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Development of an *O*^{6}-alkylguanine—DNA alkyltransferase assay based on covalent transfer of the benzyl moiety from [benzene-^{3}H] *O*^{6}-benzylguanine to the protein

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Abstract

Although it is known that (i) *O*^{6}-alkylguanine—DNA alkyltransferase (AGT) confers tumor cell resistance to guanine *O*^{6}-targeting drugs such as cloretazine, carmustine, and temozolomide and that (ii) AGT levels in tumors are highly variable, measurement of AGT activity in tumors before treatment is not a routine clinical practice. This derives in part from the lack of a reliable clinical AGT assay; therefore, a simple AGT assay was devised based on transfer of radioactive benzyl residues from [benzene-^{3}H]*O*^{6}-benzylguanine ([^{3}H]BG) to AGT. The assay involves incubation of intact cells or cell homogenates with [^{3}H]BG and measurement of radioactivity in a 70% methanol precipitable fraction. Approximately 85% of AGT in intact cells was recovered in cell homogenates. Accuracy of the AGT assay was confirmed by examination of AGT levels by Western blot analysis with the exception of false-positive results in melanin-containing cells due to [^{3}H]BG binding to melanin. Second-order kinetic constants for human and murine AGT were 1100 and 380 M^{-1} s^{-1}, respectively. AGT levels in various human cell lines ranged from less than 500 molecules/cell (detection limit) to 45,000 molecules/cell. Rodent cell lines frequently lacked AGT expression, and AGT levels in rodent cells were much lower than in human cells.

Keywords

O^6-alkylguanine—DNA alkyltransferase; assay; [Benzene-^{3}H]*O*^{6}-benzylguanine; Cloretazine; Carmustine; Temozolomide; AGT-positive and -negative cells; $B_{16}F_{10}$ melanoma; Drug binding to melanin

The cytotoxic effects of antitumor agents such as cloretazine [1], carmustine [2], and temozolomide [3] are primarily a consequence of their ability to alkylate DNA at the *O*^{6} position of guanine. The *O*^{6}-methylguanine lesion generated by temozolomide [3] and the *O*^{6}-chloroethylguanine and subsequently rearranged *N*^{1},*O*^{6}-ethan-oguanine lesions generated by carmustine [4] and cloretazine [5] are subject to repair by *O*^{6}-alkylguanine—DNA alkyltransferase (AGT).[1] By covalently transferring the alkyl moieties to the active site cysteine, AGT restores the *O*^{6} position of guanine to its native state and attenuates the cytotoxicity of guanine *O*^{6}-targeting agents [6]. The reaction is irreversible; thus, the AGT protein is inactivated during the repair process [6]. A number of in vitro and in vivo studies have established an inverse relationship between the AGT concentration and the sensitivity to guanine *O*^{6}-targeting drugs [7–9].

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The AGT content of tumor and normal tissues is highly variable [10]. In humans, liver contains the highest level of AGT, followed by small intestine and lung [11]. Histochemical studies have shown that human tumor tissues often express more AGT than adjacent normal tissues [10], whereas 22–27% of human brain tumors completely lack AGT activity [12,13]. The lack of AGT expression in tumors is due to silencing of the AGT gene through hypermethylation [14,15] possibly caused by epigenetic malfunction during tumorigenesis [16].

Because AGT protects normal host tissues from the deleterious effects of guanine O^6-targeting drugs, unlike most antineoplastic agents whose mechanisms of tumor selectivity are not well defined, a lower AGT content in tumors than in normal tissues constitutes a basis of tumor selectivity for guanine O^6-targeting drugs. Clear tumor selectivity is manifested by cloretazine being curative in AGT-negative tumors in preclinical mouse models [1,17]. In phase II clinical trials, cloretazine produced a 28% complete response rate in elderly patients with acute myeloid leukemia or high-risk myelodysplastic syndromes with modest extramedullary toxicity [18]. These results collectively point to the potential importance of AGT measurements in tumor and normal tissues before treatment with O^6-targeting drugs.

The known AGT assays use DNA reacted with *N*-methyl-*N*-nitrosourea (MNU) [11,19,20] or ^{32}P- or fluorescein-labeled double-stranded oligonucleotides containing O^6-methylguanine in a restriction endonuclease site [21,22] as substrates for AGT. These assays require multistep substrate preparation, DNA and protein hydrolysis in some cases, and analytical procedures such as HPLC and gel electrophoresis. Therefore, these assays are complex, laborious, cumbersome, and time-consuming.

In this article, we present a simple AGT assay using O^6-benzylguanine (O^6-BG), a small chemical inhibitor of AGT [23], labeled with ^{3}H in the benzyl moiety. Although ^{3}H labeling of the benzyl portion of the O^6-BG analog O^6-(*p*-hydroxy[^{3}H]methylbenzyl)guanine has been

[1]Abbreviations used

AGT	O^6-alkylguanine—DNA alkyltransferase
MNU	*N*-methyl-*N*-nitrosourea
O^6-BG	O^6-benzylguanine
[^{3}H]BG	[benzene-^{3}H]O^6-benzylguanine
[^{3}H]B–AGT	[^{3}H]benzyl residue transferred to AGT
DTT	dithiothreitol
HGPRT	hypoxanthine—guanine phosphoribosyltransferase
F-MEL	Friend murine erythroleukemia
FBS	fetal bovine serum
GM—CSF	granulocyte-macrophage colony-stimulating factor
IL-3	interleukin-3
CHX	cycloheximide
TMG	O^6-(thiophenemethyl)guanine
6-MP	6-mercaptopurine
HQ	hydroquinone
L-DOPA	L-3,4-dihydroxyphenylalanine
MSP	methylation-specific PCR

reported, an AGT assay employing this labeled compound has not been established [24]. The assay takes advantage of the transfer of a radioactive residue from a methanol-soluble small chemical substrate to a methanol-insoluble high-molecular-weight protein.

Materials and methods

Synthesis of ^{3}H-labeled O^{6}-BG

[Benzene-^{3}H]O^{6}-benzylguanine ([^{3}H]BG) was prepared with a specific activity of 46.2 Ci/mmol and with radiopurity of more than 98% by Moravek Biochemicals (Brea, CA, USA) by reaction of 2-amino-6-chloropurine with the sodium salt of [ring-^{3}H]benzyl alcohol followed by repeated purification using HPLC. The specific activity was determined by quantifying [^{3}H]BG by mass spectrometry on the final product, which was supplied in methanol at a concentration of 1 mCi/ml (21.6 μM).

The AGT assay

For assays using intact cells, cells in exponential growth were condensed to a density of 2×10^{7} cells/ml in culture medium supplemented with 20 mM Hepes (pH 7.4), and 100-μl aliquots (2×10^{6} cells) were dispensed into 1.5-ml microcentrifuge tubes. Assays were initiated by the addition of 1 μl(1 μCi) of [^{3}H]BG in the absence or presence of excess unlabeled O^{6}-BG. The addition of 0.5 μl of 50 mM unlabeled O^{6}-BG in Me_2SO was prior to that of [^{3}H]BG. The concentrations of [^{3}H]BG and unlabeled O^{6}-BG were 0.21 and 250 μM, respectively. Incubations were conducted at 37 °C for the indicated periods in open air. At the end of incubations, 240 μl of cold 100% methanol was added to denature and precipitate cellular macromolecules, and then tubes were chilled at -70 °C for 30 min. Pellets were collected by centrifugation at 12,000*g* for 3 min in an Eppendorf microcentrifuge, and supernatants were aspirated using a blunt-ended, 20-gauge, 1.5-inch needle connected to a vacuum suction flask. Pellets were suspended in 1 ml of cold 70% methanol using 5.75-inch Pasteur pipettes followed by centrifugation and aspiration of supernatants, and this washing procedure was repeated four times. Tubes were kept on ice during the washing procedure. Pasteur pipettes were prewetted with cold 70% methanol to prevent precipitates from sticking onto inner walls. The use of a vortex mixer was avoided to suspend pellets to minimize radioactive spillage from tubes onto the walls of the microcentrifuge during centrifugation. Pellets were suspended in 100 μl of 0.5% Triton X-100 aqueous solution and transferred to plastic scintillation vials. After the addition of 5 ml of SafeScint Scintillation Cocktail (American Bioanalytical, Natick, MA, USA), radioactivity was determined using a Beckman LS 6500 Scintillation Spectrometer (Fullerton, CA, USA). The amount of [^{3}H]benzyl residue transferred to AGT ([^{3}H]B—AGT) was obtained by subtracting radioactivity in the presence of unlabeled O^{6}-BG from that in the absence of unlabeled O^{6}-BG. The counting efficiency (cpm/dpm) for ^{3}H was determined by suspending nonradioactive pellets in 100 μl of 0.5% Triton X-100 containing 10,000 dpm of [^{3}H]BG and measuring the radioactivity therein.

To prepare cell homogenates, cells were washed with Hanks' balanced salt solution and suspended at a density of 5×10^{7} cells/ml in a buffer containing 50 mM Tris—HCl (pH 7.5) and 1 mM dithiothreitol (DTT), and then cell suspensions were sonicated four times in short bursts on ice using a Branson Sonifier (Danbury, CT, USA). AGT assays were then conducted as described for intact cells using 100 μl of cell homogenate equivalent to 5×10^{6} cells/assay.

Hypoxanthine—guanine phosphoribosyltransferase assay

Cytoplasmic extracts for hypoxanthine—guanine phosphoribosyltransferase (HGPRT) assays were prepared from Friend murine erythroleukemia (F-MEL) wild-type 745-PC-4 and HGPRT-deficient 745-TG-11 cells [25] as described previously [26] except that the concentration of Triton X-100 in the lysis buffer was 0.08%. Nucleotide formation from

[8-^{14}C]6-mercaptopurine (51 mCi/mmol, Moravek Biochemicals) and [^{3}H]BG was determined based on the ability of nucleotides to bind to DE81 filter discs as described previously [26].

Cell culture

All suspension cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator except that appropriate factors were supplied for factor-dependent cell lines: 10 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM—CSF) for TF-1 cells and 10% conditioned medium from WxEHI-3B cells as a source of interleukin-3 (IL-3) for Ba/-F3, FDC-P1, and B6SUtA cells. Attached cell lines were maintained in McCoy's 5A medium supplemented with 10% FBS except that Daoy, A427, and $B_{16}F_{10}$ cells were cultured in medium 199 supplemented with 10% FBS, 1 mM sodium pyruvate, 1% MEM nonessential amino acid solution, and 1% MEM vitamin solution. Human melanoma cell lines A2058 (CRL-11147), C32 (CRL-1585), and G-361 (CRL-1424) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in McCoy's 5A medium supplemented with 10% FBS, 1 mM sodium pyruvate, and 1% MEM nonessential amino acid solution.

Western blot analysis

Whole cell extracts were prepared by washing cells with Hanks' balanced salt solution and solubilizing 5×10^6 cells in 0.25 ml of 2 × Laemmli's sample buffer at 100 °C for 5 min. Then 20 μl/lane of the whole cell extract was subjected to SDS—10% PAGE. Anti-AGT antibody (MT 3.1) and anti-HSC 70 antibody (sc-1059) were obtained from NeoMarkers (Fremont, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. HSC 70, a heat shock protein whose expression was constant, was used as a loading control.

Statistical and mathematical analyses

Values are reported as triplicate averages with standard deviations unless otherwise stated, with each experiment repeated at least twice for reproducibility. IC_{50} values were derived from logistic three-parameter regression analyses using KaleidaGraph (Synergy Software, Reading, PA, USA).

Results

Rationale of AGT assay

O^6-BG was discovered by Dolan and coworkers [23] as a relatively potent small chemical inhibitor of AGT. Subsequently, Pegg and coworkers [27] reported that the reaction of [purine-8-^{3}H]*O*^{6}-BG with recombinant human AGT resulted in the stoichiometric production of [^{3}H]guanine, whereas the reaction of ^{35}S-labeled recombinant AGT with *O*^{6}-BG resulted in the formation of [^{35}S]benzylcysteine. These results have demonstrated unequivocally that (i) *O*^{6}-BG inactivates AGT by acting as a substrate to produce *S*-benzylcysteine at the active site and that (ii) AGT can act on a small chemical substrate without a DNA structure. These studies prompted us to develop an AGT assay based on the transfer of the radioactive benzyl residue from ^{3}H-labeled *O*^{6}-BG to AGT molecules. To this end, Moravek Biochemicals was commissioned to synthesize and purify [^{3}H]BG (Fig. 1).

Standardization of AGT assay

AGT assays were standardized using HL-60 human leukemia cells expressing a relatively high level of AGT as determined by Western blot analysis. Standard AGT assays consisted of incubation of cells (2×10^6 cells/100 μl) with 1 μCi of [^{3}H]BG in the absence (total binding) or presence (nonspecific binding) of more than 1000-fold excess of unlabeled *O*^{6}-BG at 37 °

C. The mean values ± standard errors (SE) for total and nonspecific binding in standard assays for 2 h in HL-60 cells were 2780 ± 380 and 300 ± 30 cpm, respectively (six independent experiments with each conducted in duplicate). The amount of [^{3}H]benzyl residue transferred to AGT was obtained by subtracting nonspecific binding from total binding.

Radioactivity recovered in the reaction product was less than 1% of the total input of radioactivity (1 μCi = 2.22 × 10^6 dpm). Thus, efficient washing of the reaction product from unreacted [^{3}H]BG was critical for accurate measurement. Centrifugation of pellets and aspiration of the supernatants using a blunt-ended, 20-gauge, 1.5-inch needle was the most effective washing method with minimum production of radioactive waste (4 × 1 ml of 70% methanol). The use of 5% trichloroacetic acid to precipitate and wash cellular macromolecules was inadequate due to a high background derived from the insolubility of O^6-BG in acidic solution. The use of glass fiber filter GF/C or GF/A discs to collect and wash pellets was also inadequate due to a high background resulting from binding of [^{3}H]BG to the filters.

Kinetics of [^{3}H]benzyl transfer to AGT

The rate of reaction of [^{3}H]BG with AGT was measured in HL-60 human and L1210/AGT1 murine cells. AGT1 cells are a derivative of L1210 cells stably transfected with a murine AGT expression plasmid [8]. Because alkylated AGT is subject to ubiquitination-dependent degradation by proteasomes [28] to eventually become acid-soluble fragments [29], the proteasome inhibitor MG132 was included in the reaction mixture. To examine the effects of continued protein synthesis on AGT levels in intact cells, the protein synthesis inhibitor cycloheximide (CHX) was also included. Fig. 2 shows that reaction of [^{3}H]BG with AGT reached plateaus at approximately 4 and 8 h in human and murine cells, respectively. The presence of MG132 produced little difference in the recovery of the radioactive product in HL-60 cells, whereas it caused a slight increase in AGT1 cells where prolonged incubation was needed to reach a plateau. The level of reaction product in the presence of CHX was approximately 10% less than that in the absence of CHX in HL-60 cells. The plateau levels, representing the amount of [^{3}H]BG reacted with all of the AGT molecules present in cells, were 3100 and 3860 cpm/2 × 10^6 cells in HL-60 and AGT1 cells, respectively. These values were translated to 17,000 and 22,000 AGT molecules/cell, respectively, using the conversion factor 895 cpm/10^{10} AGT molecules (see Fig. 1).

Determination of the second-order kinetic constant (k) enables extrapolation of the saturation value from a single time point. Because the reaction of O^6-BG and AGT follows a bimolecular displacement (S_N2-type) reaction (Eq. (1) below), the reaction rate is proportional to the product of the concentrations of AGT and [^{3}H]BG, where A_0 and B_0 are the initial concentrations of AGT and [^{3}H]BG, respectively, and $B_0 >> A_0$ (1 μCi of [^{3}H]BG corresponds to 1.3 × 10^{13} AGT molecules) (Eq. (2) below). The plot of ln(1—C/A_0) as a function of time (Eq. (3) below) gave second-order rate constants of 1100 and 380 M^{-1} s^{-1} for human and murine AGT, respectively (Fig. 2).

$$\mathrm{AGT}\,(A) + \left[{}^{3}\mathrm{H}\right] BG\,(B) \rightarrow \left[{}^{3}\mathrm{H}\right] B \cdot \mathrm{AGT}\,(C) \tag{1}$$

$$\frac{dc}{dt} = k\,[A]\,[B] = k\,[A_0 - C]\,[B_0 - C] \approx k\,[A_0 - C]\,[B_0] \because B_0 \gg C \tag{2}$$

$$\ln\left(1-\frac{C}{A_0}\right)=-k\cdot B_0\cdot t. \quad (3)$$

Effects of various purines on reaction of O^6-BG and AGT

To examine the specificity of the reaction of O^6-BG and AGT, the AGT assay was conducted in the presence of 150 μM various purines, including guanine (the leaving group of the O^6-BG/AGT reaction), 2-amino-6-chloropurine (the starting material for the synthesis of O^6-BG), hypoxanthine, and the AGT inhibitor O^6-(thiophenemethyl)guanine (TMG) [30]. These purines, except for TMG, exerted no inhibitory effects on the reaction (Fig. 3A). To compare the potencies of TMG and O^6-BG as inhibitors of AGT, HL-60 cells pretreated with these inhibitors for 1 h were subjected to AGT assays for 1 h to measure the remaining AGT activity. In keeping with the report of McElhinney and coworkers [30],TMG was three times more potent than O^6-BG; their IC_{50} values were 0.023 and 0.073 μM, respectively (Fig. 3B).

Inability of O^6-BG to serve as substrate for HGPRT

The possibility of conversion of O^6-BG to the nucleotide level by HGPRT and subsequent incorporation of O^6-BG into DNA and RNA was examined. Cytoplasmic extracts from wild-type and $HGPRT^-$ F-MEL cells were incubated with 6-mercaptopurine (6-MP) and [^{3}H]BG as substrates. 6-MP was readily converted to 6-MP nucleotide by wild-type extracts but not by $HGPRT^-$ extracts, whereas neither extract produced O^6-BG nucleotide (Fig. 4). AGT assays gave equal values in wild-type and $HGPRT^-$ F-MEL cells (Table 1), confirming that incorporation of [^{3}H]BG into DNA and RNA did not occur.

Nature of nonspecific binding

The [^{3}H]BG preparation contained less than 2% radioimpurities. Standard AGT assays using HL-60 cells performed at different temperatures for 30 min revealed that AGT functioned optimally between 37 and 45 °C, whereas nonspecific binding increased in a temperature-dependent manner (Fig. 5).

AGT assay using cell homogenates

To obtain optimal conditions, various ingredients were tested individually using HL-60 cell homogenates prepared in water. Consistent with previous findings [31], AGT activity was optimal in alkaline conditions and in the presence of 1 mM DTT, whereas the additions of high salt and protease inhibitors were detrimental (Fig. 6A). Thus, cell homogenates were prepared in buffer (pH 7.5) containing 1 mM DTT. The rate of [^{3}H]benzyl transfer in cell homogenates was indistinguishable from that in intact cells, with a k value of approximately 1100 M^{-1} s^{-1} (Fig. 6B). The recovery of AGT activity in cell homogenates was 83% (6420 cpm/5 × 10^6 cells for cell homogenates vs. 3100 cpm/2 × 10^6 cells for intact cells).

AGT content of human and rodent cell lines

The values obtained from standard AGT assays for 2 h were used to calculate saturation values (A_0). The multiplication factors defined by second-order kinetic constants were 1.2 and 2.2 for human and murine cells, respectively. The AGT content of human cells varied from less than 500 to 45,000 molecules/cell (Table 1). AGT levels were below the detection limit in three of eight leukemia cell lines. Because most human carcinoma cells are polyploid, the AGT levels in LNCaP, DU145, and MCF7 cells, if expressed based on the DNA content, were similar to the level in HL-60 cells (17,000 AGT molecules/cell).

A number of murine cell lines lacked AGT expression (Table 1). Consistent with the previous observations that AGT levels in normal mouse tissues are generally approximately 10% of those in corresponding human tissues [11], AGT-positive murine cell lines contained much lower AGT activities than human cell lines. Unexpectedly, the AGT assay produced erroneous results in $B_{16}F_{10}$ melanoma cells; the experimental values for total and nonspecific binding were 59,300 and 7910 cpm/2 × 10^6 cells, respectively. $B_{16}F_{10}$ cells were assessed as AGT negative by the lack of sensitization to cloretazine on pretreatment with 20 μM O^6-BG for 2 h in growth inhibition assays (data not shown).

Correlation between [^{3}H]BG incorporation into methanol-insoluble fraction and melanin content

Although $B_{16}F_{10}$ murine melanoma cells are highly melanotic as judged by dark brown colorization of cells, these cells gradually lose the capacity to produce melanin in serial cell passages. The amount of [^{3}H]BG incorporation into the methanol-insoluble fraction was inversely related to the passage number (data not shown). [^{3}H]BG incorporation conducted for intact $B_{16}F_{10}$ cells was unaffected by hydroquinone (HQ), an inhibitor of tyrosinase, or L-3,4-dihydroxyphenylalanine (L-DOPA), a substrate for tyrosinase, which catalyzes the production of melanin [32] (Fig. 7A). For the same passage of $B_{16}F_{10}$ cells, [^{3} H]BG incorporation conducted for cell homogenates, in which melanin polymer was dispersed by repeated sonication, was less than 15% of that in intact cells (Fig. 7A).

AGT assays were performed for three human melanoma cell lines (A2058, C32, and G-361), all of which visually lacked dark brown deposits in cells, with parallel measurement of AGT levels by Western blot analysis (Fig. 7A and B). Both A2058 and C32 cells in which specific [^{3}H]BG binding was less than 90 cpm/2 × 10^6 cells were negative in AGT expression by Western blot analysis, whereas G-361 cells in which 14,000 AGT molecules/cell were estimated in the AGT assay showed an AGT level similar to that in HL-60 cells (17,000 AGT molecules/cell) by Western blot analysis. These results imply that [^{3}H]BG incorporation correlates with the melanin (polymer) content of cells. [^{3}H]BG incorporation in $B_{16}F_{10}$ cells is reminiscent of the phenomenon that a variety of drugs and xenobiotics are selectively concentrated in pigmented tissues such as the eye, inner ear, skin, and melanoma [33].

Determination of the AGT level in $B_{16}F_{10}$ murine melanoma cells by Western blot analysis was not possible due to the inability of the anti-human AGT antibody (MT 3.1) to cross-react with murine AGT. AGT levels in human leukemia and carcinoma cell lines examined by Western blot analysis (Fig. 7B) and by the AGT assay (Table 1) were strictly correlated, confirming the accuracy of the AGT assay using [^{3}H]BG in nonmelanotic cells.

Discussion

A variety of methods exist to measure AGT activity; difficulty in quantitation of AGT levels derives in part from the fact that AGT is not an enzyme. Because a single AGT molecule produces a single reaction product, a highly sensitive method is needed to detect minute amounts of AGT present in cells. The classical AGT assay employing [^{3}H]MNU-reacted DNA lacks sensitivity because of the predominant reactivity of MNU at sites other than the O^6 position of guanine coupled with the relatively low specific activity of [^{3}H]MNU, whereas the assay employing ^{32}P-labeled oligonucleo-tides containing O^6-methylguanine is inappropriate as a clinical assay largely because of the use of ^{32}P. A nonradioactive enzyme-linked immunosorbent assay (ELISA) employing oligonucleotides containing biotinylated O^6-BG as a substrate for AGT has been reported [34]; however, the presence of a bulky molecule such as biotin at the benzyl moiety diminishes affinity for AGT [35] and quantitation falls in a narrow range (0.1–2.0) of optical density measurement. Other methods such as Western blotting and histo-chemical analysis are time-consuming and not quantitative. Methylation-specific PCR

(MSP) that examines the methylation status of the *AGT* gene promoter is currently used to correlate AGT expression with clinical efficacy of temozolomide in glioblastoma [36]. Although MSP reveals a therapeutically critical aspect of AGT expression (i.e., silencing of the *AGT* gene by hypermethylation), it does not determine actual AGT activity.

In this article, we have devised a simple assay for AGT using [^{3}H]BG as a substrate. This assay relies on the incorporation of the labeled benzyl residue from a methanol-soluble small chemical substrate into the methanol precipitable protein product AGT. Time-consuming substrate preparation and analytical procedures such as HPLC and gel electrophoresis are eliminated in this assay. The assay is superior to existing methods in many ways; it is sensitive due to the high specific activity of [^{3}H]BG (41.3 Ci/mmol), quantitative, accurate, simple, applicable to both intact cells and cell homogenates, and (importantly) suitable for routine clinical assay.

The reaction of O^6-BG and AGT follows second-order kinetics. The second-order kinetic constant (k) was 1100 M^{-1} s^{-1} for human AGT in our assay. Pegg and coworkers [27] reported a k value of 600 M^{-1} s^{-1} in their system using [purine-8-^{3}H]O^6-BG and recombinant human AGT. Because they estimated the specific activity of recombinant AGT by assuming that the protein is 100% active, the discrepancy in the k values may be the result of an overestimation of the AGT concentration. The lower k value (380 M^{-1} s^{-1}) for murine AGT in our study is consistent with the observation that murine AGT is 2- to 3-fold more resistant to inhibition by O^6-BG than human AGT [23]. These k values for O^6-BG are approximately 3000-fold lower than the reported k values for various oligonucleotide substrates containing O^6-methylguanine [37], indicating that AGT reacts with the O^6-methylguanine lesion in DNA with enormous effectiveness. As such, the reaction of [^{3}H]BG with human AGT in intact cells and homogenates took nearly 4 h to reach a plateau. The saturation level representing the total amount of AGT in intact cells or homogenates was mathematically extrapolative by simple multiplication of an experimental value from a single incubation time and a factor defined by the reaction constant. Inclusion of the proteasome inhibitor MG132 to prevent degradation of alkylated AGT was unnecessary for incubations up to 2 h. In addition, we have ruled out the possibility of [^{3}H]BG being a substrate for HGPRT and incorporation of [^{3}H]BG into DNA and RNA.

The shortcomings of this new AGT assay are as follows. First, the assay is not applicable to pigmented cells or tissues such as $B_{16}F_{10}$ melanoma in which it produces false-positive results. Natural melanins, especially eumelanins, have been shown to act as weak cation exchange polymers with the capacity to bind to metal ions and a variety of chemicals, including 2-thiouracil [38], chlorpromazine, chloroquine, haloperidol, cocaine, and polycyclic aromatic hydro-carbons [33]. Interaction of electron-rich chemicals with melanin can also be nonelectrostatic whereby the chemicals act as electron donors with melanin as the acceptor [33]. Considering the capacity of [^{3}H]BG to bind to filters such as GF/C and GF/A, it is plausible that [^{3}H]BG exhibits high affinity toward melanin. Second, a number of natural variant forms of the *AGT* gene that encode an altered protein have been reported in humans [39]. The AGT assay produces false-negative results for the polymorphism variant G160 R, which imparts high resistance to O^6-BG, although occur-rence of this variant is rare [39]. Third, and obviously, this assay is not applicable to variant forms of AGT selected for resistance to inactivation by O^6-BG [40].

McElhinney and coworkers [30] reported two O^6-(hetarylmethyl)guanines that are more potent than O^6-BG as inhibitors of AGT. Consistently, O^6-(thiophenemethyl)guanine was approximately three times more potent than O^6-BG in our assays. These compounds labeled with ^{3}H at the guanine O^6-alkyl residue are attractive candidates as substrates for AGT, producing faster reactions and reducing incubation times to reach a saturation level. However,

these compounds exhibit the unfavorable chemical feature that their half-lives in an alkaline buffer (pH 8.3) are short; for example, the half-life of O^6-(thiophenemethyl)guanine is 40 min compared with more than 48 h for O^6-BG [30].

In this article, we have described the development of a simple AGT assay applicable to clinical measurements. The availability of this assay should allow a quantification of the AGT content of tumor and normal tissues before treatment and the selection of patients with a high probability of responding to guanine O^6-targeting drugs with the least number of side effects to normal host tissues, thereby making cancer therapy by these agents as targeted and personalized as possible.

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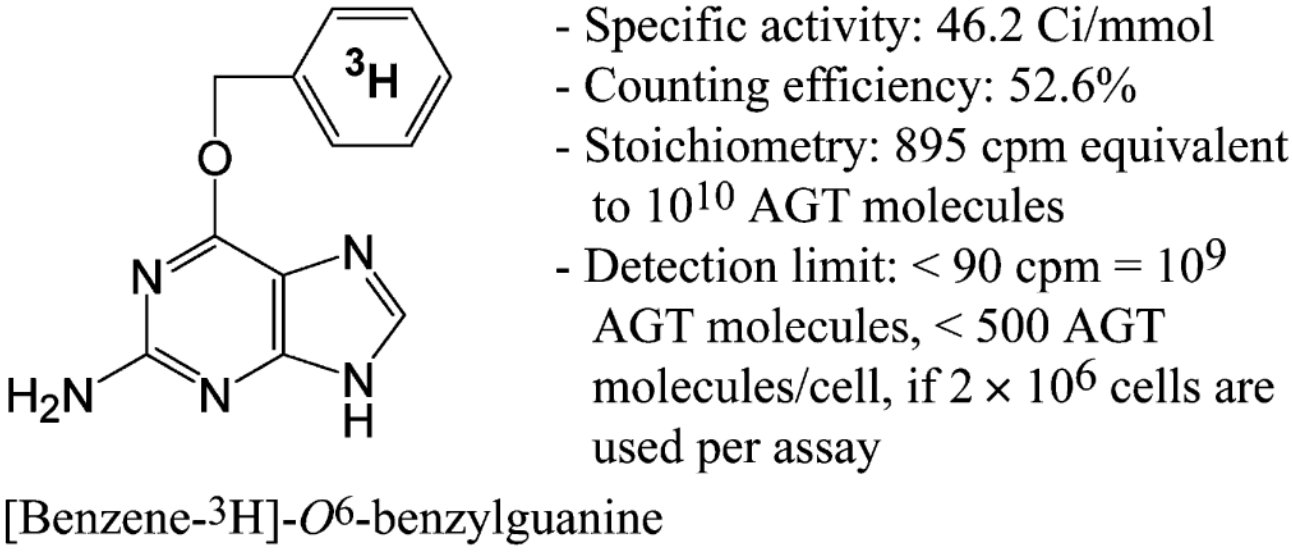

Fig. 1.

Structure of [^{3}H]BG. Counting efficiency was determined as described in Materials and methods. Stoichiometry was derived from the counting efficiency and definitions; Avogadro's number = 6.022×10^{23} mol^{-1} and 1 μCi = 2.22×10^6 dpm. Because mass spectrometry indicated that [^{3}H]BG consisted of a mixture of unlabeled, ^{3}H-mono-labeled, ^{3}H-di-labeled, and ^{3}H-tri-labeled materials, ^{3}H was placed in the center of the benzene ring to indicate that ^{3}H was located in the benzene ring of O^6-BG.

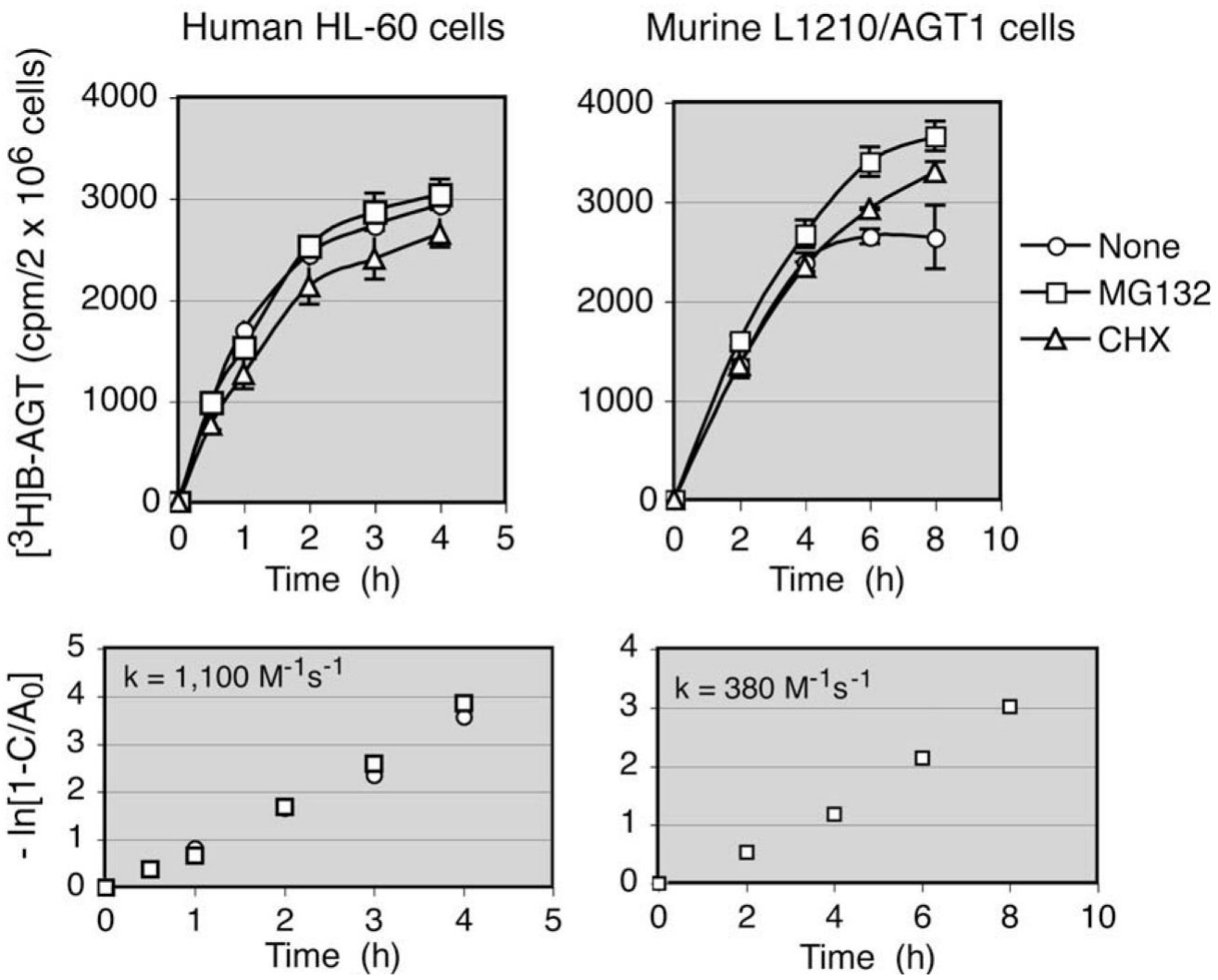

Fig. 2.

Rate of [^{3}H]benzyl transfer to AGT in intact HL-60 and L1210/AGT1 cells. Cells (2×10^6 cells/100 μl) were incubated with 1 μCi of [^{3}H]BG in the absence or presence of 20 μM MG132 or 20 μM CHX for the indicated periods of time, and radioactivity in a 70% methanol-insoluble fraction was determined. Radioactivity in the presence of excess unlabeled O^6-BG was subtracted from the total radioactivity. The bottom panels show the plots used to determine the second-order kinetic constant (*k*).

A

Addition	[^{3}H]B-AGT cpm/2 x 10^6 cells (%)
None	2,360±150 (100)
Guanine	2,370 ± 48 (100)
2-Amino-6-chloropurine	2,380 ± 77 (101)
Hypoxanthine	2,400±12 (102)
O^6-(Thiophenemethyl)guanine	6 (0)

B

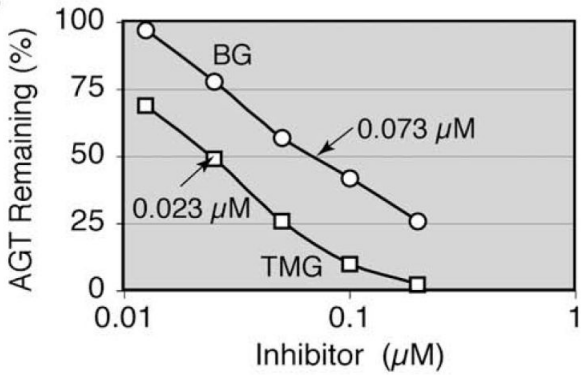

Fig. 3.

Effects of various purines on the reaction of O^6-BG with AGT. (A) HL-60 cells were incubated with [^{3}H]BG in the absence or presence of 150 μM various purines for 2 h. TMG was synthesized in our laboratory and dissolved in Me_2SO at a concentration of 20 mM. (B) HL-60 cells were exposed to O^6-BG or TMG for 1 h, followed by the AGT assay for 1 h, to measure remaining AGT activity.

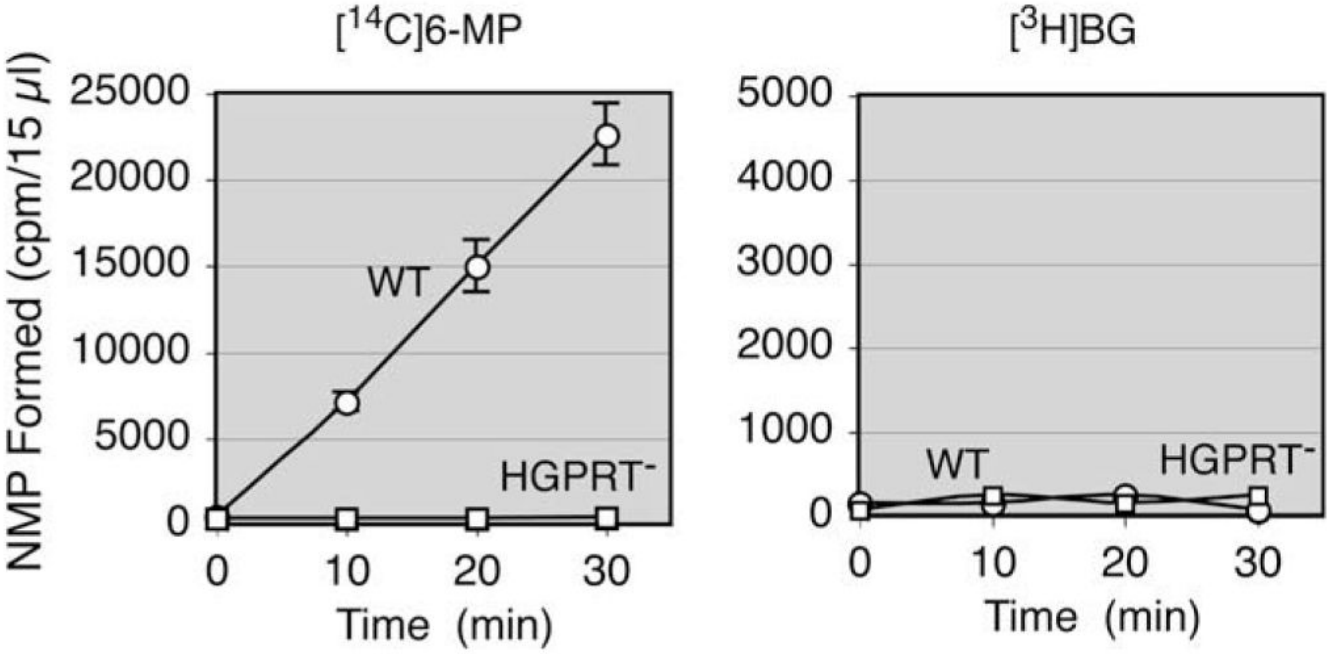

Fig. 4.

Inability of O^6-BG to serve as a substrate for hypoxanthine—guanine phosphoribosyltransferase. Cytoplasmic extracts (120 μg of protein) from wild-type (WT) and HGPRT⁻F-MEL cells were incubated with 0.2 mM [^{14}C]6-MP or [^{3}H]BG at a radioactive concentration of 2 μCi/ml and 2 mM 5-phosphoribosyl-1-pyrophosphate in a volume of 200 μl. NMP, nucleoside monophosphate.

Temp (°C)	(cpm/2 x 10^6 cells) Total	None-specific
4	282 ± 9	220 ± 26
24	870 ± 62	293 ± 26
37	1,610 ± 2	308 ± 3
45	1,780 ± 40	400 ± 19
55	1,050 ± 21	773 ± 25
65	1,280 ± 21	1,120 ± 55

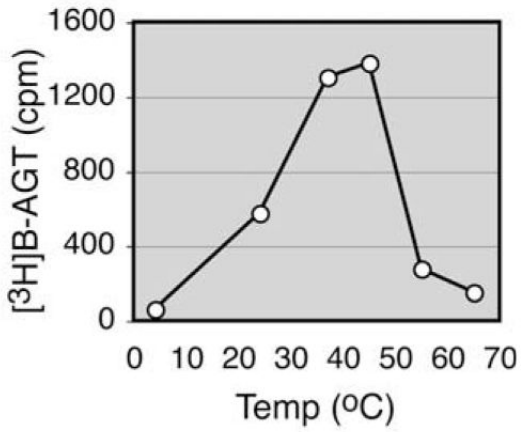

Fig. 5.

Optimal temperature for AGT activity and temperature-dependent increase in nonspecific binding. Standard AGT assays using intact HL-60 cells were conducted at various temperatures (Temp) for 30 min.

A

Addition	[^{3}H]B-AGT (cpm/100 μl)
H_2O	3,030 ± 41
50 mM NaOAc-HOAc, pH 6.5	2,980 ±110
50 mM Tris-HCl, pH 7.5	4,140 ± 75
50 mM Tris-HCl, pH 9.0	3,760±130
1 mM DTT	3,720 ± 93
5 mM DTT	3,270 ±150
0.5 mM PMSF	2,410 ±110
1 mg/ml Aprotinin	2,340 ± 87
1 mg/ml Leupeptin	2,150 ±80
0.2 M NaCl	1,850 ± 82

B

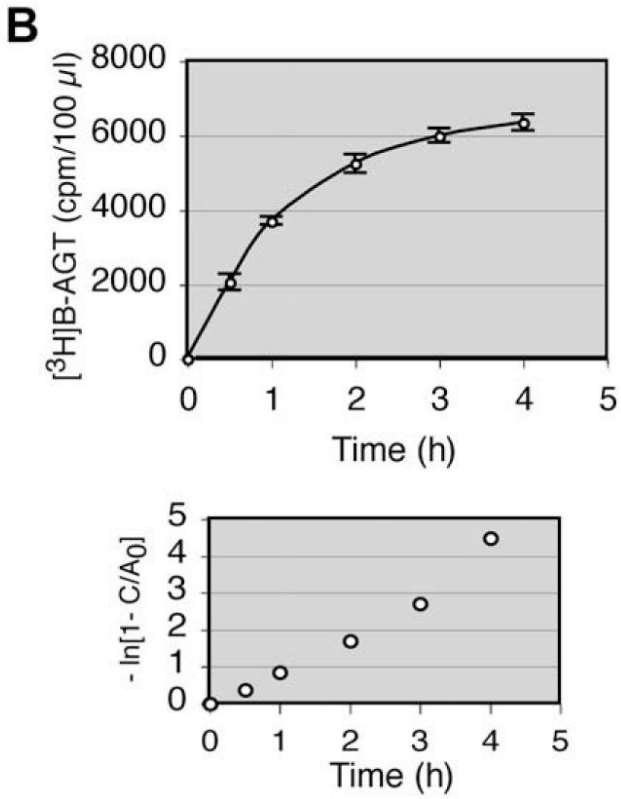

Fig. 6.

AGT assay using cell homogenates. (A) Effects of various ingredients on AGT activity were measured using HL-60 cell homogenates prepared in H_2O(5×10^6 cells/ 100 μl). PMSF, phenylmethylsulfonylfluoride. (B) Kinetics of [^{3}H]benzyl transfer using HL-60 cell homogenates (5×10^6 cells/100 μl) prepared in a buffer (pH 7.5) containing 1 mM DTT and plots to determine the second-order kinetic constant (*k*).

A

	(cpm/2 x 10^6 cells) Total	Non-specific	AGTs/cell
Intact cells			
$B_{16}F_{10}$	25,500 ± 250	3,330 ± 95	
+ 25 μM HQ	25,100 ± 300		
+ 100 μM HQ	25,800 ± 110		
+ 25 μM L-DOPA	25,900 ± 180		
Cell homogenates			
$B_{16}F_{10}$	3,260 ± 100	780 ± 8	
Human melanoma intact cells			
A2058	465 ± 38	446 ± 36	< 500
C32	394 ± 40	392 ± 5	< 500
G-361	2,830 ± 110	454 ± 5	14,000

B

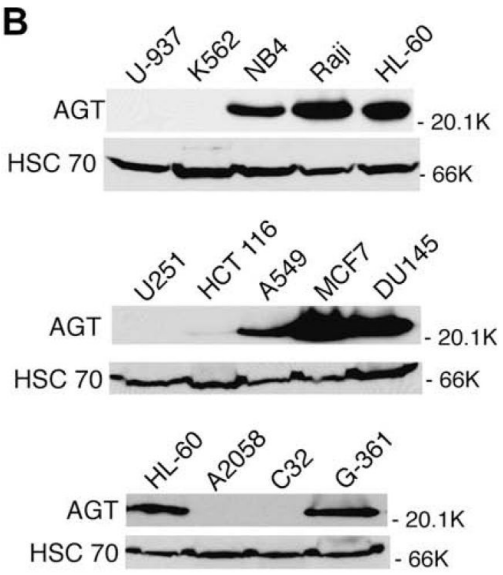

Fig. 7.

AGT assays conducted for various melanoma cell lines and the correlation between AGT levels measured by the AGT assay and Western blot analysis. (A) Standard AGT assays were performed for various human melanoma cell lines and for intact $B_{16}F_{10}$ cells in the presence of HQ or L-DOPA. HQ was dissolved in Me_2SO at a concentration of 20 mM. L-DOPA was dissolved in 0.001 N HCl at a concentration of 5 mM. (B) Whole cell extracts were prepared from various human cell lines and subjected to Western blot analysis for AGT using HSC 70 as a loading control. K, thousands (000) (far right of panel B).

Table 1

AGT levels in various human and rodent cell lines

Human AGT **$k = 1100\ M^{-1}\ s^{-1}$** **$A_0 = 1.2$ C (2 h incubation)**		**Murine AGT** **$k = 380\ M^{-1}\ s^{-1}$** **$A_0 = 2.2$ C (2 h incubation)**	
Human leukemia cell lines		Murine suspension cell lines	
U-937	<500	L1210	<500
TF-1	<500	P388	<500
K-562	<500	F-MEL	<500
NB4	8500	$HGPRT^-$ F-MEL	<500
CCRF-CEM	14,000	FDC-P1	<500
Jurkat	15,000	B6SUtA	1400
Raji	17,000	Ba/F3	3200
HL-60	17,000	WEHI-3B D^+	3400
Human carcinoma cell lines		Murine attached cell lines	
U251	<500	EMT6	<500
HCT 116	1500	NIH/3T3	2600
A427	1700	$B_{16}F_{10}$	N.M.
A549	6600		
Daoy	8800	Hamster attached cell line	
HeLa S3	22,000	CHO	<500
MCF7	42,000		
DU145	42,000	Rat attached cell line	
LNCaP	45,000	NRK	2900

Note. Values are AGT molecules/cell. AGT assays using the standard conditions were conducted for 2 h. The saturation levels (A_0) were calculated by multiplication of the experimental values (C) and the constant determined by k. N.M., not measurable due to erroneous incorporation of [^{3}H]BG.