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O^6-3-[^{125}I]iodobenzyl-2′-deoxyguanosine ([^{125}I]IBdG): synthesis and evaluation of its usefulness as an agent for quantification of alkylguanine-DNA alkyltransferase (AGT)

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Abstract—The development of O^6-(3-[^{125}I]iodobenzyl)-2′-deoxyguanosine ([^{125}I]IBdG), the glycosylated analogue of the O^6-3-iodobenzylguanine (IBG), as an agent for the in vivo mapping of the DNA repair protein alkylguanine-DNA alkyltransferase (AGT) is described. Synthesis of its tin precursor, O^6-3-trimethylstannylbenzyl-2′-deoxyguanosine (TBdG) was achieved in four steps from deoxyguanosine. Radioiodination of TBdG in a single step gave [^{125}I]IBdG in 70–85% isolated radiochemical yield. [^{125}I]IBdG bound specifically to pure AGT with an IC_{50} of 7.1 μM. From paired-label assays, [^{125}I]IBdG showed a 2- to 3-fold higher cellular uptake than [^{131}I]IBG in DAOY medulloblastoma, TE-671 rhabdomyosarcoma, SK-Mel-28 melanoma, and HT-29 colon carcinoma human cell lines. Uptake of both labeled compounds in these cell lines decreased with increasing concentrations of unlabeled O^6-benzylguanine (BG) when BG was present in the medium during incubation with the labeled compounds. Compared to BG, unlabeled IBdG diminished the uptake of [^{125}I]IBdG and [^{131}I]IBG in DAOY cells more efficiently (IC_{50} < 1 μM vs >10 μM for BG). There was no significant change in cell-bound activity of [^{125}I]IBdG and [^{131}I]IBG when BG was removed from the incubation medium before incubating cells with the tracers, suggesting that only a very small portion of radioactivity taken up by the cells is AGT bound. This was corroborated by gel-electrophoresis performed on extracts from cells treated with varying amounts of BG and then incubated with [^{125}I]IBdG in the presence of BG. No radiolabeled AGT band was discernable by phosphor-imaging, signifying low cellular AGT binding of the radiotracer. In contrast, when cell extracts were prepared from BG pre-treated cells and aliquots were incubated with [^{125}I]IBdG subsequently, the intensity of radiolabeled AGT band decreased linearly as a function of BG concentration. This suggests that the low level of [^{125}I]IBdG that binds to AGT does so in a concentration dependent manner. These data suggest that IBdG is transported across the cell membrane to a higher degree than IBG. However, to be a practical tracer for quantifying cellular AGT, considerable localization of such derivatives need to occur within the cell nucleus where AGT is present predominantly.

1. Introduction

Chemotherapeutic alkylating agents such as alkylnitrosoureas and alkyltriazenes exhibit sufficient anti-tumor activity to be used clinically for the chemotherapy of several types of cancers including glioma, lymphoma, melanoma, myeloma, and small cell lung cancers.[1–3] Their cytotoxicity arises mainly from the alkylation of O^6-position of the guanine residues in DNA, which leads to disruption of the cell cycle due to the formation of lethal DNA–DNA interstrand crosslinks.[4] It is known that as few as ten lesions are sufficient for cytotoxicity.[5]

However, failure of these treatment regimens is common and has been attributed principally to the resistance developed by tumor cells to such chemotherapeutics. The resistance is caused by the repair of the DNA alkyl lesions. A number of repair mechanisms exist in cells to reverse the consequences of naturally occurring cytotoxic and genotoxic mutations of the DNA in cells. The 22 kDa protein O^6-alkylguanine-DNA alkyltransferase (AGT) is a protein that plays an important role in cell maintenance. In normal tissues, AGT mitigates alkylation DNA damage caused either endogenously or by environmental toxins. It effects this by a suicidal transfer of the alkyl group from the O^6-position of

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alkylated guanine residues on DNA to a cysteine moiety at position 145 on the binding site of the protein, thus regenerating native DNA.[6,7] Unfortunately, this process also attenuates the therapeutic efficacy of chemoalkylating agents in cancer cells by reversing their effect on DNA. The alkylated AGT undergoes rapid ubiquitination and degradation.[8] Cellular levels of AGT are restored only by de novo synthesis and thus modulation of AGT levels has emerged as a strategy for inhibiting this repair process. Although, chemoalkylator drugs themselves may be used to deplete cellular AGT levels prior to chemotherapy in order to enhance their therapeutic efficacy, it is disfavored since this can lead to myelosuppression.[9–13] Instead, the small molecule potentiator drug O^6-benzylguanine (BG) is used commonly to achieve this goal[1,4,14,15] because it reacts readily with AGT to transfer its benzyl group to the protein. Several clinical trials have been undertaken to evaluate the AGT modulating potential of BG.[16–18]

Cellular AGT levels vary among tumor types and even within a group of patients with the same type of malignancy. Prior knowledge of tumor AGT levels is thus a sine qua non for individualizing chemotherapy protocols. Current techniques to assay cellular AGT content, which use either in vitro or ex vivo methods,[19–21] are invasive because they require obtaining a tumor biopsy. We have sought to utilize the ready reactivity of BG to AGT to design a radiolabeled analogue of BG that may be used to quantify cellular AGT content in vivo noninvasively by scintigraphic methods.

Previously, we investigated radioiodinated IBG, the 3-iodo analogue of BG, as a potential AGT imaging agent in vivo.[22,23] IBG was chosen because of its close structural similarity to BG. Radiolabeled IBG was capable of specifically labeling pure AGT. Its specific uptake in three AGT-expressing cell lines was linear with increasing cell density. In vivo, [^{131}I]IBG administered intratumorally showed uptake specific to AGT content in a TE-671 rhabdomyosarcoma xenograft model. However, both in vitro and in vivo results showed high nonspecific uptake of IBG.[23]

While BG is a good inactivator of AGT, a confounding problem is its low water solubility. Its sugar derivative, O^6-benzyl-2′-deoxyguanosine (dBG), is more water soluble and although it is 10-fold less potent than BG in inactivating AGT in vitro, its AGT modulating ability is equal to, or even better than, BG in vivo.[24,25] Compared with BG, dBG also has been shown to be relatively resistant to metabolism.[24] Based on these facts, and with the objective of creating a less lipophilic analogue of BG, we have embarked on the synthesis of the nucleoside analogue of IBG—namely, O^6-3-iodobenzyl-2′-deoxyguanosine (IBdG; Chart 1), and evaluated the potential usefulness of radioiodinated IBdG as an agent for imaging AGT.

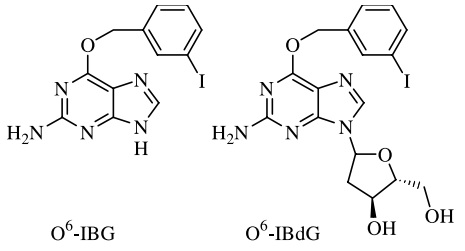
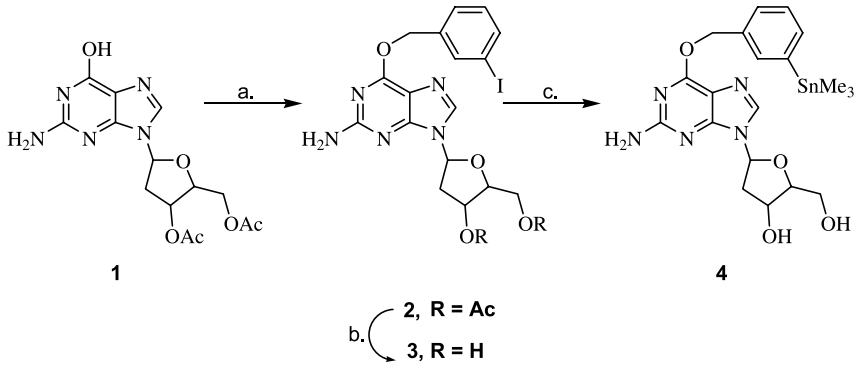


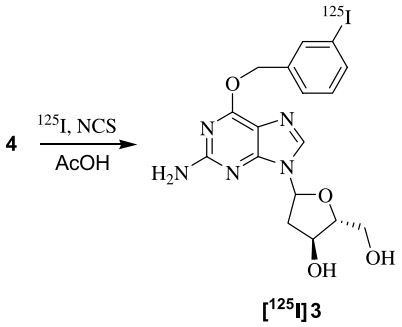
Chart 1.

2. Results

Commercially available 2′-deoxyguanosine (dG) was chosen as the starting point for the synthesis of IBdG (Scheme 1). The 3′,5′-diacetylation of dG was accomplished in quantitative yield by reaction with an excess of acetic anhydride, triethylamine, and catalytic DMAP in acetonitrile to give **1**.[26] Following the Mitsunobu protocol, 3-iodobenzyl alcohol was reacted with **1** in refluxing dioxane affording 3′,5′-diacetyl-IBdG ($(AcO)_2$-IBdG, **2**) in 50% yield. Because **2** elutes closely with TPPO, a by-product of the Mitsunobu reaction, a tedious chromatographic purification was required. Use of polymer-supported TPP obviated the need to remove TPPO from the product mixture, leading to very clean separation of **2**. Quantitative deprotection of the acetyl protecting groups of **2** to yield **3** was achieved by treatment with guanidine/guanidinium hydrochloride.[27] The trimethylstannyl analogue of **3**, namely O^6-3-trimethylstannylbenzyl-2′-deoxy-guanosine (TBdG, **4**), was obtained by a Pd-catalyzed Stille coupling of **3**



Scheme 1. Reagents and conditions: (a) 3-Iodobenzyl alcohol, TPP, DIAD; (b) guanidinium hydrochloride; (c) hexamethylditin, $Pd(TPP)_2Cl_2$.



Scheme 2.

with hexamethylditin. Radioiodination of **4** was accomplished by using *N*-chlorosuccinimide (NCS) in AcOH to give n.c.a. [^{125}I]**3** in 70–85% radiochemical yield (Scheme 2). It was possible to store [^{125}I]**3** in MeOH at −20 °C under argon for more than one month without discernable decomposition.

A binding assay of [^{125}I]IBdG and [^{131}I]IBG was performed in paired-label format in order to compare the ability of [^{125}I]IBdG to react with pure AGT with that of [^{131}I]IBG.[22,23] Different concentrations of BG were used to variably inactivate AGT. As shown in Figure 1, the binding of both [^{125}I]IBdG and [^{131}I]IBG to AGT decreased with increasing BG concentration. The percent of input dose that was bound to AGT was substantially higher for [^{131}I]IBG compared to that for [^{125}I]IBdG. This result is concordant with the observation that dBG is much less potent than BG in inactivating AGT.[6] The IC_{50} values of BG in reducing the binding of the tracers to AGT were determined to be 3.3 μM for [^{131}I]IBG and 1.4 μM for [^{125}I]IBdG. Approximately 10–15% non-specific binding (to BSA) of both tracers was also noticed. A similar trend was evident when unlabeled IBdG was used in lieu of BG to deplete AGT, in a single label experiment using just [^{125}I]IBdG (Fig. 2). The percent of [^{125}I]IBdG bound to AGT increased at 0.1 μM IBdG, owing possibly to kinetic factors, and thereafter declined steadily with increasing concentrations of unlabeled IBdG. The IC_{50} value for the reduction of [^{125}I]IBdG binding to AGT by unlabeled IBdG was 7.1 μM, which as expected was higher than that determined for BG in the earlier paired-label experiment.

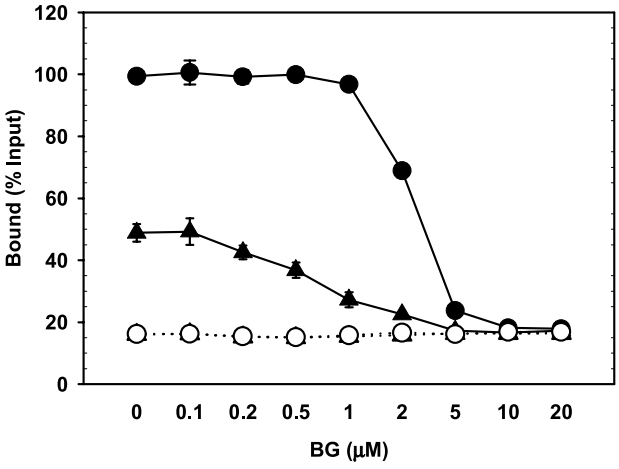


Figure 1. Paired-label binding of [^{125}I]IBdG and [^{131}I]IBG to purified AGT as a function of BG concentration. [^{125}I]IBdG (▲) and [^{131}I]IBG (●) were incubated for 30 min at 37 °C with 10 μg each of either AGT (closed) or BSA (open) (as control for non-specific binding), in the presence of increasing amounts of unlabeled BG (0–100 μM), in a Tris-buffer. The protein associated activity was determined by TCA precipitation.

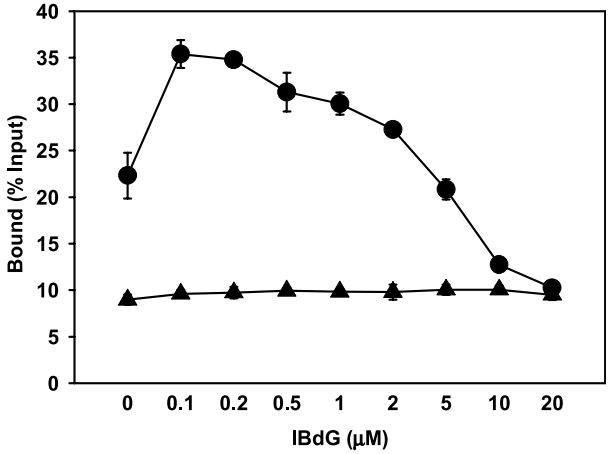


Figure 2. Binding of [^{125}I]IBdG to purified AGT as a function of unlabeled IBdG concentration. [^{125}I]IBdG was incubated for 30 min at 37 °C with 10 μg each of either AGT (●) or BSA (▲), in the presence of increasing amounts of unlabeled IBdG (0–100 μM), in a Tris-buffer. The protein associated activity was determined by TCA precipitation.

Next, the ability of [^{125}I]IBdG to bind cellular AGT was determined. Several different cell lines which express AGT were chosen for this study. The human medulloblastoma cell line DAOY, which reportedly expresses ca. 350 fmol of AGT/mg of protein,[28] was first studied. A paired-label cellular uptake experiment of [^{125}I]IBdG and [^{131}I]IBG was performed where the cells were preincubated for 4 h with varying concentrations of BG (0–100 μM) before adding the tracers. The results are expressed as the percentage of total radioactivity that is cell associated as a function of BG concentration (Fig. 3A). The uptake of both tracers decreased with increasing BG concentration, suggesting that their uptake may be related to the AGT content. Furthermore, the cellular uptake of [^{125}I]IBdG was 3-fold that of [^{125}I]IBG, and this ratio remained constant throughout almost the entire range of BG concentrations that was studied. A similar paired-label uptake assay was performed in the TE-671 human rhabdomyosarcoma cell line whose AGT content is reported to be ca. 100 fmol/mg protein.[29] As seen with DAOY cells, the uptake of both tracers also decreased with increasing concentrations of BG suggesting a possible correlation between uptake and cellular AGT levels (Fig. 3B). Again, the uptake of [^{125}I]IBdG was nearly 3-fold that of [^{131}I]IBG at 0 μM BG concentration, and the ratio remained nearly constant over various BG concentrations. The experiment was repeated with SK-Mel-28 and HT-29 cell lines and the results are shown in Figure 3C and 3D. These cells also

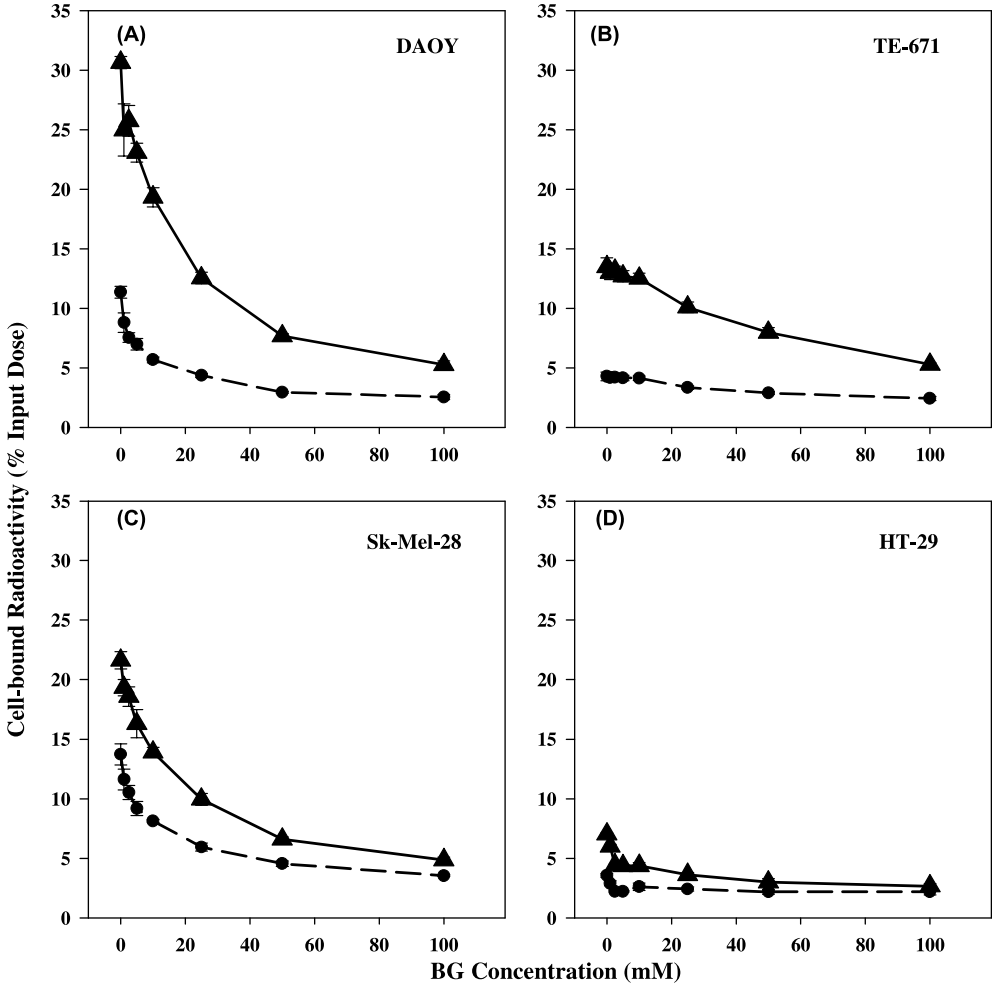


Figure 3. Paired-label cellular uptake of [^{125}I]IBdG (▲) and [^{131}I]IBG (●) as a function of BG concentration. (A) DAOY, (B) TE-671, (C) SK-Mel-28, and (D) HT-29 cells were incubated with 0–100 μM BG for 4 h, and then with ca. 100 nCi each of [^{125}I]IBdG and [^{131}I]IBG for 2 h, and the cell-bound activity was determined. Results are expressed as the percentage of input dose that was cell-bound.

demonstrated a decrease in tracer uptake with increasing BG concentrations. The difference in uptake between [^{125}I]IBdG and [^{131}I]IBG, however, was not quite as pronounced in these cell lines. Nevertheless, between a 1- and 2-fold higher uptake of [^{125}I]IBdG over [^{131}I]IBG was noticed throughout.

Parallel paired-label experiments were conducted in order to study the effect of BG in the medium on the cellular uptake of the radiotracers. Two sets of DAOY cells were pre-incubated with 0–100 μM BG for 4 h. In one set, the medium containing BG was removed, fresh medium was added, and the cells were incubated with 100 nCi each of [^{125}I]IBdG and [^{131}I]IBG for 2 h. In the second set, the medium containing BG was not removed prior to incubation with the radiotracers. As shown in Figure 4, DAOY cells incubated with the radiotracers in medium containing BG displayed the highest [^{125}I]IBdG and [^{131}I]IBG uptake at 0 μM BG, and this uptake decreased with increasing BG concentration, consistent with the observation in the prior experiments. In contrast, when BG was removed from the medium and replaced with fresh BG-free medium, the cell-bound radioactivity of [^{125}I]IBdG and [^{131}I]IBG remained virtually unchanged over the various BG concentrations. The uptake of both [^{125}I]IBdG and [^{131}I]IBG was similar at zero BG concentration in the two protocols suggesting that the replenishment of medium had no effect on tracer uptake.

Another paired-label uptake assay was conducted in DAOY cells using unlabeled IBdG (0–100 μM), instead of BG, to deplete AGT. In this case, the percent uptake of both tracers decreased far more rapidly with increasing IBdG concentration (Fig. 5) than that seen when BG was employed as the AGT inactivator. The cellular uptake of [^{125}I]IBdG decreased from 29.03 ± 0.74% to 7.16 ± 0.47% between 0 and 1 μM unlabeled IBdG concentration. And the uptake of [^{131}I]IBG dropped from 10.21 ± 0.35% to 2.71 ± 0.24% for the same IBdG concentrations. The IC_{50} values for reduction in [^{125}I]IBdG and [^{131}I]IBG binding were <1 μM each.

To determine whether the decreasing uptake of these tracers with increasing BG concentrations was related to a reduction in cellular AGT, gel electrophoresis of cell extracts from all four cell lines that were incubated with [^{125}I]IBdG was performed. Initially, cells that were

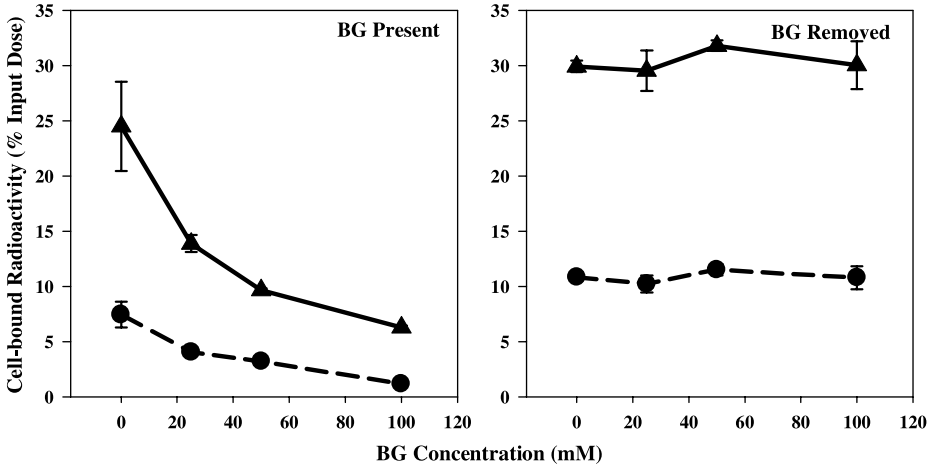


Figure 4. Effect of removal of BG-containing medium after the initial incubation on the cellular uptake of [^{125}I]IBdG (▲) and [^{131}I]IBG (●) in DAOY cells. Two sets of DAOY cells were incubated with 0–100 μM unlabeled BG for 4 h. From one set, BG-containing medium was removed and replaced with fresh BG-free medium, while the medium from the other set was not removed. The cells were subsequently incubated with ca. 100 nCi each of [^{125}I]IBdG and [^{131}I]IBG for 2 h, and the cell-bound activity was determined. Results are expressed as the percentage of input dose that was cell-bound.

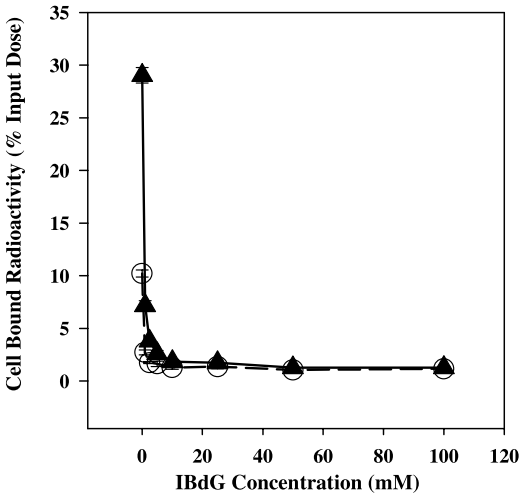


Figure 5. Paired-label cellular uptake assay of [^{125}I]IBdG (▲) and [^{131}I]IBG (○) in DAOY cells as a function of IBdG concentration. DAOY cells were incubated with 0–100 μM unlabeled IBdG for 4 h, and then with ca. 100 nCi of [^{125}I]IBdG for 2 h, and the cell-bound activity was determined. Results are expressed as the percentage of input dose that was cell-bound.

pre-treated with varying BG concentrations for 4 h were incubated with up to 25 μCi of [^{125}I]IBdG for 2 h. Cell extracts (1 mL) were made and 40 μL of these extracts were subjected to gel electrophoresis. However, even when 25 μCi of [^{125}I]IBdG was employed, no discernable radiolabeled AGT band was observed using autoradiography. Therefore, the cells were treated first with various concentrations of BG for 4 h from which 1 mL of cell extracts were made. A 100 μL portion of the extract was incubated with [^{125}I]IBdG for 2 h, and a 40 μL aliquot of this was electrophoresed on a non-reducing gel along with ^{14}C-labeled protein MW markers and radioiodinated AGT. The autoradiographs obtained by phosphor imaging are shown in Figure 6A–D. In all cases, the intensity of the band corresponding to AGT decreased with increasing concentrations of BG suggesting that the uptake of the tracers reflected the AGT content of the cells. Except in the case of HT-29 cells, only one major band corresponding to AGT was seen; HT-29 cells exhibited visible levels of binding to proteins other than AGT (Fig. 6D). Lack of labeling of proteins other than AGT by a tritium-labeled BG analogue in cells lacking AGT has been reported.[30] The BG concentrations used for this assay (0–5 μM) were substantially lower than that used for the cellular uptake assay (0–100 μM). Use of smaller concentration range was necessitated by the inability to visualize AGT bands when higher than 5 μM BG was used.

3. Discussion

Our primary goal is to develop a means of in vivo mapping of cellular AGT content in tumors. Previously, we have developed two radiolabeled derivatives of BG—O^6-(4-[^{18}F]fluorobenzyl)guanine ([^{18}F]FBG) and [^{131}I]IBG—and have evaluated their potential as AGT imaging agents.[22,23] Both of these agents demonstrated an ability to radiolabel AGT when they were incubated with the pure protein. Due to the ease of its synthesis, [^{131}I]IBG was selected for further evaluation using cell lines and xenograft models. It was observed that [^{131}I]IBG had a rather high non-specific uptake in vivo, presumably due to its lipophilicity. Although BG is used as an AGT modulator in phase I and II clinical trials,[16–18] its high lipophilicity is a major drawback. The nucleoside analogue of BG, O^6-benzyl-2′-deoxybenzyl (dBG), has been investigated as an alternative to overcome this issue.[24,25] It has been shown that dBG is 10-fold less potent than BG in inactivating AGT in cells and cell-free extracts.[31–33] However, in xenograft models, dBG is equally or even slightly more potent than BG.[24] This has been attributed to the fact that the cumulative plasma concentration of BG from dBG is higher than that obtained by administering BG itself. The

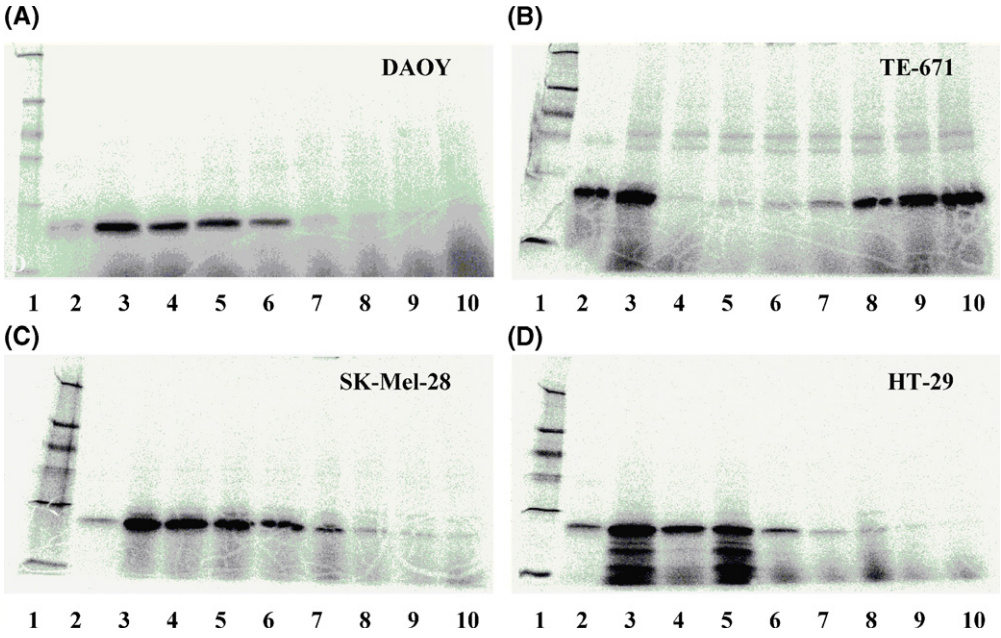


Figure 6. Cellular AGT labeling capacity of [^{125}I]IBdG as a function of BG concentration. (A) DAOY, (B) TE-671, (C) SK-MEL-28, (D) HT-29 cells. Cells were pre-incubated with none or 1–5 μM BG for 4 h. Cell extracts were prepared, and an aliquot was incubated with radiotracer for 2 h at 37 °C. A portion of this was electrophoresed on a non-reducing gel and the dried gel was subjected to phosphor imaging. For A–D, lanes 1 and 2 are C-14 standards and labeled AGT, respectively. For A, C, and D, the concentration of BG (0–5 μM) increased from left to right; for B, it decreased from left to right.

deoxyguanosine derivative metabolizes to BG, although only 20% of its metabolism follows this pathway.[32] Based on these findings, we embarked on the development of IBdG (**3**), anticipating that its non-specific binding may be lower than that of BG due to its potential higher hydrophilicity.

Unlabeled **3** was synthesized by a sequence of Mitsunobu coupling of 3-iodobenzyl alcohol with 3′,5′-diacetyl-2′-deoxyguanosine followed by deacetylation using guanidinium hydrochloride. The Mitsunobu reaction also was performed using polymer-bound TPP to avoid the cumbersome separation of the closely eluting by-product TPPO from **3**. Synthesis of 2′-deoxy-O^6-(3-trimethylstannylbenzyl)guanosine (TBdG, **4**)—the 3-trimethylstannyl precursor of **3**—was achieved by Pd-catalyzed trimethylstannylation of **3**. TPPO, arising from oxidation of the ligand TPP from the catalyst, confounded the clean isolation of **4**. This can be circumvented by stepwise stannylation of **2** followed by deacetylation of the product diacetyl-TBdG giving **4** (experimental details are not given). Radioiodination of **4** using NCS in acetic acid proceeded in high yields. The resulting no-carrier-added [^{125}I]**3** was isolated by column chromatographic separation on a reversed-phase HPLC column. This product was stored for reasonable periods of time in MeOH and exhibited no discernable deiodination or other decomposition. Nonetheless, [^{125}I]**3** was purified by either normal or reversed-phase chromatography and reconstituted in PBS, pH 7.4 just prior to its evaluation in biological assays.

It was possible to label AGT using [^{125}I]IBdG. However, the percent of input radioactivity bound to AGT was substantially lower than that seen for [^{131}I]IBG. This is consistent with the fact that BG is a better inactivator of AGT than is dBG. However, it should be pointed out that these assays were performed in the presence of calf thymus DNA. It has been shown that calf thymus DNA increases the rate of inactivation of AGT by BG.[6,30] On the other hand, it decreases this rate for some 9-substituted derivatives of BG, including dBG.[6] Our results seem to corroborate this finding. To further demonstrate this, an assay was done wherein the AGT was incubated with [^{125}I]IBdG in the presence and absence of calf thymus DNA (details are not given). The binding in the absence of DNA was 76.1 ± 1.7% compared to 62.2 ± 1.3% ($p < 0.05$) in the presence of DNA suggesting that DNA clearly negatively influenced the ability of alkyl transfer to pure AGT in the case of the nucleoside analogue, albeit to a lesser extent than that reported in the literature for unlabeled BG and its analogues.

To determine if the cellular uptake of radiolabeled IBdG was influenced by the amount of AGT in the cells, assays were performed wherein the uptake of [^{125}I]IBdG was determined (along with that of [^{131}I]IBG) in four different human tumor cell lines which were variably depleted of their AGT using BG. In all four cell lines shown (Fig. 3A–D), cellular uptake of [^{125}I]IBdG in untreated cells was between 2- and 3-fold higher than that of [^{131}I]IBG. This observation is opposite to that seen earlier with respect to the ability of these tracers to bind to pure AGT. Multiple factors may contribute to this observation, with improved transport of the sugar analogue across the cell membrane being the most likely cause.[34] Intuitively, IBdG is expected to be less lipophilic than IBG due to the presence of hydrophilic sugar moiety in its structure. The log *P* values calculated from

the octanol–water partition experiment were 2.2 ± 0.2 and 1.4 ± 0.1 for [^{125}I]IBG and [^{131}I]IBdG, respectively, suggesting that this is indeed the case. Therefore, if transport across cell membrane is exclusively via diffusion, IBG should have a higher uptake than IBdG. The opposite result that we observed suggests that an active transporter may be involved. Several transporters do exist that actively transport nucleosides and nucleobases[35–37] and one or more of these may be involved in the cellular transport of IBG and IBdG. It has been well established that BG can deplete AGT from cells in a concentration-dependent manner.[38,39] Thus, the decreasing uptake of the tracers with the increasing concentration of BG suggested that the uptake may be correlated to the AGT content of the cells.

To substantiate that this indeed was the case, gel electrophoresis of cell extracts was performed. Initially, extracts were made following incubation of BG-treated cells with the tracers. However, no significant radiotracer bound AGT bands were visible—even in cells untreated with BG—when using up to 25 μCi of [^{125}I]IBdG per flask ($\sim 10^7$ cells). To circumvent this, experiments were conducted whereby extracts were made from BG-treated cells first, followed by incubation of small aliquots of these extracts with [^{125}I]IBdG and evaluation by gel electrophoresis. Although this allowed for a more effective visualization of the AGT band, it was a less accurate representation because it excludes the role of pharmacodynamic factors, such as permeability or effect of transporters, which impinge on cell uptake and binding. The results from these experiments showed that the intensity of the AGT band decreased with increasing BG concentration, again suggesting that the cellular uptake of the tracers may be a reflection of their AGT content. However, when BG concentrations of greater than 5 μM were used, it was difficult to visualize the AGT band even under these conditions. This observation can be rationalized if one considers the effect of the presence of BG during the incubation of cells with the tracers. When BG was removed from the medium after the cells were pre-treated with it for 4 h, the uptake of the tracers did not vary as a function of the BG concentration originally added to the medium. Because cellular AGT is known to be depleted by BG in a concentration-dependent manner under the conditions used, this result leads us to suggest that the as yet unknown mechanism of radiolabeled IBG/IBdG uptake involves a transport process that can be inhibited by BG or its analogues (vide supra). From the results of Figure 4, it has to be inferred that only a very small portion of the tracers taken up by the cells binds to AGT generally present in the cell nucleus. About 2 nmol of BG per well is present when a BG concentration of 1 μM was used in the cell uptake assays. Even assuming 1000 fmol/mg of AGT in DAOY cells, the AGT in 5×10^5 cells used per well in our assay is about 400 fmol. That means only about 0.02% of the BG used needs to react with AGT to completely deplete it. If this argument can be applied to the labeled compounds as well, then the results of Figure 4 can be rationalized. An alternative and perhaps less probable explanation is the rapid regeneration of cellular AGT upon removal of BG. Although the problem of relatively rapid de novo replenishment of AGT on BG removal from the medium has been reported for VACO 6 colon cancer cells,[39] the $t_{1/2}$ for the AGT regeneration was 9 h. It remains to be determined whether the 2 h duration used for the incubation of cells with the tracers in our study was sufficient to regenerate AGT in DAOY cells. The higher uptake of [^{125}I]IBdG compared to [^{131}I]IBG in the four cell lines studied may be simply due to it being a better substrate for the putative transporter. This probably is also why unlabeled IBdG inhibited the cellular uptake of the labeled compounds more efficiently than did BG.

4. Conclusion

From the in vitro results obtained using different cell lines, [^{125}I]IBdG seemed to be a better agent than [^{131}I]IBG for labeling cellular AGT. However, it appears that only a small percent of the cellular uptake of these tracers is bound to AGT. Thus, to be successful AGT imaging agents, these molecules may have to be modified in such a way that they will be localized predominantly in the cell nucleus where AGT is present. Several approaches to achieve this goal are under way in our laboratory.

5. Experimental

5.1. General

All chemicals were purchased from Aldrich Chemical Company except where noted. Synthesis of O^6-(3-[^{125}I]iodobenzyl)guanine was performed as reported earlier.[23] Sodium [^{125}I]iodide (1200 Ci/mmol) and sodium [^{131}I]iodide (2200 Ci/mmol) in 0.1 N NaOH were supplied by Perkin–Elmer Biosciences (North Billerica, MA).

High pressure liquid chromatography (HPLC) was performed using a Beckman Gold HPLC system equipped with a Model 126 programmable solvent module, a Model 166 NM variable wavelength detector, a Model 170 radioisotope detector, and Beckman System Gold remote interface module SS420X, using 32 Karat® software. Reversed-phase HPLC was performed on a 4.6×250 mm XTerra RP_{18} (5 μm) column (Waters, Milford, MA). Normal-phase HPLC was conducted using a 4.6×250 mm Partisil (10 μm) (Alltech, Deerfield, IL) silica column. Analytical TLC was done on aluminum-backed sheets (Silica gel 60 F_{254}), and normal-phase column chromatography was performed using Silica gel 60, both obtained from EM Science (Gibbstown, NJ). Preparative thick layer chromatography was done using 20×20 cm, 1000 μm plates (Whatman, Clifton, NJ). Radio-TLC was analyzed initially using a System 200 Imaging Scanner (BioScan, Washington, DC), then cut into strips and counted using an automated gamma counter (LKB 1282, Wallac, Finland). Phosphor-imaging of electrophoresed gels was performed on a Cyclone Phosphor Scanner (Packard Bioscience Company) using Optiquant™ 4.00 software. ^{1}H NMR (300 MHz) and

^{13}C NMR (75 MHz) spectra were obtained on a Varian Mercury 300 spectrometer. Chemical shifts are reported in δ units; solvent peaks are referenced appropriately. Mass spectra were obtained on a Hewlett-Packard GC/MS/DS Model HP-5988 A instrument, an Applied Biosystems DE Pro, or a JEOL SX-102 high resolution mass spectrometer.

5.2. 3′,5′-Di-*O*-acetyl-6-*O*-(3-iodobenzyl)-2′-deoxyguanosine ((AcO)$_2$-IBdG, 2)

3′,5′-Di-*O*-acetylguanosine (**1**), which was derived by acetylation of deoxyguanosine,[26] was converted to (AcO)$_2$-IBdG (**2**) under Mitsunobu reaction conditions.[40,41] Polymer-bound TPP (2.37 g, 7.12 mmol, 3 mmol/g, Fluka) was swelled in $CHCl_3$ for 30 min. The resin was added to a solution of **1** (500 mg, 1.42 mmol) and 3-iodobenzyl alcohol (833 mg, 3.56 mmol) in 15 mL dioxane and stirred at reflux under a flow of argon. Diisopropylazadicarboxylate (DIAD, 1.21 mL, 7.12 mmol) was added dropwise to the refluxing heterogeneous mixture, resulting in a pale yellow solution, which was stirred for 3–4 h. The progress of the reaction was followed by TLC. The polymer was filtered, washed with MeOH, and the filtrate was evaporated to dryness. The non-polar components of this mixture were separated by a short-column filtration over silica gel using 0–20% EtOAc. The remainder of the product mixture was eluted using EtOAc. This crude product mixture was separated by flash column chromatography on silica gel using a 0–5% MeOH–EtOAc gradient to afford 345 mg of **2** (50%). An analytical sample was derived from this product by further preparative TLC purification. ^{1}H NMR ($CDCl_3$) δ 7.83 (s, 1H), 7.75 (s, 1H), 7.62 (d, 1H, J = 7.83 Hz), 7.44 (d, 1H, J = 7.69 Hz), 7.07 (dd, 1H, J = 7.83, 7.69 Hz), 6.27 (dd, J = 7.83, 6.2 Hz), 5.47 (s, 2H), 5.40–5.43 (m, 1H), 4.93 (br s, 2H), 4.4–4.8 (m, 3H), 2.97 (ddd, 1H, J = 6.46, 7.83, 14.28 Hz), 2.51 (ddd, 1H, J = 6.04, 8.52, 14.14 Hz), 2.12 (s, 3H), 2.07 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 170.6, 170.2, 160.7, 159.1, 153.6, 138.7, 137.5, 137.0, 127.3, 116.1, 94.1, 84.4, 82.3, 74.6, 66.9, 63.8, 36.7, 20.9, 20.8. LRMS (FAB+) m/z 568.1 (MH^+). HRMS calcd for $C_{21}H_{23}IN_5O_6$: 568.0693. Found: 568.0702.

5.3. 6-*O*-(3-Iodobenzyl)-2′-deoxyguanosine (IBdG, 3)

Deacetylation of **2** was performed by reacting a solution of **2** in CH_2Cl_2 with 0.5 mL of 1 mM guanidine/guanidinium hydrochloride in MeOH/CH_2Cl_2 at room temperature for 1 h.[27] The solvents were evaporated and the crude mixture was purified by preparative TLC using 5% MeOH/CH_2Cl_2 to afford 70 mg of **3** (36%). ^{1}H NMR ($CDCl_3$) δ 7.83 (s, 1H), 7.63 (t, 1H, J = 4.12 Hz), 7.44 (d, 1H, J = 8.10 Hz), 7.08 (dd, 1H, J = 7.69, 7.87 Hz), 6.22 (dd, 1H, J = 5.63, 9.47 Hz), 5.48 (s, 2H), 4.96 (br s, 2H), 4.75 (d, 1H, J = 5.08 Hz), 4.20 (s, 2H), 3.97 (dd, 1H, J = 1.65, 12.77 Hz), 3.01 (ddd, 1H, J = 5.34, 9.61, 14.83 Hz), 2.25 (1H, dd, J = 5.36, 13.32 Hz). LRMS (FAB^+) m/z 484.2 (MH^+). HRMS calcd for $C_{17}H_{19}IN_5O_4$: 484.0482. Found: 484.0486.

5.4. 6-*O*-(3-Trimethylstannylbenzyl)-2′-deoxyguanosine (TBdG, 4)

A solution of **3** (46 mg, 0.09 mmol), hexamethylditin (94 mg, 0.29 mmol), and $Pd(TPP)_2Cl_2$ (7 mg, 0.01 mmol) in 1 mL dioxane was refluxed for 1 h under a flow of argon. The insoluble components were filtered over a plug of Celite. The non-polar components of the reaction, consisting mainly of tin-based by-products and unreacted hexamethylditin, were separated by elution over a short column of silica with hexanes. The more polar products were eluted out using neat EtOAc and were concentrated to dryness. Preparative TLC purification using 5% MeOH/CH_2Cl_2 gave 48 mg of **4** (90%). ^{1}H NMR (CD_3OD) δ 8.02 (s, 1H), 7.60 (s, 1H), 7.43 (t, 1H), 7.31 (t, 1H), 6.31 (dd, 1H), 5.52 (s, 2H), 4.55 (t, 1H), 4.03 (d, 1H), 3.82 (dd, 1H), 3.73 (m, 1H), 2.77 (ddd, 1H), 2.33 (ddd, 1H), 0.26 (s, 9H). ^{13}C NMR (CD_3OD) δ 140.0, 136.8, 136.6, 129.5, 129.1, 89.7, 86.9, 73.2, 69.3, 63.7, 41.2, 26.5. LRMS (FAB^+) m/z 522.1 (MH^+, cluster peaks). HRMS calcd for $C_{20}H_{28}N_5O_4{}^{118}Sn$: 520.1163. Found: 520.1158.

5.5. 6-*O*-(3-[^{125}I]Iodobenzyl)-2′-deoxyguanosine ([^{125}I]3)

Radioiodination of TBdG was performed using a procedure similar to that reported for the synthesis of [^{131}I]IBG.[23] To 2–3 mCi of [^{125}I]iodide in 1–2 μL NaOH in a $\frac{1}{2}$-dram vial was added NCS (0.4 mg) in 15 μL AcOH. The vial was vortexed to mix the components. To this was added 0.2 mg **4** in 10 μL AcOH, and the reaction vial was vortexed and left at room temperature for 30 min. Evaporation of AcOH under a flow of argon gave a white residue, which was dissolved in 100 μL MeOH and injected on a reversed-phase HPLC column that was eluted with a gradient composed of 0.1% TFA/water (A) and 0.1% TFA/CH_3CN (B)—the solvent composition was changed from 5% B to 60% B over 30 min at a flow rate of 1 mL/min. The product fraction ($t_R \approx 25$ min) was purged with argon to remove much of the CH_3CN. It was further concentrated using an activated (5 mL MeOH and 30 mL water) solid phase C-18 Sep-pak® column (Waters) as follows: first, the activity was diluted with 10 mL water and passed through the column; the column was washed further with 10 mL water followed by elution with 5×0.5 mL of MeOH. The product, which eluted in MeOH fractions 3 and 4, was obtained in 70–85% radiochemical yield. The [^{125}I]IBdG could be stored in MeOH at −20 °C for more than one month without discernable decomposition. Prior to biological assays, aliquots of this activity were subjected to normal phase HPLC purification using a gradient of 1–5% MeOH/EtOAc/0.2% AcOH over 30 min. The fraction containing the product ($t_R \approx 22$ min) was evaporated to dryness under a flow of argon and reconstituted in PBS pH 7.14 for biological assays. Radiochemical purity was checked by both HPLC and TLC.

5.6. Octanol–water partition coefficient

PBS of pH 7.14 and *n*-octanol were each presaturated with the other prior to the experiment. About 500,000

CPM each of [^{131}I]IBdG and [^{125}I]IBG in 5–10 μL of PBS was added in triplicate to a mixture of 2 mL each of *n*-octanol and buffer, and the contents were mixed thoroughly by vortexing for 1–2 min. The layers were separated by centrifugation. A 50 μL aliquot of each layer in duplicate from each tube was withdrawn and counted for radioactivity, and the ratios of activities in *n*-octanol and buffer were calculated.

5.7. Cell culture conditions

DAOY (human medulloblastoma),[42] TE-671 (human rhabdomyosarcoma), SK-Mel-28 (human melanoma), and HT-29 (human colon carcinoma) cell lines were obtained from ATCC. The following cell propagation conditions were used: DAOY and TE-671 cells were grown in improved MEM Zinc option medium (GIBCO) with 2.2 g/L sodium bicarbonate, 8 mM HEPES, and 10% fetal bovine serum (FBS). SK-Mel-28 cells were grown in RPMI 1640 with 2 mM L-glutamine and 10% FBS. HT-29 cells were grown in McCoy's SA (modified) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% FBS.

5.8. Specific binding of [^{125}I]IBdG to pure AGT

O^6-Methylguanine-DNA methyltransferase (AGT, 90%, ⩾10,000 unit per milligram of protein) was purchased from Sigma. The specific binding of [^{125}I]IBdG to AGT was determined in single-label format, by using unlabeled IBdG to deplete AGT. It was also studied in a paired-label format with [^{131}I]IBG and by using BG to deplete AGT.[23] In brief, about 50,000 counts each tracer was added, in either the presence or absence of increasing concentrations of BG or IBdG (0.1 to 20 μM), to 10 μg of either AGT or BSA, the latter being a control for non-specific binding. The incubation was done in 0.1 mL of 50 mM Tris-HCl, pH 7.5, 5 mM DTT, and 0.1 mM EDTA in the presence of 10 μg of calf thymus DNA. After incubation for 30 min at 37 °C, proteins were precipitated by the addition of 200 μg of carrier BSA and 1 mL of cold 12% TCA. The precipitated proteins were collected on GF-C (Whatman) filters and washed extensively with 5% TCA. The results are expressed as the percentage of input activity retained on the filter. For each concentration, the assay was performed in triplicate.

5.9. In vitro uptake of [^{125}I]IBdG as a function of BG concentration

Paired-label cellular uptake assays were performed in several tumor cell lines (DAOY, TE-671, SK-Mel-28, and HT-29) using [^{131}I]IBG and [^{125}I]IBdG. Cells at a density of 5×10^5 per well per 2 mL of the medium (1×10^6 for TE-671) in 6-well plates were pre-incubated with 0, 1, 2, 5, 10, 25, 50, and 100 μM of BG for 4 h, in order to variably deplete their AGT content, and then co-incubated with 100 nCi of each tracer for 2 h. Afterwards, the cells were washed free of unbound radioactivity, solubilized, and counted for ^{131}I and ^{125}I activity in an automated gamma counter. The cell-associated activity was plotted as a function of the concentration of BG. In order to determine whether presence of BG in the medium after the 4 h period had an effect on tracer uptake, the cells were washed following the pre-incubation with various BG concentrations, fresh medium was added, and the cells were then incubated with 100 nCi each of the two tracers. The cell-bound activity was counted and plotted as above.

5.10. In vitro uptake of [^{125}I]IBdG as a function of IBdG concentration

Paired-label cellular uptake assays were performed in DAOY cells using [^{131}I]IBG and [^{125}I]IBdG. The experiment was performed in identical fashion to that above but using varying concentrations of unlabeled IBdG to deplete AGT variably. The cell-associated activity was plotted as a function of the concentration of IBdG.

5.11. Determination of cellular AGT level by Gel Electrophoresis and phosphor imaging

Cells were pre-incubated with various concentrations of BG (0–5 μM) for 4 h and cell extracts were prepared from the treated cells in a final volume of 1 mL. Aliquots (100 μL) of the gel extracts were incubated with [^{125}I]IBdG for 30 min of which a 40 μL portion was subjected to gel electrophoresis under non-reducing conditions using a 15% gel. Pure AGT labeled with [^{125}I]IBdG (see above) and C-14 labeled protein standards were used for reference. The gels were then imaged in a phosphor-imager to visualize the bands.

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