text{C}_{23}\text{H}_{27}\text{F}_3\text{N}_7\text{O}_5$ ($\text{M}^+ + \text{H}^+$) 538.202 found 538.2017 [M^+].

^1H -NMR (400 MHz, MeOD) δ [ppm] 2.39 (m, 1H, 2'), 2.76 (m, 1H, 2''), 3.08 (s, 3H, $\text{N}(\text{CH}_3)_2$), 3.14 (s, 3H, $\text{N}(\text{CH}_3)_2$), 3.73 (dd $J = 12$ Hz, $J = 3$, 1H, 5'), 3.79 (dd $J = 12$ Hz, $J = 3$, 1H 5''), 4.01 (m, 1H, 4'), 4.43 (s, 2H, $\text{Bz}-\text{CH}_2\text{NH}$), 4.56 (m, 1H, 3'), 5.57 (s, 2H, $\text{Bz}-\text{CH}_2\text{O}$), 6.41 (dd, 1H $J = 8$ Hz, $J = 6$ Hz, 1'), 7.29 (d, 2H $J = 8$ Hz, $\text{Bz}-\text{H}_{\text{arom}}$), 7.48 (d, 2H $J = 8$ Hz, $\text{Bz}-\text{H}_{\text{arom}}$), 8.24 (s, 1H, 8), 8.65 (s, 1H, $\text{CH}=\text{N}$).

^{13}C NMR (100 MHz, MeOD) δ [ppm] 34.58 (C-2'), 40.62, 40.73 (2C, $\text{N}(\text{CH}_3)_2$), 43.14 ($\text{Bz}-\text{CH}_2\text{NH}_2$), 62.54 (C-5'), 68.02 ($\text{Bz}-\text{CH}_2\text{O}$), 71.88 (C-3'), 85.38 (C-1'), 88.63 (C-4'), 116.77 (q $J = 286.7$ Hz, CF_3), 117.60 (C-5), 128.01 (2C_{arom}), 128.56 (2C_{arom}), 136.77 (C-8), 137.57 (C_{q-arom}), 140.62 (C_{q-arom}), 153.42 (C-4), 158.94 (q, $J = 37$ Hz COCF_3), 159.6 (C-2), 160.41 (C-6), 162.60 ($\text{CH}=\text{N}$).

^{19}F -NMR (376 MHz, MeOD): δ [ppm] -77.16 (CF_3).

2.6. Preparation of 5'-O-(4,4'-dimethoxytrityl)-[N^2 -(N , N' -dimethylamino)- O^6 -(N -4-((hydroxymethyl)benzyl)-trifluoroacetamide)-2'-deoxyguanosine (**6**)

A solution of (90 mg, 167 μmol s) of product (**5**) was dried three times with anhydrous pyridine (3×5 mL) and finally dissolved in anh. pyr (4 ml) and placed under argon. Then, 125 mg (1.4 eq, 0.32 mmols) of DMT-Cl and 14 mg (0.5 eq, 0.115 mmols) of DMAP were added. The reaction was stirred under argon over night at room temperature and protected from the light. The product formation was followed up by TLC. Then, the reaction was quenched with 1 ml of MeOH and the solvent was evaporated. The crude product was purified by flash chromatography over silica gel (DCM 100%, DCM:MeOH 1-10% + 2% Et_3N) to give 117 mg (83%, solid) of compound **6**. TLC R_f =0.68 (DCM:MeOH 95:5).

^1H -NMR (400 MHz, CDCl_3) δ [ppm] 2.49 (m, 1H, 2'), 2.65 (m, 1H, 2''), 2.92 (s, 6H, $\text{N}(\text{CH}_3)_2$), 3.41 (m, 2H, 5' 5''), 3.66 (s, 6H, $\text{DMT}-\text{O}-\text{CH}_3$), 4.14 (m, 1H, 4'), 4.40 (s, 2H, $\text{Bz}-\text{CH}_2\text{NH}$), 4.58 (m, 1H, 3'), 5.44 (s, 2H, $\text{Bz}-\text{CH}_2\text{O}$), 6.33 (t, 1H $J = 6$ Hz, 1'), 6.66 (d, 2H $J = 8$ Hz, $\text{DMT}-\text{H}_{\text{arom}}$), 6.68 (d, 2H $J = 8$ Hz, $\text{DMT}-\text{H}_{\text{arom}}$), 7.06-7.34 (m, 13H $\text{DMT}-\text{H}_{\text{arom}}$, $\text{Bz}-\text{H}_{\text{arom}}$), 7.87 (s, 1H, 8), 9.32 (s, 1H, $\text{N}=\text{CH}$).

^{19}F -NMR (376 MHz, CDCl_3): δ [ppm] -75.56 (CF_3).

2.7. Preparation of 5'-O-(4,4'-dimethoxytrityl)-[N^2 -(N , N' -dimethylamino)- O^6 -(N -4-

((hydroxymethyl)benzyl)-trifluoroacetylmidate)-2'-deoxyguanosine-3'-O-(N,N-diisopropyl-2-cyanoethyl phosphoramidite (7)

A solution of product **(6)** (117 mg, 136 μ mol) was dissolved in anhydrous CH_2Cl_2 under argon and at 0°C . Then, DIPEA (95 μL , 540 μmol , 4 eq) and 2-cyanoethyl diisopropylphosphoramidochloride (50 μL , 204 μmol) were added. After 15 min the reaction was allowed to reach r.t and stirred for 1 hour. The reaction was quenched with brine, extracted with CH_2Cl_2 , dried (MgSO_4) and concentrated. The resulting oil was used directly without further purification for oligonucleotide synthesis. TLC $R_f=0.68$ (hexane/EtOAc 20:70 + + 2% Et_3N).

^1H -NMR (400 MHz, CDCl_3) δ [ppm] 1.25 (s, 6H, $\text{NCH}(\text{CH}_3)_2$), 1.26 (s, 6H, $\text{NCH}(\text{CH}_3)_2$), 2.50 (m, 1H, 2'), 2.65 (m, 3H, 2'', CH_2CN), 2.92 (s, 6H, $\text{N}(\text{CH}_3)_2$), 3.24 (m, 2H, 5' 5''), 3.41 (m, 4H, $\text{CH}_2\text{CH}_2\text{OP}$, $\text{NCH}(\text{CH}_3)_2$), 3.66 (s, 6H, DMT-O-CH_3), 4.14 (m, 1H, 4'), 4.42 (s, 2H, $\text{Bz-CH}_2\text{NH}$), 4.58 (m, 1H, 3'), 5.54 (s, 2H, $\text{Bz-CH}_2\text{O}$), 6.33 (t, 1H $J = 6$ Hz, 1'), 6.65 (dd, 4H $J = 7$ Hz, $\text{DMT-H}_{\text{arom}}$), 7.06-7.34 (m, 13H $\text{DMT-H}_{\text{arom}}$, $\text{Bz-H}_{\text{arom}}$), 7.87 (s, 1H, 8), 9.32 (s, 1H, $\text{CH}=\text{N}$).

^{13}C NMR (100 MHz, MeOD) δ [ppm] 20.45 (CH_2CN), 24.47, 24.52, 24.54, 24.59 (4C, $\text{P-NCH}(\text{CH}_3)_2$), 39.28 (C-2'), 43.18 (2C, $\text{N}(\text{CH}_3)_2$), 43.31 (2C, $\text{C}(\text{CH}_3)_2$), 43.55 ($\text{Bz-CH}_2\text{NH}_2$), 55.16 (2C, DMT-OCH_3), 57.94 ($\text{CH}_2\text{CH}_2\text{OP}$), 63.48 (C-5'), 68.31 ($\text{Bz-CH}_2\text{O}$), 74.38 (C-3'), 84.59 (C-1'), 85.80 (C-4'), 86.43 (DMT-C_q), 113.11 (4C, DMT-C_3), 117.46 (CF_3), 117.69 (CN), 119.15 (C-5), 127.28 (2C, C_{arom}), 128.05 (2C, C_{arom}), 128.18, 128.72, 129.93, 129.95 (9C, $\text{DMT-C}_{3\text{-arom}}$), 135.05 (2C, $\text{DMT-C}_{q\text{-arom}}$), 135.56 ($\text{Bz-C}_{q\text{-arom}}$), 135.97 (C-8), 140.12 ($\text{Bz-C}_{q\text{Arom}}$), 140.70 ($\text{DMT-C}_{q\text{Arom}}$), 151.45 (2C, $\text{DMT-C}_{q\text{Arom}}$), 152.50 (C-4), 158.47 (2C, COCF_3 , C-2), 160.57 (C-6), 162.56 ($\text{N}=\text{CH}$).

^{19}F -NMR (376 MHz, CDCl_3): δ [ppm] -75.56 (CF_3).

^{31}P -NMR (161 MHz, CDCl_3): δ [ppm] 148.83.

3. Synthesis and purification of oligonucleotides

3.1. Oligonucleotide synthesis

The oligonucleotide sequences were synthesized at 1 μmol scale using the standard protocols (Table 1). The incorporation of the O^6 -(N-4-((hydroxymethyl)benzyl)-trifluoroacetylmidate)-2'-deoxyguanosinephosphoramidite ($^{\text{Bz}}$ dG) in the sequence was carried out manually with a coupling time of 15 min, obtaining a yield of 75%. The incorporation of the $^{\text{Q}}$ dU-nucleobases to the complementary sequences was automatically performed on the synthesizer. In all the syntheses we used dimethylformamidino-protected guanine phosphoramidite (**7**). The syntheses were completed using the DMT-OFF protocol, except for the sequence containing $^{\text{Bz}}$ dG, which was synthesized in DMT-ON mode to help with the purification.

3.2. Oligonucleotides deprotection and purification.

After oligonucleotide synthesis, the solid supports were transferred to screw-cap vials and incubated with a solution of concentrated aqueous ammonia overnight at room temperature, and

additional 15 min incubation at 55°C were needed for the sequence containing ^{Bz}dG. The solutions were then filtered using sterile cotton and transferred into a 2 mL eppendorf tube. The solutions were evaporated using a nitrogen system to remove the ammonia. The resulting products were desalted by Sephadex G-25 using water as eluent.

The sequence containing ^{Bz}dG was purified by reversed-phase HPLC using the DMT-ON protocol in a Nucleosil 120 10-C18 10 μm (250x8 mm) column with a flow rate of 3 mL/min and an increasing gradient of ACN (15% to 80%) over 0.1 M aqueous TEAA pH 6.5, during 20 minutes. The retention time for the oligonucleotide was 10.23 min. The pure fractions were combined and evaporated to dryness. The obtained residues were detritylated by adding 1 mL of 80% acetic acid solution for 30 min at room temperature. The deprotected oligonucleotides were desalted and further purified by a second round of chromatography, using the DMT-OFF protocol: 20 minutes of linear gradient from 5% to 50% ACN over 0.1 M aqueous TEAA pH 6.5. The yield and purity obtained for the products was around 85% in all cases. The length and homogeneity of the oligonucleotide were verified by MALDI-TOF (table 1).

The DNA-strand concentration was determined by absorbance measurements (260 nm) and its extinction coefficients. Oligonucleotide samples were kept at 4 °C until further use.

3.3. Preparation of the fluorescent ^FdG oligonucleotide.

The 5'-ATCTTCTC^{Bz}GATTCA-3' oligonucleotide was left to react with fluorescein isothiocyanate (FITC) through its free amino group as follows. 5 O.D. of the oligonucleotide were dissolved in 250 μL of an aqueous solution of 0.2 M NaHCO₃ (pH 9) and 10 eq of a solution of FITC solved in 250 μL DMF were added and left to react at rt for 8h. Then, 100 μL of an aqueous solution of 0.2 M NaHCO₃ (pH 9) and an additional 10 equiv of FITC in 100 μL of DMF were added and the mixture was left to react overnight at rt. The mixtures were concentrated to dryness and the residue resuspended in 1 mL of water. The solution was purified by Sephadex G-25 and analyzed by RP-HPLC using an XBridge OST C18 2.5 μm column. The coupling efficiency was determined by HPLC analysis (86 %). The purified oligomers were analyzed by MS (MALDI-TOF), see table 1.

DNA duplexes were obtained by annealing equimolar concentrations of the control, ^{Bz}dG and ^FdG sequences with the complementary oligonucleotide strands at 72 °C for 5 min and then allowing them to slowly cool down to room temperature.

4. hAGT expression and purification

In vitro assays were carried out using recombinant hAGT, overexpressed and purified as previously described.¹³ Briefly, hAGT protein was expressed in the *E. coli* strain Rosetta, induced by adding 1 mM IPTG and left to express for 4 h at 30 °C. The pellet was disrupted by sonication and centrifuged. The supernatant was filtered, loaded into a HiTrapTM FF column and eluted with an Imidazole gradient (20-500 mM) in the following buffer of 350 mM NaCl, 20 mM Tris pH 8, and 1 mM BME. Finally, the protein was loaded into a Superdex 75 16/60 being the buffer 200 mM NaCl, 20 mM Tris pH 8.0, 10 mM DTT and 0.1 mM EDTA. The protein was concentrated to 2 mg/ml in this buffer and kept at -20 °C in the presence of 40 %

glycerol. The same protocol was used for the purification of the inactive mutant hAGT-C145S expressed in the *E. coli* strain BL21. This mutant was used as negative control in many experiments.

5. Thermal denaturation studies.

Melting curves of all the possible pairs of complementary oligonucleotides were measured by monitoring the absorbance hyperchromicity at 260 nm. The sample was heated over the range 8–80 °C at a heating rate of 1°C/min. The buffer solution used was 10 mM sodium phosphate pH 7.0 and 100 mM NaCl. Sample concentration was around 4 µM. Each sample was allowed to equilibrate at the initial temperature for 5 min before the melting experiment began. The melting temperatures (T_m) are the average value of at least one pair of experiments. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computer fitting of the first derivative of absorbance with respect to $1/T$.

6. Circular Dichroism

The CD spectra of all the possible pairs of complementary oligonucleotides were registered at 25°C over a range of 220–320 nm, with a scanning speed of 50 nm·min⁻¹, a response time of 4s, data pitch of 0.5 nm, and a bandwidth of 1nm. The duplexes concentration was 2 µM in a buffer solutions of 10 mM sodium phosphate pH 7.0 and 100 mM NaCl. The samples were annealed before recording of spectra.

7. HPLC analysis of hAGT dealkylation.

In order to measure the dealkylation of ^FdG, 142 pmol and 350 pmol of the duplexes were incubated with 40 pmol of full-length-hAGT to a final volume of 400 µl in a reaction buffer (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM DTT, 5 mM EDTA). The reaction was incubated for 90 min at 25°C and stopped by heating the samples at 72 °C for 5 min. The reaction products were analyzed by HPLC on a Nucleosil analytical column at 60°C to facilitate the duplex dissociation. The HPLC flow rate was 1 ml/min, and a gradient of 10–40% acetonitrile in 20 min was used.

8. Fluorescence assay for hAGT activity.

The fluorescence assay was performed with full-length hAGT and the hAGT-C145S (inactive mutant) was used as a negative control. The reaction was accomplished in a total volume of 50 µl in each well, incubating increasing concentrations of hAGT (50, 100 and 200 nM) in reaction buffer (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM DTT, 5 mM EDTA, 20 mM KCl) at 25°C. The assay was initiated by the addition of 5 µl of fluorophore-quencher duplex substrate (10 nM, 0.5 pmol) and this solution was then placed in a microplate reader system. Fluorescence was measured every minute for 20 min at excitation and emission wavelengths of 485 and 535 nm, respectively. Averages over three readings were taken for each condition tested. Each experiment was performed in triplicates.

Both duplexes were used to measure hAGT repair activity. However, the Oligo-^FdG: C ^QdU duplex showed a higher increase in fluorescence relative to the Oligo-^FdG:Mismatch-^QdU duplex, and was used for all experiments.

Table S1: Sequence of oligonucleotides and its characterization by MALDI-TOF

Name	Sequence	Mass found (expected)
Oligo- ^{Bz} dG	5'-ATC TTC TC ^{Bz} G ATT CA-3'	4310.9 (expected 4306,94)
Oligo- ^F dG	5'-ATC TTC TC ^F G ATT CA-3'	4702.2 (expected 4695.31)
Control	5'-ATC TTC TCG ATT CA-3'	4190.0 (expected 4187.77)
Complementary	5'-TGA ATC GAG AAG AT-3'	4333.8 (expected 4332.77)
Complementary- ^Q dU	5'-TGA A ^Q dU C GAG AAG AT-3'	4741.2 (expected 4740.38)
Mismatch-T	5'-TGA ATT GAG AAG AT-3'	4348.2 (expected 4347.77)
Mismatch- ^Q dU	5'-TGA AT ^Q dU GAG AAG AT-3'	4757.0 (expected 4755.39)

Sequences of the oligonucleotides used in the development of the hAGT fluorescence assay. ^{Bz}**dG** represents O⁶-benzyl-2'-deoxyguanosine. ^F**dG** corresponds to the O⁶- Fluorescein-benzyl-2'-deoxyguanosine, where fluorescein is a fluorophore group. ^Q**dU** represents a modified T with the quencher group (Dabcyl). Mass spectrometry analysis (MALDI-TOF) and expected mass.

Table S2. Melting temperatures of the duplexes.

Name	XA	ZW	Tm (260 nm)
Control:Complementary	GA	CT	54.6
Control: Mismatch-T	GA	TT	45.0
Oligo- ^{Bz} dG:Complementary	^{Bz}GA	CT	33.3
Oligo- ^{Bz} dG:Mismatch-T	^{Bz}GA	TT	36.4
Oligo- ^{Bz} dG:Mismatch- ^Q dU	^{Bz}GA	^QdUT	40.2
Oligo- ^{Bz} dG: C ^Q dU	^{Bz}GA	C^QdU	41.3
Oligo- ^F dG:Complementary	^FGA	CT	33.0
Oligo- ^F dG: C ^Q dU	^FGA	C^QdU	30.3
Oligo- ^F dG:Mismatch-T	^FGA	TT	27.9
Oligo- ^F dG:Mismatch- ^Q dU	^FGA	^QdUT	34.3
nd: not determined.			

Figure S1: CD spectra of the different duplexes. A) CD spectra of complementary duplexes. B) CD of the mismatch duplexes. All the experiments were performed at 25°C in 10 mM sodium phosphate pH 7.0 and 100 mM NaCl with a 2 μ M concentration of duplexes.

Figure S2: Denaturation curves of duplexes Oligo-^FdG:Mismatch-^QdU and Oligo-^FdG: C^QdU at 260 nm. All the experiments were performed in 10 mM sodium phosphate pH 7.0 and 100 mM NaCl with a 4 μ M concentration of duplexes.

Figure S3: HPLC profiles of the control sequences. **A)** Mixture of the unmodified dG oligonucleotide sequence with its complementary strand (Control:Complementary). **B)** Mixture of the fluorescein-benzyl-modified oligonucleotide sequence with its complementary strand. (Oligo-FdG:Complementary). The HPLC analyses were performed at 60 °C to ensure duplex dissociation.

Figure S4: Control experiments of the fluorescence assay. The background fluorescence of the double stranded sequence is observed in the absence of hAGT and in the presence of an inactive mutant. An increase of fluorescence is only observed in the presence of the same concentration of active hAGT (150 nM). The reactions were performed in reaction buffer (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM DTT, 5 mM EDTA, 20 mM KCl) at 25°C.

Figure S5. Kinetic data of figure 2 (main text). The emission of fluorescence corresponding to 3 concentrations of hAGT (10, 20 and 30 nM) is measured for 20 minutes. The reactions were performed in reaction buffer (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM DTT, 5 mM EDTA, 20 mM KCl) at 25°C.

^1H -NMR, ^{13}C -NMR ^{19}F -NMR and ^{31}P -NMR spectra