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Synthesis and biological evaluation of glucuronide prodrugs of the histone deacetylase inhibitor CI-994 for application in selective cancer chemotherapy

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ABSTRACT

Two glucuronide prodrugs of the histone deacetylase inhibitor CI-994 were synthesized. These compounds were found to be soluble in aqueous media and stable under physiological conditions. The carbamoyl derivatisation of CI-994 significantly decreased its toxicity towards NCI-H661 lung cancer cells. Prodrug incubation with β-glucuronidase in the culture media led efficiently to the release of the parent drug and thereby restoring its ability to decrease cell proliferation, to inhibit HDAC and to induce E-Cadherin expression.

1. Introduction

Recently, the inhibition of histone deacetylases (HDACs), a family of enzymes which play a fundamental role in the regulation of gene expression,[1] has emerged as a new strategy in cancer chemotherapy. During the last decade, an increasing number of structurally diverse small molecule HDAC inhibitors including hydroxamates, carboxylates, benzamides, thiol derivatives, cyclic peptides and hydrophilic ketones, have been studied as potential therapeutic agents.[2–5] These investigations demonstrated that several HDAC inhibitors exhibited potent antitumour activities in the course of the treatment of solid and haematological malignancies. As a consequence, there are well over 100 clinical trials ongoing with at least 13 different HDAC inhibitors as monotherapy or in combination with other anticancer drugs.[6] In October 2006, SAHA **1** (Zolinza™) (Fig. 1) became the first HDAC inhibitor approved by the Food and Drug Administration for the treatment of patients with cutaneaous T-cell lymphoma.

CI-994 **2** (Fig. 1) is a potent member of the benzamide class of HDAC inhibitors that has demonstrated significant antitumour activity against a broad spectrum of murine, rat and human tumour models.[7–9] This compound is currently progressing through clinical trials in combination with other standard anticancer agents such as carboplatin, paclitaxel,[10] capecitabine[11] or gemcitabine.[12] Although CI-994 is a promising chemotherapeutic agent, it induces adverse events including thrombocytopenia, anemia and neutropenia. Another major drawback linked to its use in cancer chemotherapy is its lack of aqueous solubility. Within this framework the design of non-toxic hydrophilic prodrugs that could deliver CI-994 predominantly in the vicinity of the tumour seems to be an interesting alternative in order to enhance the therapeutic index of this HDAC inhibitor. Such an approach has recently been proposed for the selective targeting of SAHA.[13]

Toward this end, we have undertaken the study of the two glucuronide prodrugs **3** and **4** (Fig. 2). Indeed, several glucuronide prodrugs have already been selectively activated by β-glucuronidase, either present in high concentration in necrotic tumour areas (PMT)[14] or previously targeted to the tumour sites (ADEPT,[15] GDEPT[16]), and consequently demonstrated superior efficacy in vivo compared to standard chemotherapy.[17] These results were attributed to the increased drug deposition and retention in the tumour connected with reduced anticancer agent concentration in normal tissues, considerably lowering the destruction of normal cells.

Prodrug **3** includes a nitrobenzylphenoxy carbamate linker[18] which has been employed successfully to release either anticancer drug such as doxorubicine[19] or magnetic resonance imaging contrast agent.[20] According to these results, this linker should allow easy recognition of **3** by β-glucuronidase and, after enzymatic cleavage of the glycosidic bond should rapidly liberate CI-994 via an 1,6 elimination as depicted in Figure 2.

On the other hand, for prodrug **4** CI-994 is attached directly to the glucuronide moiety through a carbamate functional group.

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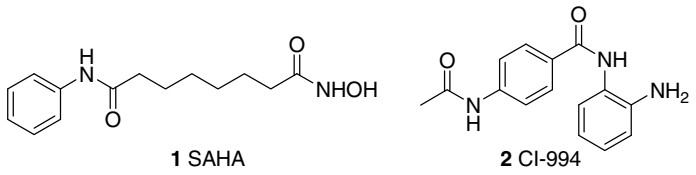


Figure 1. SAHA and CI-994.

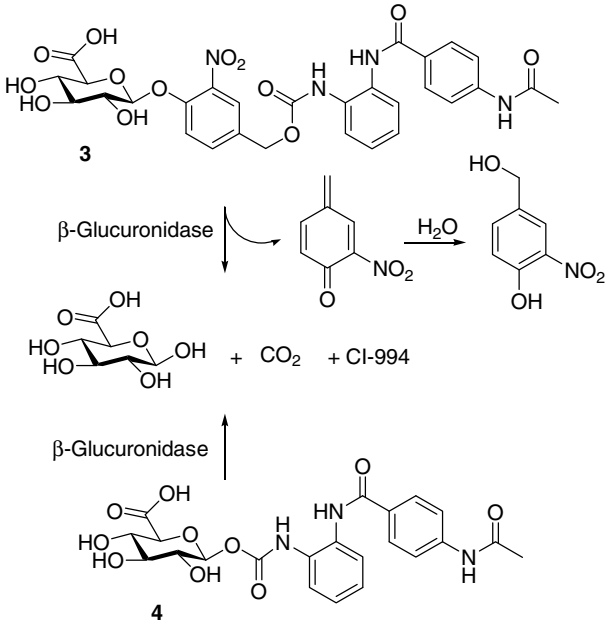
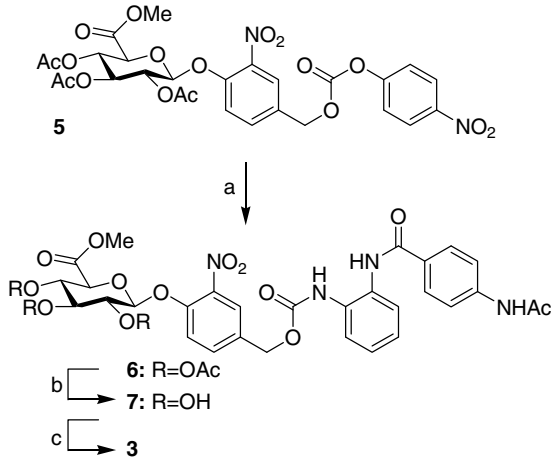


Figure 2. Mechanism of CI-994 release from either prodrug **3** and **4** in the presence of β-glucuronidase.



Scheme 1. Reagents and conditions: (a) CI-994, pyridine, DMF, rt, 3 days, 60%; (b) MeONa/MeOH (0.3 equiv.), 0 °C to rt, 14 h, 40%; (c) NaOH, acetone, −40 °C, 15 min, quant.

With this design, we anticipated that **4** would be an excellent substrate for the enzyme since *N*-phenyl β-glucuronyl carbamate was proved to be hydrolyzed by β-glucuronidase at a rate comparable to that of *p*-nitrophenyl β-glucuronide.[21] Thus, enzymatic hydrolysis should conduct to the expulsion of the carbamic acid of the drug that should lead rapidly to the free aniline after decarboxylation (Fig. 2).

In both cases, we hypothesised that (1) the carbamoyl derivatisation of the amino group of CI-994 may prevent its ability to inhibit HDACs and therefore may decrease its toxicity toward normal cells and, (2) the hydrophilic character of the glucuronide moiety should enhance the water solubility of the drug allowing i.v. administration.

2. Results and discussion

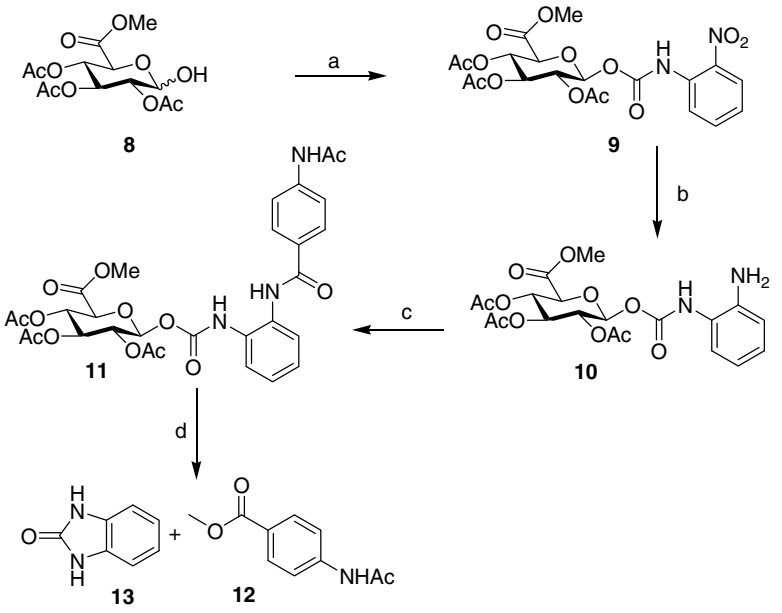
2.1. Chemistry

Prodrug **3** was prepared in three steps from the readily accessible activated carbonate **5**[19] (Scheme 1). First, CI-994 was condensed with **5** in the presence of pyridine to give the carbamate **6** in 60% yield. The *O*-acetyl groups of **6** were then deprotected with MeONa in MeOH to afford **7** (40%). Finally, saponification of the methyl ester using NaOH produced the target prodrug **3** in quantitative yield.

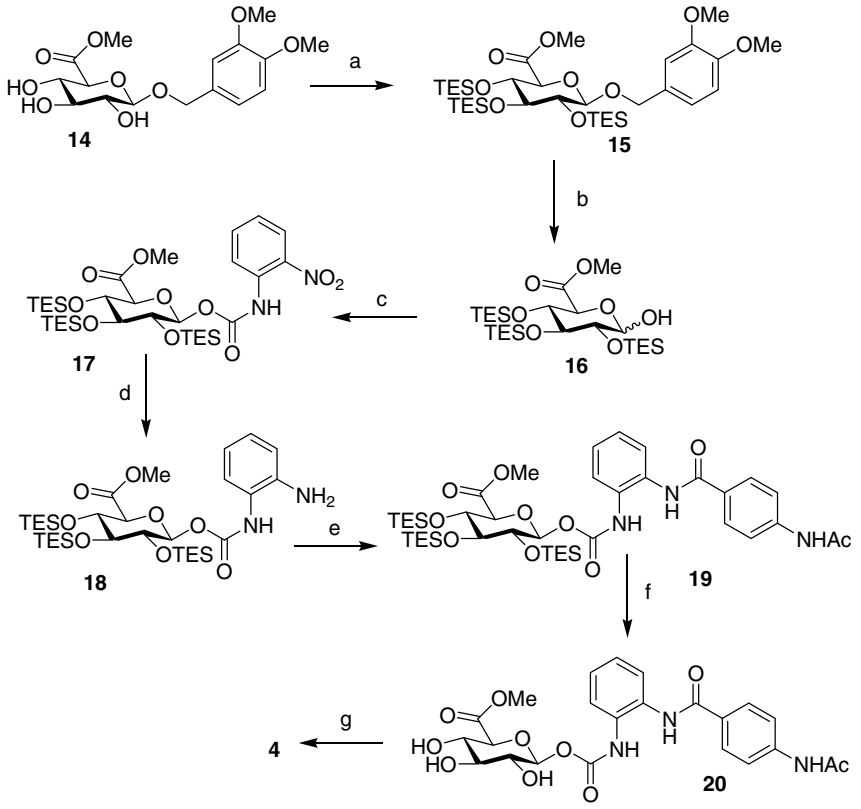
In order to access to the requested glucuronide prodrug **4**, our initial efforts focused upon the synthetic route illustrated in Scheme 2. Thus, the β-*O*-glucuronyl carbamate **9** was synthesized by coupling the well known glucuronide **8** with commercially available 2-nitrophenyl isocyanate in a very high β-diastereoselectivity using the elegant method developed earlier by Leenders et al. (86%, e.d. 97%).[22] The compound **9** was then converted quantitatively to the amine **10** by reduction of the nitro group (H_2, Pd/C). The latter reacted with 4-acetamidobenzoic acid in the presence of EDC and HOBt in DMF to yield the protected prodrug **11** (63%). In spite of several attempts, using various amounts of MeONa in MeOH (from 0.1 to 1 equiv.) cleavage of the acetate groups always failed and resulted mainly in the formation of the methyl 4-acetamidobenzoate **12** and the cyclic urea **13** along with unidentified products. Other standard basic deprotection conditions including KCN/MeOH, K_2CO_3/MeOH, Et_3N/MeOH, $Ba(OH)_2$/MeOH, NaOH/acetone or LiOH/H_2O led to similar results. Further attempts, using acidic reagents such as HF–BF_3/MeOH or $TiCl_4$/MeOH, generated complex mixtures. These results appeared surprising since such a problem was not observed during the deprotection steps of prodrug **3**. However, although the reasons of this failure remain unclear, we considered it preferable to turn our attention to other protecting groups.

At this stage, triethylsilyl (TES) ether appeared as an interesting alternative since such protective groups can be removed under mild acidic conditions.[23] For that purpose, we have first prepared the key intermediate **16** in two steps as illustrated in Scheme 3.

Thus, the protected glucuronide **15** was obtained in quantitative yield by treating the hydroxyl free carbohydrate derivative **14**[24] with triethylsilyltriflate and pyridine in CH_2Cl_2. The deprotection of the anomeric center was then performed with DDQ to afford the 1-hydroxyl-free glucuronide **16** as a mixture of diastereoisomers (90%). This compound was then coupled to the 2-nitrophenyl isocyanate using the same methodology as described above.[22] Under these conditions the carbamate **17** was obtained in 88% yield. Unfortunately, in this case no diastereoselectivity has been observed. Nevertheless, a careful flash column chromatography permitted to isolate a pure fraction of the β-anomer which allowed



Scheme 2. Reagents and conditions: (a) 2-nitrophenyl isocyanate, Et_3N, toluene, 0 °C, 2.5 h, 86%, e.d. 97%; (b) H_2, Pd/C 10%, EtOAc/EtOH 1:1, rt, 1 h, quant.; (c) 4-acetamidobenzoic acid, EDC, HOBt, DMF, rt, 24 h, 63%; (d) MeONa/MeOH (from 0.1 to 1 equiv.), 0 °C.



Scheme 3. Reagents and conditions: (a) TESOTf, pyridine, CH_2Cl_2, 0 °C to rt, 72 h, quant.; (b) DDQ, H_2O/CH_2Cl_2 (1: 10), rt, 4 h, 90%; (c) 2-nitrophenyl isocyanate, Et_3N, toluene, 0 °C, 2.5 h, 88%; (d) H_2, Pd/C 10%, EtOH, rt, 1 h, quant.; (e) 4-acetamidobenzoic acid, EDC, DMAP, DMF, rt, 12 h, 80%; (f) HCOOH, EtOAc, rt, 14 h, 77%; (g) NaOH, acetone, −40 °C, 80%.

us to pursue our investigations in this direction. Reduction of the nitro group of **17** undertaken under H_2 atmosphere in the presence of a catalytic amount of Pd/C led to the aniline **18** in quantitative yield. The coupling of **18** with 4-acetamidobenzoic acid was achieved using EDC and DMAP in DMF to give the protected prodrug **19** in 80% yield. The crucial deprotection of the 2,3,4-hydroxyl groups of **19** was carried out with HCOOH in ethyl acetate at room temperature over a period of 14 h. As expected, under such conditions the glucuronide **20** was isolated in good yield after purification by flash column chromatography (77%). It is worth

mentioning that neither the acetamide **12** nor the cyclic urea **13** was formed in this case. Finally, the cleavage of the methyl ester of compound **20** was realized with NaOH in acetone at −40 °C to produce the prodrug **4** in 80% yield.

2.2. Solubility and stability

The aqueous solubility of the glucuronide prodrugs **3** and **4** was measured in phosphate buffer (0.02 M) at pH 7 and compared to that of the corresponding drug. Under these conditions, CI-994 exhibited a poor solubility (0.08 mg/mL) whereas both prodrugs were perfectly soluble at a concentration of 1 mg/mL which is compatible with i.v. administration.

The stability of both prodrugs was examined by incubating these compounds in a 0.02 M phosphate buffer (pH 2.1 or 7) or in cell culture media supplemented with 10% foetal calf serum at 37 °C. The evolution of each solution was followed by HPLC (UV detection) over a period of 72 h. As expected, no detectable decomposition of **3** or **4** has been observed in the course of these experiments.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of each compound was first conducted in the presence of an excess of enzyme which is always the case in the course of an ADEPT protocol (**3** or **4**: 0.1 mg/mL; *Escherichia coli* β-glucuronidase: 133 U/mL). Both prodrugs were readily cleaved by β-glucuronidase and quantitative conversion of either **3** or **4** to CI-994 was achieved in less than 5 min. Further experiments were undertaken using a lower concentration of the *E. coli* enzyme (5 U/mL) in order to compare kinetics of drug release from glucuronides **3** or **4** (Fig. 3).

These experiments showed that CI-994 was liberated slightly faster starting from prodrug **4** than prodrug **3**. However, kinetic profiles were very similar and both compatible with either an ADEPT or a PMT strategy. The absence of major differences between the kinetics for **3** and **4** seems to indicate that the linker decomposition step of prodrug **3** is not rate-determining.

2.4. Biological evaluations

Compounds **3** and **4** were then tested for their anti-proliferative activity on NCI-H661 non-small cell lung cancer cells after 48 h treatment. IC_{50} value was 20 μM for CI-994 (Fig. 4). In contrast, prodrugs **3** and **4** did not exhibit any anti-proliferative activity on these cells, except a slight decrease of proliferation at high dose

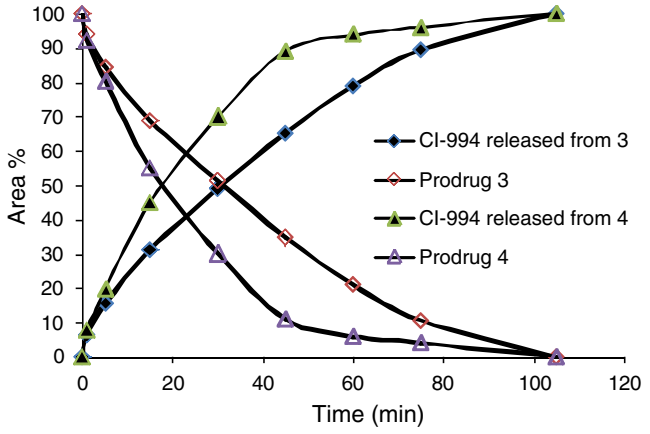


Figure 3. Enzymatic cleavage of prodrugs **3** and **4**. Prodrug concentration: 0.1 μmol mL^{-1}, enzyme concentration 5 U mL^{-1}.

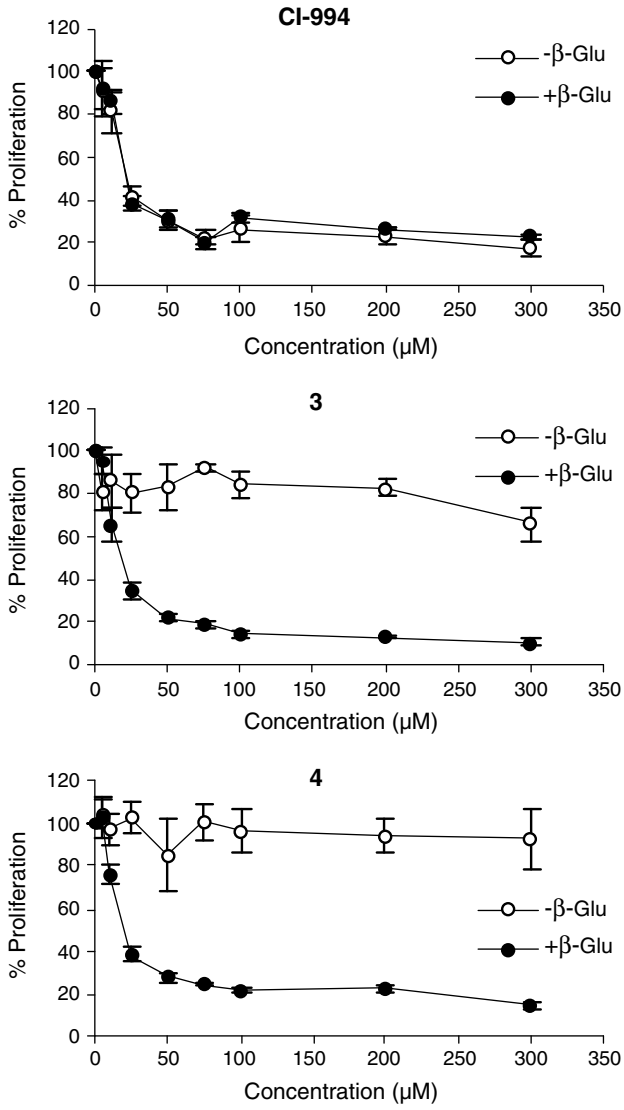


Figure 4. Proliferative activity of NCI-H661 cells treated during 48 h with the indicated compounds with (black dot) or without (white dot) β-glucuronidase. Three experiments were done in triplicate. Standard deviation is indicated.

(300 μM) for **3**. This demonstrated that the carbamoyl derivatisation of the amino group of CI-994 reduces significantly its toxicity. Thus, the use of glucuronide prodrugs such as **3** or **4** in the course of a tumour targeting strategy may prevent the adverse events recorded with the free drug. However, addition of β-glucuronidase (40 U/mL) in the culture media induced a dramatic anti-proliferative effect with **3** and **4** showing that these two glucuronide prodrugs behaved with similar activity than CI-994 in the presence of the enzyme (IC_{50} = 20 μM). These latter results can be unambiguously attributed to an efficient release of the drug in the culture media therefore restoring its initial activity.

The HDAC inhibition was further estimated by Western blot for histone H4 acetylation (Fig. 5). As previously shown, Trichostatin A (TSA), a well known HDAC inhibitor, induced histone H4 acetylation.[25] This HDAC inhibition was also detected for CI-994 but not for **3** or **4** when incubated alone. Again, after β-glucuronidase treatment, **3** and **4** were able to induce histone H4 acetylation to similar level obtained with the same concentration of CI-994.

Next, we tested the ability of the compounds to induce E-Cadherin expression in NCI-H661 cells. E-Cadherin is a cell surface transmembrane protein that plays a major role in epithelial cell adhesion and connects the extracellular environment to the con-

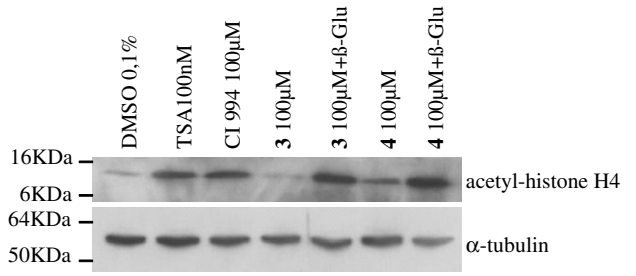


Figure 5. Western blot analysis for histone H4 acetylation. NCI-H661 cells were treated with the indicated compounds for 6 h. α-tubulin is used as loading controls. Representative of three independent experiments.

tractile cytoskeleton. E-Cadherin loss is one of the hallmark of the epithelial–mesenchymal transition (EMT) that plays a critical role in tumor progression and invasion, leading to distant metastasis.[26,27] E-Cadherin loss is also associated with resistance to EGFR inhibitors and poor prognosis in lung cancer.[28,29] Therefore, restoring E-Cadherin expression has therapeutical interest. This induction can be achieved by TSA treatment in NCI-H661 cells.[30] In our experiment, we found E-Cadherin expression with CI994 and **3** and **4** after β-glucuronidase treatment, whereas prodrugs alone did not show any activity on E-Cadherin expression (Fig. 6).

3. Conclusion

In summary, we have reported the synthesis of the first CI-994 glucuronide prodrugs to date aimed at β-glucuronidase activation in ADEPT or PMT strategies. Such prodrugs were at least 12.5 times more soluble in aqueous media compared to the parent drug and exhibited good stability under physiological conditions. In contrast with CI-994, prodrugs **3** and **4** have no activity on cell proliferation, histone acetylation and E-Cadherin expression in our cellular model. On the other hand, when incubated in the presence of β-glucuronidase, CI-994 was efficiently release and consequently its antitumour properties were restored. All these results suggested that both glucuronide prodrugs **3** and **4** possess the necessary prerequisites for further in vivo investigation in the course of a tumour targeting strategy such as ADEPT or PMT.

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 300 DPX Bruker. The chemical shifts are expressed in part per million (ppm) relative to TMS ($\delta = 0$ 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.

Optical rotations were measured on a Schmidt + Haensch Polartronic HH-8 polarimeter, in a 1 dm cell. Melting points were measured on a Büchi Melting Point B-545 instrument and were uncorrected. 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 hot gun. Flash column chromatography was performed using Macherey-Nagel silica gel 60 (15–40 µm).

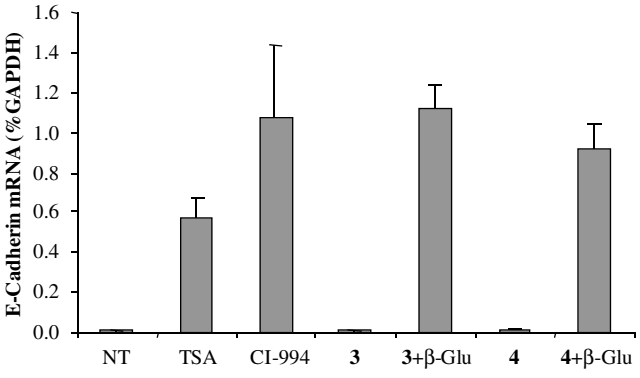


Figure 6. E-Cadherin mRNA level was measured by quantitative real-time RT-PCR on NCI-H661 cells grown 16 h with the indicated compounds. This experiment was done 2 times and each RT PCR in duplicate. Values are expressed in % of GAPDH expression. Bars, SD.

4.2. Synthesis and characterization of described compounds

4.2.1. 4-Acetamido-*N*-(2-aminophenyl)-benzamide or CI-994 (2)

To a solution of 4-acetamidobenzoic acid (500 mg, 2.79 mmol, 1 equiv.) in dry DMF (10 mL) was added benzene-1,2-diamine (904 mg, 8.37 mmol, 3 equiv.) followed by EDC (695 mg, 6.63 mmol, 1.3 equiv.) and a catalytic amount of DMAP (34 mg, 0.28 mmol, 0.1 equiv.). After stirring overnight, the mixture was concentrated under vacuum. Dichloromethane was added and the reaction mixture was placed in the fridge overnight. The solution was filtered and washed with hot dichloromethane to afford **2** as a white solid (609 mg, 2.26 mmol, 81%): m.p. 216.1 °C; ^1H NMR (DMSO, δ) 10.21 (bs, 1H), 9.57 (bs, 1H), 7.94 (d, 2H, $J = 8.5$ Hz), 7.69 (d, 2H, $J = 8.6$ Hz), 7.15 (d, 1H, $J = 7.5$ Hz), 6.96 (t, 1H, $J = 7.1$ Hz), 6.78 (d, 1H, $J = 7.4$ Hz), 6.59 (t, 1H, $J = 7.3$ Hz), 4.89 (bs, 2H), 2.09 (s, 3H); ^{13}C NMR (DMSO, δ) 169.1, 165.1, 143.5, 142.5, 129.1 (*2), 129.0, 127.0, 126.7, 123.8, 118.4 (*2), 116.6, 116.5, 24.2.

4.2.2. 3,4,5-Triacetoxy-6-{4-[2-(4-acetylamino-benzoylamino)-phenylcarbamoyloxymethyl]-2-nitro-phenoxy}-tetrahydro-pyran-2-carboxylic acid methyl ester (6)

Carbonate **5** (1 g, 1.54 mmol, 1 equiv.) and CI-994 **2** (830 mg, 3.08 mmol, 2 equiv.) were dissolved in DMF (20 mL). Pyridine (0.31 mL, 3.85 mmol, 2.5 equiv.) was added and the solution was stirred at room temperature for 72 h. The mixture was then hydrolysed with distilled water and extracted three times with chloroform. The organic layer was washed with HCl (1 M) twice, dried over $MgSO_4$ and evaporated to dryness. The residue was purified by flash chromatography (90/10 AcOEt/PE and 100% AcOEt). Product **6** was isolated as a white powder with a yield of 60% (720 mg, 0.92 mmol): m.p. 112.5 °C; $[\alpha]_D^{20}$: +8 (c 0.11, $CHCl_3$); ^1H NMR (CD_3COCD_3, δ) 9.52 (bs, 1H), 9.48 (bs, 1H), 8.58 (bs, 1H), 7.95 (m, 3H), 7.78–7.52 (m, 6H), 7.21 (m, 2H), 5.67 (d, 1H, $J = 7.7$ Hz), 5.46 (t, 1H, $J = 9.4$ Hz), 5.25 (m, 4H), 4.68 (d, 1H, $J = 9.7$ Hz), 3.69 (s, 3H), 2.15 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H); ^{13}C NMR (CD_3COCD_3, δ) 170.2, 169.9, 169.6, 169.5, 167.7, 166.1, 155.5, 149.8, 144.1, 142.2, 134.7, 134.1, 132.9, 131.8, 129.9, 129.8 (*2), 127.1, 126.8, 126.1, 125.6, 125.4, 119.8 (*2), 119.7, 100.2, 73.2, 72.5, 71.5, 70.3, 66.1, 53.4, 24.8, 20.8 (*3); MS (ESI) $[M+Na]^+$ for $C_{36}H_{36}O_{16}N_4Na$ = 803.8.

4.2.3. 6-{4-[2-(4-Acetylamino-benzoylamino)-phenylcarbamoyloxymethyl]-2-nitro-phenoxy}-3,4,5-trihydroxy-tetrahydro-pyran-2-carboxylic acid methyl ester (7)

Compound **6** (987 mg, 1.26 mmol, 1.0 equiv.) was dissolved in distilled MeOH (40 mL). The solution was cooled to 0 °C and MeO-Na (20 mg, 0.37 mmol, 0.3 equiv.) was added. The reaction mixture was stirred during 2 h and then allowed to stand to room temper-

ature during 14 h. The reaction was neutralised with IRC 50 during 20 min. The mixture was then filtered and evaporated to dryness. Flash chromatogtraphy (6/94, 8/92 and 10/90 MeOH/CH_2Cl_2) gave compound **7** (330 mg, 0.5 mmol) as a white solid with a yield of 40%: m.p. 144.1 °C; $[\alpha]_D^{20}$: +0.17 (*c* 0.1, MeOH); ^{1}H NMR (MeOD, δ) 7.81 (m, 3H), 7.64 (d, 2H, *J* = 8.7 Hz), 7.49 (m, 3 H), 7.29 (d, 1H, *J* = 8.7 Hz), 7.22 (m, 2H), 5.15 (m, 3H), 4.07 (d, 1H, *J* = 9.6 Hz), 3.60 (s, 3H), 3.51–3.47 (m, 3H), 2.15 (s, 3H); ^{13}C NMR (MeOD, δ) 170.8, 169.6, 166.8, 155.3, 149.4, 142.4, 140.8, 133.4, 131.9, 131.7, 129.0, 128.4 (*2), 126.5, 126.2, 125.5, 124.6, 121.6, 121.3, 119.1 (*2), 117.5, 101.0, 75.9, 75.6, 73.2, 71.5, 65.2, 51.8, 22.9; MS (ESI) $[M+Na]^+$ for $C_{30}H_{31}O_{13}N_4Na$ = 678.5.

4.2.4. 6-{4-[2-(4-Acetylamino-benzoylamino)-phenylcarbamoyloxymethyl]-2-nitro-phenoxy}-3,4,5-trihydroxy-tetrahydro-pyran-2-carboxylic acid (3)

To a solution of methyl ester **7** (52 mg, 0.08 mmol, 1.0 equiv.) in acetone (3 mL) cooled to −30 °C was added NaOH (0.8 mL, 1 M, 0.8 mmol, 10 equiv.). After stirring for 15 min, the starting material has totally disappeared. The solution was neutralised with IRC 50 for 20 min. Evaporation of the filtrate under vacuum afforded compound **3** in quantitative yield as a white solid (51 mg, 0.079 mmol): m.p. 161.6 °C; $[\alpha]_D^{20}$: +0.21 (*c* 0.1, MeOH); ^{1}H NMR (D_2O, δ) 7.81 (m, 3H), 7.64 (d, 2H, *J* = 8.7 Hz), 7.49 (m, 3H), 7.29 (d, 1H, *J* = 8.7 Hz), 7.22 (m, 2H), 5.15 (m, 3H), 4.07 (d, 1H, *J* = 9.6 Hz), 3.60 (s, 3H), 3.51–3.47 (m, 3H), 2.15 (s, 3H); ^{13}C NMR (MeOD, δ) 170.8, 169.6, 166.8, 155.3, 149.4, 142.4, 140.8, 133.4, 131.9, 131.7, 129.0, 128.4, 126.5, 126.2, 125.5, 124.6, 119.1, 117.5, 101.0, 75.9, 75.6, 73.2, 71.5, 65.2, 51.8, 22.9; HRMS Calcd for $C_{29}H_{27}O_{13}N_4Na_2$ $[M-H+2Na]^+$ = 685.1370, Found: $[M-H+2Na]^+$ = 685.1380. Anal Calcd for $C_{29}H_{28}N_4O_{13}\cdot 1.5H_2O$: C 52.18, H 4.68, N, 8.39; Found: 52.10, H 4.75, N 8.14.

4.2.5. 3,4,5-Triacetoxy-6-(2-nitro-phenyl-carbamoyloxy)-tetrahydro-pyran-2-carboxylic acid methyl ester (9)

To a solution of glucuronide **8** (2.5 g, 7.49 mmol) in anhydrous toluene (150 mL) and Et_3N (3.2 mL, 22.5 mmol, 3 equiv.) cooled to 0 °C was added slowly (over a period of 1 h) a solution of 2-nitrophenyl isocyanate (7.49 mmol, 1 equiv.) in toluene (60 mL). After 2 h, the mixture was evaporated and subjected to flash column chromatography (60/40, EP/AcOEt) to furnish **9** as a yellow solid (3.2 g, 86%, d.e. β = 97%, 6.4 mmol): m.p. 154.2 °C; $[\alpha]_D^{20}$: −22 (*c* 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, δ) 9.97 (bs, 1H), 8.50 (d, 1H, *J* = 7.4 Hz), 8.23 (d, 1H, *J* = 8.5 Hz), 7.67 (t, 1H, *J* = 7.2 Hz), 7.22 (t, 1H, *J* = 7.4 Hz), 5.86 (d, 1H, *J* = 7.5 Hz), 5.30 (m, 3H), 4.26 (d, 1H, *J* = 9.0 Hz), 3.75 (s, 3H), 2.06 (s, 9H); ^{13}C NMR ($CDCl_3$, δ) 170.3, 169.8, 169.5, 167.2, 150.9, 136.9, 136.4, 134.3, 126.3, 123.8, 121.3, 93.0, 73.2, 71.8, 70.0, 69.1, 53.4, 20.9, 20.8, 20.7; MS (ESI) $[M+Na]^+$ for $C_{20}H_{22}N_2O_{13}Na$ = 521.6.

4.2.6. 3,4,5-Triacetoxy-6-(2-amino-phenyl-carbamoyloxy)-tetrahydro-pyran-2-carboxylic acid methyl ester (10)

A solution of compound **9** (1.0 g, 2.0 mmol) in EtOH/AcOEt (v/v, 1/1, 30 mL/30 mL) was hydrogenated over 10% palladium/carbon (30 mg) at room temperature for 1 h. The catalyst was filtered through a pad of celite and the filtrate was evaporated in vacuo to give the desired product **10** as a white solid in quantitative yield (936 mg, 2.0 mmol): m.p. 145.1 °C; $[\alpha]_D^{20}$: +21 (*c* 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, δ) 7.32 (d, 1H, *J* = 7.2 Hz), 7.03 (t, 1H, *J* = 7.5 Hz), 6.97 (s, 1H), 6.79 (m, 2H, *J* = 7.8 Hz), 5.81 (d, 1H, *J* = 7.7 Hz), 5.28 (m, 3H), 4.23 (d, 1H, *J* = 9.5 Hz), 3.75 (s, 2H), 3.73 (s, 3H), 2.05 (s, 9H), ^{13}C NMR ($CDCl_3$, δ) 170.3, 170.2, 170.0, 167.5, 152.4, 140.6, 127.2, 125.3, 123.5, 119.5, 117.7, 93.0, 72.9, 72.1, 70.4, 69.4, 53.4, 21.0, 20.9, 20.8; MS (ESI) $[M+Na]^+$ for $C_{20}H_{24}N_2O_{11}Na$ = 491.3.

4.2.7. 3,4,5-Triacetoxy-6-[2-(4-acetylamino-benzoylamino)-phenyl-carbamoyloxy]-tetrahydro-pyran-2-carboxylic acid methyl ester (11)

To a solution of 4-acetylamino-benzoic acid (717 mg, 4.0 mmol, 2.0 equiv.) in dry DMF (4 mL) was added compound **10** (940 mg, 2.0 mmol, 1.0 equiv.), followed by EDC (997 mg, 5.2 mmol, 2.6 equiv.) and hydroxybenzotriazole hydrate (703 mg, 5.2 mmol, 2.6 equiv.). After stirring during 72 h at room temperature, the mixture was quenched by addition of a saturated NH_4Cl solution (10 mL) and extract with AcOEt/benzene (v/v, 2/1, 20 mL) twice. The organic phase was washed consecutively with water and saturated brine (10 mL each). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash chromatography (80/20, AcOEt/EP), yielding the coupling compound **11** as a white solid (800 mg, 1.27 mmol): m.p. 125.5 °C; $[\alpha]_D^{20}$: +4 (*c* 0.21, $CHCl_3$); ^{1}H NMR ($CDCl_3$, δ) 8.99 (bs, 1H), 8.34 (bs, 1H), 8.03 (bs, 1H), 7.90 (d, 2H, *J* = 8.4 Hz), 7.60 (d, 2H, *J* = 8.7 Hz), 7.55 (m, 1H), 7.42 (dd, 1H, *J* = 2.9 Hz, *J*′ = 7.0 Hz), 7.07 (m, 2H), 5.76 (d, 1H, *J* = 8.0 Hz), 5.30 (t, 1H, *J* = 9.2 Hz), 5.03 (t, 1H, *J* = 6.5 Hz), 4.94 (t, 1H, *J* = 9.1 Hz), 4.20 (d, 1H, *J* = 9.7 Hz), 3.67 (s, 3H), 2.11 (s, 3H), 1.95–2.05 (s, 9H); ^{13}C NMR ($CDCl_3$, δ) 170.2, 170.0, 169.9, 169.5, 167.8, 166.8, 152.0, 149.3, 142.0, 130.8, 129.3 (*2), 129.2, 126.9, 126.5, 125.6, 123.3, 119.8 (*2), 92.9, 72.7, 71.9, 70.0, 69.2, 53.6, 24.9, 20.9 (*3); MS (ESI) $[M+Na]^+$ for $C_{29}H_{31}N_3O_{13}Na$ = 652.5.

4.2.8. 6-(3,4-Dimethoxy-benzoyloxy)-3,4,5-tris-triethylsilanoloxy-tetrahydro-pyran-2-carboxylic acid methyl ester (15)

TESOTf (15.3 mL, 67.2 mmol, 6.0 equiv.) was added to a stirred solution of the triol **14** (4 g, 11.2 mmol, 1.0 equiv.), anhydrous pyridine (50 mL, 618 mmol) and anhydrous dichloromethane (285 mL) at 0 °C. At the end of the addition, the reaction was allowed to reach to room temperature. After complete conversion of starting material (72 h), the solvents were removed in vacuo and the residue was poured into a mixture of dichloromethane and saturated Na_2CO_3 solution (25 mL). The organic phase was washed with distilled water (25 mL), dried, filtered and concentrated in vacuo. The resulting residue was purified by flash chromatography (15/85, AcOEt/PE) to yield the fully protected glucuronide **15** (7.85 g, 11.2 mmol) as a clear oil: $[\alpha]_D^{20}$: −21 (*c* 0.19, $CHCl_3$); ^{1}H NMR ($CDCl_3$, δ) 6.89 (d, 1H, *J* = 1.7 Hz), 6.79 (dd, 1H, *J* = 8.2 Hz, *J*′ = 1.7 Hz), 6.71 (d, 1H, *J* = 8.2 Hz), 4.84 (d, 1H, *J* = 11.2 Hz), 4.67 (d, 1H, *J* = 6.1 Hz), 4.34 (d, 1H, *J* = 11.2 Hz), 4.13 (m, 2H), 3.79 (s, 3H), 3.78 (s, 3H), 3.67 (s, 3H), 3.60 (m, 2H), 0.81 (m, 27H), 0.51 (m, 18H); ^{13}C NMR ($CDCl_3$, δ) 170.7, 149.1, 148.8, 130.4, 121.0, 112.0, 110.9, 101.6, 78.8, 78.1, 77.9, 73.4, 72.1, 56.2, 56.1, 52.5, 7.3 (*3), 7.2 (*3), 7.1 (*3), 5.4 (*3), 5.2 (*3), 5.1 (*3); MS (ESI) $[M+Na]^+$ for $C_{34}H_{64}O_9Si_3Na$ = 723.8.

4.2.9. 6-Hydroxy-3,4,5-tris-triethylsilanyloxy-tetrahydro-pyran-2-carboxylic acid methyl ester (16)

To a solution of the fully protected glucuronide **15** (7.8 g, 11.1 mmol, 1.0 equiv.) in dichloromethane/water (v/v, 10/1, 200 mL/20 mL) was added DDQ (5.05 g, 22.3 mmol, 2.0 equiv.). The resulting mixture was stirred at room temperature for 4 h. The reaction was poured into a saturated $NaHCO_3$ solution (100 mL) and extracted with dichloromethane (3 × 100 mL). The organic phases were dried over $MgSO_4$ and concentrated under vacuum. Purification by silica gel chromatography (5/95 and 10/90 AcOEt/EP) afforded glucuronide **16** (5.5 g, 10.0 mmol, 90%) as a colourless oil: $[\alpha]_D^{20}$: +24 (*c* 0.15, $CHCl_3$); ^{1}H NMR ($CDCl_3$, δ) 5.24 (dd, 1H, *J* = 10.9 Hz, *J*′ = 2.7 Hz), 4.28 (d, 1H, *J* = 3.0 Hz), 3.90 (t, 1H, *J* = 3,3 Hz), 3.72 (t, 1H, *J* = 4.1 Hz), 3.59 (s, 3H), 3.45 (t, 1H), 0.81 (m, 27H), 0.51 (m, 18H); ^{13}C NMR ($CDCl_3$, δ) 170.8, 89.8,

75.8, 73.2, 71.8, 71.4, 52.4, 7.3 (*3), 7.2 (*3), 7.1 (*3), 5.4 (*3), 5.2 (*3), 5.1 (*3); MS (ESI) $[M+Na]^+$ for $C_{25}H_{54}O_7Si_3Na$ = 573.9.

4.2.10. 6-(2-Nitro-phenylcarbamoyloxy)-3,4,5-tris-triethylsilanoloxy-tetrahydropyran-2-carboxylic acid methyl ester (17)

To a solution of glucuronide **16** (5.5 g, 10.0 mmol, 1.0 equiv.) in anhydrous toluene (165 mL) and Et_3N (7 mL, 50 mmol, 5 equiv.) cooled to 0 °C was added slowly (over a period of 1 h) a solution of 2-nitrophenyl isocyanate (1.8 g, 11.1 mmol, 1.1 equiv.) in toluene (60 mL). After stirring overnight at room temperature, the mixture was evaporated and subjected to flash column chromatography (4/96, 5/95, 7/93, 9/91 and 10/90 AcOEt/PE) to furnish compound **17** (6.2 g, 8.7 mmol, d.e. β = 4%) with 88% of yield: $[\alpha]_D^{20}$: −15 (*c* 0.22, $CHCl_3$); ^1H NMR ($CDCl_3$, δ) 9.98 (s, 1H), 8.60 (t, 1H, *J* = 8.6 Hz), 8.22 (d, 1H, *J* = 8.5 Hz), 7.64 (t, 1H, *J* = 8.5 Hz), 7.16 (t, 1H, *J* = 8.4 Hz), 6.06 (d, 1H, *J* = 6.1 Hz), 4.50 (d, 1H, *J* = 2.2 Hz), 4.34 (bs, 1H), 3.86 (dd, 1H, *J* = 6.1 Hz, *J′* = 1.8 Hz), 3.83 (dd, 1H, *J* = 4.1 Hz *J′* = 2.0 Hz), 3.75 (s, 3H), 0.81 (m, 27H), 0.51 (m, 18H); ^{13}C NMR ($CDCl_3$, δ) 169.6, 151.6, 136.4, 136.0, 135.0, 126.0, 121.8, 120.9, 95.2, 78.7, 76.3, 74.1, 72.6, 52.3, 7.3 (*3), 7.2 (*3), 7.1 (*3), 5.4 (*3), 5.2 (*3), 5.1 (*3); MS (ESI) $[M+Na]^+$ for $C_{32}H_{58}O_{10}N_2Si_3Na$ = 737.7.

4.2.11. 6-(2-Amino-phenylcarbamoyloxy)-3,4,5-tris-triethylsilanoloxy-tetrahydropyran-2-carboxylic acid methyl ester (18)

A solution of compound **17** (580 mg, 0.81 mmol) in EtOH (25 mL) was hydrogenated over 10% palladium/carbon (50 mg) at room temperature for 50 min. The catalyst was filtered through a pad of celite and the filtrate was evaporated to dryness. The residue was purified by flash column chromatography (10/90, 15/85 and 20/80 AcOEt/PE) to yield the desired product **18** (465 mg, 0.68 mmol, 84%) as a colourless oil: $[\alpha]_D^{20}$: +18 (*c* 0.12, $CHCl_3$); ^1H NMR ($CDCl_3$, δ) 7.31 (d, 1H, *J* = 7.7 Hz), 7.05 (t, 1H, *J* = 7.6 Hz), 6.79 (t, 2H, *J* = 7.9 Hz), 6.43 (bs, 1H), 6.02 (d, 1H, *J* = 6.2 Hz), 4.48 (bs, 1H), 4.27 (bs, 1H), 3.80 (m, 2H), 3.75 (s, 3H), 0.95 (m, 27H), 0.59 (m, 18H); ^{13}C NMR ($CDCl_3$, δ) 170.3, 153.2, 140.8, 127.1, 125.5, 124.0, 119.8, 117.8, 95.1, 79.0, 77.2, 75.3, 72.9, 52.6, 7.1 (*3), 6.9 (*3), 6.8 (*3), 5.3 (*3), 5.0 (*3), 4.9 (*3); MS (ESI) $[M+Na]^+$ for $C_{32}H_{60}O_8N_2Si_3Na$ = 707.5.

4.2.12. 6-[2-(4-Acetylamino-benzoylamino)-phenylcarbamoyloxy]-3,4,5-tris-triethylsilanoxy-tetrahydropyran-2-carboxylic acid methyl ester (19)

To a solution of 4-acetylamino benzoic acid (1.57 g, 8.77 mmol, 2.0 equiv.) in dry DMF (15 mL) was added the amine **18** (3.0 g, 4.39 mmol, 1.0 equiv.) followed by EDC (2.19 g, 11.4 mmol, 2.6 equiv.) and DMAP (50 mg, 0.41 mmol, 0.1 equiv.) at room temperature. After stirring overnight, the mixture was quenched by addition of a saturated NH_4Cl solution (25 mL) and extracted with AcOEt/benzene (v/v, 2/1, 40 mL) twice. The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash column chromatography (50/50, AcOEt/PE) yielding the coupling compound **19** (3.0 g, 3.55 mmol, 81%) as a colourless oil: $[\alpha]_D^{20}$: −18 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, δ) 9.05 (bs, 1H), 8.50 (bs, 1H), 7.94 (d, 2H, *J* = 8.5 Hz), 7.73 (bs, 2H), 7.64 (d, 2H, *J* = 8.7 Hz), 7.41 (d, 1H, *J* = 7.3 Hz), 7.22 (t, 1H, *J* = 6.7 Hz), 7.13 (t, 1H, *J* = 7.04 Hz), 6.08 (d, 1H, *J* = 7.2 Hz), 4.51 (s, 1H), 4.28 (s, 1H), 3.80 (s, 1H), 3.78 (s, 1H), 3.66 (s, 3H), 1.99 (s, 3H), 1.0–0.80 (m, 27H), 0.65–0.54 (m, 18H); ^{13}C NMR ($CDCl_3$, δ) 170.4, 170.1, 166.4, 153.2, 142.0, 131.7, 129.6, 129.1, 128.9, 127.1, 126.7, 125.6, 124.0, 119.8, 95.3, 79.4, 77.0, 75.4, 72.9, 52.6, 24.7, 7.2 (*6), 7.1 (*3), 5.3 (*3), 5.1 (*6); MS (ESI) $[M+Na]^+$ for $C_{41}H_{67}O_{10}N_3Na$ = 868.1.

4.2.13. 6-[2-(4-Acetylamino-benzoylamino)-phenylcarbamoyloxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid methyl ester (20)

The glucuronide **19** (415 mg, 0.49 mmol) was stirred overnight in ethyl acetate/formic acid (3/2, 6 mL). The solvents were removed in vacuo and the residue was purified by flash column chromatography (10/90, 13/87 and 15/85, $MeOH/CH_2Cl_2$) to yield the triol **20** (190 mg, 3.8 mmol, 78%) as a white solid: m.p. 178.1 °C; $[\alpha]_D^{20}$: +2 (*c* 0.1, MeOH); ^1H NMR (MeOD, δ) 7.94 (d, 2H, *J* = 8.7 Hz), 7.72 (d, 2H, *J* = 8.7 Hz), 7.59 (d, 1H, *J* = 7.5 Hz), 7.54 (dd, 1H, *J* = 7.2 Hz, *J′* = 1.7 Hz); 7.29–7.20 (m, 2H), 5.55 (d, 1H, *J* = 7.9 Hz), 4.01 (d, 1H, *J* = 9.2 Hz), 3.74 (s, 3H), 3.60–3,44 (m, 3H), 2.16 (s, 3H); ^{13}C NMR (MeOD, δ) 172.4, 171.3, 168.8, 155.2, 144.1, 144.0, 133.0, 131.9, 130.3, 130.2, 128.1, 127.8, 127.2, 126.0, 120.9, 120.8, 97.3, 77.6, 77.5, 73.9, 73.3, 53.4, 24.5; MS (ESI) $[M+Na]^+$ for $C_{23}H_{25}O_{10}N_3Na$ = 526.3.

4.2.14. 6-[2-(4-Acetylamino-benzoylamino)-phenylcarbamoyloxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid (4)

To a solution of compound **20** (190 mg, 0.38 mmol, 1 equiv.) in methanol (14 mL) cooled to −40 °C was added NaOH 1 M (1.9 mL, 1.9 mmol, 5 equiv.). After 15 min of stirring, the starting material has totally disappeared. The solution was neutralised with IRC50 for 20 min. The resin was removed by filtration and the filtrate was evaporated to dryness. The compound **4** was isolated as a white solid (149 mg, 0.30 mmol) with a yield of 80%: m.p. 194 °C; $[\alpha]_D^{20}$: +9 (*c* 0.1, MeOH); ^1H NMR (D_2O, δ) 7.65 (d, 2H), 7.40 (bs, 3H), 7.28–7.18 (m, 3H), 5.37 (d, 1H, *J* = 7.7 Hz), 3.71 (d, 1H, *J* = 9.0 Hz), 3.51–3.40 (m, 3H), 2.03 (s, 3H); ^{13}C NMR (D_2O, δ) 177.9, 175.6, 171.7, 157.3, 143.8, 133.9, 132.5, 132.3, 131.4 (*2), 130.6, 129.8, 129.5, 127.9, 123.2 (*2), 97.8, 79.3, 78.0, 74.5, 74.4, 26.0; HMRS Calcd for $C_{22}H_{22}O_{10}N_3Na_2$ $[M-H+2Na]^+$ = 534.1101, Found: $[M-H+2Na]^+$ = 534.1098. Anal Calcd for $C_{22}H_{23}N_3O_{10}\cdot 2H_2O$: C 50.29, H 5.18, N, 8.00; Found: 50.24, H 4.68, N 7.72.

4.3. Biological assays

4.3.1. HPLC analysis

Analytical HPLC was carried out using a Dionex Ultimate 3000 System with UV variable wavelength detector. Compounds **2**–**4** analysis and enzymatic hydrolysis analysis were performed on a reverse phase column chromatography (Acclaim$^{(R)}$ 120, C18, 250 × 4.6 mm, 5 μm, 120 Å) using a mobile phase (1 mL/min) of CH_3CN/H_2O(0.2%TFA) 3:7. Retention time for compounds **3**, **4** and **2** were 9, 3.88 and 3.68 min , respectively. Peak area and calibration curves were obtained with Dionex Chromeleon software.

4.3.2. Compound solubility

Ten milligrams of **2** were dissolved in 1 mL of DMSO and several dilutions with phosphate buffer (0.02 M, pH 7) were realized to obtain a calibration curve in a range of 0.1–0.025 mg/mL. Then, the solubility of compound **2** was determined in phosphate buffer (0.02 M, pH 7). The sample was filtered through a 0.45 μm Milipore filter, diluted in HPLC phase, and analyzed by HPLC.

4.3.3. Compound stability

Prodrugs **3** and **4** were placed in phosphate buffer (0.02 M, pH 2.1 or 7) and 10% foetal calf serum at 37 °C for a period of 72 h. HPLC analysis showed no detectable degradation of these compounds in these conditions.

4.3.4. Enzymatic cleavage of prodrugs

Escherichia coli β-glucuronidase was purchased from Sigma–Aldrich (reference: G8162). Prodrugs **3** and **4** (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 and sample were analyzed by HPLC after 5 min.

4.3.5. Kinetic of drug release

Prodrugs **3** and **4** (1.5 μmol/mL) were incubated with *E. coli* β-glucuronidase (5 U/mL) in phosphate buffer (0.02 M, pH 7) at

37 °C. Aliquots of 50 μL were taken at 0, 1, 5, 15 min and then every 15 min and diluted in 450 μL of HPLC mobile phase and analyzed by HPLC.

4.3.6. Cell culture

The NCI-H661 non-small cell lung cancer cells were grown in RPMI 1640 plus 10% foetal calf serum (Invitrogen) at 37 °C and under 5% CO_2. When indicated, β-glucuronidase (Sigma, G8162) was added at 40 U/ml in the culture media.

4.3.7. Cell proliferation

The Cell Proliferation Kit II (XTT; Roche) was used to assess cell proliferation. This assay is based on the cleavage of XTT by metabolic active cells resulting in the production of an orange formazan dye that is quantified by spectrophotometry. Assays were carried out essentially as described by the manufacturer. Briefly, 4×10^3 cells/well were plated in 100 μl of media in a 96-well plate. Cells were grown for 24 h before adding the compound at the indicated concentration. After 48 h treatment with or without β-glucuronidase in the media, 50 μl of the XTT labeling mixture were added per well. Cells were further incubated for additional 4 h at 37 °C before reading the absorbance at 480 nm.

4.3.8. Western blot analysis

Cells (5×10^5) were treated with the compounds for 6 h with or without β-glucuronidase in the media, for histone H4 acetylation analysis. Cells were lysed in 200 μl of the electrophoresis loading buffer from Laemmli and sonicated. Protein lysates were resolved by SDS–PAGE, followed by Western blot. Primary antibodies were rabbit polyclonal anti-acetylated histone H4 antibody (1/500; Upstate) or mouse monoclonal anti-α-tubulin antibody (1/2000; Sigma). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Amersham) were used at 1/5000 dilution. Detection was performed with ECL (Perkin–Elmer).

4.3.9. E-Cadherin expression

Cells were treated during 16 h with the indicated compounds (at 100 nM for TSA, and 100 μM for CI994, **3** and **4**). Total RNA was extracted using the RNA Total Isolation Kit (Promega). Reverse transcription-PCR (RT-PCR) was done with SuperScript II (Invitrogen) using the procedure supplied by the manufacturer. Gene expression was assessed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by quantitative real-time PCR with the GeneAmp 5700 Sequence Detection System and SYBR Green chemistry (Applied Biosystems). Primer sequences were as follows: GAPDH, 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-GGCATG GACTGTGGTCATGAG-3′ and E-Cadherin, 5′-CGGGAATGCAGTTGA GGATC-3′ and 5′-AGGATGGTGTAAGCGATGGC-3′.

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