Differential Degradation Rates of Inactivated Alkyltransferase in Blood Mononuclear Cells and Tumors of Patients after Treatment with O^6-Benzylguanine[1]

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ABSTRACT

O^6-Alkylguanine-DNA alkyltransferase (AGT) repairs O^6-alkylating DNA adducts generated by alkylating therapeutic agents. Therefore, AGT activity may be an important marker of tumor and normal tissue sensitivity to chemotherapeutic agents and a predictor for the success of chemotherapeutic regimens. It is rapidly inactivated by O^6-benzylguanine (BG) that mimics its substrates, O^6-methylguanine and O^6-chloroethylguanine DNA adducts. In a Phase I clinical trial, BG was given in increasing doses (from 10 to 120 mg/m^2) by 1-h infusion. We previously reported depletion of AGT activity, and in this report, we demonstrate the relationship between degradation of BG-inactivated AGT protein and the depletion of AGT activity in peripheral blood mononuclear cells (PBMCs) and tumor samples obtained by computed tomography-guided cutting needle biopsy from patients prior to BG and either 2 or 18 h after BG. In PBMCs, BG inactivated AGT activity by over 95–100% at the end of a 1-h infusion, and depletion was maintained for 18 h. In contrast, AGT protein remained almost unchanged for up to 18 h after BG, suggesting that inactivated AGT proteins remain immunoreactive and are not rapidly degraded in PBMCs. In patient tumor biopsies, AGT activity was depleted ~90% 2 h after BG. Tumor AGT protein levels were reduced to ~40% of pretreatment values when detected by either Western blot or immunohistochemistry staining. In tumor samples obtained 18 h after BG, >95% inactivation of tumor AGT activity was observed at BG doses of 36–80 mg/m^2, and complete depletion of tumor AGT activity occurred at 120 mg/m^2 BG. However, residual AGT protein (5–10% of baseline) was detectable in all tumor samples. Therefore, the degradation of BG-inactivated AGT protein appeared to be much more rapid in tumors than that in PBMCs, which may impact on AGT regeneration rates as well. Because degradation of BG-inactivated AGT takes place slowly, antibody-based measurements of AGT protein correlate poorly with depletion of AGT activity immediately after BG. Thus, biochemical activity measurements remain the appropriate monitor of AGT during therapeutic modulation. These data provide the first and conclusive evidence of differential degradation rates of inactivated AGT in PBMCs and tumors of patients after treatment with BG and suggest that immunoreactive AGT measurements in PBMCs are a poor surrogate for AGT activity in tumor tissue.

INTRODUCTION

AGT[3] is a ubiquitous DNA repair protein. It repairs DNA alkylating adducts at the O^6-position of guanine by a single reaction of transferring an alkyl group to an internal cysteine residue resulting in self-inactivation. This unique property renders AGT as an ideal target for biochemical modulation of antitumor drug resistance (1–4). BG, a direct substrate for AGT, is a potent inhibitor of human AGT (5–7). It has been consistently shown that BG sensitizes AGT$^+$ tumor cells (colon cancer, breast cancer, lung cancer, medulloblastoma, and glioblastoma) *in vitro* and in xenograft models to the cytotoxic effect of alkylating agents, including 1,3-bis(2-chloroethyl)-1-nitrosourea, chlorozotocin, clomesone, streptozotocin, procarbazine, dacarbazine, and temozolomide (8–11).

The therapeutic efficacy of using BG to inactivate AGT followed by 1,3-bis(2-chloroethyl)-1-nitrosourea has been evaluated in clinical trials (12). We reported on our clinical trial previously in which AGT activity was measured in PBMCs and in tumor biopsies of patients before and after escalating doses of BG. Baseline AGT activity showed marked intersubject variability (12). BG rapidly and completely inhibited AGT activity in both PBMCs and tumors. Higher doses of BG were required to inhibit AGT activity completely in tumor biopsy samples than in PBMCs. The depletion of AGT activity by BG in PBMCs correlated poorly with that in tumor tissue from the same patient.

The measurements of native AGT protein using immunoreactive analysis, such as Western blotting, IHC, and fluorescence-activated cell sorting, were thought to correlate closely

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[3] The abbreviations used are: AGT, alkyltransferase; BG, O^6-benzylguanine; PBMC, peripheral blood mononuclear cell; IHC, immunohistochemistry; MGMT, O^6-methylguanine-DNA methyltransferase; HPLC, high-performance liquid chromatography.

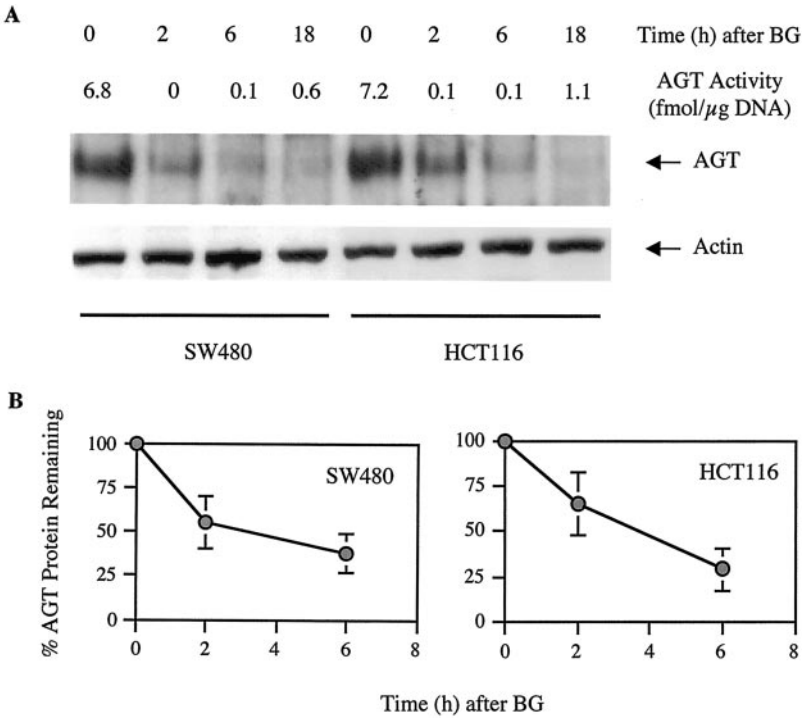

Fig. 1 *A*, the levels of AGT protein in colon cancer cell lines treated with 25 μM BG. *B*, the rate of degradation of BG-inactivated AGT proteins in colon cancer cell lines. Results represent the means from at least three independent experiments; *bars*, SD.

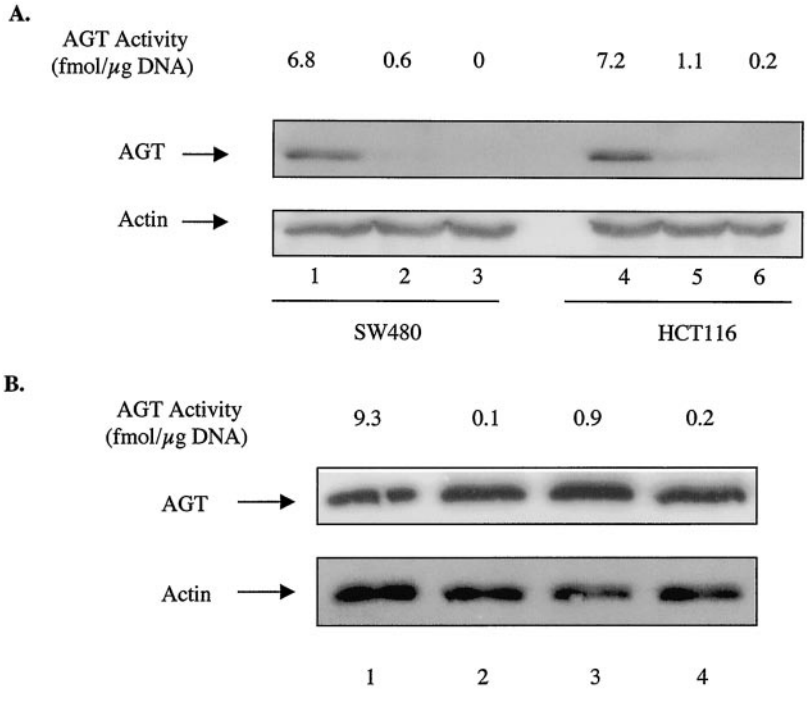

Fig. 2 *A*, the generation of AGT activity and protein after depletion by BG in colon cancer cells. *Lanes 1* and *4*, AGT protein levels in cells without treatment; *Lanes 2* and *5*, AGT protein level in cells 18 h after treatment with BG (25 μM for 2 h); *Lanes 3* and *6*, AGT protein levels in cells treated with cycloheximide (50 μg/ml) for 18 h after exposure to BG. *B*, comparison of AGT activity with protein after BG treatment in the human PBMC *in vitro* study. *Lane 1*, AGT activity and protein level in cells without treatment; *Lane 2*, AGT activity and protein level in cells treated with BG (25 μM) for 2 h; *Lane 3*, AGT activity and protein level in cells 18 h after treatment with BG; *Lane 4*, AGT activity and protein level in cells treated with cycloheximide (50 μg/ml) for 18 h after exposure to BG.

with AGT biochemical activity (13, 14). However, we have found that after treatment with BG *in vitro*, both AGT by fluorescence-activated cell sorting and Western blot assays failed to distinguish active form inactive protein (15). The protein levels did not reflect the levels of activity after inhibition of AGT by BG because of the slow rate of degradation of BG-inactivated AGT protein (15). Heretofore, there have been no previous studies correlating AGT activity with AGT protein levels in human tissues after treatment with BG and alkylating agents. Thus, it is unclear whether immunoreaction-based de-

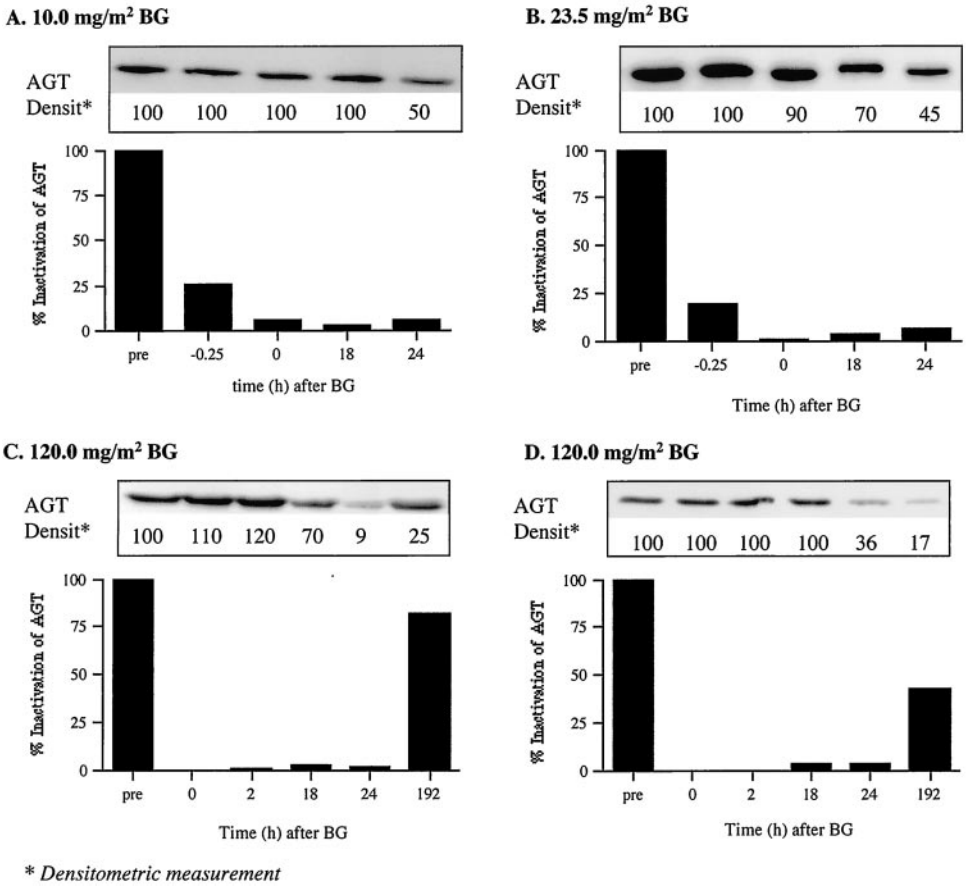

Fig. 3 Comparison of AGT activity (by HPLC method) and protein levels (by Western blot assay) in PBMCs of patients treated with different doses of BG. *A,* AGT activities and protein levels in PBMCs obtained from a patient treated with 10 mg/m^2 BG. *B,* AGT activities and protein levels in PBMCs obtained from a patient treated with 23.5 mg/m^2 BG. *C,* AGT activities and protein levels in PBMCs obtained from a patient treated with 120 mg/m^2 BG. *D,* AGT activities and protein levels in PBMCs obtained from a patient treated with 120 mg/m^2 BG.

tection assays of AGT protein accurately predict AGT activity in the clinical setting.

Using Western blot and IHC analyses of AGT protein and HPLC-based measurements of AGT enzymatic activity, we studied the correlation between the depletion of AGT activity and the disappearance of BG-inactivated AGT protein in both PBMCs and tumor biopsies taken from patients treated with BG in a Phase I clinical trial. The objectives of this study were to determine whether the depletion of AGT protein by BG in PBMCs predicts that in tumor tissue and whether detection of AGT protein after BG inactivation can be used as an indicator of biochemical AGT modulation.

MATERIALS AND METHODS

Measurement of AGT Activity in Tumor Tissue Biopsies and PBMCs

As described previously (12, 16), the activity of AGT in tumor tissue and PBMC extracts was measured as removal of the [^{3}H]methyl adduct from the O^6 position of guanine in DNA by incubating tissue extracts with DNA substrate. Briefly, substrate [^{3}H]methyl-DNA was prepared by reacting calf thymus DNA with [^{3}H]methylnitrosourea. The specific activity of [^{3}H]-methylated DNA was defined based on AGT activity. Calf thymus DNA served as a negative control, and a MGMT-transfectant cell extract was used to construct the standard curves.

Preparation of Tumor Tissue and PBMC Extracts. To prepare tumor tissue extracts, a small piece of tumor segment (about 2 × 3 mm) was placed in 250 μl of cell extract buffer [containing 70 mM HEPES (pH 7.8), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 25 μM spermidine] and sonicated three times for 10 s at 4°C to complete cell disruption using a microsonicator equipped with a 3/32-inch diameter probe (Branson Ultrasonics Corporation, Danbury, CT). PBMCs from whole blood samples were separated by Ficoll-Hypaque isopyknic gradient. The PBMC extract was prepared by the same procedure as described as above. Aliquots (20 μl) for DNA quantification were removed, and tumor tissue and PBMC extracts were centrifuged at 13,000 rpm for 2 min to remove cellular debris. Protein concentration was determined by Bio-Rad protein assay.

Enzyme Assay. Fifty to 250 μg of proteins from tumor tissue or PBMC extracts were incubated with [^{3}H]methyl DNA in cell extract buffer in the total volume of 300 μl for 1 h at 37°C. The reaction mixture contained excess substrate DNA, such that total AGT activity could be determined. The reaction was stopped with 7.5% trichloroacetic acid at 4°C for 30 min. The precipitate was collected by centrifugation at 13,000 rpm for 2 min and washed with 300 μl of 80% ethanol. Methylated purines were liberated from precipitated DNA during hydrolysis with 150 μl of 0.1 N HCl at 80°C for 1 h and then neutralized to pH 7 with 375 μl of 0.04 M Tris base. [^{3}H]O^6-methylguanine

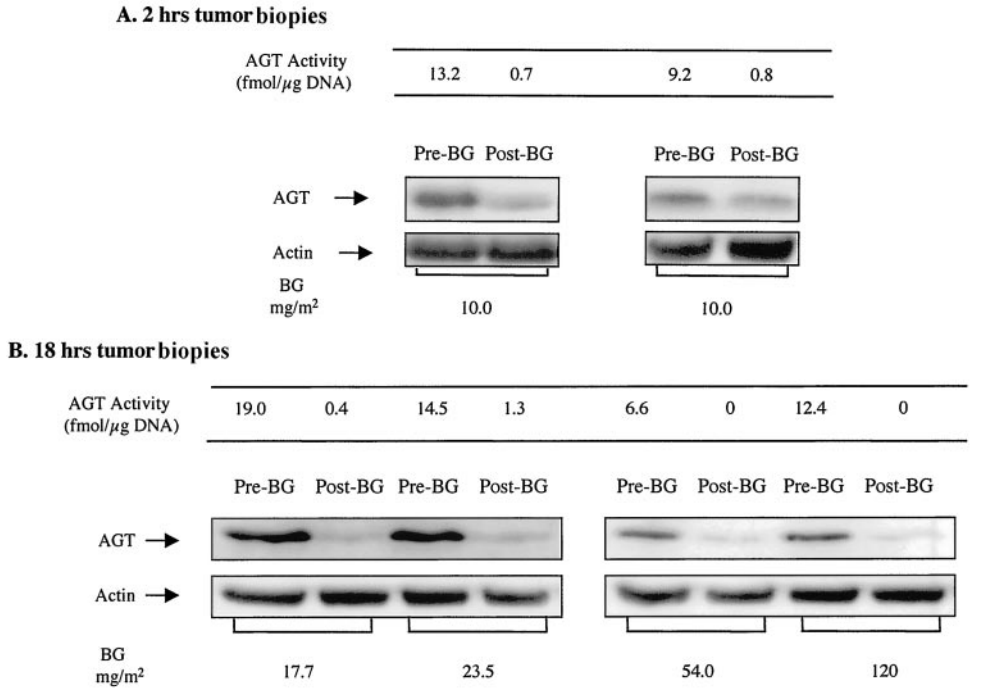

Fig. 4 Comparison of AGT activity (by HPLC method) and protein levels (by Western blot assay) in tumors of patients treated with different dose of BG. *A,* AGT activities and protein levels in tumor samples obtained from 2 patients at 2 h after BG treatment with doses of 10 mg/m^2. *B,* AGT activities and protein levels in tumor samples obtained from 4 patients at 18 h after BG treatment with doses of 17.7, 23.5, 54.0, and 120 mg/m^2, respectively.

and N^7-methylguanine (internal standard) present in the supernatant were separated by reverse-phase HPLC (12.5 cm and 5 μM Partisil column; Whatman, Inc., Clifton, NJ) with UV absorbance at 260 nm. The methylated DNA adducts were quantitated by liquid scintillation counting (16).

Western Blotting for AGT Protein

Aliquots (25 μg of protein quantitated by Bio-Rad Protein Assay) from the same samples, either tumor tissue or cell extracts that were analyzed for AGT activity assay, were resolved by SDS-PAGE gels (12% acrylamide) for AGT protein measurement. Proteins were transferred onto polyvinylidene difluoride membranes, using a Bio-Rad mini *Trans*-Blot cell for 1 h at 100 V. The blotted membranes were blocked with 5% dry milk in TBS buffer and then probed for 2 h with a monoclonal human MGMT antibody (mT 3.1; Kamiya Biomedical Co., Seattle, WA). After three 5-min washes with TBS-TW20, the blots were incubated with a secondary antibody, antimouse HRPO-anti IgG, for 1 h. Antibody binding was visualized by enhanced chemiluminescence according to the manufacturer's instructions (15).

AGT Immunohistochemistry

Tumor tissues were fixed in Carnoy (60% ethanol, 30% chloroform, and 10% acetone) for 90 min and then transferred to 70% ethanol. Paraffin-embedded sections were brought to water, and endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol. Sections were treated with 0.1% Triton X-100 in PBS for 10 min and incubated with the antihuman MGMT antibody mT 23.2 (kindly provided by Dr. T. P. Brent, St. Jude Children's Hospital, Memphis, TN) overnight at 4°C. After PBS washes, sections were incubated with the peroxidase-labeled goat antimouse antibodies for 1 h. The peroxidase reaction was developed with diaminobenzidine for 10 min. Omission of the primary antibody was performed as control (13).

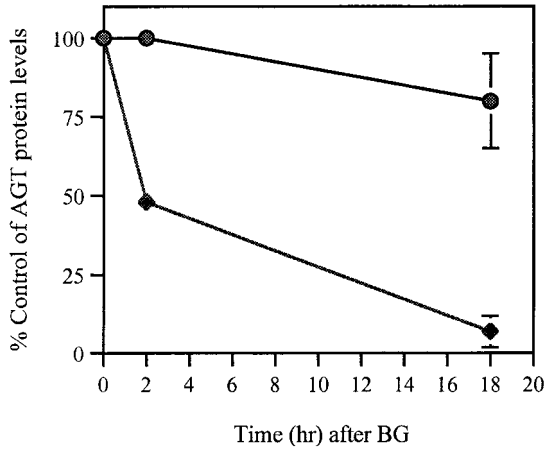

Fig. 5 Comparison of the rate of degradation of BG-inactivated AGT protein levels between PBMCs and tumors from patients treated with BG. ●, AGT proteins in PBMCs. Results are the mean value of PBMC AGT from 8 patients treated with different doses of BG; ◆, AGT proteins in tumors. Results at the 2-h time point are the mean values of tumor AGT from 3 patients treated with 10 mg/m^2 BG, whereas results at the 18-h time point are the mean values of tumor AGT protein from 8 patients treated with different doses of BG. *Bars,* SD.

RESULTS AND DISCUSSION

Determination of the Degradation Rate of AGT Protein after Inactivation by BG in Preclinical Studies. The preclinical studies were performed to determine whether the dif-

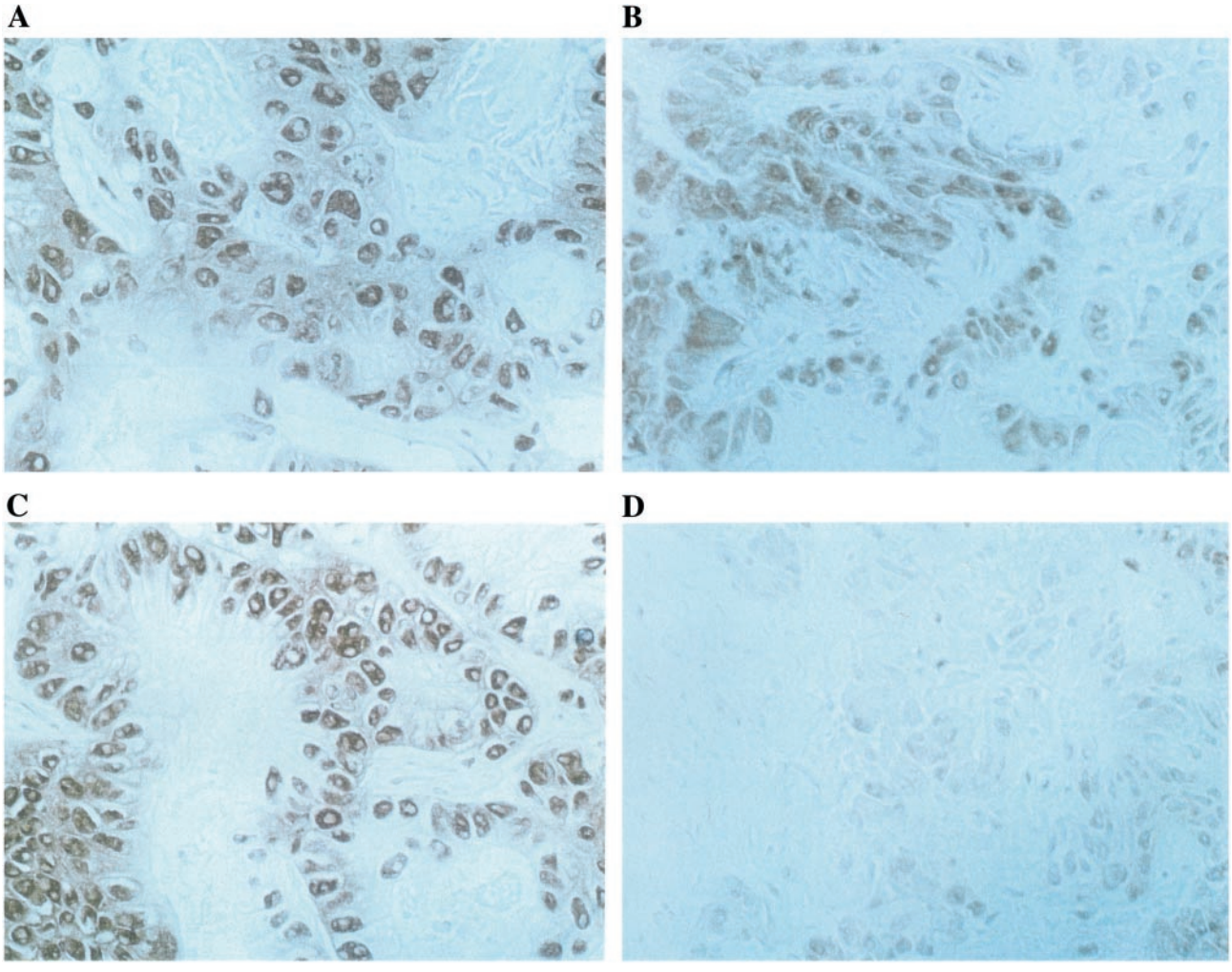

Fig. 6 Immunohistochemical staining for AGT expression in tumor biopsies obtained from patients before and after treatment with BG. *A*, BG pretreatment. *B*, 2 h after BG treatment. *C*, BG pretreatment. *D*, 18 h after BG treatment.

ferential rate of degradation of AGT proteins exhibits between human colon cancer cell lines and human PBMCs. We treated two colon cancer cell lines, SW480 and HCT116, with BG for 2 h at a concentration of 25 μM, equivalent to 6000 ng/ml. In clinical trials (12), BG elimination from plasma obeyed a two-compartment model with $t_{1/2}$ of 0.1 and 0.6 h. Plasma 8-oxo-BG, the major active metabolite of BG with similar AGT inactivation kinetics to BG, reached a maximum concentration of 5940 ng/ml 90 min after the end of BG infusion. Thus, we expect that the concentrations of BG used in *in vitro* studies have a similar effect on AGT inactivation and degradation to those observed *in vivo*. The two colon cancer cell lines had similar levels of AGT activity (6.8 fmol/μg DNA in SW480 and 7.2 fmol/μg DNA in HCT116, respectively) that were rapidly and completely inactivated by BG (limitation of AGT measurement is 0.1 fmol/μg DNA by HPLC; Fig. 1). The time-dependent degradation of BG-inactivated AGT protein was observed in these two cell lines (Fig. 1*A*). Half-life for the disappearance of inactivated-protein was 3 h (Fig. 1*B*). We also tested regeneration of AGT after BG treatment, as we then did with clinical samples. After BG treatment (for 2 h), cells were washed and incubated for 18 h in the presence or absence of cycloheximide (50 μg/ml) to inhibit protein synthesis. AGT activity recovered to 10–15% of the control levels 18 h after BG removal, whereas no AGT activity was detected in cells treated with cycloheximide (Fig. 2*A*). Similarly, Western blot showed that low levels of AGT protein were detected 18 h after BG (5–10% of control) in cells without cycloheximide treatment, but no AGT protein was seen 18 h after BG in cells treated with cycloheximide. This suggests that new AGT proteins were slowly synthesized during the 18-h time period after BG inactivation of AGT. In contrast, the intact AGT proteins in human PBMCs (Fig. 2*B*) remained until 18 h after BG depletion in samples treated with and without cycloheximide.

Comparison between AGT Protein and AGT Activity in Patient PBMCs before and after BG Treatment. We have reported previously that BG efficiently inactivates AGT activity in PBMCs in 30 patients who were treated with increasing doses of BG (12). To determine whether immunoreactive AGT protein levels can be used as a predictor of the effect of BG inactivation,

we compared AGT activity kinetics with the rate of AGT protein degradation in PBMCs of patients. Because the methods used for the measurement of AGT protein in this study are unable to distinguish BG-inactivated AGT protein from an active one, the protein level may reflect the sum of protein synthesis and degradation. Fig. 3 shows the activity and protein levels of AGT in PBMCs obtained from four different patients. At doses of 10 mg/m^2 (Fig. 3*A*) and 23.5 mg/m^2 (Fig. 3*B*), BG efficiently and rapidly inhibited AGT activity with >90% inactivation at the end of the 1-h infusion. Twenty-four h later, AGT activity began to recover. Similar results were observed in PBMCs from 2 patients treated with progressively higher doses of BG, up to 120 mg/m^2 (Fig. 3, *C* and *D*). At these doses, BG completely depleted AGT activity at the end of infusion (indicated as 0 h), and 2–4% of basal AGT activities was detected at 24 h after BG. By 8 days (192 h), AGT activities had recovered up to 40–80% of basal levels. In contrast, BG-inactivated AGT proteins (quantitated by densitometric measurement) were measurable in PBMCs throughout this time period in all tested PBMC samples but were inconsistent with data of AGT activity. At 18 h after treatment, inactivated AGT proteins remained almost intact in a BG dose-independent fashion, and at 24 h, detected AGT proteins were 40–50% of control levels after treatment with lower doses of BG (Fig. 1, *A* and *B*) and were 9–36% of control AGT protein levels (Fig. 1, *C* and *D*) at the highest doses of BG (120 mg/m^2). Thus, loss of protein was much slower than loss of AGT activity in PBMCs, and the degradation of inactivated AGT proteins by higher doses of BG appeared to be faster than that observed at lower BG doses. Whether the extent of BG binding to AGT results in a conformation change leading to ubiquitin-dependent proteolysis (17, 18) is suggested but not proven by these data.

AGT Activity *versus* AGT Protein in Patient Tumor Biopsies before and after BG Treatment. In tumor biopsies, 18 h after BG (dose range from 10 to 80 mg/m^2), >90% of basal AGT activity was inhibited, and complete depletion of AGT activity was observed in tumors of all patients treated with 120 mg/m^2 BG. As shown in Fig. 4, AGT protein was degraded to <10% control levels in all tested tumor biopsies (Fig. 4*B* shows four representative tumors of eight total tested tumor biopsies obtained 18-h after BG). In contrast, in tumor biopsies obtained 2 h after BG, 35–40% of intact AGT protein was detected (Fig. 4*A*), although <10% AGT activity was present. Although tumor biopsies were not performed at time points between 2 and 18 h, it is reasonable to assume a constant rate of protein degradation after inactivation and a much lower rate of protein synthesis, resulting in net loss of immunoreactive protein. Because of the fact that BG-induced total loss of immunoreactive AGT protein in human tumors was somewhat correlated with loss of AGT activity, we used the mean values of AGT protein levels in samples from patients treated with different doses of BG but obtained at either 2 or 18 h for tumors and multiple time points for PBMCs to construct crude decay curves for the rate of degradation of AGT proteins in tumors compared with that in PBMCs. We pooled data from different BG doses to develop median effect curves. This was reasonable because in tumor tissues, the rate of degradation of BG-inactivated AGT protein was dependent on time course not BG dose, whereas very stable AGT protein levels were observed up to 18 h in PBMCs from all patients treated with different doses of BG. The half-life ($t_{1/2}$) for degradation of BG-inactivated AGT protein (Fig. 5) was ≈2 h in tumors ($n = 3$ for biopsy samples collected 2 h after BG and $n = 8$ for biopsy samples collected 18 h after BG), compared with greater than 20 h in PBMCs ($n = 8$; $P < 0.01$). Thus, degradation of BG-AGT protein was faster in tumor tissues than in PBMCs of patients receiving BG.

AGT protein in tumors was also analyzed by IHC. The tumor tissues from untreated patients expressed high levels of AGT (Fig. 6, *A* and *C*). Tumor biopsy obtained 2 h after BG showed a reduced AGT protein staining compared with untreated tumor sample from the same patient (Fig. 6, *A* and *B*). AGT expression was diminished significantly in the tumor from the patient 18 h after treatment with BG, but residual protein was still detected (Fig. 6, *C* and *D*). These results were consistent with those obtained by Western blotting analysis.

In the present studies, we show from the results of both preclinical and clinical samples that AGT activity was rapidly lost after BG in both PBMCs and tumors but that new AGT protein was slowly generated in PBMCs compared with tumors. These observations are consistent with those of our previous studies (11, 18). It has been suggested that the degradation of the consumed AGT proteins provide a signal for the synthesis of additional protein (4, 14). Thus, the differential regeneration of AGT protein between human tumors and PBMCs may result from the differences in proteolysis of inactivated AGT protein in these two tissues (14, 17). Because inactivated AGT protein is targeted for polyubiquitination and subsequent degradation by the ubiquitin-proteasome pathway (17) and because AGT phosphorylation may function to down-regulate or inactivate AGT (19), it is possible that AGT phosphorylation is linked with ubiquitination and enhances protein degradation (20, 21). Thus, tumor cells may have more active ubiquitin-dependent proteasome, kinases, and phosphatases compared with PBMCs. The more rapid AGT protein degradation and regeneration in human tumor tissues compared with PBMCs may indicate that tumors are at an inherent advantage in synthesis of AGT resulting in drug resistance. Furthermore, slow resynthesis of AGT in resting PBMCs (18) suggests that the rate of AGT degradation and synthesis may be also related to cellular proliferation.

In summary, in this study, we compared biochemical modulation of AGT by BG in patient tissues using an AGT activity assay and antibody-mediated methods for detection of AGT protein. In a BG clinical Phase I trial, we have found, for the first time, that there is differential depletion and regeneration of AGT between PBMCs and tumor tissues of patients treated with BG. The data also provide important information regarding the evaluation of PBMC AGT as a marker of tumor AGT and as a pharmacodynamic end point. AGT activity assays appear much more reliable than immunodetection methods.

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