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PAPER

A new cyclopamine glucuronide prodrug with improved kinetics of drug release†

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We prepared a new glucuronide prodrug of cyclopamine designed to target selectively the Hedgehog signalling pathway of cancer cells. This prodrug includes a novel self-immolative linker bearing a hydrophilic side chain that can be easily introduced *via* "click chemistry". With this design, the prodrug exhibits reduced toxicity compared to the free drug on U87 glioblastoma cells. However, in the presence of β-glucuronidase, the prodrug conducts to the quick release of cyclopamine thereby restoring its antiproliferative activity.

Introduction

Recently, aberrant activation of the Hedgehog (Hh) signalling pathway[1] has been observed in a wide range of malignancies such as breast,[2] prostate,[3] gastric,[4] lung[5] and brain[6] tumours. Ever since then, many efforts have been devoted to the discovery of small-molecule Hh inhibitors for cancer chemotherapy.[7] Cyclopamine **1** is a natural alkaloid isolated from *Veratrum californicum* which was the first Hh inhibitor to be identified (Fig. 1).[8] This compound is a potent antagonist of the Hh pathway which inactivates Smoothened (Smo) by binding to its heptahelical bundle.[9] Cyclopamine already demonstrated antitumor activity in the course of preclinical and clinical evaluations.[10]

Although this Hh inhibitor is a promising chemotherapeutic agent, cyclopamine could induce serious damage in normal tissues since somatic stem cells are also Hh-dependent. Moreover, the use of compound **1** *in vivo* is limited by its poor aqueous solubility.[11] In order to circumvent these drawbacks, our group[12] and others[13] have proposed to develop water-soluble prodrugs programmed to deliver cyclopamine selectively in the vicinity of the tumour. Within this framework, we studied the glucuronide prodrug **2a** (Fig. 1, R═H) designed to release cyclopamine in the presence of β-glucuronidase, an enzyme that has been detected at high level in necrotic areas of numerous tumours.[14] This specificity of the tumor microenvironment has been already exploited to activate enzyme-responsive glucuronide prodrugs exclusively in malignant tissues. To date, several glucuronide prodrugs led to

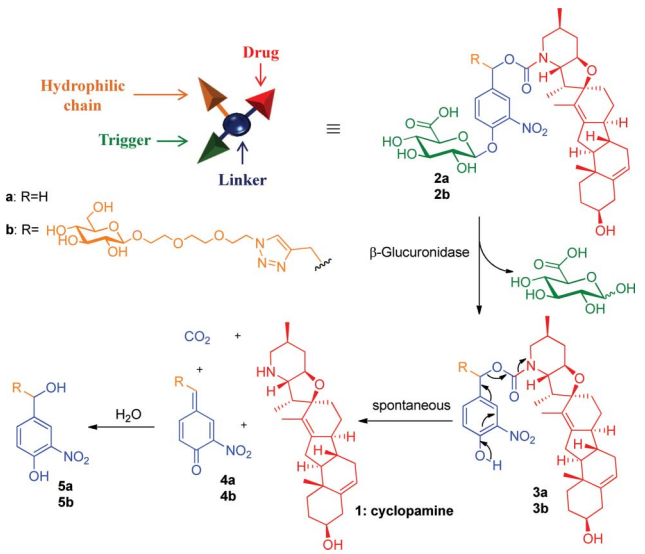

Fig. 1 β-Glucuronidase-catalysed drug release mechanism.

superior therapeutic efficacy compared to standard treatment demonstrating the validity of this targeting strategy.[15]

In our previous study, derivatisation of cyclopamine in the form of prodrug **2a** resulted in a non-toxic compound. However, incubation of the glucuronide **2a** with β-glucuronidase triggered the clean release of the drug through the mechanism depicted in Fig. 1 thereby restoring its antiproliferative activity towards U87 human glioblastoma cells. In our design, we included a nitrobenzylphenoxy carbamate linker[16] between the glucuronide and the bulky cyclopamine in order to allow a good recognition of the carbohydrate substrate by the enzyme. As expected, in the presence of β-glucuronidase the glycosidic bond was rapidly cleaved to generate the phenol **3a**. The latter underwent a 1,6-elimination followed by a spontaneous decarboxylation leading to the full expulsion of cyclopamine within 28 h. During this

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experiment, we observed the precipitation of the linker-drug intermediate **3a** as soon as it was produced by the enzymatic hydrolysis. Therefore, the kinetics of drug release was limited by the gradual solubilisation of **3a** in aqueous media.

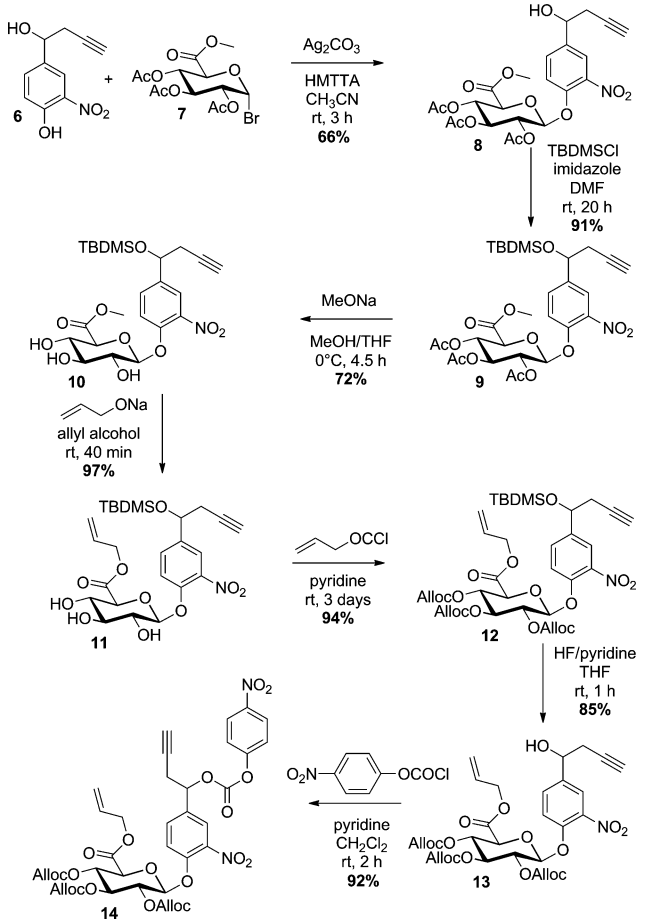
The slow release of cyclopamine from the phenol **3a** could be problematic in the course of a tumour-activated prodrug therapy. Indeed, in such an approach it is well admitted that the liberation of the active compound has to occur quickly after the enzymatic activation step in order to avoid the diffusion of the linker-drug intermediate outside of the tumour site.[16] In this context, it seems worthwhile to develop new cyclopamine glucuronide prodrugs with improved kinetics of drug release. Thus, we decided to pursue our investigations by the study of the prodrug **2b** composed of a glucuronide trigger, the potent cyclopamine and a self-immolative linker bearing a glycosylated poly(ethylene glycol) side chain[17] (Fig. 1). With this design, we anticipated that enzymatic hydrolysis of **2b** will yield the phenol **3b** which will be readily water soluble thanks to the presence of the hydrophilic side chain. Under such circumstances, cyclopamine should be eliminated faster from the intermediate **3b** than from its weakly soluble analogue **3a**.

Results and discussion

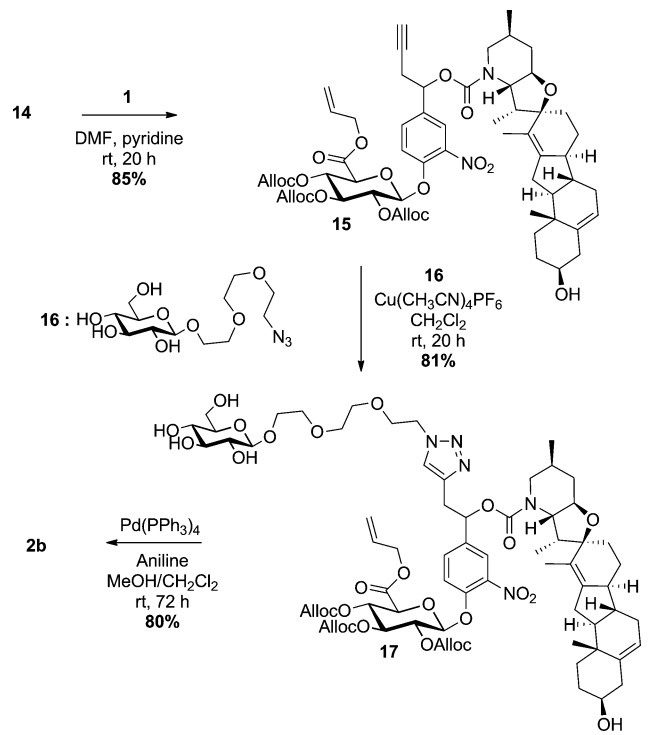
Prodrug **2b** was prepared in ten steps starting from a racemic mixture of the readily accessible nitrophenol **6**[18] (Scheme 1). First, stereoselective glycosylation of **6** was carried out with the bromo-glucuronide **7**[19] under Koenigs-Knorr conditions in the presence of silver carbonate as the catalyst (66%). The resulting β-glucuronide **8** was then treated with *tert*-butyldimethylsilyl chloride and imidazole to produce the silyl ether **9** in 91% yield.

At this stage, the protecting groups of the carbohydrate moiety were modified through a three step strategy to give the fully allyl protected derivative **12**. This choice was motivated by a recent study described by Schmidt and co-workers who demonstrated that both allyl ester and carbonates are compatible with the presence of either alkali- or acid-sensitive anticancer drugs.[20] Furthermore, the entire deprotection of the glucuronide can be achieved in a one step procedure under mild conditions at the end of the synthesis. Thus, this synthetic strategy will limit the number of steps after the introduction of the expensive cyclopamine **1** on the linker unit. The *O*-acetyl groups of compound **9** were first removed using catalytic amount of MeONa to afford the hydroxyl free derivative **10** (72%). Transesterification of the methyl ester with sodium allylate produced the allyl ester **11** in 97% yield. The three allyl carbonates were then introduced in the presence of 30 equivalents of allyl chloroformate employing pyridine as solvent. After 3 days under these conditions, the fully allyl protected glucuronide **12** was obtained in an excellent yield of 94%. Cleavage of the silyl ether was undertaken with HF/pyridine to furnish the alcohol **13** which was subsequently activated in the form of the 4-nitrophenyl carbonate **14** (92%).

Coupling between carbonate **14** and cyclopamine **1** gave the clickable derivative **15** in 85% yield (Scheme 2). The "click chemistry" reaction was then carried out at room temperature in the presence of the azide **16**[21] using $Cu(CH_3CN)_4PF_6$ as a catalyst. Under these conditions, the triazole **17** was prepared in 81% yield after purification by flash column chromatography. The prodrug **2b** was finally obtained as a mixture of two diastereoisomers by the



Scheme 1 Synthesis of the carbonate **14**.



Scheme 2 Synthesis of prodrug **2b** from carbonate **14**.

cleavage of protecting groups using catalytic amount of $Pd(PPh_3)_4$ and two equivalents of aniline (80%).

First, the stability of **2b** was examined in phosphate buffer (0.02 M, pH = 7.2) at 37 °C. No decomposition of prodrug **2b** was detected after 24 h under these conditions. Enzymatic hydrolysis was then conducted with *E. coli* β-glucuronidase and monitored by HPLC/MS (Fig. 2).

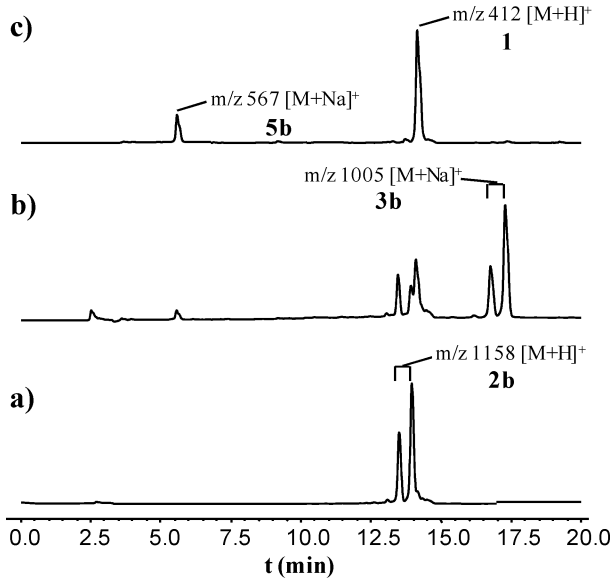


Fig. 2 Enzymatic hydrolysis of the two diastereoisomers of prodrug **2b** with *E. coli* β-glucuronidase in phosphate buffer (0.02 M, pH 7.0) at 37 °C. a) $t = 0$ min; b) $t = 10$ min; c) $t = 120$ min.

The glycosidic bond of prodrug **2b** was rapidly cleaved (within 30 min) showing that the glucuronide trigger is readily accessible by the enzyme, despite the presence of bulky moieties such as cyclopamine and the glycosylated poly(ethylene glycol) side chain attached on the linker unit. Only ten minutes after the addition of β-glucuronidase, cyclopamine **1** and the intermediate **3b** were detected in the medium (Fig. 2b). The latter totally disappeared in less than two hours leading to the clean release of the drug along with the formation of the benzyl alcohol **5b** (Fig. 2c). All together, these results confirmed that the disassembly of prodrug **2b** proceed through the self-immolative mechanism illustrated in Fig. 1. Cyclopamine was expelled significantly faster from prodrug **2b** than from our previous glucuronide **2a** (<2 h *versus* 28 h). In contrast with its analogue **3a**, the phenol **3b** did not precipitate in the reaction mixture. As expected, this intermediate was fairly water soluble thanks to the hydrophilic side chain thereby allowing the quick release of cyclopamine.

Prodrug **2b** was then tested for its anti-proliferative activity on U87 glioblastoma cells after a 5-day treatment (Fig. 3). When incubated alone in the culture medium, prodrug **2b** did not affect viability of cells whereas the free drug was highly toxic with an IC_{50} value of 16.5 µM. This result indicated that derivatisation of cyclopamine in the form of prodrug **2b** markedly reduced its anti-proliferative activity. On the other hand, addition of β-glucuronidase in the culture medium induced a dramatic anti-proliferative effect with an IC_{50} value close to that obtained for cyclopamine (IC_{50} = 24.5 µM). This can be unambiguously attributed to the release of the drug in the culture medium

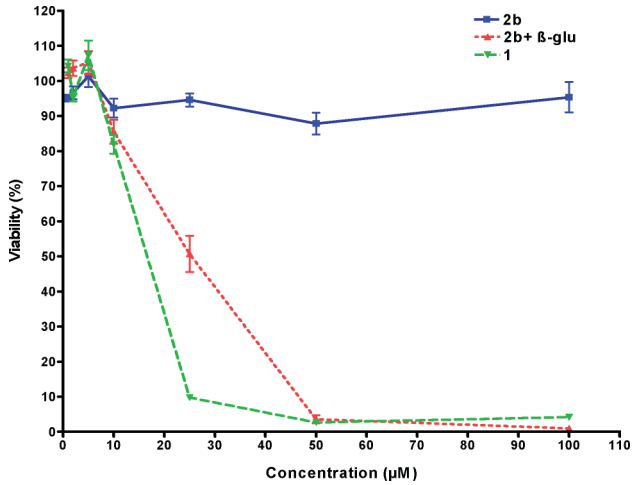


Fig. 3 Viability of U87 cells treated during 5 days with prodrug **2b** in the absence or presence of β-glucuronidase (β-glu, 40 U/mL) or with cyclopamine **1**. Values were obtained from two independent experiments, each performed in hexaplicate and are expressed as mean ± SEM.

as previously observed in the course of enzymatic hydrolysis experiments.

Conclusions

In summary, we prepared a novel non-toxic prodrug of cyclopamine bearing a hydrophilic side chain introduced *via* "click chemistry" on the self-immolative linker. When activated by β-glucuronidase, this prodrug exhibits improved kinetics of drug release compared to its previous analogue which included a less water soluble linker. Furthermore, incubation of the prodrug in the presence of the activating enzyme restores its antiproliferative activity on U87 glioblastoma cells. All these results suggest that this new glucuronide prodrug possesses the necessary prerequisites for further *in vivo* investigation in the course of a tumor targeting strategy.

Experimental

Preparation of compound 8

A solution of 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTTA) (6.3 mL, 23.1 mmol) and Ag_2CO_3 (33.7 g, 122.3 mmol) in anhydrous CH_3CN (33 mL) was stirred during 2 h at room temperature. Nitrophenol **6** (4.56 g, 22 mmol) and bromo-glucuronide **7** (11.31 g, 33 mmol) were added at 0 °C, and the solution mixture was stirred for 4 h at room temperature. The reaction was quenched with water and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with HCl 1 M, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting crude material was purified by column chromatography over silica gel (petroleum ether/AcOEt 6/4, 5/5, 4/6) to afford **8** (7.62 g, 0.84 mmol, 66%) as a mixture of two diastereoisomers (pale yellow solid). R_f 0.56 (petroleum ether/AcOEt 50/50). mp = 67.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.04 (s, 6H), 2.10 (s, 4H), 2.62 (m, 2H), 3.38 (bs, 1H), 3.74 (s, 3H), 4.28 (d, 1H, J = 9 Hz), 4.92 (m, 1H), 5.25–5.32 (m, 4H), 7.34 (d, 1H, J = 8.6 Hz), 7.60 (m, 1H), 7.87 (d, 1H, J = 2.1 Hz);

^{13}C NMR (100 MHz, $CDCl_3$) δ 20.5, 29.2, 53.1, 68.7, 70.1, 70.6, 71.1, 71.8, 72.3, 79.8, 99.5, 119.4, 122.7, 131.5, 139.1, 140.7, 148.0, 166.8, 169.5, 170.1; HRMS (ESI) $[M+Na]^+$ m/z 546.1228 (calcd for $C_{23}H_{25}NO_{13}Na$: 546.1224); $[M+K]^+$ m/z 562.0997 (calcd for $C_{23}H_{25}NO_{13}K$: 562.0963).

Preparation of compound 9

Imidazole (2.4 g, 35.2 mmol) and TBDMSCl (5.3 g, 35.2 mmol) were dissolved in dry DMF (4 mL). The solution was stirred for 0.5 h and a solution of **8** (9.23 g, 17.6 mmol) in DMF (14 mL) was added. After stirring at room temperature for 20 h, water (100 mL) was added; the layers were separated and the aqueous layer was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (CH_2Cl_2/petroleum ether 50/50; 75/25) to give **9** (10.32 g, 91%) as a mixture of two diastereoisomers (pale yellow solid). R_f 0.60 (petroleum ether/AcOEt 60/40). mp = 59.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ –0.04 (s, 3H), 0.09 (2 s, 3H), 0.89 (2 s, 9H), 1.99 (s, 1H), 2.06 (s, 6H), 2.13 (s, 3H), 2.42–2.62 (m, 2H), 3.74 (s, 3H), 4.22 (m, 1H), 4.82 (t, 1H, J = 7.0 Hz), 5.19–5.23 (m, 1H), 5.30–5.37 (m, 3H), 7.33 (2d, 1H, J = 8.7 Hz), 7.55 (m, 1H), 7.80 (d, 0.5H, J = 2.1 Hz), 7.84 (d, 0.5 H, J = 2.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ –4.8, –4.6, 18.3, 20.7, 25.8, 30.9, 53.2, 68.9, 70.3, 71.3, 72.1, 72.2, 72.7, 80.3, 99.9 and 100.0, 119.4 and 119.9, 122.8, 131.3 and 131.4, 140.3 and 140.4, 141.0, 148.4, 166.9, 169.4, 169.5, 170.2; HRMS (ESI) $[M+Na]^+$ m/z 660.2083 (calcd for $C_{29}H_{39}NO_{13}SiNa$: 660.20829); $[M+K]^+$ m/z 676.1819 (calcd for $C_{29}H_{39}NO_{13}SiK$: 676.18223).

Preparation of compound 10

9 (2.6 g, 4.08 mmol) was dissolved in THF (48 mL) and methanol (96 mL). The mixture was cooled at 0 °C and sodium methoxyde (220 mg, 4.08 mmol) was added. Sodium methoxide (110 mg, 2.04 mmol) was added twice after 1 h and 2 h of stirring. The mixture was stirred again for 1 h and was hydrolyzed with IRC-50 acidic resin. The mixture was then filtrated and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (CH_2Cl_2, MeOH 99/1; 98/2; 97/3) to give **10** (1.51 g, 72%) as a mixture of two diastereoisomers (white solid). R_f 0.33 (CH_2Cl_2/MeOH 95/5). mp = 62.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ –0.05 (2 s, 3H), 0.09 (2 s, 3H), 0.89 (2 s, 9H), 1.98 (m, 1H), 2.42–2.62 (m, 2H), 3.76–3.85 (m, 5H), 3.94 (t, 1H, J = 9.7 Hz), 4.10 (2d, 1H, J = 9.7 Hz), 4.81 (2t, 1H, J = 6.3 Hz), 5.01 (2d, 1H, J = 7.3 Hz), 7.34 (2d, 1H, J = 8.7 Hz), 7.59 (dd, 1H, J = 2.1 Hz, J = 8.7 Hz), 7.88 (d, 0.5H, J = 2.1 Hz), 7.90 (d, 0.5H, J = 2.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ –4.8, –4.6, 18.3, 25.8, 30.8, 53.2, 71.0, 71.3, 72.1 and 72.2, 73.0, 74.7, 75.0, 80.4, 102.8 and 102.9, 118.3 and 118.6, 123.3, 132.1 and 132.2, 139.7 and 139.8, CD_3OD 140.0, 149.6 and 149.7, 169.1; HRMS (ESI) $[M+Na]^+$ m/z 534.1766 (calcd for $C_{23}H_{33}NO_{10}SiNa$: 534.17659); $[M+K]^+$ m/z 550.1503 (calcd for $C_{23}H_{33}NO_{10}SiK$: 550.15053).

Preparation of compound 11

To a solution of **10** (390 mg, 0.76 mmol) in allylic alcohol (12 mL) was added dropwise a solution of sodium allylate 0.125 M (1.15 mL, 0.145 mmol). The mixture was stirred 40 min and was hydrolyzed with IRC-50 acidic resin, filtrated and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (CH_2Cl_2/MeOH 98/2) to give **11** (399 mg, 97%) as a mixture of two diastereoisomers (pale yellow solid). R_f 0.75 (CH_2Cl_2/MeOH 95/5). mp = 55.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ –0.04 (2 s, 3H), 0.10 (2 s, 3H), 0.89 (2 s, 9H), 1.99 (2t, 1H, J = 2.6 Hz), 2.49–2.60 (m, 2H), 3.50–3.81 (m, 2H), 3.95 (m, 1H), 4.07 (2d, 1H, J = 9.6 Hz), 4.75 (m, 2H), 4.83 (t, 1H, J = 6.4 Hz), 4.96 (d, 1H, J = 6.8 Hz), 5.30 (m, 1H), 5.38 (m, 1H), 5.97 (m, 1H), 7.38 (2d, 1H, J = 8.6 Hz), 7.60 (m, 1H), 7.90 (d, 0.5H, J = 2.1 Hz), 7.93 (d, 0.5H, J = 2.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ –4.8, –4.7, 18.3, 25.8, 30.9, 66.7, 70.9, 71.3, 72.2, 73.0, 74.6, 74.9, 80.3, 103.2, 118.8 and 119.0, 119.5, 123.3, 131.0, 132.1, 140.0, 149.6, 168.3; HRMS (ESI) $[M+Na]^+$ m/z 560.1922 (calcd for $C_{25}H_{35}NO_{10}SiNa$: 560.19224); $[M+K]^+$ m/z 576,1664 (calcd for $C_{25}H_{35}NO_{10}SiK$: 576.16618).

Preparation of compound 12

11 (1.3 g, 2.42 mmol) was dissolved in dry pyridine (12 mL). The mixture was cooled at 0 °C and allyl chloroformiate (7.07 mL, 72.54 mmol) was added dropwise. The mixture was stirred 72 h at room temperature and was hydrolyzed with aqueous 1 M HCl (40 mL). The mixture was extracted three times with ethyl acetate and the combined organic layers were dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (petroleum ether/AcOEt 80/20) to give **12** (1.80 g, 94%) as a mixture of two diastereoisomers (pale yellow solid). R_f 0.84 (petroleum ether/AcOEt 60/40). mp = 53.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ –0.05 (s, 3H), 0.09 (2 s, 3H), 0.89 (2 s, 9H), 1.98 (2t, 1H, J = 2.6 Hz), 2.41–2.61 (m, 2H), 4.31–4.34 (m, 1H), 4.59–4.70 (m, 6H), 4.74–4.72 (m, 2H), 4.81 (2t, 1H, J = 6.5 Hz), 5.24–5.40 (m, 12H), 5,82–6,00 (m, 4H), 7.31 (2d, 1H, J = 8.5 Hz), 7.55 (2t, 1H, J = 8.9 Hz), 7.84 (d, 0.5H, J = 2.1 Hz), 7.86 (d, 0.5H, J = 2.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ –4.8, –4.7, 18.2, 25.8, 30.8, 67.0, 69.2, 69.3, 69.6, 71.2, 72.0 and 72.1, 72.3, 72.5, 74.0, 75.1, 80.3, 99.8, 118.7, 119.0, 119.1, 119.3, 119.4, 123.0, 130.9, 131.0, 131.1, 131.3, 131.4, 140.1, 140.2, 140.6, 140.7, 148.4 and 148.5, 153.5, 154.0, 165.7; HRMS (ESI) $[M+Na]^+$ m/z 812.2556 (calcd for $C_{37}H_{47}NO_{16}SiNa$: 812.2556), $[M+K]^+$ m/z 828.2930 (calcd for $C_{37}H_{47}NO_{16}SiK$: 828.2957).

Preparation of compound 13

To a solution of **12** (370 mg, 0.47 mmol) in dry THF (5.7 mL) was added dropwise HF/pyridine 70% (1.8 mL). The mixture was stirred 1 h at room temperature and poured into 100 mL of ice-cold saturated aqueous $NaHCO_3$. The mixture was extracted three times with ethyl acetate. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (petroleum ether/AcOEt 70/30) to afford **13** (272 mg, 85%) as mixture of two diastereoisomers (white solid). R_f 0.62 (petroleum ether/AcOEt 60/40). mp = 61.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.10 (t, 1H, J = 2.6 Hz), 2.51 (bs, 1H), 2.61–2.64 (m, 2H), 4.32 (d, 1H, J = 9.1 Hz), 4.59–4.69 (m, 6H), 4.72 (m, 2H), 4.90 (t, 1H, J = 6.2 Hz), 5.22–5.39 (m, 12H), 5,81–6,00 (m, 4H), 7.34 (d, 1H, J = 8.6 Hz), 7.57 (m, 1H), 7.87 (d, 0.5H, J = 2.1 Hz), 7.89 (d, 0.5H, J = 2.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.6; 67.0, 69.3, 69.4, 69.7,

70.8, 72.1, 72.4, 72.5, 74.0, 75.0, 79.5, 99.8, 119.2, 119.3, 119.4, 119.5, 122.9 and 123.0, 131.0, 131.1, 131.2, 131.3, 131.4, 138.8, 140,9 and 141.0, 148.7, 153.6, 154.1, 165.7; HRMS (ESI) $[M+Na]^+$ m/z 698.1692 (calcd for $C_{31}H_{33}NO_{16}Na$: 698.16915); $[M+K]^+$ m/z 714.1430 (calcd for $C_{31}H_{33}NO_{16}K$: 714.14309).

Preparation of compound 14

To a solution of **13** (262 mg, 0.38 mmol) in dry dichloromethane (4 mL) were added *para*-nitrophenol chloroformiate (198 mg, 0.77 mmol) and pyridine (77 µL, 0.96 mmol). The mixture was stirred 2 h at room temperature and saturated aqueous $NaHCO_3$ was added. The layers were separated and aqueous layer was extracted three times with dichloromethane. The combined organic layers were washed three times with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude product was purified by column chromatography over silica gel (petroleum ether/AcOEt 70/30) to afford **14** (293 mg, 92%) as a mixture of two diastereoisomers (white solid). R_f 0.79 (petroleum ether/AcOEt 60/40). mp = 52.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 (t, 1H, J = 2.6 Hz), 2.82–2.97 (m, 2H), 4.35 (d, 1H, J = 8.65 Hz), 4.68–4.70 (m, 6H), 4.72 (d, 2H, J = 5.8 Hz), 5.24–5.39 (m, 12H), 5.80 (t, 1H, J = 6.6 Hz), 5.83–6.00 (m, 4H), 7.36–7.40 (m, 3H), 7.63 (dd, 1H, J = 2.2 Hz, J = 8.6 Hz), 7.95 (d, 1H, J = 2.2 Hz), 8.27 (d, 2H, J = 8.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.2, 67.0, 69.2, 69.4, 69.6, 72.2, 72.3, 72.5, 73.9, 74.9, 77.2, 77.6, 99.3, 119.0, 119.1, 119.4, 119.5, 121.8, 123.9, 125.4, 130.9, 131.0, 131.1, 131.2, 132.5, 133.4, 140.7, 145.6, 149.6, 151.6, 153.5, 154.0, 155.2, 165.5; HRMS (ESI) $[M+Na]^+$ m/z 863.1755 (calcd for $C_{38}H_{36}N_2O_{20}Na$: 863.17536), $[M+K]^+$ m/z 879.1478 (calcd for $C_{38}H_{36}N_2O_{20}K$: 879.1493).

Preparation of compound 15

Anhydrous pyridine (0.06 mL, 1.5 eq.) was added dropwise to a solution of **14** (0.41 g, 0.48 mmol) and cyclopamine (0.2 g, 0.48 mmol) in DMF (5.6 mL). The mixture was stirred for 20 h at room temperature and the crude mixture was concentrated *in vacuo*. Purification by flash chromatography on silica gel (CH_2Cl_2/MeOH 98/2) afforded **15** (0.46 g, 85%) as a mixture of two diastereoisomers. R_f 0.77 (CH_2Cl_2/MeOH 98/2). mp = 121.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95–1.45 (m, 14H), 1.50–2.45 (m, 21H), 2.76 (m, 1H), 2.89 (m, 1H), 3.06 (m, 1H), 3.27 (m, 1H), 3.57 (m, 2H), 4.33 (m, 1H), 4.72 (m, 8H), 5.37 (m, 14H), 5.91 (m, 5H), 7.35 (m,1H), 7.56 (m, 1H), 7.88 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.54, 13.55, 14.20, 17.46, 18.67, 19.29, 20.64, 21.06, 24.59, 25.35, 26.42, 29.03, 31.07, 31.36, 32.56, 36.54, 36.96, 38.14, 38.31, 41.55, 41.77, 41.9, 49.2, 51.99, 59.65, 60.41, 63.10, 66.93, 69.16, 69.27, 69.52, 69.61, 71.8, 71.92, 72.3, 72.92, 73.85, 74.88, 78.47, 85.14, 99.56, 119.01, 119.24, 119.30, 119.32, 119.41, 119.46, 121.82, 122.63, 123.47, 126.33, 130.84, 130.94, 131.05, 131.16, 135.67, 140.77, 141.58, 143.41, 148.84, 149.07, 153.46, 153.95, 156.35, 165.58, 165.62; HRMS (ESI) $[M+Na]^+$: m/z 1135.4625 (calcd. for $C_{59}H_{72}N_2O_{19}Na$: 1135.46215).

Preparation of compound 17

To a solution of **15** (0.46 g, 0.41 mmol) and **16** (0.14 g, 1 eq.) in anhydrous CH_2Cl_2 (14.7 mL) was added tetrakis(acetonitrile)copper(I) hexafluorophosphonate (1 eq., 0.14 g). The resulting mixture was stirred at room temperature for 20 h. After removing the volatiles under reduced pressure, the crude material was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 90/10) to afford **17** (0.47 g, 70%) as a mixture of two diastereoisomers. R_f 0.21 (CH_2Cl_2/MeOH 90/10). mp = 133.5 °C. ^{1}H NMR (400 MHz, CD_3OD, 313 K) δ 0.65–1.3 (m, 15H), 1.4–2.4 (m, 19H), 3.1–3.4 (m, 24H), 4.49 (m, 1H), 4.78 (m, 15H), 5.5 (m, 12H), 6.11 (m, 5H), 7.60 (m, 1H), 7.77 (m, 1H), 7.96 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 10.89, 13.99, 15.58, 19.07, 19.76, 19.93, 20.58, 21.21, 21.40, 25.69, 28.51, 29.28, 29.95, 30.77, 32.09, 33.44, 37.74, 37.87, 38.09, 39.49, 42.58, 43.44, 49.93, 50.12, 50.58, 53.39, 61.13, 62.80, 64.12, 67.87, 68.06, 68.48, 69.71, 70.11, 70.30, 70.39, 71.40, 71.67, 72.48, 72.66, 72.86, 73.36, 73.99, 75.03, 75.54, 76.76, 78.0, 86.81, 89.83, 100.06, 104.52, 119.04, 119.31, 119.56, 122.72, 127.58, 132.68, 132.73, 132.82, 132.90, 141.77, 143.15, 143.29, 144.55, 148.53, 149.83, 150.05, 155.01, 155.22, 155.29, 155.47, 158.14, 167.32, 167.67; HRMS (ESI) $[M+Na]^+$: m/z 1472.6115 (calcd. for $C_{71}H_{95}N_5O_{27}Na$: 1472.61066).

Preparation of compound 2b

To a solution of **17** (0.21 g, 0.147 mmol) in MeOH/CH_2Cl_2 (10/90, 5 mL) was added tetrakis(triphenylphosphine)palladium(0) (17 mg, 0.0147 mmol) and aniline (0.03 mL, 0.29 mmol). Total deprotection was observed after stirring at room temperature for 24 h (HPLC analysis, Method A). Solvents were removed under reduced pressure. The resulting solid was washed three times in CH_2Cl_2 and collected by filtration (0.136 g, 80%), (85% purity HPLC analysis). High degree of purity for compound **2b** was obtained using preparative-reverse phase HPLC (0.067 g, purity > 95%). Retention times for the two diastereoisomers of 2b are 13.57 and 13.83 min (for HPLC conditions see HPLC analysis). mp = 149.5 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 0.73–1.05 (m, 13H), 1.13–1.38 (m, 8H), 1.38–1.90 (m, 20H), 2.10–2.35 (m, 8H), 2.70 (m, 1H), 3.1 (m, 1H), 3.3 (m, 1H), 3.36 (m, 1H), 3.47–3.75 (m, 14H),3.84–3.87 (m, 4H), 3.99 (m, 1H), 4.30 (dd, 1H, J = 7.7 Hz, J = 2.2 Hz), 4.51 (sl, 2H), 5.10 (m, 1H), 5.37 (sl, 1H), 6 (m, 1H), 7.50 (m, 1H), 7.58 (m, 1H), 7.70 (s, 0.5H), 7.76 (s, 0.5H), 7.78 (m, 1 H). ^{13}C NMR (100 MHz, CD_3OD) δ 10.60, 10.72, 13.83, 18.99, 21.27, 25.66, 29.33, 29.89, 30.79, 32.02, 32.08, 33.41, 37.74, 39.46, 42.56, 42.78, 43.47, 51.37, 53.41, 62.80, 69.71, 70.45, 71.44, 71.66, 72.50, 73.29, 74.51, 75.08, 76.39, 77.03, 78.03, 86.86, 102.33, 104.45, 122.69, 125.38, 127.57, 133.04, 141.51, 143.17, 144.58, 151.22, 158.24; HRMS (ESI) $[M - H]^-$ m/z 1156.5195 (calcd. for $C_{56}H_{78}N_5O_{21}$: 1156.51948).

HPLC analysis

Analytical HPLC was carried out using a Dionex Ultimate 3000 System with UV variable wavelength detector. Compounds **17**, **2b**, cyclopamine analysis and enzymatic hydrolysis analysis were performed on a reverse phase column chromatography (**Method A**: Acclaim ® 120, C18, 250×4,6 mm, 5 µm, 120 Å; Flow 1 mL min^{-1}.; mobile phase CH_3CN, H_2O+0.2% TFA, 20/80: 100). Retention time for compounds **17**, **2b** and cyclopamine **1** are 22.88, 13.57–13.83 and 14.3 respectively. Peak area and calibration curves were obtained with Dionex Chromeleon software.

Stability

Compound **2b** (0.1 mg, 0.8 μmol) was incubated in 1 mL of phosphate buffer (0.02 M, pH 7.0) at 37 °C. Stability was monitored by analytical HPLC using Method A. HPLC analysis showed no detectable degradation of compound **2b** during 24 h under these conditions.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with commercially available β-glucuronidase from *Escherichia coli* (purchased from Sigma Aldrich ref. G8162). Prodrug **2b** (1 μmol) was incubated with *Escherichia coli* (133 U/mL) in phosphate buffer (0.02 M, pH 7) at 37 °C and sample were analyzed by HLPC/MS.

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^{-1} streptomycin (Invitrogen). Cells were incubated in a humidified 95% air/5% CO2 controlled atmosphere at 37 °C.

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 24 h of 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-ulfophenyl)-2*H*-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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