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Preliminary communication

Study of a cyclopamine glucuronide prodrug for the selective chemotherapy of glioblastoma

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ABSTRACT

The first glucuronide prodrug of the hedgehog signaling inhibitor cyclopamine was synthesized. The carbamoyl derivatisation of cyclopamine significantly decreased its toxicity towards the U87 human glioblastoma cell line. However, when the prodrug was incubated with β-glucuronidase in the culture media, the active drug was efficiently released thereby restoring its anti-proliferative activity.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults with a very poor prognosis of patients (median survival ranges from 9 to 12 months), linked to its highly invasive characteristics and to the lack of efficient treatment of these tumors [1,2]. Hence, there is an urgent need to develop novel therapeutic strategies against GBM. These cancers, also referred as type IV astrocytoma, derive from brain astrocytes or neural progenitor cells [3,4]. Astrocytic commitment and growth lie on a number of cytokines, trophic and developmental factors. Among them are the hedgehog (Hh) family of proteins which control cell proliferation and differentiation in processes that range from insect segmentation and limb formation to vertebrate neural tube development and bone differentiation [5]. Hh factors act on target cells through binding to a transmembrane glycoprotein receptor called patched (Ptc), associated to a co-receptor, called smoothened (Smo), a member of the G-protein coupled receptors (GPCR) superfamily. In the absence of Hh ligand, Ptc represses Smo activation. Hh binding to Ptc alleviates its repression on Smo, leading to activation of the Smo/Ptc complex and of its associated downstream effectors, particularly the Gli family of transcription factors which play prominent roles in this pathway. In vertebrates, three Gli proteins (Gli1, 2, 3) have been characterized [6,7]. Overexpression of Gli2 appears to be sufficient to generate and maintain the malignant process in some cancer types such as basal cell carcinoma [8].

Alteration of function and/or expression of components of the Hh pathway has been demonstrated in several cancer types. Ptc mutations have been associated to medulloblastoma and generally, dysfunction of the Hh pathway is involved in development and progression of several human tumors resembling primitive precursor cells, deriving from the skin, lung, prostate, gastrointestinal tract and brain, including GBM [8–12]. Interestingly, Gli1 was originally identified long ago as a gene amplified in a human malignant glioma [13]. Blockade of the Hh pathway in all these cancer types thus appears like a promising therapeutic strategy.

Prominent molecules to target this pathway are represented by the alkaloid substance cyclopamine and its derivatives (Fig. 1). Cyclopamine was initially discovered as the teratogenic agent from *Veratrum californicum* (the hellebore or corn-lily) causing holoprosencephaly (or cyclopia) in lambs, a syndrome associated with severe alterations in the development of the central nervous system and of the facial skeleton. Several studies suggest that cyclopamine specifically inhibits Smoothened (Smo) activity and thus interrupts the activated Hh–Gli pathway in cancer cells [14–17]. Recent reports confirm the effectiveness of cyclopamine to eradicate GBM in experimental models *in vitro* or *in vivo*. Viable

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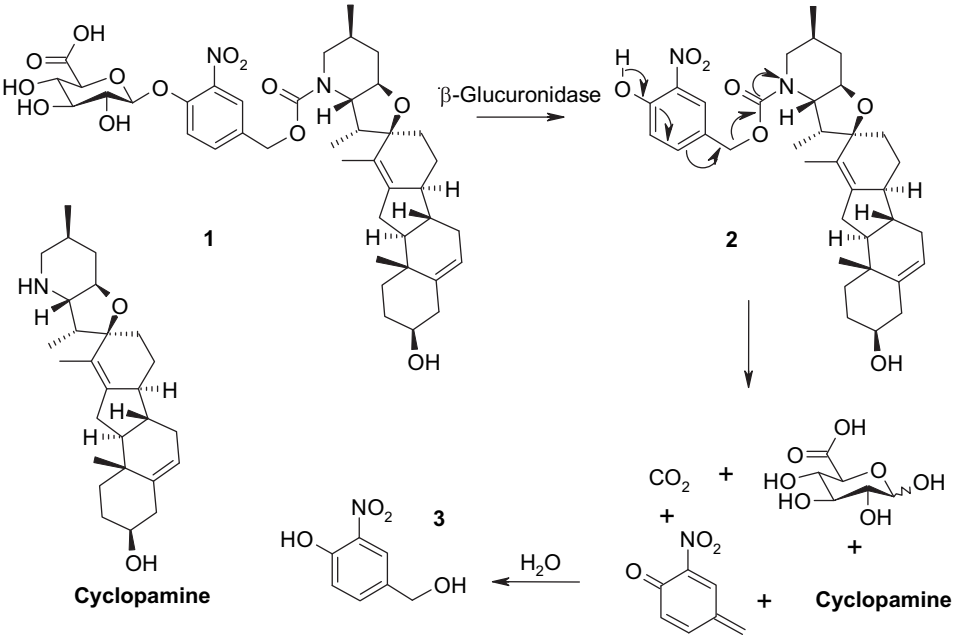


Fig. 1.

GBM cells injected intracranially following Hedgehog blockade by cyclopamine were no longer able to form tumors in athymic mice. The data also indicate that a cancer stem cell population critical for ongoing growth had been removed [18,19]. The specificity of cyclopamine for the Hh pathway is also its main pitfall, due to its potential "on-target" toxicity in somatic stem cells of the patient which are also Hh-dependent.

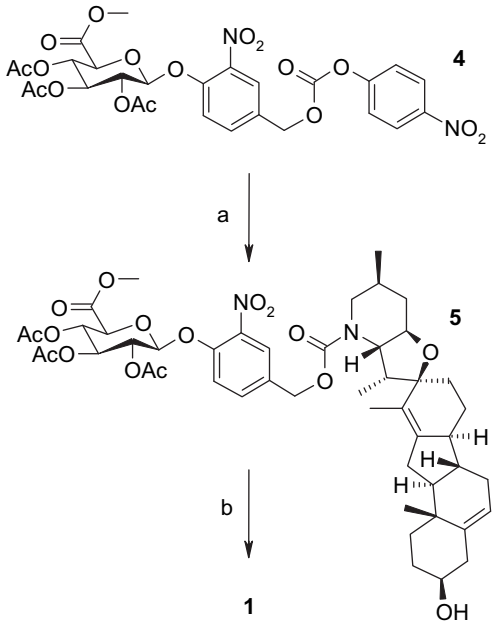
In order to avoid this drawback, we propose the study of the glucuronide prodrug **1** designed to deliver cyclopamine predominantly in the vicinity of the tumor (Fig. 1) [20]. Indeed, this prodrug could be selectively activated by β-glucuronidase present in high concentration in necrotic areas of neoplasia [21]. The selectivity of such an approach has already been demonstrated *in vivo* with several glucuronide prodrugs which exhibited superior therapeutic efficacy in various tumour xenograft models compared to standard chemotherapy [22–24]. Thus, since elevated level of β-glucuronidase has been observed in human GBM [25], the use of **1** should increase cyclopamine concentration in the tumor while reducing its delivery in normal tissues.

Prodrug **1** includes a nitrobenzylphenoxy carbamate linker between the enzyme substrate and the drug [26,27]. With this design, the carbohydrate moiety will be substantially far away from cyclopamine to allow an easy recognition of **1** by β-glucuronidase. Enzymatic hydrolysis of the glycosidic bond should generate the phenol intermediate **2** which may then induce the release of the free drug through a 1,6 elimination process as illustrated in Fig. 1.

2. Results and discussion

2.1. Chemistry

Prodrug **1** was prepared starting from the well-known activated carbonate **4** (Scheme 1) [28]. The latter was first coupled with commercially available cyclopamine in the presence of pyridine to afford the carbamate **5** in 76% yield. At this stage, deprotection of the glucuronide moiety remains to be carried out to obtain the expected compound **1**. However, instability of cyclopamine in acidic media [29] rules out acid-catalysed solvolysis of the ester functional groups. Moreover, as pointed out earlier by Schmidt and co-workers, the one step deprotection of 2,3,4-tri-*O*-acetyl-glucuronyl methyl esters is problematic under basic conditions due to the formation of the 4,5-unsaturated byproduct [30]. Consequently, cleavage of the protecting groups is usually achieved in two steps including acetates transesterification followed by hydrolysis of the methyl ester. Recently, we discovered a mild and efficient one step procedure for the deprotection of 2,3,4-tri-*O*-acetyl-glucuronyl methyl esters with 3 equivalents of dibutyltin oxide (Bu_2SnO) in methanol [31]. This method appeared to be general and tolerant of several functional groups such as carbamates, acetals or allyl ethers. Thus, when applied to



Scheme 1. Reagents and conditions: (a) Cyclopamine, pyridine (3 equiv.), DMF, rt, 16 h, 76% (b) Bu_2SnO (3 equiv.), MeOH, reflux, 24 h, 81%.

the deprotection of the glucuronide derivative **5** this new procedure allowed the synthesis of prodrug **1** in 81% yield after purification by flash column chromatography.

2.2. Stability and enzymatic hydrolysis

The stability of **1** was examined in both phosphate buffer (0.02 M, pH = 7.2) and cell culture media supplemented with 10% foetal calf serum at 37 °C. No decomposition of the prodrug was detected after 48 h under these conditions. Enzymatic hydrolysis was then carried out with an excess of β-glucuronidase and monitored by HPLC (Fig. 2). As expected after addition of the enzyme, rapid disappearance of **1** was observed generating the phenol intermediate **2**. This was followed by the clean decomposition of **2** which led to the release of cyclopamine together with the formation of the 4-hydroxy-3-nitrobenzyl alcohol **3** resulting from nucleophilic addition of water to the corresponding methylene quinone (Fig. 1).

2.3. Biological evaluations

The decrease of the growth rate of U87 human GBM cells by cyclopamine has been previously reported *in vitro* and *in vivo* [18,19]. Prodrug **1** was then evaluated for its anti-proliferative activity on U87 cells after a 5-day treatment (Fig. 3). When incubated alone in the culture media, prodrug **1** did not affect viability of cells, except at the highest tested dose (200 μM). This result demonstrated that the carbamoyl derivatisation of the amino group of cyclopamine significantly reduced its toxicity. However, in the presence of β-glucuronidase (40 U/ml), **1** dramatically decreased the viability of U87 cells with an IC_{50} value of 21 μM similar to that obtained for cyclopamine (IC_{50} = 15.5 μM). This can be unambiguously attributed to an efficient release of the drug in the culture media as previously observed in the course of enzymatic hydrolysis experiments.

3. Conclusion

In summary, we have prepared the first glucuronide prodrug of cyclopamine. This prodrug exhibited reduced toxicity compared to the free drug on U87 glioblastoma cells. On the other hand, enzymatic cleavage of the glucuronide moiety by β-glucuronidase released efficiently cyclopamine thereby restoring its initial anti-proliferative activity. In the light of these results, this prodrug seems to be a good candidate for further *in vivo* investigations.

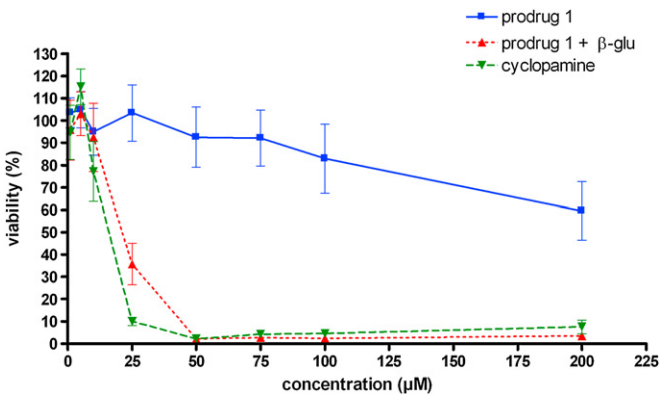


Fig. 3. Viability of U87 cells treated during 5 days with prodrug 1 in the absence (—■—) or presence (···▲···) of β-glucuronidase or with cyclopamine (— ▼ —). Values were obtained from two independent experiments, each performed in hexaplicate and are expressed as mean ± SD.

4. Experimental

4.1. General chemistry methods

All reactions were performed under N_2 atmosphere. Solvents used were of HPLC quality and chemicals were of analytical grade. ^1H and ^{13}C NMR were performed on an Avance 400 Bruker. The chemical shifts are expressed in part per million (ppm) relative to TMS (δ = 0 ppm) and the coupling constant J in hertz (Hz). NMR multiplicities are reported using the following abbreviations: b = broad, s = singulet, d = doublet, t = triplet, q = quadruplet, m = multiplet. The reaction progress was monitored on precoated silica gel TLC plates Macherey-Nagel ALUGRAM® SIL G/UV_{254}. (0.2 mm silica gel 60 Å). Spots were visualized under 254 nm UV light and/or by dipping the TLC plate into a solution of 3 g of phosphomolibdic acid in 100 mL of ethanol followed by heating with a heat gun. Flash column chromatography was performed using Macherey-Nagel silica gel 60 (15–40 μm).

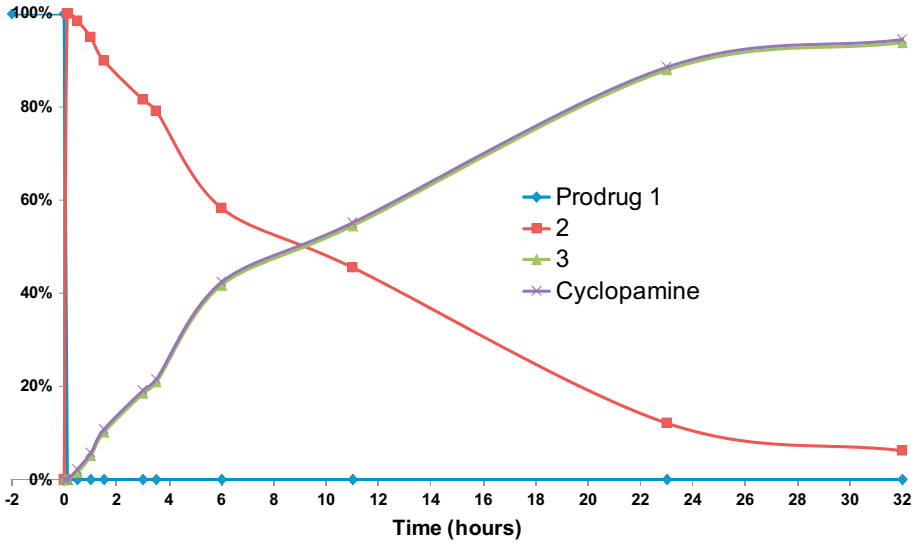


Fig. 2. Enzymatic cleavage of prodrug 1 (0.1 mg.mL^{-1}); β-glucuronidase (133 U.mL^{-1}) was introduced at t = 0 h.

4.2. Synthesis and characterization of described compounds

4.2.1. Synthesis and characterization of compound **5**

Carbonate **4** (0.077 g, 0.12 mmol) and cyclopamine **1** (0.050 g, 0.12 mmol) were dissolved in DMF (3.7 mL). Pyridine (0.03 mL, 0.36 mmol) was added and the solution was stirred at room temperature for 12 h. The mixture was then hydrolyzed with a saturated NH_4Cl solution and extracted three times with EtOAc. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was purified by flash chromatography (30/70 and 50/50 EtOAc/PE). Product **5** was isolated as a white powder in 76% yield (0.085 g, 0.092 mmol). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.90 (m, 3H); 0.95 (m, 3H); 1.21 (m, 8H); 1.48 (m, 2H); 1.70 (m, 12H); 2.05–2.03 (2s, 6H); 2.08 (s, 3H); 2.15 (m, 5H); 2.30 (m, 1H); 2.80 (m, 2H); 3.14 (m, 1H); 3.46 (m, 2H); 3.50 (m, 1H); 3.70 (s, 3H); 4.14 (d, 1H, $J = 7.5$ Hz); 5.12 (s, 2H); 5.28 (m, 5H); 7.39 (d, 1H, $J = 8.7$ Hz); 7.54 (dd, 1H, $J = 8.7$ Hz, $J = 2$ Hz); 7.81 (d, 1H, $J = 2$ Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 10.47, 13.52, 14.20, 18.67, 20.44, 20.52, 20.59, 21.01, 24.58, 28.31, 29.03, 31.36, 31.44, 32.59, 35.11, 36.53, 38.13, 41.77, 49.20, 50.96, 51.98, 53.07, 53.44, 60.40, 65.33, 68.68, 70.16, 71.01, 71.77, 72.58, 85.02, 99.71, 120.11, 121.82, 124.56, 126.36, 133.05, 133.33, 141.22, 141.64, 143.41, 148.73, 157.3, 162.55, 166.71, 169.26, 169.32, 170.02. ESI-MS: m/z $[M + H]^+$ 923.

4.2.2. Synthesis and characterization of prodrug **1**

Compound **5** (0.085 g, 0.092 mmol) was dissolved in MeOH (2.5 mL). After the addition of dibutyltin oxide (0.067 g, 0.28 mmol) the mixture was heated at reflux overnight. The mixture was then evaporated and subjected to flash column chromatography (10/90 and 30/70 MeOH/CH_2Cl_2) to furnish the expected prodrug **1** as a white solid in 81% yield (0.057 g, 0.075 mmol). ^{1}H NMR (CD_3OD/$(CD_3)_2CO$ 3/2) δ (ppm): 0.90 (m, 3H); 1.00 (m, 8H); 1.04 (m, 1H); 1.24 (m, 5H); 1.43 (m, 1H); 1.55 (m, 2H); 1.65 (s, 3H); 1.77 (m, 3H); 1.91 (m, 2H); 2.13 (m, 3H); 2.26 (m,4H); 2.83 (m, 1H); 3.00 (m, 1H); 3.24 (m, 1H); 3.30 (m, 1H); 3.43 (m, 1H); 3.61 (m, 5H); 3.91 (d, 1H, $J = 7.5$ Hz); 5.17 (bs, 3H); 5.37 (bs, 1H); 7.55 (d, 1H, $J = 8.7$ Hz); 7.65 (dd, 1H, $J = 8.7$ Hz, $J = 2$ Hz); 7.87 (d, 1H, $J = 2$ Hz). ^{13}C NMR (CD_3OD/$(CD_3)_2CO$ 3/2) δ (ppm): 177.9, 158.9, 151.1, 144.0, 143.2, 141.6, 134.9, 132.4, 127.8, 125.6, 122.5, 119.0, 102.4, 86.2, 77.6, 76.1, 74.4, 73.6, 73.2, 72.3, 66.6, 64.4, 53.3, 51.6, 50.0, 49.2, 43.3, 42.8, 42.6, 39.4, 37.6, 35.8, 33.4, 32.1, 32.0, 29.8, 25.6, 20.9, 19.0, 13.8, 10.8. ESI-MS: m/z $[M - H]^-$ 781.

4.3. HPLC analysis

Analytical HPLC was carried out using a Dionex P680 System with UV variable wavelength detector and DEDL PLS-ELS 2100 detector. Enzymatic hydrolysis analysis were performed on a reverse phase column chromatography (Chromolite Performance, RP18E, 100×4.6 mm) using a mobile phase (1 mL/min) of CH_3CN/H_2O + 0.1%TFA (gradient from 1:9 to 10:0). Retention time for compounds **1**, **2**, **3** and cyclopamine were 9.02, 11.36, 5.27 and 6.44 min respectively. Peak area and calibration curves were obtained with Dionex Chromeleon software.

4.4. Stability and enzymatic hydrolysis

4.4.1. Compound stability

Prodrugs **1** was placed in phosphate buffer (0.02 M, pH 2.1 or 7) and 10% foetal calf serum at 37 °C for a period of 48 h. HPLC analysis showed no detectable degradation of this compound in these conditions.

4.4.2. Enzymatic cleavage of prodrug **1**

Escherichia *coli* β-glucuronidase was purchased from Sigma–Aldrich (reference: G8162). Prodrug **1** (0.1 mg/mL) were incubated with *E. coli* β-glucuronidase (133 U/mL) in phosphate buffer (0.02 M, pH 7) at 37 °C. Aliquots of 20 μL were taken at indicated time and analyzed by HPLC.

4.5. Cell culture

The U87 human glioblastoma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™ I and sodium pyruvate (Invitrogen), supplemented with 10% fetal calf serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Cells were incubated in a humidified 95% air/5% CO_2 controlled atmosphere at 37 °C.

4.6. Cell proliferation

Cell viability was evaluated using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). U87 cells were plated in 96-well plates at a density of 400 cells/well in 100 μL medium. After a 24 h incubation, medium was replaced by medium containing the prodrug ± β-glucuronidase (40 U/mL) or cyclopamine. Control cells were incubated in the presence of DMSO (used for prodrug or cyclopamine solubilization) ± β-glucuronidase. Cell viability was determined after 5 days of treatment by adding 20 μL of CellTiter 96® Aqueous One Solution Reagent into each well 3 h before measuring the optical density. Metabolically active cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into a coloured formazan product that was measured in a spectrophotometric microplate reader at 490 nm. The OD of control cells was considered as the 100 value.

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