Engineering Substrate Specificity of O^6-Alkylguanine-DNA Alkyltransferase for Specific Protein Labeling in Living Cells

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Fusion proteins of human O^6-alkylguanine-DNA alkyltransferase (AGT) can be specifically labeled with a wide variety of synthetic probes in mammalian cells; this makes them an attractive tool for studying protein function. However, to avoid undesired labeling of endogenous wild-type AGT (wtAGT), the specific labeling of AGT fusion proteins has been restricted to AGT-deficient mammalian cell lines. We present here the synthesis of an inhibitor of wtAGT and the generation of AGT mutants that are resistant to this inhibitor. This enabled the inactivation of wtAGT and specific labeling of fusion proteins of the AGT mutant in vitro and in living cells. The ability to specifically label AGT fusion proteins in the presence of endogenous AGT, after brief incubation of the cells with a small-molecule inhibitor, should significantly broaden the scope of application of AGT fusion proteins for studying protein function in living cells.

Introduction

Characterizing protein function in living cells requires studying the localization and translocation of the protein of interest, its interactions with other biomolecules, its post-translational modifications, and conformational changes. Considering the complexity of protein function and the limitations of the currently available methods to address it, there is a generally acknowledged need for the development of new and innovative tools to study proteins in the living cell. One promising approach is based on the specific labeling of proteins with chemically diverse compounds that allow characterization and probing of the function of the protein of interest.[1] Currently, the majority of labeling approaches rely on the expression of the protein of interest as a fusion protein with an additional polypeptide, a so-called tag, that mediates the labeling of the fusion protein.[2–8] Such a chemical approach to exploit fusion proteins has the potential to equip the protein of interest with properties that cannot be genetically encoded, thereby complementing the more traditional tags such as autofluorescent proteins. We have recently introduced a general method for the specific labeling of fusion proteins of mutants of human O^6-alkylguanine-DNA alkyltransferase (AGT) using O^6-benzylguanine (BG) derivatives (Scheme 1).[3] The labeling is highly specific with respect to AGT but promiscuous with respect to the label, as substitutions at the 4-position of the benzyl ring do not significantly influence the reaction with AGT. We have used this approach for the labeling of AGT fusion proteins with different fluorophores and with ligands that mediate the interaction of AGT fusion proteins with other proteins.[3, 9, 10] The most serious limitation to the application of this approach in mammalian cell lines is that, in order to avoid unwanted background labeling, cells deficient in endogenous wild-type AGT (wtAGT) must be used. Up to now, human and hamster wtAGT have been reported to react with BG derivatives used for protein labeling.[3, 10] We have previously generated a mutant of human wtAGT, termed ^{GE}AGT, that carries the mutations Asn157Gly and Ser159Glu and has significantly increased activity against BG derivatives.[11] When expressed in normal cell lines at relatively high concentrations, ^{GE}AGT fusion proteins are labeled preferentially.[10] However, the scope of the approach would be significantly broadened if AGT mutants could be generated that allowed for a reliable and specific labeling of AGT fusion proteins in all mammalian cell lines. Here, we present the synthesis of a new inhibitor of human wtAGT and the generation of an AGT mutant that is resistant to this inhibitor. This allows the selective inactivation of human wtAGT while simultaneously labeling AGT fusion proteins.

Results and Discussion

Our strategy to achieve selective labeling of AGT fusion proteins in the presence of human wtAGT (referred to as wtAGT,

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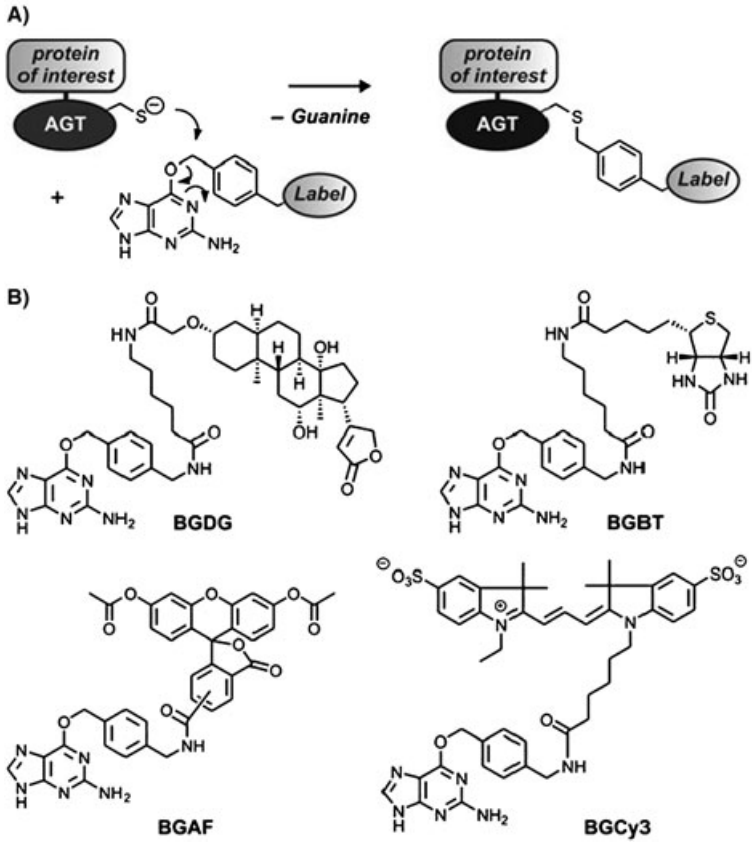


Scheme 1. Labeling of AGT fusion proteins. A) General mechanism for the labeling of AGT fusion proteins with O^6-benzylguanine (BG) derivatives. B) Structure of BG derivatives used for labeling with biotin (BGBT), digoxigenin (BGDG), fluorescein (BGAF), and Cy3 (BGCy3).[3,22] The fluorescein derivative was synthesized as a diacetate in order to increase its membrane permeability. In the cell, the diacetate of fluorescein is readily hydrolyzed to fluorescein.

unless noted otherwise) requires an AGT mutant resistant to an irreversible inhibitor of wtAGT. To this end, we envisioned blocking parts of the active site of AGT through appropriate mutations so that the resulting mutant protein would be resistant to an inhibitor that occupies this space. To identify an appropriate structure as well as complementary residues in the active site of AGT, we relied on the large body of work published on the mechanism and structure of AGTs.[12–15] It is now firmly established that AGTs operate by a "base-flipping" mechanism.[15,16] Structural and mechanistic studies have revealed that an invariant arginine residue (Arg128, numbering based on human wtAGT), which is located at the N terminus of a so-called recognition helix, is inserted into the gap in the DNA double helix that is generated by base flipping. The recognition helix is part of a helix–loop–helix motif that mediates the interaction with the DNA minor groove.[15] The flipped-out nucleotide is bound along the axis of the recognition helix; this allows the reactive cysteine residue (Cys145) to react with the alkylated base. In all known AGT sequences, a glycine or alanine (Gly131 in wtAGT) follows at position (i+3) after the invariant arginine. It is believed that this residue in the recognition helix is in contact with the deoxyribose of the flipped-out nucleotide (Figure 1).[15] It has also been suggested that the failure of wtAGT to repair alkylated ribonucleotides results from steric interactions between the 2′-hydroxyl group and Gly131.[15] Based on these data, we reasoned that replacement of Gly131 and Gly132 in wtAGT with bulkier residues should make the resulting mutant resistant to N^9-substituted O^6-alkylguanine derivatives. Therefore, we first synthesized N^9-cyclopentyl-O^6-(4-bromothenyl)guanine (CG) as a potential irreversible inhibitor of wtAGT (Scheme 2). We chose O^6-(4-bromothenyl)guanine instead of BG as a lead structure, since it has previously been shown that O^6-(4-bromothenyl)guanine is a more potent inhibitor of wtAGT than BG.[17] The cyclopentyl ring was chosen as substituent for the N^9 position as it sterically mimics the deoxyribose. Indeed, wtAGT and ^{GE}AGT are readily inactivated by CG in vitro, the IC_{50} in a competition assay with substrate BGBT (0.5 μM; Scheme 1) was 0.5 μM (Figure 2, Table 1). These data demonstrate that CG is an efficient inhibitor of wtAGT.

In order to generate AGT mutants resistant to inactivation by CG, we used a combinatorial approach based on phage display of AGT. We have previously demonstrated that AGT phage display can be used to select for mutants with increased activity against BG. As a starting point for this study, we chose the mutant ^{GE}AGT, which exhibits approximately 20-fold higher activity towards BG derivatives than wtAGT.[11] Codons for residues Gly131, Gly132, Met134, and Arg135 of ^{GE}AGT were randomized by using saturation mutagenesis. After transformation into phagemid pAK100, this resulted in an AGT library of 2×10^5 independent clones.[18] Met134 and Arg135 were included in the randomization, as these two residues make contact with the nucleobase and mutations at

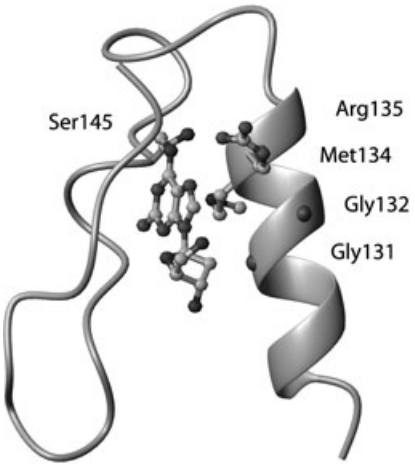
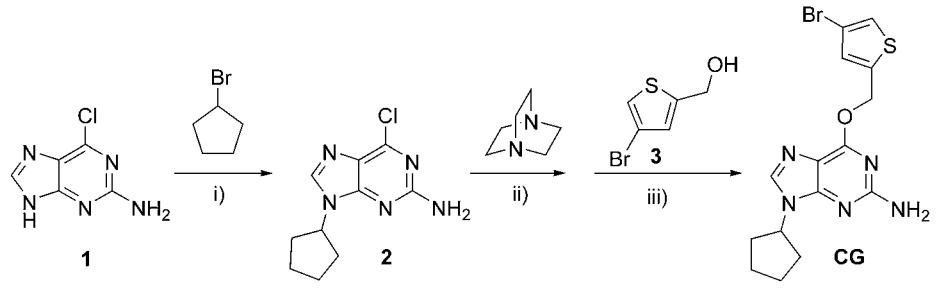


Figure 1. Binding of extrahelical O^6-methylguanosine in the active site of AGT. The structure of the active site of the Cys145Ser mutant ^{Ser145}AGT in complex with DNA containing O^6-methylguanine (PDB ID 1T38) is shown.[15] Residues Gly131, Gly132, Met134, Arg135, and Ser145 as well as the alkylated nucleoside are highlighted; the DNA and other side chains are omitted for clarity.



Scheme 2. Synthesis of CG. i) NaOMe, dimethylacetamide, 100 °C, overnight; ii) DABCO), DMF, RT, 3 h; iii) DBU, DMF, RT, overnight.

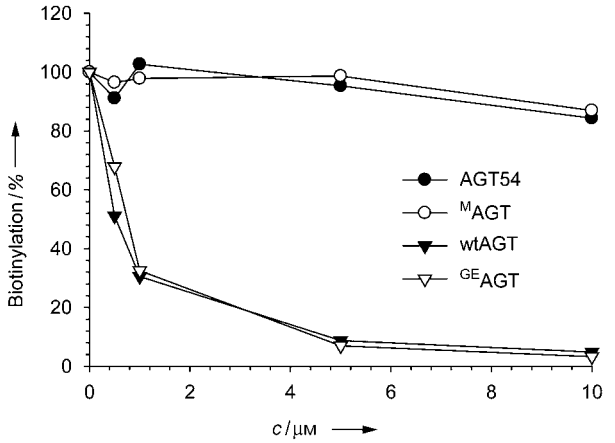


Figure 2. Inhibition of GST–AGT biotinylation by CG. GST fusion proteins (0.5 μM) were incubated with BGBT (0.5 μM), and varying concentrations of CG and biotinylation were detected by Western blotting by using a NeutrAvidin–peroxidase conjugate. The signal observed in the absence of CG was set to 1.

Table 1. Sequences of selected AGT mutants at randomized residues, their activities (k_{obs}) as GST–AGT fusion proteins against BGBT and their IC_{50} values of CG (incubation at 0.5 μM of BGBT).[a]

	Residue				k_{obs} [s^{-1} M^{-1}][b]	IC_{50} [μM][b]
	131	132	134	135	BGBT	CG
wtAGT	G	G	M	R	400	0.5
GEAGT	G	G	M	R	8000	0.7
AGT53	V	H	L	R	10000	n.d.
AGT54	K	T	L	S	12000	≫10
AGT57	Q	V	L	S	n.d.	n.d.
AGT58	M	T	M	V	n.d.	n.d.
AGT59	V	M	L	Q	n.d.	n.d.
MAGT[c]	K	T	L	S	2000	≫10

[a] All mutants are based on GEAGT (N157G, S159E). [b] Standard deviations of k_{obs} and IC_{50} are below 20%. [c] MAGT possesses the following additional mutations: Cys62Ala, Gln115Ser, Gln116His, Lys125Ala, Ala127Thr, Arg128Ala, Cys150Asn, Ser151Ile, Ser152Asn, and is truncated after position 182. n.d. = not determined.

these positions might therefore increase activity against BG derivatives that are used for labeling (Figure 1). For the selections, the phage library was incubated with substrate BGDG (Scheme 1) to label the active AGT with digoxigenin. This allowed for the subsequent enrichment of the corresponding phages by using magnetic beads that were covered with anti-digoxigenin antibodies. After four rounds of selection for activity against BGDG, five clones were analyzed by DNA sequencing (Table 1). At positions 131 and 132, a variety of different residues were found, all of which were sterically more demanding than glycine or alanine. The observed variability at those positions after selections against BGDG supports the hypothesis that the conservation of Gly131 in natural AGTs reflects the steric requirements for accommodating the deoxyribose of the flipped-out base. At position 134, either a leucine or methionine was found. The conservation of a hydrophobic residue at this position is in agreement with the fact that Met134 in wtAGT is mostly buried in the interior of the protein and that it also makes hydrophobic contact with the nucleobase. As also observed for residues 131 and 132, no consensus sequence at position 135 can be deduced from the analysis of the five clones. Two of the clones, AGT53 and AGT54, were then expressed and purified as glutathione *S*-transferase (GST) fusion proteins. Both proteins possessed activity against BGBT that was equal to or greater than that of the parental clone GEAGT (Table 1). The slightly more active GST–AGT54 was then tested for inactivation by CG (Figure 3, Table 1). In contrast to

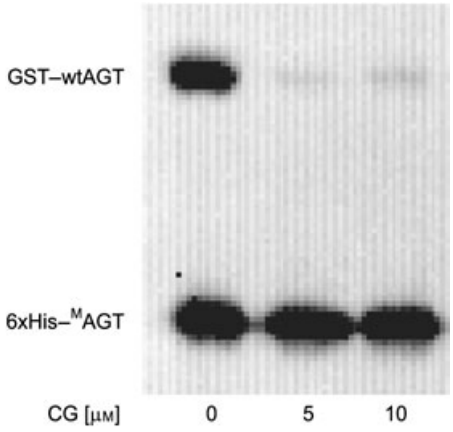


Figure 3. Specific labeling of 6×His–MAGT in the presence of GST–wtAGT. A mixture of 6×His–MAGT and GST–wtAGT (0.2 μM each) was incubated with BGBT (0.5 μM) in the presence or absence of varying concentrations of CG. Biotinylation was detected by Western blotting by using a NeutrAvidin–peroxidase conjugate.

GST–wtAGT and parental clone GST–GEAGT, its reaction with BGBT (0.5 μM) was not significantly affected at CG concentrations of up to 10 μM. This indicates that the binding site for the cyclopentyl ring in GST–AGT54 is indeed blocked

(Figure 2). Measurements at higher concentrations of CG were not possible due to the relatively low solubility of this compound (20 μM). Before testing further applications of AGT54, we engineered the mutant with respect to three properties that are important for application in protein labeling: i) Suppression of any significant activity against alkylated guanine in DNA to avoid unwanted inactivation before labeling, ii) removal of nonessential cysteines Cys62 and Cys150 to increase its stability under oxidizing conditions, and iii) reduction in the size of the protein. To suppress activity against alkylated guanine in DNA and also to decrease the affinity of the protein towards DNA in general, we randomized the codons of five residues (Gln115, Gln116, Cys150, Ser151, and Ser152) believed to be important for the sequence-independent interaction of AGTs with DNA in parental clone ^{GE}AGT.[13–15] After three rounds of selections with the substrate BGDG and phage display, a clone (AGT56) with the following mutations was isolated: Gln115Ser, Gln116His, Cys150Asn, Ser151Ile, and Ser152Asn. These mutations were then introduced into AGT54. In addition, we simultaneously introduced the mutations Lys125Ala, Ala127Thr, and Arg128Ala, which have previously been shown to disrupt the interaction of wtAGT with DNA.[9] Furthermore, Cys62 was mutated to Ala, and the last 25 C-terminal residues, which do not affect the activity of wtAGT, were deleted; this resulted in the final mutant referred to as ^{M}AGT. An overview of all mutations found in ^{M}AGT relative to wtAGT is shown in Table 1.

The combined effects of these eight mutations and the truncation of AGT54 caused only a minor (sixfold) drop in activity towards BGBT (Table 1) and did not affect the newly acquired resistance to CG (Figure 2, Table 1). To determine whether these mutations had reduced the activity towards alkylated DNA, we tested protein labeling with the fluorophore BGCy3 (Scheme 1) in the presence of increasing concentrations of a BG-containing double-stranded oligonucleotide (BG-oligonucleotide). In this assay, treatment of AGT with BGCy3 results in a threefold increase in fluorescent intensity, whereas treatment of AGT with the oligonucleotide abolishes this increase in fluorescence.[10] wtAGT is reported to react with BG-oligonucleotides at least 10^4-fold faster than with BG itself.[12] In agreement with the data on wtAGT, the labeling of GST–^{GE}AGT with BGCy3 is completely abolished by BG-oligonucleotide (Figure 4A, D). It was thought that mutations at positions 131 and 132 would impair the activity of GST–AGT54 with DNA substrates. This was indeed the case, although significant activity with the BG-oligonucleotide remained (Figure 4B, D). GST–^{M}AGT, however,

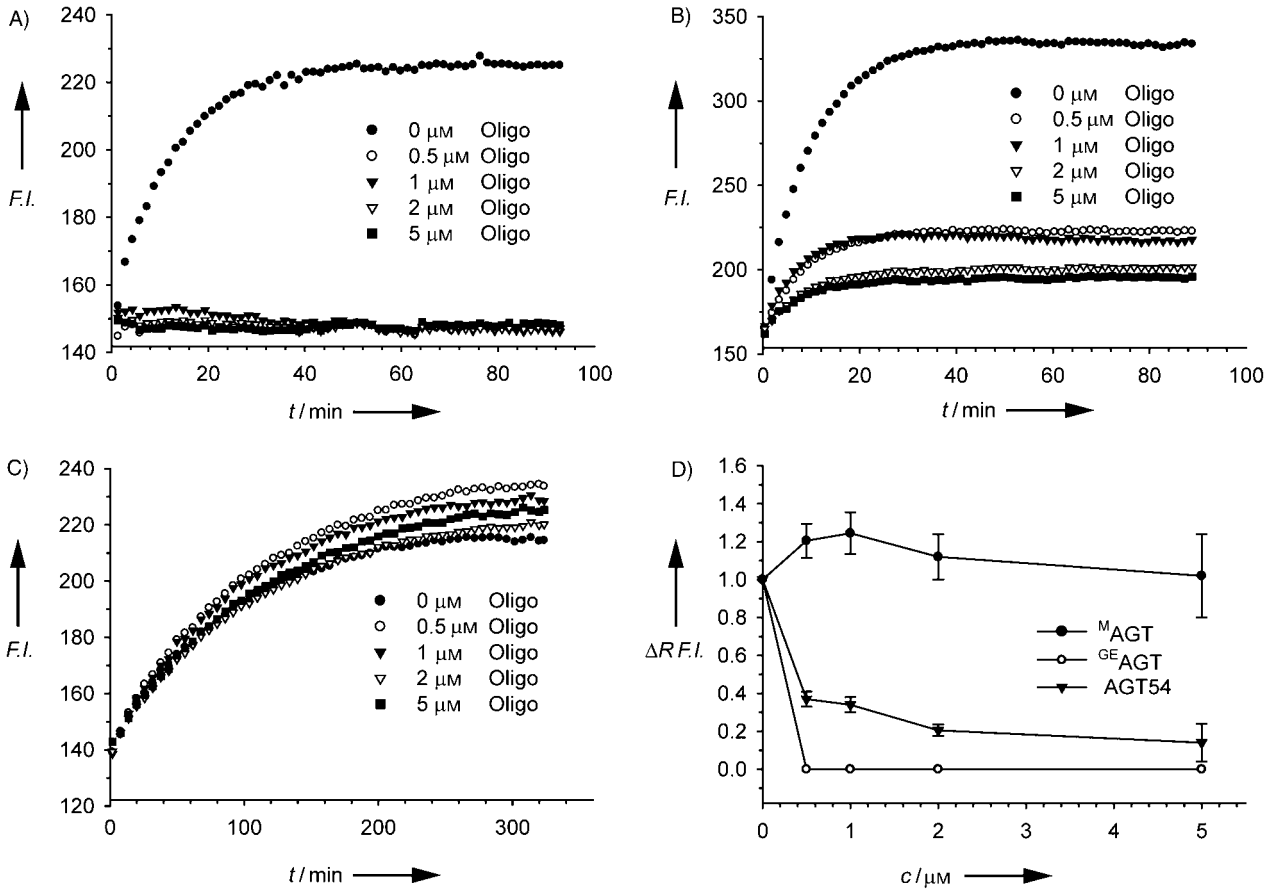


Figure 4. Treatment of AGT mutants with BGCy3 in the presence of varying concentrations of a 22-mer BG-oligonucleotide. Reactions were analyzed by following the increase in fluorescence intensity (F.I.) after treatment of AGT with BGCy3 in the presence of varying concentrations of BG–oligonucleotide. A) GST–^{GE}AGT; B) GST–AGT54; C) GST–^{M}AGT; D) graphical representation of the data in A–C. Differences in relative fluorescence intensity ($\Delta R F.I.$) at t_∞ and t_0 were plotted as a function of BG–oligonucleotide concentration. Values measured in the absence of oligonucleotide were arbitrarily set to 1.

has no detectable activity with BG-oligonucleotides in this assay; this demonstrates that the additional mutations introduced, efficiently suppress reactivity with DNA substrates (Figure 4C, D).

Next, we investigated if the low reactivity of ^{M}AGT versus CG could be exploited for the selective labeling of ^{M}AGT in the presence of wtAGT. To this end, we incubated an equimolar mixture of hexahistidine-tagged ^{M}AGT (6×His–^{M}AGT) and GST–wtAGT (0.2 μM each) with either BGBT (0.5 μM) or with a mixture of BGBT (0.5 μM) and CG (10 μM). Incubation with BGBT alone resulted in simultaneous biotinylation of both proteins to about the same extent (Figure 3). However, incubation with a mixture of BGBT and CG lead to an almost complete inhibition of GST–wtAGT labeling (5% of signal compared to measurements in absence of CG), whereas labeling of 6×His–^{M}AGT was barely affected (95% of signal compared to measurements in absence of CG). These data indicate that ^{M}AGT can be selectively labeled in the presence of wtAGT in vitro.

We then used fluorescence labeling with substrate BGAF (Scheme 1) in living cells to determine whether the combination of CG and ^{M}AGT fusion proteins led to highly specific labeling and reduced background fluorescence from endogenous AGT. To examine improvements in specificity without the complication of variable levels of endogenous AGT and to compensate for the higher activity of ^{M}AGT compared to wtAGT, we transiently coexpressed ^{M}AGT and ^{W160}AGT fusion proteins in an AGT-deficient Chinese hamster ovary (CHO) cell line. ^{W160}AGT contains the single mutation G160W, which increases its activity towards BG threefold to a level similar to that of ^{M}AGT.[3] First, we transiently transfected AGT-deficient CHO cells with a plasmid that directed the expression of ^{M}AGT fused to β-galactosidase (^{M}AGT–βGal). Incubation of the cells with BGAF (5 μM) led to fluorescent staining of the cytosolic ^{M}AGT–βGal (Figure 5A). By preincubating the transfected cells with BG (5 μM for 10 min), the fluorescence labeling was completely suppressed, whereas preincubation with CG (5 μM for 10 min) did not significantly affect the fluorescent labeling. Next, cells were transiently transfected with a plasmid that expressed ^{W160}AGT fused to a nuclear localization sequence (^{W160}AGT–NLS_3). Incubation with BGAF led to specific fluorescent labeling of the fusion protein, and, unlike ^{M}AGT–βGal, this staining could be suppressed by brief preincubation with either BG or CG (5 μM for 10 min). We then cotransfected cells with plasmids that coexpress cytosolic ^{M}AGT–βGal and nuclear localized ^{W160}AGT–NLS_3. If cotransfected cells were preincubated with CG, fluorescent labeling of cytosolic ^{M}AGT–βGal was evident, whereas no significant labeling was observed for the nuclear localized ^{W160}AGT–NLS_3 (Figure 5A). These data indicate that ^{M}AGT fusion proteins can be labeled in the presence of wtAGT in living cells. To verify that the observed selectivity is independent of the localization of the fusion proteins, we reversed the localization of the two AGT mutants by transiently expressing ^{W160}AGT–βGal and ^{M}AGT–NLS_3 fusion proteins. Essentially, repeating the experiments described above allowed us to demonstrate that nuclear-localized ^{M}AGT–NLS_3 can be selectively fluorescently labeled in the presence of cytosolic ^{W160}AGT–βGal in CHO cells by preincubation with CG (Figure 5B). In the above experiments, cells were incubated for short periods of time with CG. To investigate whether the inhibitor possessed any cytotoxic properties, we incubated HeLa cells for 24 h with CG (10 μM). Under these conditions, no obvious effects on cell growth and morphology were detected.

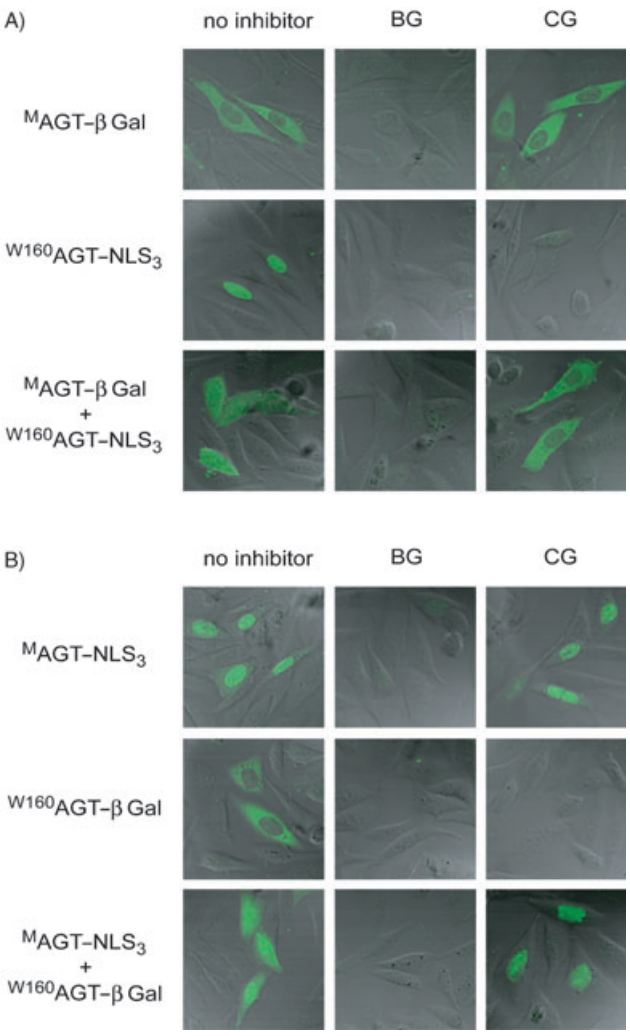


Figure 5. Selective fluorescent labeling of ^{M}AGT fusion proteins in living cells in the presence of ^{W160}AGT fusion protein. A) Fluorescein labeling of transiently (co)expressed ^{M}AGT–βGal and ^{W160}AGT–NLS_3 by using BGAF, with or without preincubation with BG or CG. B) Fluorescein labeling of transiently (co)expressed ^{M}AGT–NLS_3 and ^{W160}AGT–βGal by using BGAF, with or without preincubation with BG or CG. In A and B, cells were first incubated with or without inhibitor (5 μM, 10 min) and then with BGAF (5 μM, 20 min). Fluorescence and differential interference contrast (DIC) images of confocal micrographs are overlaid.

In summary, we present here the synthesis of an inhibitor of wtAGT and the generation of an AGT mutant that is resistant to this inhibitor. Brief incubations of living mammalian cells with low concentrations of the inhibitor allow for the efficient inactivation of wtAGT and the subsequent specific labeling of AGT-fusion proteins in the presence of inactivated wtAGT. The resistance of the generated mutants to the N^9-substituted guanine derivative confirms the hypothesis that steric requirements are the main reason for the conservation of Gly131 in the recognition helix of AGTs. Introduction of additional muta-

tions in regions believed to be important for DNA binding further decreases the affinity of AGT towards alkylated DNA and shifts the ratio of the activities against free BG and BG-oligonucleotide by at least five orders of magnitude towards free BG. Together, the properties of the AGT mutants generated significantly broaden the scope of applica'ions for the labeling of AGT fusion proteins with chemically diverse compounds, as they allow for specific labeling in different genetic backgrounds and are therefore an important step towards the establishment of the method as a general tool in functional proteomics.

Experimental Section

Standard chemicals were purchased from Fluka or Sigma–Aldrich. Enzymes for recombinant DNA work were purchased from MBI Fermentas (Nunningen, Switzerland) or New England Biolabs (Bioconcept, Allschwil, Switzerland).

***N*^{9}-cyclopentyl-6-chloroguanine (2)**: Cyclopentyl bromide (200 mg, 1.34 mmol) was added to a suspension of 6-chloroguanine (**1**; 228 mg, 1.34 mmol) in dimethylacetamide (2 mL). This was followed by the addition of NaOMe (144 mg, 2.67 mmol), which became soluble in the chloroguanine. The solution was stirred at 100 °C, overnight. The solvent was evaporated, and the residue was adsorbed on silica gel (1 g). Purification by flash chromatography (ethyl acetate/petrol ether, 1:1) yielded 140 mg (44 %) of the desired product. ^{1}H NMR ($CDCl_3$): $\delta = 7.81$ (s, 1 H), 5.03 (br s, 2 H), 4.77 (quin, $J = 7.5$ Hz, 1 H), 2.24 (m, 2 H), 1.94 (m, 4 H),1.79 ppm (m, 2 H); ^{13}C NMR ($CDCl_3$): $\delta = 24.0$, 32.6, 56.1, 125.8, 140.8, 151.3, 153.9, 158.9 ppm; ESI-MS: *m/z* (%): calcd for $C_{10}H_{12}ClN_5+H^+$: 238.08; found: 238.28 (100).

(4-Bromothiophen-2-yl)methanol (3): This product was synthesized by using slight modifications of a previously published protocol.[19] $NaBH_4$ (1.11 g, 29.31 mmol) was added to a solution of 4-bromothiophene-2-carbaldehyde (5.00 g, 26.17 mmol) in propan-2-ol (70 mL). The reaction mixture was stirred for 2 h at RT. A saturated solution of NH_4Cl (15 mL) was then added, and the suspension was filtered. The filtrate was concentrated in vacuo, dissolved in CH_2Cl_2, dried over $MgSO_4$, and concentrated again. The residue was purified by flash column chromatography (ethyl acetate/petrol ether, 1:10) to give 4.55 g (90 %) of **3**. ^{1}H NMR ($CDCl_3$): $\delta = 7.19$ (s, 1 H), 6.94 (s, 1 H), 4.78 (d, 2 H, $J = 5.8$ Hz), 2.07 ppm (br s, 1 H).

***N*^{9}-cyclopentyl-*O*^{6}-(4-bromothenyl)guanine (CG)**: 1,4-diazabicyclo-[2.2.2]octane (DABCO; 71 mg, 0.63 mmol) was added to a solution of *N*^{9}-cyclopentyl-6-chloroguanine (50 mg, 0.21 mmol) in dimethylformamide (DMF, 1.3 mL). The reaction mixture was stirred for 3 h at RT. A solution of **3** (49 mg, 0.25 mmol) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU; 96 mg, 94 μL, 0.63 mmol) in DMF (0.7 mL) was then added to the reaction mixture. The solution was stirred at RT overnight. The product was purified by flash column chromatography (ethyl acetate/petrol ether, 1:9→3:7). Yield: 25 mg (30 %) over two steps; ^{1}H NMR ($CDCl_3$): $\delta = 7.66$ (s, 1 H), 7.18 (s, 1 H), 7.11 (s, 1 H), 5.64 (s, 2 H), 4.87 (br s, 2 H), 4.76 (quin, 1 H, $J = 7.5$ Hz), 2.21 (m, 2 H), 1.92 (m, 4 H), 1.77 ppm (m, 2 H); ^{13}C NMR ($CDCl_3$): $\delta = 24.0$, 32.8, 55.7, 61.8, 109.2, 116.0, 124.1, 131.0, 138.0, 140.1, 154.6, 158.7, 160.3 ppm. ESI-MS *m/z* (%) calculated for $C_{15}H_{16}BrN_5OS+H^+$: 394.03; found: 394.36 (100).

Library construction and phage selection: Residues 131, 132, 134, 135 (library 1) and 115, 116, 150, 151, 152 (library 2) were randomized by overlap extension PCR by using primers **1**–**10** (see Supporting Information for list of primers) with ^{GE}AGT as the template. Primers **1**, **2**, **5**, and **10** contain *Sfi*I restriction sites; primer **3** contains the randomized bases for randomization at positions 131, 132, 134, and 135; primer **7** contains the randomized bases for randomization at positions 115 and 116; primer **9** contains the randomized bases for randomization at positions 150, 151, and 152. The PCR products were ligated into phage display vector pAK100 and electroporated into *E. coli* XL-1Blue (Stratagene, USA). This yielded libraries that contained at least 2.5×10^5 independent clones each.[18] Phage selections were performed as described by using the following reaction times and substrate concentrations for the four selection rounds.[11] Selections with library 1: 1 μM BGDG for 6 min in the first round; 1 μM BGDG for 2 min in the second round; 1 μM BGDG for 45 s in the third round; 0.01 μM BGDG for 40 s in the fourth round. Selections with library 2: 1 μM BGDG for 5 min in the first round; 1 μM BGDG for 5 min in the second round; 90 nM BGDG for 4 min in the third round; 90 nM BGDG for 1 min in the fourth round.

Generation of ^{M}AGT: Using overlap extension PCR and primers **11**–**22** the gene expressing ^{M}AGT was generated by starting from the gene expressing ^{GE}AGT. ^{M}AGT possesses the following mutations relative to wtAGT: Cys62Ala, Gln115Ser, Gln116His, Lys125-Ala, Ala127Thr, Arg128Ala, Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Cys150Asn, Ser151Ile, Ser152Asn, Asn157ly, and Ser159Glu. In addition, the part of the gene following the codon for Gly182, which encodes the last 25 amino acids of wtAGT, was deleted.

Characterization of AGT mutants: The genes of mutants isolated after phage selections, were amplified by PCR and either subcloned into pGEX-2T (Amersham Biosciences, Otelfingen, Switzerland) for expression as a GST–AGT fusion, or into pET15b (Novagen, Lucerne, Switzerland) for expression as a 6×His–AGT fusion protein. Expression and purification of the proteins was preformed as described.[11] For the measurements of the reaction rates between the AGT mutants and BGBT, protein (0.2 μM–0.4 μM) was incubated with BGBT (1 μM, 0.5 % DMSO final concentration) in reaction buffer (50 mM HEPES, pH 7.2, 1 mM DTT, 200 μg mL^{-1} BSA) at 24 °C, and aliquots were taken at defined times. The aliquots were quenched with BG (100 μM final concentration) and analyzed with Western blotting by using a NeutrAvidin–peroxidase conjugate (Pierce, Lausanne, Switzerland) and a chemiluminescent peroxidase substrate (renaissance reagent plus; Perkin–Elmer). The intensities of the bands on the Western blot were analyzed with an image station (440CF, Kodak) and the data were fitted to a pseudo first-order reaction model. Second-order rate constants were obtained by dividing the pseudo first-order rate constants by the concentration of BGBT.

Competition assays: To measure the labeling of AGT mutants in the presence of CG, AGT mutants (0.5 μM final concentration) were incubated with BGBT (0.5 μM final concentration) and different concentrations of CG (0, 0.5, 1, 5, 10 μM final concentrations) in reaction buffer for 45 min. Reactions were quenched by addition of 2×SDS buffer (20 % glycerol, 3% SDS, 100 mM Tris, pH 7.4, 5 mM mercaptoethanol) and incubated for 2 min at 95 °C. Samples were analyzed by Western blotting as described above.

To measure the labeling of AGT mutants in the presence of BG-oligonucleotide, AGT mutants (0.2 μM final concentration) were incubated with BGCy3 (0.5 μM final concentration) and different concentrations of BG-oligonucleotide (0, 0.5, 1, 2, 5 μM final concentrations) in reaction buffer (20 % glycerol, 3% SDS, 100 mM Tris, pH 7.4, 5 mM mercaptoethanol) in microtiter plates (black 96-well

plate, Greiner bio-one). Fluorescence was measured (excitation 519 nm, emission 572 nm) every 30 s for 3.5 h on a Spectramax Gemini plate reader (Molecular Devices, Basel, Switzerland). The sequence of the 22-mer oligonucleotide was: 5′-GTGGTGGGCAGCT-XAGGCGTGG-3′, where X corresponds to the O^6-benzylated nucleoside.[20] The base opposite to X in the complementary strand was C.

Construction of mammalian expression vectors: For the expression of ^{W160}AGT–βGal, the gene of ^{W160}AGT was PCR amplified with primers **23** and **24** from a plasmid that contained ^{W160}AGT–HA-NLS_3. The product was ligated into a *Nhe*I/*Bgl*II digested mammalian-expression plasmid that contained the ^{GE}AGT–βGal gene; ^{GE}AGT was thus replaced.[10] For ^{M}AGT–βGal and ^{M}AGT–NLS_3 expression ^{M}AGT was PCR amplified with the primers **23** and **25** and inserted into the *Nhe*I/*Bgl*II sites of the vector pECFP-Nuc (Clontech, Basel, Switzerland) or a mammalian expression plasmid containing the β-galactosidase gene.[10]

Fluorescence labeling in CHO cells: CHO-9-neo-C5 cells deficient of AGT were used for transient transfection.[21] The day before transfection, cells cultured in F-12 (Ham) nutrient mixture (Invitrogen, Basel, Switzerland), which contained fetal bovine serum (FBS, 10%), penicillin (0.5 units mL^{-1}; Invitrogen) and streptomycin (0.5 μg mL^{-1}, Invitrogen), were seeded on sterile Petri dishes (diameter 26 mm). Cells were transfected by using the calcium phosphate-precipitation method. DNA (3 μg) in $CaCl_2$ (100 μL, 250 mM) was pipetted drop-wise into 100 μL of a solution containing NaCl (280 mM), KCl (1 mM), Na_2HPO_4 (1.4 mM), dextrose (1 g L^{-1}), and HEPES (10 mM, pH 7.2). After 5 min the mixture was added to the cells, and, after 4 h incubation at 37 °C, under 5% CO_2, the medium was removed. Glycerol (10%) in phosphate-buffered saline (PBS; 2 mL) was then incubated with the cells for 1 min at RT. The cells were then washed with PBS and incubated for 24 h in F-12 (Ham) nutrient mixture (10% FBS, 0.5 units mL^{-1} penicillin, 0.5 μg mL^{-1} streptomycin) at 37 °C and 5% CO_2.

For the labeling experiments, adherent cells in plastic Petri dishes were incubated for 10 min with 5 μM CG or BG in PBS containing DMSO (0.5%) at RT to quench either ^{W160}AGT or ^{W160}AGT and ^{M}AGT. BGAF was then added (final concentration 5 μM), and the cells were incubated for 20 min at RT. The cells were then washed with PBS (3×) and incubated at RT for 30 min before being imaged in PBS.

Laser-scanning confocal micrographs were recorded by using a 488 nm argon-laser line on a Leica DM RXA2 microscope with a 63× water objective. Emission was recorded at 520–550 nm. Differential interference contrast (DIC) optics was used to image unlabeled cellular structures. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorescent probes and cell damage or morphological changes. Images of cells incubated with BG or CG or without inhibitor were acquired with identical microscope settings.

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