Selective Release of a Cyclopamine Glucuronide Prodrug toward Stem-like Cancer Cell Inhibition in Glioblastoma

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Abstract
Recent data suggest that inhibition of the Hedgehog pathway could be a therapeutic target for glioblastoma. Alkaloid cyclopamine inhibits Hedgehog signaling, depleting stem-like cancer cells derived from glioblastoma. However, this compound is toxic for somatic stem cells, preventing its use for clinical applications. In this study, we tested a derivatization product of cyclopamine in the form of cyclopamine glucuronide prodrug (CGP-2). This compound was used in vitro and in vivo toward glioblastoma-initiating cells (GIC). Results obtained in vitro indicate that CGP-2 is active only in the presence of β-glucuronidase, an enzyme detected in high levels in necrotic areas of glioblastomas. CGP-2 decreased proliferation and inhibited the self-renewal of all GIC lines tested. Hedgehog pathway blockade by 10 μmol/L of CGP-2 induced a 99% inhibition of clonogenicity on GICs, similar to cyclopamine treatment. Combination of CGP-2 with radiation decreased clonogenic survival in all GIC lines compared with CGP-2 alone. In a subcutaneous glioblastoma xenograft model, a two-week CGP-2 treatment prevented tumor growth with 75% inhibition at 8 weeks, and this inhibition was still significant after 14 weeks. Unlike cyclopamine, CGP-2 had no detectable toxic effects in intestinal crypts. Our study suggests that inhibition of the Hedgehog pathway with CGP-2 is more effective than conventional temozolomide adjuvant, with much lower concentrations, and seems to be an effective therapeutic strategy for targeting GICs. Mol Cancer Ther; 13(9); 1–11. ©2014 AACR.

Introduction
Glioblastoma multiforme (GBM; World Health Organization grade IV glioma) is the most frequent and aggressive primary brain tumor in adults. Standard treatment consists of surgical resection of tumor, followed by adjuvant radiation therapy and chemotherapy using temozolomide, an oral alkylating agent. However, GBM remains one of the most fatal and least successfully treated solid tumors with a median survival of 14.6 months with current treatment (1). Studies have suggested that the progression of GBM is associated with presence in the tumor of a small subpopulation of cells with stem cell characteristics called “glioblastoma-initiating cells” (GIC; refs. 2, 3). These reports have indicated that tumorigenic cells in GICs were restricted to the CD133+ population. However, recent studies have revealed that CD133-negative cells isolated from GBM could also be tumorigenic (4–6) and may in fact represent interconvertible phenotypic states of the same cell population giving rise to a heterogeneous GIC population (4). These cells are highly resistant to conventional chemotherapeutic drugs, including temozolomide (7, 8) and mediate tumor recurrence following radiation therapy (9). Therefore, complete eradication of GICs might be a prerequisite for successful therapeutic strategies.

Signaling pathways involved in the proliferative and self-renewal capacities of stem cells are a potential target in attempts to reduce relapse and improve patient survival. Transient activation of the Hedgehog (HH) and Wnt pathways is known to promote stem cell self-renewal in normal tissues, whereas continuous activation is associated with initiation of many types of cancer such as skin (10), lung (11), prostate (12), gastrointestinal tract (13), breast (14), and brain cancer, including GBM (10, 15). Sandberg and colleagues

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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recently identified alterations of Wnt and HH signaling pathways as important events conferring self-renewal potential and tumorigenic properties to GICs (16). In addition, it has been shown that the HH pathway is implicated in glioma resistance to alkylating agents and the maintenance of GICs (17–19).

Cyclopamine is a natural alkaloid from Veratrum californicum (California corn-lily) that is known to inhibit the HH pathway by direct binding on the heptahelical bundle of Smo (20–22). This compound exhibits antiproliferative activity on glioma cell lines (17). Recent data have demonstrated that blockage of the HH pathway by cyclopamine reduces proliferation, neurosphere formation, self-renewal, and tumorigenicity of GICs with or without radiation (18, 19). Furthermore, pretreatment of GICs with cyclopamine has inhibited tumor formation and growth in athymic mice (18). In addition, cyclopamine was recently evaluated for its antitumor activity in preclinical and clinical trials (23).

Although cyclopamine seems to be a promising chemotherapeutic agent, this drug could be highly toxic for somatic stem cells in normal tissues like brain, which are also HH dependent. In an attempt to circumvent this drawback, Hamon and colleagues have recently designed glucuronide prodrugs programmed to deliver cyclopamine selectively in the tumor microenvironment (24–26). In fact, several studies have shown that glucuronide prodrugs can be activated by β-glucuronidase present in high concentrations in necrotic areas of most solid tumors, including glioblastomas (27). β-Glucuronidase is secreted extracellularly in tumors by inflammatory cells such as neutrophils and macrophages, while in healthy tissues, its activity is confined to lysosomes (28, 29). This enzymatic specificity of the tumor microenvironment allows glucuronide prodrugs to discriminate between malignant and normal tissues. As expected, the derivatization of cyclopamine in the form of cyclopamine glucuronide prodrugs (CGP) significantly reduced its toxicity. When incubated with β-glucuronidase, CGPs led to release of the free drug, thereby restoring its antiproliferative activity against U87-MG glioblastoma cell line. In light of these promising preliminary results, it seemed worthwhile to pursue evaluation of this targeting strategy as a potential alternative means of glioma treatment. Indeed, this therapeutic approach can be used in a systemic way since it has been recently reported that the blood–brain barrier (BBB) is disturbed in GBM (30, 31). Newly formed blood vessels in GBM display abnormal structure and loss of expression of tight junctions and claudin-3 causing structural and functional alterations of the BBB (32). These alterations allow chemotherapeutics to reach the bulk of the tumor. Furthermore, the BBB permeability can also be increased using ultrasound technology allowing transient opening and drug delivery without neural cells damage (33). Within this framework, the aim of the present study is to appraise the effect of the CGP-2 on glioma-initiating cells in vitro and in vivo.

Materials and Methods

Cyclopamine glucuronide prodrug production

The CGP-2 (Supplementary Fig. S1) was selected as the best candidate for this study due to its improved aqueous solubility compared with its previous analogue CGP-1 described by Hamon and colleagues (24) CGP-2 was prepared following the previously described synthetic strategy (25). The compound was then purified using preparative high-performance liquid chromatography before biologic evaluation (purity > 95%). In our experiments, CGP-2 was dissolved in DMSO.

GIC cell lines, H9-NSC, and cell culture

Tumor samples were obtained within 30 minutes after surgical resection from 4 adult patients with glioblastoma (GIC-2, GIC-3, GIC-6, and GIC-12). The methodology for isolation and characterization of these cells has been described in details in our earlier reports (34–36). All GIC lines were assessed for self-renewal, differentiation, and in vitro clonogenicity by limiting dilution assays. In addition, tumorigenicity and stemness properties of GBM-derived initiating cells were evaluated by xenograft experiments in nude mice. Cells derived from all four tumors were cultured as proliferative nonadherent spheres in neurobasal medium (NBE) supplemented with 20 ng/mL of basic fibroblast growth factor (bFGF; Life Technologies), 20 ng/mL of EGF (Life Technologies), and culture supplements N2 (100X, Life Technologies) and B27 (50X, Life Technologies). The molecular characteristics, including MGMT promoter methylation, EGFR copy number, IDH1, IDH2, EGFR-variant III, p53, PTEN status as well as LOH at loci 1p36, 19q13, 9p21, and 10q23, of GICs and their corresponding tumors are notified in Supplementary Table S1. Human neural stem cells (H9-NSCs) are derived from NIH-approved H9 (WA09) human embryonic stem cells (Life Technologies). Cells were cultured following the manufacturer’s instructions.

Reverse transcription quantitative PCR

Total RNA was extracted from tumors and GICs by RNAqueous-4PCR Kit (Life Technologies) according to the manufacturer’s instructions. First-strand cDNA synthesis was carried out on 1-μg samples of total RNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). SHH, PTCH1, SMO, GLI1, and GLI3mRNA expressions were determined using TaqMan gene expression assays Hs00179843_m1, Hs00181117_m1, Hs00170665_m1, Hs0110768_m1, and Hs00609233_m1 (Life Technologies), respectively. qRT-PCR was carried out in duplicate and performed using the TaqMan master mix (Life Technologies) on a 7900HT Fast system (Life Technologies). SHH, PTCH1, SMO, GLI1, and GLI3mRNA expressions were normalized to GAPDH mRNA levels and determined by the \( \frac{C_{\text{DD}}}{C_{0}} \) method.
Cell viability assays

The CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used to determine the effect of cyclopamine (Biovalley), temozolomide (Interchem), and CGP-2 on cell viability. Cells were plated in 96-well plates at a density of $5 \times 10^4$ cells per well in 90-μL medium. After 24 hours of incubation, 10 μL of CGP-2 ± β-glucuronidase (40 IU/mL, Sigma-Aldrich), cyclopamine, or temozolomide was added to each well. Control cells were treated with DMSO or β-glucuronidase. Cell viability was determined after 5 days of treatment. The quantification of viable cells was performed at 492 nm with a microplate reader (Dynex Technologies). The IC50 value was calculated as the drug concentration required to inhibit cell viability by 50% compared with control (DMSO or β-glucuronidase).

Cell proliferation assays

Cells were plated in 96-well plates at a density of $5 \times 10^4$ cells per well in 90-μL medium. After 24 hours of incubation, 10 μL of CGP-2 (at IC50 concentration) + β-glucuronidase (40 IU/mL) were added to each well. Control cells were treated with DMSO + β-glucuronidase. The quantification of viable cells was performed at 492 nm with a microplate reader at indicated times.

Clonogenic assays in methycellulose

GIC cells ($4 \times 10^4$ cells) were plated in 30-mm Petri dish (Greiner Bio One) in 1 mL of complete methycellulose medium S1 (Stem Cell Technologies) and supplemented with B27 (50X), N2 supplements (100X), bFGF, and EGF (0, 1 μg/mL). GIC-2, -3, -6, and -12 had consistently low plating efficiencies (7.4%, 2.8%, 8.1%, and 7.8%, respectively) and therefore required high seeding concentrations to provide sufficient number of cells to be scored after treatment. Cells were treated with CGP-2 (5 and 10 μmol/L + 40 IU/mL of β-glucuronidase), cyclopamine (5 and 10 μmol/L), or temozolomide (200 and 800 μmol/L). After 21 days of incubation, colonies consisting of more than 50 cells were counted under an inverted microscope. The experiment was repeated three times. For radiation combination, the cells were plated in methycellulose, treated with CGP-2 (IC50 concentration + 40 IU/mL of β-glucuronidase) or temozolomide (IC50 concentrations), and irradiated using an Elekta Synergy Beam Modulator (electron energies 18 MeV) at a dose of 2 Gy and colonies were counted after 21 days.

Limiting dilutions and neurosphere-initiating cell assays

To determine the effect of CGP-2 and temozolomide on the frequency of neurosphere-initiating cells (NS-IC), we performed limiting dilution assays using single cell populations immediately after dissociation of neurospheres. Final cell dilutions ranged from 1 cell per well to 100 cells per well (IC50 concentrations). After a 21-day incubation, the fraction of wells not containing neurosphere was determined at each plating density. The fraction of negative wells was plotted against the number of cells per well. The number of cells required to form one neurosphere, which reflects the frequency of cancer-initiating cells in the entire population, was determined as described previously (34, 35).

Annexin V and flow cytometry

GICs were seeded 24 hours before treatment with 500 μmol/L temozolomide or 30 μmol/L CGP-2 with β-glucuronidase (40 IU/mL). After 5 days, cells were stained with Annexin V and 7-aminoactinomycin D (7-AAD) using a FITC Annexin V apoptosis detection kit (BD Biosciences) following the manufacturer’s instructions. The cells were washed twice with PBS and stained with FITC-conjugated Annexin V (5 μL) and 7-AAD (7 μL, BD Biosciences) in 100 μL of binding buffer [10 mmol/L Hepes/NaOH (pH7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2] for 15 minutes at room temperature in the dark. Apoptosis was measured immediately by flow cytometry on a FACS Canto II (BD Biosciences). Data analysis was performed using FACS Diva software (BD Biosciences). A total of 10,000 events were analyzed in one typical experiment.

Mouse xenografts

Female athymic Swiss nude mice were used at 9 weeks of age (Crl:nu-Foxn1nu, Charles River). All of the mice were maintained in a conventional-specific pathogen-free facility and received standard laboratory rodent food and water ad libitum. All experiments were conducted in accordance with institutional guidelines for animal use and care. GIC-2 cells ($1 \times 10^5$) were cultured as previously described (see cell culture section). The cells were dissociated, suspended in NBE, and injected subcutaneously with a 26-gauge needle into the left flank of nude mice. Tumors were observed at injection site (>4 mm) after 130 days in 8 of 18 mice and collected when their size reached 1000 mm². They were then removed, finely chopped, and dissociated into single cell suspension. Cells were grafted subcutaneously into the left flank of female nude mice at a concentration of $3 \times 10^5$ cells. Mice were randomly allocated to the treatment groups when tumor volume reached 42 mm³. They were anesthetized with isoflurane (1.5%–3%) and a drop of ophthalmic anesthetic (0.5% proparacaine hydrochloride ophthalmic solution, Sigma-Aldrich) was placed on the eye that would receive the injection. Cyclopamine (4 mg/kg), CGP-2 (50 mg/kg), or vehicle (20% DMSO) was injected in the retro-orbital sinus with a 0.5-inch insulin needle and syringe once every 3 days (5 injections).

To determine tumor growth, tumor size was estimated with calipers every week. Tumor volume ($V$) was calculated as $V = (a \times b^2)/2$, where $a$ is the long tumor axis and $b$ is the short tumor axis. Mice were sacrificed when tumors reached 17 mm as the longest axis (~2,000 mm³) or on appearance of the first clinical signs (weight loss). Subcutaneous tumors were removed, fixed in 4% paraformaldehyde, embedded in paraffin, cut in 3 μm-thick sections,
and stained with hematoxylin and eosin (H&E). IHC staining to examine GFAP expression in subcutaneous tumors and patient was performed using GFAP mouse monoclonal antibody (1/200; Dako).

Results

Heterogeneous expression of the HH pathway components in glioblastoma-initiating cells

The HH pathway was shown to be active in a subset of primary GBM and established GBM cell lines (18). To evaluate HH pathway expression in GICs, we examined mRNA expression of pathway receptors SMO and PTCH1, ligand SHH and targets GLI1 and GLI3 by real-time RT-PCR in four GICs. Expression of these genes was then compared with human H9-NSCs and to the corresponding tumors. The HH pathway receptors SMO and PTCH1 mRNA were detected at various levels, in line with previous results from Bar and colleagues 2007(18), with a lower expression level of PTCH1 as compared with SMO. SMO and PTCH1 were most highly expressed in GIC-3 and GIC-6. Analysis of GLI1 and GLI3 targets revealed different expression profiles with higher levels of GLI1 transcript in GIC-6 and GIC-12. Very low levels of SHH mRNA expression were detected in our GICs. A lack of SHH mRNA expression had previously been observed in adherent cell lines as well as in high-grade gliomas (18). These observations corroborate previous results obtained in GBM-derived neurospheres (18, 19) and suggest that the HH pathway is present in these cells. SMO, PTCH1, and GLI1 were similarly expressed in GICs and H9-NSCs, whereas GLI3 and SHH expressions were higher in H9-NSCs (Fig. 1A). In the corresponding tumors, the HH pathway receptor SMO and targets GLI1 and GLI3 expressions were comparable with GICs. Interestingly, PTCH1 mRNA level was 2- to 3-fold higher in tumors than the corresponding GIC. In these tumors, SHH mRNA expression was similar to that of GICs except for GBM-12 showing increased expression (Fig. 1B).

Cyclopamine glucuronide prodrug inhibits viability of GICs

We initially examined the effect of cyclopamine on GICs viability. As expected, cyclopamine treatment resulted in reduced viability of GICs. The viability of these cells decreased after 5 days of treatment with IC50 values of 24, 33, 29, and 24 μmol/L for GIC-2, -3, -6, and -12, respectively (Table 1). We then evaluated the effect of CGP-2 on GIC viability. The cells were treated for...
5 days either with the prodrug alone or in combination with \( \beta \)-glucuronidase, and MTS assays were performed. CGP-2 had no effect on GIC viability when used alone. In contrast, when prodrug was combined with \( \beta \)-glucuronidase, the viability of GICs markedly decreased \((P < 0.001)\), with IC\(_{50}\) values between 25 and 29 \( \mu \)mol/L (Fig. 2A). We further examined the effect of CGP-2 (IC\(_{50}\)) in combination with \( \beta \)-glucuronidase on GICs proliferation. Analysis of growth curves showed a reduction of proliferation in all GICs treated with CGP-2 (Fig. 2B; \( P < 0.001)\). Nevertheless, at IC\(_{50}\) concentration (30 \( \mu \)mol/L), the prodrug had no effect on H9-NSCs in the absence of \( \beta \)-glucuronidase (Supplementary Fig. S2).

To analyze the mechanisms of cell death, we carried out Annexin staining of GICs after treatment with 30 \( \mu \)mol/L of CGP-2 \( \beta \)-glucuronidase. After 5 days of treatment, all GICs underwent apoptosis at various levels. As shown in Fig. 2C, in the absence of CGP-2, similar basal levels of apoptosis were present in GIC-2, -3, and -12 except for GIC-6. After 5 days of treatment with CGP-2, the percentage of apoptotic cells had risen significantly from 19% to 99% after treatment with \( \beta \)-glucuronidase (Fig. 2D).

To determine the effect of CGP-2 on the frequency of "NS-IC", we performed limiting dilution assays using single-cell populations immediately following neurosphere dissociation. The frequency of NS-IC, corresponding to the number of cells required to generate at least one tumor sphere per well was 1/17, 1/25, 1/16, and 1/42 for GIC-2, -3, -6, and -12, respectively (Table 2). Treatment of cells with IC\(_{50}\) doses of CGP-2 resulted in a decrease of NS-IC frequency to 1/321 for GIC-3, 1/1,710 for GIC-6 (\( P < 0.001\)), 1/735 for GIC-2, and 1/661 for GIC-12 (\( P < 0.05\)).

Taken together, these data show that derivatization of cyclopamine in the form of the glucuronide prodrug CGP-2 turned off its activity. Furthermore, the presence of \( \beta \)-glucuronidase was necessary to restore cyclopamine’s biologic properties, which include GIC proliferation inhibition, clonogenicity, and neurosphere initiation.

**CGP-2 in combination with radiation**

The standard Stupp protocol for the treatment of glioblastoma includes tumor resection followed by chemotherapy with temozolomide and concomitant radiation therapy. We first compared the effect of temozolomide with CGP-2. We have previously shown that the intended clinical dose of temozolomide (50 \( \mu \)mol/L) has no effect on GIC proliferation (35). Dose–response curves of temozolomide on GIC-2, -3, -6, and -12 showed IC\(_{50}\) values of 474, 787, 830, and 864 \( \mu \)mol/L, respectively (Table 1). As observed with CGP-2, temozolomide also inhibits colony formation. Colony growth was significantly reduced in all GIC lines in the presence of 200 \( \mu \)mol/L temozolomide (24%–50%; Fig. 3A) and growth inhibition using 800 \( \mu \)mol/L temozolomide ranged from 47% to 83% (\( P < 0.001\)). The effect of temozolomide on NS-IC frequency was limited as frequency was 1/735 for GIC-2 and 1/661 for GIC-6 (\( P < 0.001\)).

**Table 1. IC\(_{50}\) values after 5 days of cyclopamine, CGP-2, or temozolomide treatments**

<table>
<thead>
<tr>
<th>GIC</th>
<th>IC(_{50}) values of cyclopamine (( \mu )mol/L)</th>
<th>IC(_{50}) values of CGP-2 (( \mu )mol/L)</th>
<th>IC(_{50}) values of temozolomide (( \mu )mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIC-2</td>
<td>24 ± 1.5</td>
<td>25 ± 0.2</td>
<td>474 ± 47</td>
</tr>
<tr>
<td>GIC-3</td>
<td>33 ± 1.9</td>
<td>26 ± 1.8</td>
<td>787 ± 54</td>
</tr>
<tr>
<td>GIC-6</td>
<td>29 ± 1.6</td>
<td>29 ± 3.9</td>
<td>830 ± 40</td>
</tr>
<tr>
<td>GIC-12</td>
<td>24 ± 3.2</td>
<td>27 ± 4.6</td>
<td>864 ± 1.7</td>
</tr>
</tbody>
</table>

NOTE: IC\(_{50}\) values of cyclopamine and CGP-2 were much lower than temozolomide IC\(_{50}\) values. IC\(_{50}\) values were calculated as the mean drug concentration required for inhibiting cell proliferation by 50% compared with control (mean ± SD).
0.05) when cells were treated with IC_{50} concentrations. Furthermore, we did not observe any significant reduction in NS-IC frequency in GIC-3 and GIC-12 (1/164 and 1/63, respectively; Table 2). Taken together, these data indicate that CGP-2 treatment (in the presence of β-glucuronidase) was more effective than temozolomide at much lower concentrations as it led to enhanced anti-proliferative activity, inhibition of colony formation in GICs, and reduced NS-IC frequency.

We went on to investigate whether or not CGP-2 in combination with radiation was more potent in reducing GIC survival than the conventional adjuvant temozolomide. Following 2 Gy radiation, even though clonogenic survival of all GIC lines decreased significantly beyond 21 days (P < 0.001), the population of resistant cells remained higher than 70% for GIC-3 and GIC-6, 60% for GIC-12, and 50% for GIC-2 (Fig. 3B). Combined temozolomide treatment (IC_{50} concentrations) with radiation reduced the number of resistant cells in comparison with radiation alone or temozolomide alone (P < 0.001) with their population ranging from 18% to 39%. When combined with radiation, IC_{50} doses of CGP-2 drastically reduced clonogenic survival of all GIC lines compared with CGP-2 alone (Fig. 3B; P < 0.001) or radiation alone as the population of resistant cells dropped below 10% for GIC-2, -3, -6 and 16% for GIC-12. Combination of CGP-2 with radiation was clearly more potent than the conventional temozolomide adjuvant (P < 0.001). Additional tests performed
on GIC-2 and GIC-6 cells indicate that the combination of CGP-2 with temozolomide or temozolomide and radiation reduced drastically the number of resistant cells ($P < 0.001$; Fig. 3B).

**In vivo effects of CGP-2**

To test the antitumoral activity of CGP-2 in vivo, we first established subcutaneous glioblastoma xenografts in athymic Swiss nude mice (see Materials and Methods). As shown in Fig. 4B, tumor xenografts that originated by injecting GICs (ii) shared the same histologic properties as the original tumors (i); H&E section of xenografts from patients showed pseudopalisading surrounding areas of necrosis center (NC). These tumors were characterized by high cellularity, nuclear atypia, and mitotic activity (iii and iv), which reflected the patient’s original tumor.

**Table 2. Effects of CGP-2 and temozolomide treatment on NS-IC assay**

<table>
<thead>
<tr>
<th>GIC</th>
<th>NS-IC frequency control</th>
<th>NS-IC frequency after CGP-2 treatment</th>
<th>NS-IC frequency after temozolomide treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIC-2</td>
<td>1/17 (±4)</td>
<td>1/735 (±28; $^aP &lt; 0.05$)</td>
<td>1/735 (±46; $^aP &lt; 0.05$)</td>
</tr>
<tr>
<td>GIC-3</td>
<td>1/25 (±3)</td>
<td>1/321 (±55; $^aP &lt; 0.001$)</td>
<td>1/164 (±6)</td>
</tr>
<tr>
<td>GIC-6</td>
<td>1/16 (±1)</td>
<td>1/1710 (±725; $^aP &lt; 0.001$)</td>
<td>1/661 (±16; $^aP &lt; 0.05$)</td>
</tr>
<tr>
<td>GIC-12</td>
<td>1/42 (±5)</td>
<td>1/86 (±2; $^aP &lt; 0.05$)</td>
<td>1/63 (±2)</td>
</tr>
</tbody>
</table>

*NOTE: Treatment with CGP-2 and temozolomide resulted in a significant decrease of NS-IC frequency ($^a, P < 0.05$; $^b, P < 0.001$; calculated with a two-tailed unpaired Student t test). Final cell dilutions yielding 37% of negative wells correspond to the dilution at which there is one NS-IC per well (mean ± SD).*

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**Figure 3.** CGP-2 and temozolomide in combination with radiation. A, colony formation was measured by clonogenicity assay after 21 days following 200 and 800 μmol/L temozolomide treatment. The number of colonies formed was markedly reduced by temozolomide. B, combined CGP-2 with radiation was more effective than combined temozolomide treatment. Each set of results was obtained from two independent experiments. Experiments were performed in triplicate and expressed as mean ± SD. $^***, P < 0.001$, calculated with a two-tailed unpaired Student t test.
Patient's tumors (v) and xenografts (vi) expressed the astrocyte-differentiation marker GFAP. When the tumors reached a volume of 42 mm\(^3\), mice were injected through the retro-orbital venous sinus with either 100 μL of vehicle (20% DMSO/80% water), cyclopamine (4 mg/kg), CGP-2 (50 mg/kg), or vehicle control DMSO (n = 4, 20% v/v) were injected when tumor reached 42 mm\(^3\). CGP-2 delayed tumoral development for 14 weeks. Results are expressed as mean ± SD. B, H&E section of patient’s original glioblastoma GBM-2 (i) and of subcutaneous glioblastoma xenografts. GIC-2 derived from GBM showed pseudopalisading (ii, black arrowheads) surrounding areas of necrosis center (NC), high cellularity, nuclear atypia, and mitotic activity (iii and iv, white arrowhead). These histologic characteristics reflect the corresponding original patient tumors. Patient tumors (v) and xenografts (vi) were positive for GFAP staining. C, representative section of intestinal crypts from prodrug-treated mice showing normal histology. One of four cyclopamine-treated mice showed apoptotic cells (black arrowheads) in small intestinal crypts. Original magnifications: x200 (ii, v, vi, vii) x400 (i, iv, viii).

Figure 4. CGP-2 prevented growth of subcutaneous glioblastoma xenografts without side effects on intestinal crypts. A, 1 x 10^6 GIC-2 cells derived from a patient with GBM (GBM-2) were subcutaneously injected into athymic mice. Cyclopamine (n = 4, 4 mg/kg), CGP-2 (n = 4, 50 mg/kg), or vehicle control DMSO (n = 4, 20% v/v) were injected when tumor reached 42 mm\(^3\). CGP-2 delayed tumoral development for 14 weeks. Results are expressed as mean ± SD. B, H&E section of patient’s original glioblastoma GBM-2 (i) and of subcutaneous glioblastoma xenografts. GIC-2 derived from GBM showed pseudopalisading (ii, black arrowheads) surrounding areas of necrosis center (NC), high cellularity, nuclear atypia, and mitotic activity (iii and iv, white arrowhead). These histologic characteristics reflect the corresponding original patient tumors. Patient tumors (v) and xenografts (vi) were positive for GFAP staining. C, representative section of intestinal crypts from prodrug-treated mice showing normal histology. One of four cyclopamine-treated mice showed apoptotic cells (black arrowheads) in small intestinal crypts. Original magnifications: x200 (ii, v, vi, vii) x400 (i, iv, viii).
For this reason, only 4 mice were included in each treatment arm. As shown in Fig. 4A, treatment with cyclopamine or CGP-2 prevented tumor growth with 75% inhibition at 8 weeks compared with DMSO-treated mice. However, even though short-term protection was observed with both compounds, long-term follow-up observations indicated a noticeable resumption of tumor development in cyclopamine and CGP-2–treated mice with 70% inhibition after 11 weeks but less than 50% after 14 weeks. Median tumor volumes for cyclopamine and CGP-2–treated mice were similar to the DMSO control group beyond 20 weeks. Previous reports from van den Brink and colleagues (37, 38) indicate that cyclopamine treatment disturbed enterocyte maturation in colonic epithelium with decreased proliferation in the crypts of the small intestine and increased proliferation of the gastric epithelium. To determine whether or not intravenous injection of cyclopamine or CGP-2 affected intestinal crypt morphology, we performed H&E staining of the crypt area. As shown in Fig. 4C, no morphologic change occurred in the small intestine of prodrug-treated mice (vii), but cell apoptosis in the crypts of the small intestine was observed in 1 of the 4 mice treated with cyclopamine (viii). Quantitative analysis of intestinal toxicity revealed the presence of heterogenous pathologic areas in a cyclopamine-treated mouse containing up to 10% of apoptotic cells. In contrast, in CGP-2–treated mice, apoptotic cell number was far below 1%.

Discussion

The HH pathway is critical to maintenance of stem cell self-renewal in many tissues. Its aberrant hyperactivation is found in different types of cancer, particularly in glioblastoma (15, 17). Cyclopamine was the first Hh inhibitor to be identified (20) and its mechanism of action is well established (21). A number of preclinical and clinical studies have been performed using cyclopamine and synthetic SMO antagonists (23, 39). Studies using disease animal models have reinforced the potential of cyclopamine as an anticancer drug (19, 40–43). Indeed, cyclopamine has the capacity to inhibit proliferation of GICs in vitro and to reduce their tumorigenicity in vivo (18, 19). It nonetheless exhibits poor solubility, while rapid clearance and nonspecific toxicity limit its usefulness as a chemotherapeutic agent. It consequently appeared worthwhile to develop a new cyclopamine-based drug with both reduced toxicity toward healthy tissues and an improved pharmacokinetic profile. Recently, Renoux and colleagues (25) designed a new water-soluble glucuronide cyclopamine prodrug (CGP-2) enabling the β-glucuronidase–catalyzed release of the drug to overcome its unselective toxicity. They demonstrated the validity of this in vitro approach on a U87-MG glioma cell line. As an extension of this type of preliminary approach, the aim of our study was to test the effect of CGP-2 on GICs in vitro and on xenografted tumors in vivo.

In all four of the tested GIC lines and in the corresponding tumors, the key components required to transduce HH signaling were present and heterogeneously expressed in accordance with previous reports (18, 19). SHH ligand mRNA was weakly expressed in GICs, a finding that partially explained the higher expression of GLI3 compared with GLI1; although transcription of GLI1 is silenced in the absence of SHH ligand, GLI3 can still be expressed (44). Furthermore, inhibition of GLI3 has been shown to revert the antiproliferative and proapoptotic effects of cyclopamine, which were thereby mediated (19).

As expected, CGP-2 is active only in the presence of β-glucuronidase and has the capacity to decrease proliferation and to prevent the self-renewal and clonogenicity of all GIC lines tested in vitro. In the range of concentrations tested, CGP-2 is more active than temozolomide. In accordance with previous work by Beier and colleagues (45), the intended clinical doses of temozolomide (50 µmol/L in plasma and 10 µmol/L in cerebrospinal fluid; refs. 36, 37) are not sufficient to prevent proliferation. As regards, the clonogenic properties of GIC lines in vitro, the above-mentioned effects have been observed only at pronouncedly higher concentrations (500 µmol/L).

The preliminary experiments we conducted in a subcutaneous xenograft model also indicate that similar CGP-2 antitumoral activity is observed in vivo. Histologic analysis of xenografted tumors showed that the mouse model recapitulated most of the typical features described in GBM. Intravenous administration of cyclopamine and CGP-2 significantly prevented tumor growth in mice over 8 weeks, but the effect could not be sustained on a long-term basis. Results were nevertheless very promising because the prodrug was administered only over a period of 2 weeks; in the future, it could be worthwhile to try to determine the effects of CGP-2 over the course of long-term treatment. In addition, unlike cyclopamine, no toxicity signs were noted in mice treated with the prodrug, in accordance with locally controlled delivery procedures. Furthermore, in vitro and in vivo results indicate that derivatization of cyclopamine in the form of CGP-2 fully abolished its off-target toxicity but did not alter its efficacy on tumor cells. With a favorable toxicity profile and promising efficacy, CGP-2 would appear to be a good candidate for testing in clinical applications. In addition, our results provide in vitro evidence of the relatively high efficiency of CGP-2 combined with 2 Gy radiation.

Over recent years, numerous efforts have been devoted to enhancing the efficacy of β-glucuronidase-responsive prodrugs. For instance, Juan and colleagues (29) demonstrated that antiangiogenic agents can synergize the anti-tumor activity of glucuronide prodrugs by selectively enhancing concentration of the activating enzyme in the tumor microenvironment. More recently, the concept of glucuronidase-responsive albumin-binding prodrugs seemed to represent a promising approach to limiting the rapid renal clearance observed with previous glucuronide prodrugs (46). These novel approaches could be used in
the near future to increase the therapeutic potential of CGIs.

To sum up, our experimental results suggest that targeting of the Hh pathway could represent a valid strategy and serve as an adjuvant to radiotherapy in the treatment of patients with GBM. In this regard, a phase II trial is ongoing to test GDC-0449 (Vismodegib), an oral small-molecule SMO inhibitor, in patient with recurrent GBM (clinicaltrials.gov; NCT00980343) GDC-0449. This drug was recently approved by US Food and Drug Administration for advanced and metastatic basal cell carcinoma (47). In addition Rudin and colleagues have reported that GDC-0449 induced a rapid but transient regression of the tumor in a patient with metastatic medulloblastoma (48). Likewise, our strategy which permits an efficient targeting of tumoral cells in a necrotic environment could be of interest for delivering such type of molecule.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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