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ORIGINAL ARTICLE

O^4-benzylfolic acid inactivates O^6-alkylguanine-DNA alkyltransferase in brain tumor cell lines

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Abstract
Purpose The DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT), is a primary source of tumor resistance to agents such as temozolomide and chloroethylnitrosoureas that form DNA lesions at the O^6-position of guanines. To increase the efficacy of these drugs, pseudosubstrate inactivators of AGT such as O^6-benzylguanine have been developed. A novel inactivator of AGT, O^4-benzylfolic acid (O^4-BFA), has been reported which is more potent and water soluble than O^6-benzylguanine. Previous studies have suggested that uptake of O^4-BFA is mediated by the folate receptor (FR), and, thus, its use may be limited to cells expressing FR.
Methods We measured AGT activity in cell extracts from a panel of brain tumor cells exposed to O^4-BFA. Inactivation of AGT by O^4-BFA was measured in cells grown without folic acid as well as in cells grown in folic acid-containing media. Competitive binding studies were performed using purified FR to determine its affinity for O^4-BFA.
Results The observed IC_{50} for O^4-BFA in brain tumor cell lines ranged from 0.2 to 1.3 μM for cells grown in media containing 2.3 μM folic acid. At this concentration, folic acid would saturate the FR and the FR would be unable to take up O^4-BFA. When cells were grown in folic acid free media, there was at most a 50% decrease in the observed IC_{50}s, indicating that the FR was not essential for O^4-BFA uptake. Competitive binding studies using purified FR confirmed that the IC_{50} for O^4-BFA is ~180 times greater than folic acid, i.e., it has a very weak affinity for FR.
Conclusion These results indicate that O^4-BFA has potentially broad use as an inactivator of AGT as its use is not limited to tumors expressing high levels of FR.

Keywords AGT · Folate receptor · Malignant glioma · Medulloblastoma

Abbreviations

AGT	O^6-alkylguanine-DNA-alkyltransferase
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
O^6-BG	O^6-benzylguanine
O^4-BFA	O^4-benzylfolic acid
FR	Folate receptor

Introduction

The prognosis for a patient with malignant glioma remains bleak, with a median survival of 12–15 months

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[27]. Current therapy for malignant glioma employs temozolomide, an imidazole tetrazinone whose mechanism of action is similar to that of dacarbazine, whereby it forms the active methylating agent, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide [12]. After an initial observation that patients with high grade gliomas responded to temozolomide [19, 20], a phase 2 trial of temozolomide prior to radiation therapy in the treatment of newly diagnosed high grade glioma demonstrated striking activity [10], a result that has subsequently been confirmed in both recurrent [32, 33] and newly diagnosed tumors [26, 27, 32, 33]. Nevertheless, it was clear from these studies that a significant proportion of tumors do not respond to temozolomide therapy.

The antitumor activity of temozolomide is a direct consequence of DNA methylation. Temozolomide produces three major methyl adducts in DNA, N^7-methylguanine, N^3-methyladenine, and O^6-methylguanine. Of these, the primary cytotoxic lesion is O^6-methylguanine although there is some evidence that *N*-methylpurines also contribute to temozolomide toxicity [17]. During replication or repair synthesis, DNA polymerase pairs thymine rather than cytosine with O^6-methylguanine. DNA mismatch repair recognizes this and initiates unsuccessful repair of the mismatch. Repetitive cycles of futile mismatch repair lead to growth arrest and apoptosis [3]. Resistance to temozolomide by tumors has been attributed primarily to two mechanisms: direct removal of the methyl adduct on the O^6 position of guanine by O^6-alkylguanine-DNA alkyltransferase (AGT) [6, 9, 29] and tolerance to the DNA methylation because of a defect in DNA mismatch repair [8, 16].

Because defects in DNA mismatch repair appear to be relatively rare in adult gliomas [1], elevated levels of AGT are believed to be more critical to the response to temozolomide in these tumors. Recently, a large study using the methylation status of the AGT promoter, where a methylated promoter correlates with low expression of AGT, found a relationship between AGT expression and survival in patients with newly diagnosed glioblastoma multiforme treated with surgery, radiation, and temozolomide [27]. AGT can be pharmacologically depleted by the use of pseudosubstrates as its reaction with O^6-methylguanine transfers the methyl to an active site cysteine, inactivating the protein [15]. One such agent that has been used clinically is O^6-benzylguanine (O^6-BG). In an early study, the combination of carmustine and O^6-BG proved to be limited by the marked enhancement of carmustine-induced myelosuppression [11, 22]. Temozolomide is far less myelosuppressive than carmustine, although in combination with O^6-BG could be given at only 50% of the dose used for monotherapy. Even with this limitation, restoration of temozolomide sensitivity was observed in 15% of patients with anaplastic astrocytoma [23].

The use of O^6-BG in therapy of brain tumors, particularly for regional delivery, is limited by its inactivating potency and low solubility in water. Recently, a group of 2-amino-O^4-benzylpteridine derivatives has been described as potent inactivators of AGT [18]. One of these, O^4-benzylfolic acid (O^4-BFA), has solubility in water of 10–20 mg/ml as compared to the 0.1 mg/ml of O^6-BG and a 40-fold increase in AGT depletion in vitro compared to O^6-BG. In this report, we demonstrate that O^4-BFA inactivated cellular AGT in a broad range of brain tumor cell lines and that cellular uptake is not likely to be mediated by the folate receptor.

Materials and methods

Reagents

O^4-benzylfolic acid was the gift of Robert C. Moschel (National Cancer Institute, Frederick, MD, USA) and was synthesized as described previously[18]. It was prepared as a 10 mM stock in 20 mM Tris base and stored at −140°. Tritiated folic acid, 2-deoxy-D-glucose and probenecid were obtained from Sigma (St. Louis, MO, USA). Tritiated folic acid (range of 30–40°C mmol) was purchased from Moravek (Brea, CA, USA). Cell culture reagents were from Invitrogen (Carlsbad, CA, USA).

Cell culture

LN-18 and T98G were obtained from the Duke University Cell Culture Facility and cultured in Dulbecco's modified Eagle's medium and Eagle's minimum essential medium with Earle's salts, respectively, containing 10% fetal bovine serum. DAOY, D341 MED and D54 TR were cultured in Improved MEM Zinc Option (Richter's modification) supplemented with 10% fetal bovine serum [5]. The temozolomide-resistant line D54 TR was generated by treating D54 MG with serially increasing amounts of temozolomide. For experiments comparing the effectiveness of O^4-BFA in folic acid free versus folic acid containing medium, cells were propagated in RPMI 1640 without folic acid containing 10% dialyzed fetal bovine serum or in RPMI 1640 containing 10% fetal bovine serum. MA104 cells (green monkey kidney tubule cells) were cultured in folic acid free medium as previously described [13, 14, 28].

AGT assay

The AGT activity of the cells was determined as previously described [4]. The enzyme activity was defined as the femtomoles of O^6-[^{3}H]methylguanine removed from ^{3}H-methylated calf thymus DNA per milligram protein. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.

Binding of O^4-BFA by folate receptor

Analysis of O^4-BFA binding to folate receptor (FR) was done in two ways. First, a competitive radio-ligand binding assay using partially purified human placental FR in a cell free system was performed as follows [2, 13, 14, 28]. Using methodology detailed previously [2, 13] increasing amounts of either folic acid or O^4-BFA were added to vials containing approximately 4 pmol of [^{3}H]-folic acid in a buffer of 0.05 M potassium phosphate, pH 7.5, containing 1 mg/ml of charcoal treated albumin and then approximately 3 pmol of folic acid binding activity (folate receptor) was added (final volume was 0.5 ml). After 30 min room temperature incubation, the bound and free ligand were separated by absorption of the latter using charcoal coated with albumin. In order to facilitate data analysis, instead of merely presenting bound [^{3}H]-folic acid versus competitor, a reciprocal plot in which the dpm bound in a sample/dpm in vial with no competitor was calculated and graphed versus the competitor. This normalizes the data and allows a linear regression to be done [2]. At $Y = 2$, X is the value at which the competitor blocks 50% of the binding of the radioligand (i.e., IC_{50}). We also measured direct inhibition of binding of radiolabeled folic acid to the surface of MA104 cells in vitro. Using methodology well detailed [2, 13, 14], MA104 cells that bind approximately 2 pmol of folic acid/10^6 cells were incubated with increasing concentrations of O^4-BFA for one hour at 37° and then 10 nM [^{3}H]-folic acid was added for 10 min to saturate surface receptor. Again, the graph is a reciprocal plot of radioligand bound versus O^4BFA so that at $Y = 2$, X equals the IC_{50} for the competing compound. These experiments were performed in duplicate and the data presented are representative of several independent determinations. The observed error was less than 10% between the points.

Inhibition of cell growth

Cell viability after exposure to temozolomide was determined as follows. Cells (D54 TR) were trypsinized and incubated with or without 10 μM O^4-BFA for 4 h at 37°. Cells were pelleted and suspended at a concentration of 5,000–10,000 cells per ml. Temozolomide was added at the indicated concentrations from a 100 mM stock (in DMSO) and the cells were plated in quadruplicate in 96 well plates at 0.1 ml per well. After 7 days, the culture medium was replaced and 10 μl of Cell Counting Kit-8 (Dojindo, Gaithersburg, MD, USA), which contains a water soluble formazan dye, were added. After additional incubation for 2–3 h at 37°, the absorbance was read using a BioTek (Winooski, VT, USA) Synergy HT plate reader set at a wavelength of 450 nm. The data presented is representative of four independent experiments.

Statistical analysis

GraphPad Prism 4.0 was used to determine IC_{50} values using a sigmoidal dose response plot and to determine statistical significance of results.

Results

Time course of AGT inactivation by O^4-BFA

To determine how rapidly cellular AGT was inactivated, we treated the DAOY medulloblastoma cell line with 0.5, 2, or 8 μM O^4-BFA and harvested the cells over a 6 h period. The AGT activity of the cellular extracts prepared at each time point was assayed. As shown in Fig. 1, inactivation of AGT by 0.5 μM O^4-BFA begins to plateau at 4 h, and the AGT activity is almost completely inactivated by 2 and 8 μM O^4-BFA at 1 and 2 h, respectively.

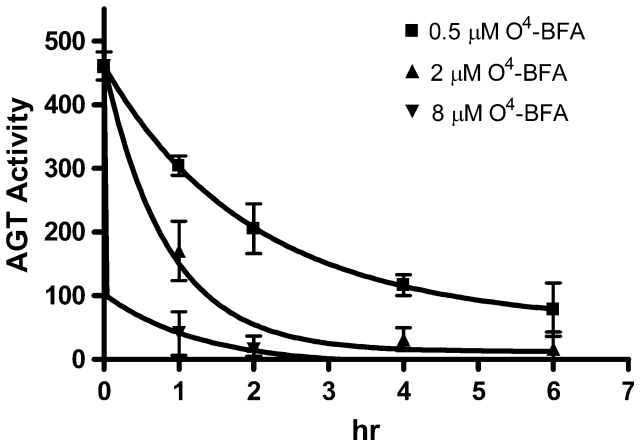

Fig. 1 Time course of AGT inactivation by O^4-BFA. DAOY cells were treated with 0.5, 2, or 8 μM O^4-BFA in RPMI containing 10% fetal bovine serum and harvested at the indicated times. AGT levels were determined as described in Methods. Error bars represent standard deviation

Inactivation of AGT by O^4-BFA in multiple cell lines

We have investigated the inactivation of AGT by O^4-BFA in the medulloblastoma cell lines, DAOY and D341 MED, the glioblastoma multiforme cell lines, T98G and LN-18, and an in vitro-derived, temozolomide-resistant malignant glioma cell line, D54 TR. (The parent cell line of D54 TR, D54 MG, has AGT activity below the limits of detection). As shown in Table 1, the IC_{50} values ranged from 0.3 to 1.3 μM when the cells were grown in normal media which contains 2.3 μM folic acid. At this concentration of folic acid, the FR is saturated.

A previous study had suggested that O^4-BFA was taken up by the α-FR [18]. Since all the FR would be saturated with folic acid at 2.3 μM folic acid and unavailable for uptake of O^4-BFA, to assess any possible role for the FR in uptake, we also measured the IC_{50} for O^4-BFA inactivation of AGT in cells grown in folic acid free medium (Table 1). The IC_{50} values ranged from 0.14 to 1.45 μM and were generally lower than the values for the each cell line grown in folic acid. We compared these results with the inactivation of AGT by O^4-BFA in a cell line that expresses high levels of α-FR, the KB carcinoma cell line. Again, the observed IC_{50} was somewhat less in cells grown in folic acid free medium although this difference did not reach statistical significance. The IC_{50} was similar for KB cells maintained in folic acid free medium for 10 or 16 days, 0.13 and 0.16 μM, respectively.

Table 1 Concentrations of O^4-BFA required to inactivate 50% of cellular AGT (IC_{50}) in cells grown in the presence or absence of folic acid

Cell line	Cell type	IC_{50} (μM): cells grown in 2.3 μM folic acid	IC_{50} (μM): cells grown in absence of folic acid
DAOY	Medulloblastoma	0.21	0.17
D341 MED	Medulloblastoma	0.32	0.43
D54 TR	Malignant glioma	0.27	0.14
LN-18	Glioblastoma	0.82	0.63
T98G	Glioblastoma	1.45	1.26
KB	Carcinoma	0.16	0.15

Cells were grown at least 48 h in the absence of folic acid before treatment with O^4 -BFA. Cells were treated for 4 h with O^4 -BFA prior to harvesting. Data is the average of at least two determinations of each IC_{50} value

Binding of O^4-BFA by folate receptor

In order to more precisely determine the potential importance of the FR for O^4-BFA uptake by FR positive cells, we first directly compared the binding of O^4-BFA and folic acid by determining an IC_{50} for both compounds in a cell free binding assay using human placental FR. As seen in Fig. 2a, when increasing amounts of folic acid are incubated with a fixed amount of [^{3}H]-folic acid, 50% inhibition of binding was calculated to be 3.39 pmol of folic acid which was as expected since the amount of the radiolabeled ligand was approximately 3 pmol. However, 50% inhibition by O^4-BFA was not attained until 625 pmol (≈1.25 μM) of O^4-BFA (Fig. 2b). The ratio of O^4-BFA/folic acid inhibition is approximately 180/1. Since the O^4-BFA solution was 0.73% folic acid by HPLC and spectral analysis, the results here could simply be the result of the folic acid contamination of the O^4-BFA as that would calculate to a folic acid level of 4.5 pmol, which is very similar to the 3.39 determined to be the IC_{50} for folic acid. Similar results were obtained when either O^4-BFA or folic acid were pre-incubated with MA104 cells in vitro (Fig. 3). O^4-BFA was incubated with the cells for 1 h in order to saturate cell surface receptors and then the cells were exposed to a short pulse of [^{3}H]-folic acid. As compared to near complete blocking of binding at a concentration of 2–5 nM folic acid, 50% inhibition of binding by O^4-BFA was calculated to be approximately 265 nM.

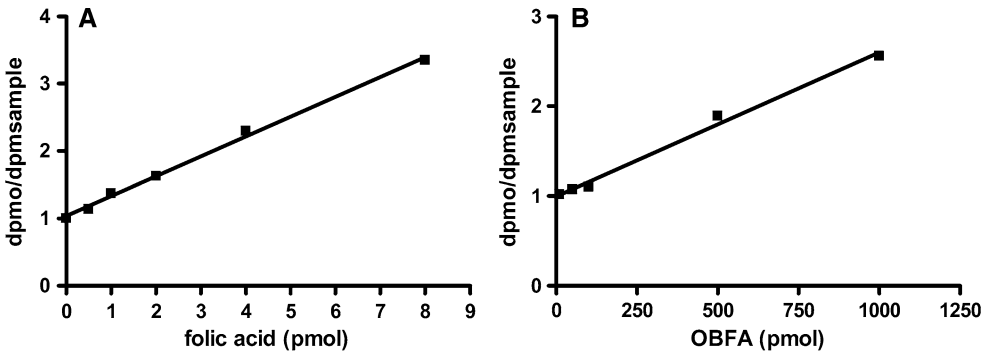

Fig. 2 Competitive binding of folic acid (panel **a**) or O^4-BFA (panel **b**) with [^{3}H]-folic acid by human placental folate receptor. The IC_{50} for folic acid was 3.39 pmol and for O^4-BFA, 625 pmol

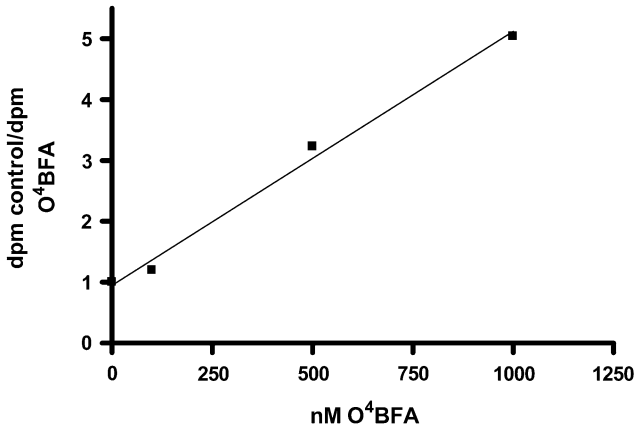

Fig. 3 Inhibition of binding of [^{3}H]-folic acid by O^4-BFA on MA104 cells. Half inhibition by O^4-BFA was calculated to be approximately 265 nM. Folic acid blocks nearly all binding at only 2 nM (data not shown)

Effect of O^4-BFA on cell growth inhibition by temozolomide

The cell line, D54 TR, was derived by treating D54 MG with serially increasing levels of temozolomide. As a result of this, the D54 TR cells express high levels of AGT (approximately 800 fmol/mg) whereas the parent line expresses negligible levels of AGT (data not shown). As shown in Fig. 4, pretreatment of D54 TR with either O^6-BG or O^4-BFA had no long term effect on cell growth. At 400 μM temozolomide, the drug had little effect on cells that were not pretreated with an AGT pseudosubstrate. At this concentration of temozolomide, inactivation of cellular AGT with either O^6-BG or O^4-BFA caused a significant decrease in cell growth.

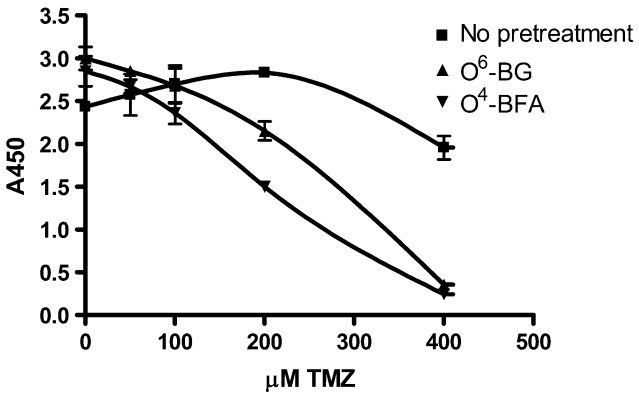

Fig. 4 Effects of the AGT inactivators, O^4-BFA and O^6-BG, on growth inhibition of D54 TR cells by temozolomide (TMZ)

Discussion

DNA alkylators such as temozolomide, nitrosoureas, and dacarbazine are widely used in the treatment of glioblastoma, astrocytoma, melanoma, and other neoplasias. Resistance to these agents is frequently linked to expression of the DNA repair protein, AGT. To improve the efficacy of these drugs, small molecule inactivators of AGT have been developed. The prototypical inactivator is O^6-BG which serves as a pseudosubstrate for AGT. After binding O^6-BG, AGT transfers the benzyl moiety to its active site cysteine, leading to ubiquination of the protein followed by degradation [21, 31]. In this manner, O^6-BG pharmacologically ablates the AGT protein from cells. Cotreatment of O^6-BG with temozolomide or BCNU increased their ability to inhibit tumor growth in xenograft studies [7, 25]. Unfortunately, in clinical studies with O^6-BG and BCNU, increased myelotoxicity was observed so that the dose of BCNU had to be lowered below an effective concentration [22]. Less myelotoxicity was seen in a clinical trial of the combination of O^6-BG and temozolomide [23]. An alternative inactivator of AGT that is currently in clinical trials is lomeguatrib [6-(4-bromo-2-thienyl) methoxy purin-2-amine]. The combination of this drug with alkylating agents has also been associated with increased myelotoxicity [24].

Additional inactivators of AGT have been recently described including O^4-BFA [18]. This compound, when compared to O^6-BG, has increased solubility in water and increased potency in vitro against AGT. Because O^4-BFA markedly increased the sensitivity of KB cells, which express high levels of FR, to BCNU, but, for cell lines with lower levels of FR expression, had less effect on BCNU sensitivity, it was suggested that its use would be restricted to tumors with high levels of FR expression. This could prove problematic for its use in increasing the efficacy of alkylating agents in brain tumors, as FR expression has previously been shown to be heterogeneous in these tumors [30]. Our in vitro studies indicated that O^4-BFA has a very low or even no significant affinity for FR compared to folic acid and the naturally occurring serum folate, 5-methyltetrahydrofolate, (sub-nanomolar and nanomolar respectively vs. μM). Thus, it would seem unlikely that FR plays a critical role vis-à-vis specifically targeting FR positive cells compared to FR negative cells if incubated in the physiological range of serum folate (only 20–40 nM). Indeed, when the effects of O^4-BFA were compared in cells grown in folic acid-free medium versus normal (2.3 μM folic acid) medium, there was, at

most, a twofold increase in concentration of O^4-BFA required to inactivate AGT in the presence of folic acid.

Previously characterized inactivators of AGT have exhibited some degree of myelotoxicity when administered with DNA alkylators. A possible method for avoiding this toxicity is regional depletion of AGT by intratumoral administration or by such means as dosing directly into a resection cavity. Preliminary studies using O^6-BG have not shown any increased response to temozolomide or BCNU in tumor-bearing rats (unpublished results). Because of its increased solubility in water, O^4-BFA may be particularly effective in such a strategy since local concentrations at levels sufficient to inactivate AGT in any residual tumor tissue can be easily achieved. The utility of this approach is currently under investigation.

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