A New $O^6$-Alkylguanine-DNA Alkyltransferase Inhibitor Associated with a Nitrosourea (Cystemustine) Validates a Strategy of Melanoma-Targeted Therapy in Murine B16 and Human-Resistant M4Beu Melanoma Xenograft Models

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ABSTRACT

Chemoresistance to $O^6$-alkylating agents is a major barrier to successful treatment of melanoma. It is mainly due to a DNA repair suicide protein, $O^6$-alkylguanine-DNA alkyltransferase (AGT). Although AGT inactivation is a powerful clinical strategy for restoring tumor chemosensitivity, it was limited by increased toxicity to nontumoral cells resulting from a lack of tumor selectivity. Achieving enhanced chemosensitization via AGT inhibition preferably in the tumor should protect normal tissue. To this end, we have developed a strategy to target AGT inhibitors. In this study, we tested a new potential melanoma-directed AGT inhibitor [2-amino-6-(4-iodobenzyloxy)-9-[4-(diethylamino)ethylcarbamoylbenzyl]purine; IBgBZ] designed as a conjugate of $O^6$-(4-iododbenzyl)guanine (IBg) as the AGT inactivator and a $N,N$-diethylaminooethylenebenzamido (BZ) moiety as the carrier to the malignant melanocytes. IBgBZ demonstrated AGT inactivation ability and potentiation of $O^6$-alkylating agents (cystemustine, a chloroethylnitrosourea) in M4Beu highly chemoresistant human melanoma cells both in vitro and in tumor models. The biodisposition study on mice bearing B16 melanoma, the standard model for the evaluation of melanoma-directed agents, and the secondary ion mass spectrometry imaging confirmed the concentration of IBgBZ in the tumor and in particular in the intracytoplasmic melanosomes. These results validate the potential of IBgBZ as a new, more tumor-selective, AGT inhibitor in a strategy of melanoma-targeted therapy.

Malignant melanoma remains an increasingly prevalent cancer worldwide, despite emphasis on prevention and recent advances in early diagnosis (Miller and Minh, 2006). When this highly metastatic cancer is disseminated, chemotherapy remains the most efficient modality of treatment commonly using $O^6$-alkylating agents (nitrosoureas and tetrazines). However, only 15 to 20% of patients are responsive, principally because melanoma is highly chemoresistant (Tsutsu-mida et al., 2005).

Melanoma chemoresistance to $O^6$-alkylating agents is mainly mediated by the DNA repair protein $O^6$-alkylguanin-DNA alkyltransferase (AGT or ATase), also termed $O^6$-methylguanine-DNA methyltransferase (Passage et al., 2006). AGT repairs the $O^6$-alkylguanine lesions by a stoichiometric and irreversible transfer of the $O^6$-alkyl group to the cysteine 145 residue in the active site (Juillerat and Juillerat-Jeanneret, 2007). The protein is thereby totally inactivated, and restoration of AGT activity then requires de novo synthesis. On the basis of this suicide mechanism of action, several pseudosubstrates derived from 6-substituted guanines were developed to inactivate AGT (McElhinney et al., 2003). Although various highly specific AGT inhibitors are now available [$O^6$-benzylguanine (Bg) and $O^6$-(4-bromoethyl)guanine (BTg)], clinical trials in association with an $O^6$-alkylating agent [chloroethylnitrosourea (CENU) or tetrazine] showed increased side effects, requiring the reduction

ABBREVIATIONS: AGT, $O^6$-alkylguanine-DNA alkyltransferase; Bg, $O^6$-benzylguanine; BTg, $O^6$-(4-bromoethyl)guanine; CENU, chloroethylnitrosourea; IBgBZ, 2-amino-6-(4-iodobenzyloxy)-9-[4-(diethylamino)ethylcarbamoylbenzyl]purine; IBg, $O^6$-(4-iododbenzyl)guanine; BZ, $N,N$-diethylaminooethylenebenzamido; HPLC, high-pressure liquid chromatography; cystemustine, $N$-[2-chloroethyl]-N-[2-(methylsulfonyl)ethyl]-N'-nitrosourea; DMSO, dimethyl sulfoxide; ID, injected dose; SIMS, secondary ion mass spectrometry.
of a therapeutic agent dose to suboptimal levels (Quinn et al., 2002; Ranson et al., 2006). This emerging clinical limitation led to the development of innovative strategies to reduce the dose-limiting toxicity of the treatments.

Our approach is based on O\textsuperscript{a}-alkylating agent chemotherapy in combination with a melanoma-selective AGT inhibitor. Our aim was to sensitize tumor cells to the therapeutic O\textsuperscript{a}-alkylating agents while preventing toxic side effects by maintaining the DNA repair process (i.e., normal AGT levels) in healthy tissues. We had previously developed a series of AGT inhibitors derived from O\textsuperscript{a}-alkyl/aralkyl guanosine or 2’-deoxyguanosine that enhanced resistant malignant melanoma sensitivity to CENUs both in vitro and in animal models (Cussac et al., 1994a; Debiton et al., 1997; Mounetou et al., 1997; Buchdahl et al., 1998). Like others inhibitors, they were widely distributed in the organism and could deplete AGT in various normal tissues (Cussac et al., 1994b). To achieve melanoma-selectivity, we designed new AGT inhibitors [2-amino-6-(4-iodobenzyloxy)-9-[4-(diethylamino)ethylcarbamoylbenzyl]guanine (IBgBZ), conjugating the O\textsuperscript{a}- (4-iododbenzyl)guanine (IBg) and the N,N-diethylaminomethylenbenzamido (BZ) moieties as a carrier to the malignant melanocytes. Indeed, we developed \textsuperscript{[125I]}benzamide derivatives as imaging agents, currently under Phase III clinical evaluation as radiopharmaceuticals for the diagnosis of disseminated melanoma (Michelot et al., 1993; Moreau et al., 1993). They form a reversible BZ-melanin complex, resulting in a high concentration in malignant melanocytes (Labarre et al., 2002; Guerquin-Kern et al., 2004). This property suggested that the BZ moiety would be a useful tool as a carrier to melanoma.

The present study was conducted as a proof-of-concept for melanoma-targeted therapy and assessed, in vitro and in vivo, the AGT inhibition, the O\textsuperscript{a}-alkylating agent (CENUs) antineoplastic activity enhancement, and the tumoral melanin tissue affinity of the new conjugate (IBgBZ) in murine and human-resistant melanoma.

**Materials and Methods**

**Drugs.** \textsuperscript{[125I]}IBgBZ was labeled with high specific activity by radioiododestannylation in the presence of 37 MBq (1 mCi) of non-carrier-added sodium \textsuperscript{[125I]}iodide (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and peracetic acid in aqueous ethanol adapted from a previously described procedure (Mounetou et al., 1995). \textsuperscript{[125I]}IBgBZ was obtained in 85% radiochemical yield and 98% purity after high-pressure liquid chromatography (HPLC) purification. The reaction was initiated by nonradioactive sodium iodide, and IBgBZ was fully identified by proton and carbon nuclear magnetic resonance and mass spectrometry. The detailed syntheses will be described in a forthcoming article.

Cystemustine was synthesized by previously described procedures (Madelmont, 1994) and supplied by Orphachem (Clermont-Ferrand, France).

**Cell Culture.** The human melanoma M4Beu cells (obtained from Dr. J. F. Dore´) (Jacubovich and Dore´, 1979) and the B16-F0 melanoma (no. CRL-6322; American Type Culture Collection, Manassas, VA) were cultured in GlutaMAX (Fisher Scientific Bioblock, Illkirch, France) (Eagle’s minimum essential medium with glutamine) supplemented with 10% fetal calf serum and solutions of vitamins, sodium pyruvate, nonessential amino acids, and gentamicin at 37°C in a 5% CO\textsubscript{2} humidified incubator.

**Determination of AGT Activity.** The AGT assay was performed on protein extracts of M4Beu cells treated in vitro by IBgBZ, using a previously described procedure (Marchenay et al., 2001). Increasing amounts of M4Beu cell protein extracts were incubated with \textsuperscript{[3H]}N-methyl-N-nitrosourea-methylated calf thymus DNA substrate for 1 h at 37°C. The substrate was then acid hydrolyzed, and the alkylated bases released in the sample were separated by reverse-phase HPLC (HP 1100 series; Hewlett Packard, Les Ulis, France) using a C18 Uptisphere column (5 μm, 25 × 4.6 mm) eluted at a flow rate of 1.5 ml/min by a 20 mM NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} (pH 5.0) mobile phase containing 6% methanol (v/v) for 11 min and 15% methanol (v/v) for 14 min. The radioactivity of column effluents was automatically measured in a Packard flow scintillation analyzer (500TR series; PerkinElmer, Courtabeuf, France). AGT activity was determined by measuring the disappearance of \textsuperscript{[125I]}N-methylguanamine from the substrate and was expressed in femtomoles of methyl groups transferred to protein per milligram of total protein present in the sample.

To measure in vivo AGT activity after drug exposure in vivo, a group of mice with tumors that reached approximately 500 mm\textsuperscript{3} were treated with IBgBZ by i.p. injection. The animals were euthanized by CO\textsubscript{2} inhalation at different times after injection. The tumors were removed and flash frozen in liquid nitrogen and stored at −80°C. Increasing amounts of M4Beu cell protein extracts were analyzed as described above.

**Cytotoxicity Tests.** For colony-forming assays, M4Beu cells were plated in six-well multidishes (150 cells/dish) and allowed to adhere for 20 h before treatment. The cells were treated with a culture medium containing increasing concentrations of IBgBZ (1–25 μM) dissolved in dimethyl sulfoxide (DMSO) before dilution in fresh culture medium, and they were incubated for various times. For combination treatment tests, cystemustine (50 μM) diluted in fresh culture medium was added 4 h after IBgBZ (2.5 or 5 μM) and incubated for 2 h. After the last treatment, the treated medium was replaced by fresh medium, and the cells were grown for 14 days at 37°C in a CO\textsubscript{2} incubator. Dishes were rinsed with phosphate-buff ered saline, and the cells were fixed with methanol and stained with 0.2% crystal violet solution. The plating efficiency of cells not treated with drugs was approximately 70%. Colonies (>50 cells) were counted. The surviving fraction (percentage of survival) was calculated as the ratio of treated to untreated cells (control) × 100.

**In Vivo Biodistribution Study.** For tissue distribution analyses, 6-week male C57BL/6Jco mice (Charles River Laboratories, L’Arbresle, France) were inoculated with 5 × 10\textsuperscript{5} melanoma B16 cells by dorsal s.c. injection. When the tumor diameter reached approximately 0.5 cm, the animals received an i.v. injection of \textsuperscript{[125I]}IBgBZ (0.74 MBq, 20 μCi, 10 mg/kg) dissolved in DMSO and 0.9% sodium chloride. Mice were euthanized by CO\textsubscript{2} inhalation at 1 h, 3 h, 5 h 30 min, 6 h, and 48 h after drug administration and were immediately frozen by immersion in liquid nitrogen. They were then embedded in a 2% carboxymethylcellulose gel, and the block was sagittally sectioned at −22°C with a Reichert-Jung Cryopolycut cryo-microtome (Reichert Jung, Heidelberg, Germany). The selected 40-μm-thick slices were taken using no. 810 Scout band tape (3M, St. Paul, MN), dried for 48 h at −22°C, fixed on a hard-bound sheet, and introduced into an Ambis 4000 detector (Scanalitics/CSP, San Diego, CA) to quantify the radioactivity in selected organs (n = 6 for each time studied) (Labarre et al., 1999). The final results are expressed as percentages of injected dose (ID) per gram of tissue.

**Excretion Study.** The excretion study was conducted using 6-week-old male C57BL/6Jco mice given \textsuperscript{[125I]}IBgBZ as described above and housed in metabolic cages (Charles River Laboratories), allowing separate collection of feces and urine at 24, 48, and 72 h after administration. Radioactivity was measured in a gamma counter (Minaxi 5530; Packard, Rungis, France).

**In Vivo Stability.** Plasma, urine, rehydrated feces, and organs homogenized in a Potter blender (Fisher Scientific Bioblock) were sampled, extracted three times with methanol, and centrifuged at 2000g for 10 min at 4°C. The recovery of radioactivity ranged from 80 to 85%. After evaporation, the dry residues were dissolved in the mobile phase. Chromatography (HPLC) analysis was performed on

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an HP 1100 system with a UV/visible detector (Hewlett Packard) equipped with a reverse-phase Kromasil column (C18, 5 μm, 250 × 4.6 mm; Hewlett Packard) and connected to a 500TR flow scintillation analyzer (Packard Instrument S.A., Rungis, France). The flow rate was 1 ml/min with a gradient mobile phase starting from a methanol/0.4% NH₄OH mixture:70/30 (v/v) to 100/0 at 10 min to 15 min.

Secondary Ion Mass Spectrometry Analysis. Secondary ion mass spectrometry (SIMS) imaging was performed using a NanoSIMS-50 Ion Microprobe ( Cameca, Gennevilliers, France). B16 murine melanoma cells were intravenously injected into male C57BL/6Jco mice to obtain, within 12 days, tumor cell colonies in lungs mimicking pulmonary micrometastases. After IBgBZ administration, mice were euthanized at 1 h, 3 h, and 5 h 30 min. Typical sample preparation for SIMS analysis was described previously (Guerquin-Kern JL et al., 2005). Image processing was performed using ImageJ (W. S. Rasband, United States National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/), a public-domain Java image-processing program, to obtain proper colocalization of the observed structures on the processed maps for all of the ion species, and allowed further correlation with light microscope images.

In Vivo Antitumor Tests. All experiments were carried out in accordance with the recommendations of the Institute of Laboratory Animal Resources (1996). Swiss nu/nu male mice (Charles River Laboratories) that were anesthetized by isoflurane inhalation received inoculations with 5 × 10⁶ M4B eu cells by abdominal s.c. injection. When tumors reached approximately 150 mm³ in size, the mice were randomly assigned to groups of six and were treated three times every 4 days with IBgBZ by i.p. injection and/or with cystemustine by i.v. injection. Cystemustine was dissolved in 0.9% sterile sodium chloride. IBgBZ was dissolved in DMSO and added to 0.9% sodium chloride. The day before the beginning of treatment was denoted as day 0. Each group of six mice was treated on day 1, 5, and 9. Twice a week, tumor diameters were recorded by caliper measurement, and tumor volume was calculated using the formula: length × width² × 0.5. Twenty-four days after the beginning of the treatment (day 24), the mice were euthanized. Tumor volumes were heterogeneous. Accordingly, the results are expressed as the factor of tumor volume increase and calculated as follows: (tumor volume on day x – tumor volume on day 0)/tumor volume on day 0.

Results
In Vitro Inactivation of Cellular AGT and CENU Potentiation. In vitro evaluation was performed on human melanoma M4B eu cells because they contained a high constitutive AGT level (800 fmol of active AGT/milligram protein), and they were highly resistant to O⁶-alkylating agents (Mounetou et al., 1997).

Figure 1A shows the AGT activity that we observed in M4B eu cells by assay of methylated DNA substrate after IBgBZ treatments at 2.5 or 5 μM for 2 or 4 h. AGT levels of treated cells decreased compared with control, depending on the treatment duration rather than the dose of IBgBZ used over the range tested. IBgBZ deactivated AGT from 50 to 60% after 2 h to more than 80% after 4 h. These results demonstrate that IBgBZ is an active substrate of AGT and allows cellular AGT inactivation.

Cytotoxicity was determined in colony-forming assays, a very sensitive method used to detect the killing effects at cell levels. Potentiation of the antiproliferative activity of O⁶-alkylating agents was tested with a CENU: cystemustine (Madelmont, 1994; Cure et al., 1999). The concentration needed to inhibit 50% of cell growth compared with untreated cells (IC₅₀) was first evaluated for IBgBZ alone. The values calculated from dose-response curves were 17.7 ± 3.4 and 9.4 ± 1.8 μM after 2 h and 4 h of treatment, respectively. The two doses selected for the in vitro evaluation (i.e., 2.5 and 5 μM), where IBgBZ exerts a strong inhibitory effect, were confirmed as low toxic. As shown in Fig. 1B, no significant decrease of survival rate was observed for the M4B eu cells treated by IBgBZ alone at these doses. The cytotoxicity evaluation of cystemustine alone (50 μM) or in combination with IBgBZ (2.5 or 5 μM) is shown in Fig. 1B. The antiproliferative activity of cystemustine was significantly increased when the cells were pre-exposed to IBgBZ at 2.5 or 5 μM for 4 h. These results were consistent with the marked reduction in AGT cellular levels observed at the same times (Fig. 1A). In addition, as observed for the AGT inactivation, cystemustine activity potentiation was not IBgBZ dose-dependent in the
range tested. Indeed, increasing the IBgBZ dose from 2.5 to 5 μM did not cause a significant decrease in M4Beu cell survival (Fig. 1B). Taken together, these results show that IBgBZ was effective in vitro for AGT inhibition and CENU activity potentiation.

Biodisposition and In Vivo Stability. To test the affinity of IBgBZ for melanoma tumors, a tissue distribution study was first conducted in C57BL/6 male mice bearing B16 murine melanoma xenograft. It is the standard model used in our laboratory to evaluate tumor affinity for melanoma-imaging agents (Moreau et al., 1993; Labarre et al., 2002). In addition, it allows formation of the pulmonary colonies needed for subsequent SIMS experiments (Guerquin-Kern et al., 2004). The radioactive tissue concentrations of mice treated with [125I]BgBZ (0.74 MBq, 20 μCi, 10 mg/kg) determined at 5 min, 30 min, and 1, 3, 6, and 48 h postinjection are given in Table 1. In addition, a mouse whole-body autoradiography of tissue distribution of the radioactivity at 1 h

TABLE 1

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after Injection</th>
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<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Brain</td>
<td>0.52 ± 0.19</td>
</tr>
<tr>
<td>Lung</td>
<td>19.46 ± 0.32</td>
</tr>
<tr>
<td>Liver</td>
<td>18.80 ± 0.85</td>
</tr>
<tr>
<td>Kidney</td>
<td>24.75 ± 2.78</td>
</tr>
<tr>
<td>Blood</td>
<td>17.48 ± 0.47</td>
</tr>
<tr>
<td>Tumor</td>
<td>2.05 ± 0.46</td>
</tr>
</tbody>
</table>

* Results are expressed as percentage of ID per gram of tissue as mean ± S.D. (n = 6 sections).

N.D., not detectable.

Fig. 2. Representative whole-body autoradiography of a mouse bearing B16 melanoma cell xenograft, 1 h after i.v. injection of [125I]BgBZ.

These results clearly indicate that IBgBZ reached the melanoma tumor mainly in its intact form, and it was particularly concentrated in this target tissue between 1 and 3 h postinjection.

SIMS Analysis. SIMS was used to determine the subcellular distribution of IBgBZ (Guerquin-Kern et al., 2004). Images showing one B16 melanoma cell colony 3 h after IBgBZ injection and histological sections in the same optical field are shown in Fig. 3. SIMS allows direct identification of chemical elements with high sensitivity and specificity and can be applied to visualize elemental distribution (chemical mapping). Figure 3A shows the distribution of phosphorus (mass, 31), and the condensed chromatin near the nuclear envelope was clearly identified. Figure 3B represents the distribution of iodine (mass, 27), i.e., IBgBZ and/or iodinated-derived compounds. The histological image (Fig. 3C) reveals that melanosomes, the specialized organelles containing melanin small grains and clusters, were largely distributed in the cellular cytoplasm. Superimposition of the melanosome and the iodine signal was observed, indicating a perfect colocalization of IBgBZ and/or iodinated-derived compounds with melanin polymers (Fig. 3, B and C). These data provide further evidence that IBgBZ was rapidly internalized in the melanocytes.

In Vivo Antitumor Tests and Intratumoral AGT Inhibition. Because AGT expression in B16 cells was very weak, the chemoresistant M4Beu line was used. To evaluate the antitumor efficacy of the systemustine/IBgBZ combination and intratumoral AGT activity in vivo, M4Beu melanoma-resistant cells were established as xenografts in nude mice. Tumors appeared within 8 to 10 days. In vivo, intratumoral AGT activity of mice from 1 to 24 h after treatment with IBgBZ at the therapeutic dose (50 mg/kg) are shown in Fig. 4B. An initial 30% decrease in the AGT tumor level was observed at 1 to 3 h after treatment followed by an additional drop from 50 to 60% after 3 to 6 h. Initial activity was recovered at 24 h after treatment, consistent with the required de novo AGT resynthesis delay (Passagne et al., 2006). These data show that IBgBZ reached its molecular target (i.e., AGT) in the tumor in an active form.

Considering these results, the treatment regimens of the antitumor tests were adjusted. Mice were treated with IBgBZ (50 mg/kg) at 3 or 6 h before the injection of systemustine (15
mg/kg), i.e., when the AGT level had decreased in the tumor. To clearly demonstrate the effects of IBgBZ in the combination regimen, a noncurative dose of cystemustine (15 mg/kg) was chosen. When tumors were established (100–150 mm$^3$ in size), each group of mice was treated on day 1, 5, and 9. On day 24, when the mean tumor diameter exceeded 10 mm for the vehicle-treated control animals, the study was stopped. Tumor responses are shown in Fig. 4A. Compared with vehicle control, IBgBZ given alone had no significant effect on tumor growth during the study period. Although cystemustine alone increased growth delay, the IBgBZ/cystemustine combinations produced greater antitumor effects. Tumor growth progression was slowed by 50% over the 24 days when IBgBZ was added. The benefits of the combination were mostly apparent after 18 days as the tumor volume in mice began to diverge. However, at the end of the study, the tumor regrowth rate in animals treated with the combination regimens was slower than in those treated with the single agent (cystemustine). Finally, the difference in tumor growth between cystemustine alone and the two combination regimens began to diverge. However, at the end of the study, the tumor regrowth rate in animals treated with the combination regimens was slower than in those treated with the single agent (cystemustine). Finally, the difference in tumor growth between cystemustine alone and the two combination regimens was only significant for the mice treated with IBgBZ 3 h after the injection of cystemustine (p < 0.05). The lower efficacy of the 6-h pretreatment was probably due to the AGT resynthesis observed between 6 and 24 h (Fig. 4A).

### Discussion

New melanoma treatments with both improved tumor selectivity and lower toxicity are urgently needed. To address this issue, strategies based on the selective suppression of AGT activity in malignant cells were considered.

To date, various approaches have been developed. Genetic chemoprotection of hematopoietic and progenitor stem cells with mutant AGT has been explored (Southgate et al., 2006). However, this approach does not consider extra-hematopoietic toxicities, and it needs to be optimized before any clinical application can be envisioned. New generations of AGT inhibitors with improved tumor selectivity have also been developed (Juillerat and Juillerat-Jeannerat, 2007). They were designed as conjugates of a first-generation AGT inhibitor (Bg, BTg, and 2-amino-O$^4$-benzylpteridine) and a ligand of a high-expression, tumor-specific receptor or transporter (n-glucose, glucuronic acid, and fol acid) (Kaina et al., 2004; Nelson et al., 2004; Wei et al., 2005). Finally, to date, none of these conjugation approaches has demonstrated any successful in vivo tumor selectivity.

In this study, our goal was to develop a melanoma-targeted therapy combining an O$^6$-alkylating agent (cystemustine) and a new guanine conjugate, IBgBZ, able to achieve AGT inactivation more selectively in melanoma tumors. The three essential constituent parts of this compound are as follows: 1) a Bg moiety as the AGT inhibitor; 2) a BZ moiety at the N-9 position of Bg as a carrier to the melanoma tumor; and 3) an iodine atom at the 4-position of the O$^6$-benzyl group of Bg as a potential radiation emitter when replaced by a suitable radioisotope (e.g., iodine 131). It would provide an opportunity to combine the effects of radionuclide therapy (Larson and Krenning, 2005) and chemotherapy (i.e., radiochemotherapy), where DNA lesions caused by chemotherapeutic agents would be synergized by local radiation damage induced by the radiiodine atom, preferentially in the melanoma tumor. To validate our targeting strategy, two melanoma models were used. For biodisposition and SIMS studies, the B16 murine melanoma model validated by our previous work on melanoma-imaging agents was used (Moreau et al., 1993; Labarre et al., 2002; Guerquin-Kern et al., 2004). Because AGT levels in the B16 murine melanoma cell line were very low, pharmacological evaluation was performed using M4Beu human melanoma, a model that exhibited a high AGT activity and was chemoresistant to O$^6$-alkylating agents such as CENUs (Cussac et al., 1994a; Mounetou et al., 1997).

First, we verified the in vitro AGT-inhibitory activity of BgBZ in M4Beu human-chemoresistant melanoma cells. Although the conjugation of the BZ vector at the N-9 position of Bg decreased AGT inhibition, as described in previous structure-activity relationship studies (McElhinney et al., 2003), IBgBZA was still an efficient inhibitor (70–80%), and it enhanced M4Beu melanoma cell sensitivity to cystemustine with no intrinsic toxicity. The activity was affected by N-9 substitution more than by O$^6$-benzyl para-substitution by halogen atom (McElhinney et al., 2003). However, N-9 and, to a lesser extent, N-2 are the only positions in Bg that are tolerated for conjugation without total loss of activity. AGT inactivation was also observed in the tumor xenograft of the treated animal, showing that IBgBZ reached the targeted tissue in vivo in an active form.

Biodisposition studies in mice bearing B16 murine melanoma demonstrated that IBgBZ concentrated in the tumor up to 5 to 6% of the injected dose per gram of tissue at 1 to 3 h.
postinjection. The maximal tumor/blood ratio (3.4) was reached at 3 h postinjection. At this time, 70% of the dose was detected as the intact IBgBZ. SIMS analysis at the same time yielded further evidence of IBgBZ concentration in the intracellular melanin-rich organelles (melanosomes). This result is consistent with our previous experiments showing that BZ derivatives concentrate in the melanosome and form a reversible melanin-BZ complex (Guerquin-Kern et al., 2004). Because nonconjugated parenteral inhibitors did not display any tissue selectivity (Cussac et al., 1994b; Vaidyanathan et al., 2004), these observations prove that the BZ moiety allows melanoma cell targeting.

Pharmacological studies in nude mice bearing the established M4Beu tumor confirmed that IBgBZ potentiated the cystemustine antitumor action in vivo. Among the protocols tested, the most effective was a 3-h IBgBZ pretreatment followed by a cystemustine injection at days 1, 5, and 9. The tumoral growth of the mice treated by the IBgBZ/cystemustine combination was significantly lower than those treated by cystemustine alone at the same dose. This result was correlated with a decrease in intratumoral AGT levels in mice treated with the combination. The in vivo 3-h pretreatment was probably more efficient because the optimum activity of the cystemustine and the lowest AGT levels were concomitant. When cystemustine was injected 6 h after the inhibitor, the antitumoral activity may have occurred when AGT levels start to increase again, resulting in a limited efficacy. However, the regimen schedules could be optimized. First, the significant efficacy of the inhibitor 3 h pretreatment versus the 6-h pretreatment suggests that cystemustine could be injected earlier. Second, the tumor regrowth observed from day 18, i.e., after a 10-day cessation of the treatment, suggests that an additional treatment cycle starting between days 13 to 15 may improve the efficacy of the combination.

In conclusion, this study describes the first melanoma-targeted AGT inhibitor, IBgBZ. The results confirm the interest of our approach based on the combination of a tumor-specific AGT inhibitor to an O\textsuperscript{6}-alkylating agent for a melanoma-targeted therapy. Future development will focus on the following: 1) optimization of inhibitor/cystemustine treatment schedules in correlation with tumoral AGT assays; 2) improvement of IBgBZ properties (selectivity and AGT inhibition) by structural modulation of the inhibitor and/or the vector moieties; and 3) validation of the combined radiochemotherapy action after replacement of the iodine 127 atom by the iodine 131 radioisotope in IBgBZ.

**References**


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