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Synthesis of oligonucleotides carrying fluorescently labelled O^6-alkylguanine for measuring hAGT activity

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ABSTRACT

O^6-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 O^6-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.

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 O^6 position of guanines.[2–9] In particular, the alkylating agent 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) initially attacks at the O^6 guanine position, causing its cross-link with the opposite cytosine, blocking DNA replication and producing G_2/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 O^6-alkylguanine DNA alkyltransferase (hAGT or MGMT) is responsible for removing alkyl adducts from the O^6 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 reactions[26–30] 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.

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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 O^6-benzylguanine (^{F}dG) and the quencher was introduced in a very close position of the complementary strand of the duplex (^{Q}dU-complementary or ^{Q}dU-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.

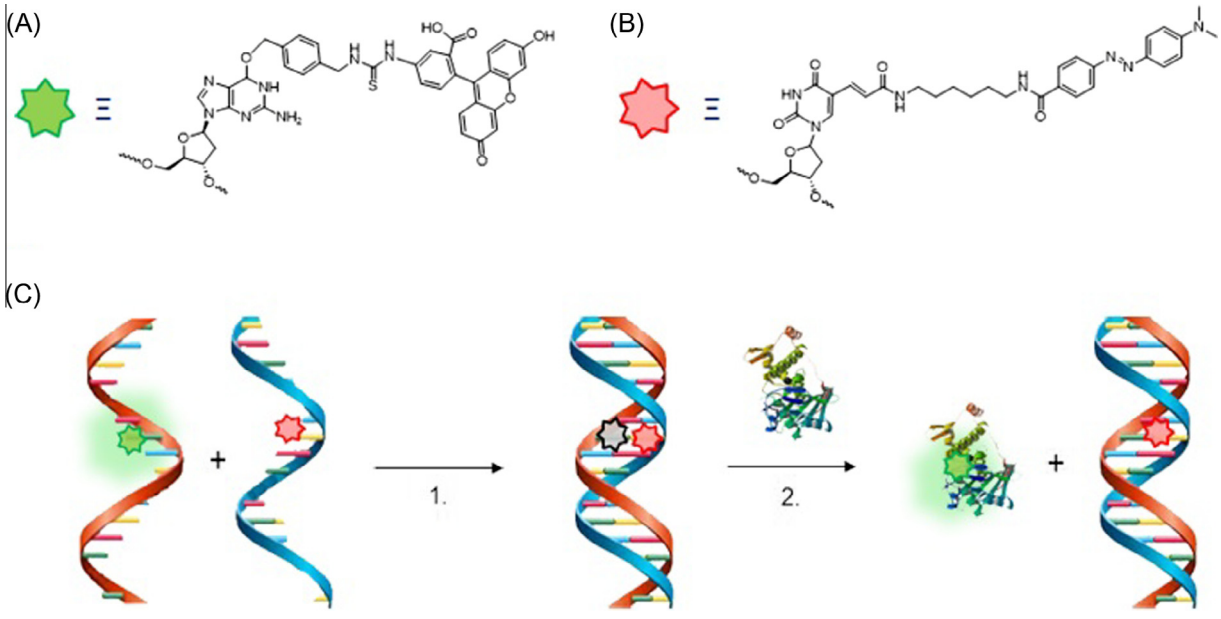
The ^{Bz}dG oligonucleotide was prepared using the appropriate modified 2′-deoxyguanosine phosphoramidite (**7**) in the automated DNA synthesizer using standard protocols.[37]

Derivative **7** was prepared from 2′-deoxyguanosine and the protected *N*-(4-(hydroxymethyl)benzyl)-trifluoroacetamide (**1**) (Scheme 2). Its incorporation at the O^6 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 O^6-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 O^6-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 ^{F}dG, 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.

First, to measure the effect of these modifications in the duplex formation, the thermal denaturation curves of all the modified and unmodified duplexes were studied at 260 nm (see Table S2 and Fig. S2 at Supplementary Data). In general, we observed destabilization of the duplex in the presence of the bulky substituent in the O^6 position of the central guanine, compared to the controls. However, both duplexes are stable at 30 °C. The duplex structures 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 Fig. S1, SD). Based on these results we decided to perform the hAGT activity assays at 25 °C that is below the melting temperature to ensure that the oligonucleotides are in the duplex form.

Efficient reaction with hAGT requires the ^{F}dG modification to correctly accommodate into the active site. This brings the CH_2 which is attached at the O^6 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 ^{F}dG sequence for 90 min at 25 °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. 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



Scheme 1. (A) Chemical structure of the O^6-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.

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