Journal of Medicinal Chemistry

Brief Article

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Synthesis and Antitumor Efficacy of a β-Glucuronidase-Responsive Albumin-Binding Prodrug of Doxorubicin

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Supporting Information

ABSTRACT: In this paper we describe the synthesis and biological evaluation of the first β-glucuronidase-responsive albumin-binding prodrug designed for the selective delivery of doxorubicin at the tumor site. This prodrug leads to superior antitumor efficacy in mice compared to HMR 1826, a well-known glucuronide prodrug of doxorubicin that cannot bind covalently to circulating albumin. Furthermore, this compound inhibits tumor growth in a manner similar to that of doxorubicin while avoiding side effects induced by the free drug.

INTRODUCTION

Despite several years of intensive research devoted to the discovery of novel anticancer agents, chemotherapy is still not entirely effective for the treatment of many solid tumors. Most of the drugs used clinically act by antiproliferative mechanisms and lack any intrinsic selectivity, leading to severe adverse effects due to the destruction of normal tissues. Therefore, the development of more selective therapeutic approaches has become a major goal in medicinal chemistry.

Over the past 2 decades, numerous glucuronide prodrugs were investigated with the aim to deliver potent cytotoxic agents exclusively in the vicinity of the tumor.[1−3] Such enzyme-responsive systems can be activated selectively by β-glucuronidase located in high concentration in the microenvironment of a wide range of solid tumors including lung, breast, and gastrointestinal tract carcimomas.[4] The validity of this targeting strategy was demonstrated in mice with several glucuronide prodrugs that showed superior antitumor efficacy associated with reduced toxicity compared to standard chemotherapy.[5] However, the potential of this approach is limited by the rapid elimination of glucuronide prodrugs by the kidneys. As a result, the administration of very high doses is usually required to achieve a significant therapeutic effect, therefore representing a major problem for their clinical use. Under these circumstances, the design of novel glucuronide prodrugs exhibiting improved half-life is of interest to enhance the efficiency of this promising drug delivery system.

In another approach, the passive targeting of tumor anatomical features was also studied for the selective deposition of anticancer agents in malignant tissues. Indeed, tumor vasculature is characterized by tortuous, irregularly shaped and leaky blood vessels. These anomalies combined with the lack of lymphatic drainage allow the passive accumulation and retention of macromolecular drug carriers in solid tumors, which are too large to pass the endothelial barrier of healthy tissues (the enhanced permeability and retention of macromolecules in solid tumors is known as the EPR effect[6]). Taking advantage of the ERP effect, Kratz and co-workers developed highly innovative albumin-binding prodrugs[7] that demonstrated remarkable antitumor activity.[8] The originality of this targeting strategy relies on the in vivo formation of a macromolecular drug carrier resulting from the selective and rapid coupling of a maleimide-containing prodrug with the cysteine 34 position of circulating albumin after intravenous administration.[8a] In addition to their discriminating accumulation in malignant tissues, these macromolecules exhibit a favorable pharmacokinetic profile due to the prolonged half-life of albumin in the body. Within this framework, INNO-206 (formerly DOXO-EMCH), an albumin-binding prodrug of doxorubicin, is currently being assessed clinically.[9]

In this paper, we report the synthesis and biological evaluation of the first β-glucuronidase-responsive albumin-binding prodrug **1** (Scheme 1). This compound includes a glucuronide trigger, the potent doxorubicin, and a self-immolative linker[10] bearing a poly(ethylene glycol) side chain terminated by a maleimide functional group.[11] After intravenous administration, the in situ binding of prodrug **1** to the thiol at the cysteine 34 position of plasmatic albumin via Michael addition will produce the macromolecular drug carrier **2**. Therefore, the larger size of the glucuronide **2** should prevent the rapid renal clearance observed with β-glucuronidase-responsive prodrugs developed so far. Once in targeted tumor tissues, the β-glucuronidase-catalyzed cleavage of the

Received: March 13, 2012
Published: April 19, 2012

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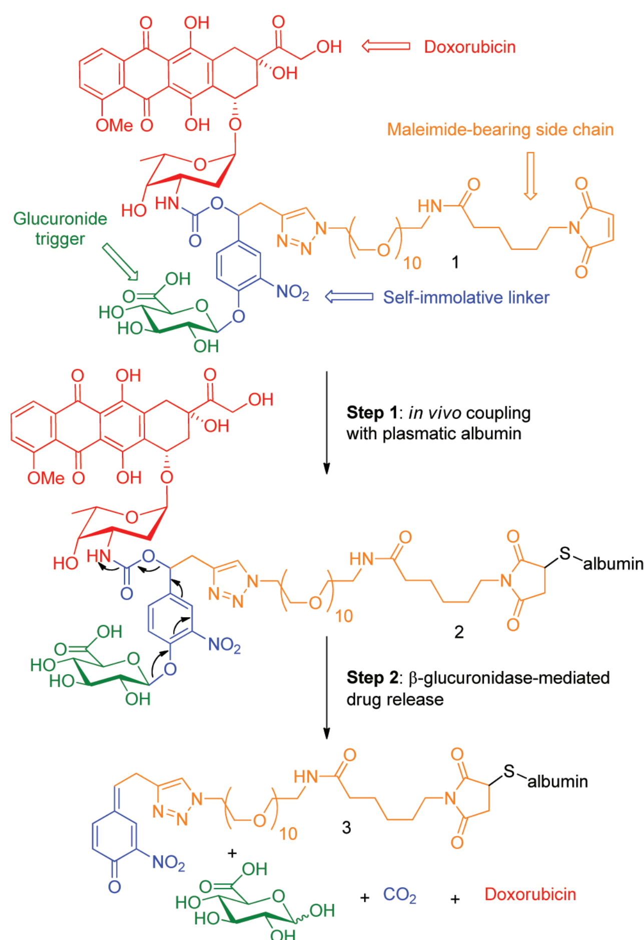
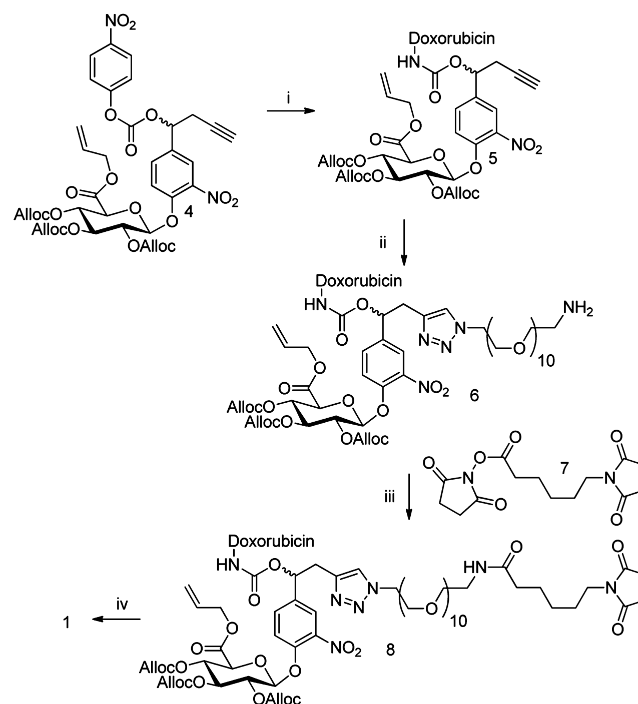
Scheme 1. Principle of Tumor Targeting with Maleimide-Containing Glucuronide Prodrug 1



Scheme 2. Synthesis of Prodrug 1[a]



[a]Reagents and conditions: (i) doxorubicin, Et_3N, HOBt, DMF, rt, 12 h, 79%; (ii) $NH_2CH_2(CH_2OCH_2)_{10}CH_2N_3$, $Cu(CH_3CN)_4PF_6$, CH_2Cl_2, rt, 16 h; (iii) $(CH_3)_2SO$, rt, 16 h, 57% (two steps); (iv) $Pd(PPh_3)_4$, aniline, THF/H_2O (9:1), rt, 4 h, 40% after purification by preparative chromatography.

glycosidic bond will trigger the release of doxorubicin in a stringently controlled fashion through the self-immolative mechanism depicted in Scheme 1. The results obtained in this study show that prodrug **1** leads to a higher antitumor efficacy than HMR 1826,[12] a well-known glucuronide prodrug of doxorubicin that cannot bind to circulating albumin. Furthermore, this compound inhibits tumor growth in similar a manner to that of doxorubicin while avoiding side effects recorded with the free drug when administered at its maximal tolerate dose.

■ RESULTS AND DISCUSSION

Chemistry. The synthesis of prodrug **1** was carried out starting from the fully allyl protected glucuronide **4** which is readily accessible as a mixture of two diastereoisomers through a multistep strategy recently described in the literature (Scheme 2).[3d] With this design, the cleavage of both allyl ester and carbonates can be realized at the very end of the synthesis in a one-step procedure under mild conditions that are compatible with the presence of either alkali- or acid-sensitive moieties such as doxorubicin or a maleimide. Thus, coupling between the activated carbonate **4** and doxorubicin undertaken via nucleophilic substitution in the presence of Et_3N and HOBt gave the alkyne **5** in 79% yield. The latter was then placed in CH_2Cl_2 with commercially available *O*-(2-aminoethyl)-*O*′-(2-azidoethyl)nonaethylene glycol and $Cu(CH_3CN)_4PF_6$ in order to form the triazole **6** through the well-known copper(I)-catalyzed azide−alkyne 1,3-cycloaddition (CuAAC).[13] After 16 h under these conditions, the reaction mixture was washed with an aqueous solution of EDTA, the solvent was removed, and the crude residue was engaged in the next step without any further purification. In this case, reaction of the primary amine of derivative **6** with the *N*-hydroxysuccinimide ester **7** afforded the protected glucuronide **8** in 57% yield over two steps. Finally, the expected prodrug **1** was obtained by the cleavage of protecting groups performed with $Pd(PPh_3)_4$ as a catalyst and 2 equiv of aniline (40%, purity of >95% after purification by preparative chromatography).

Biological Results. To demonstrate that the macromolecular drug carrier **2** can be formed spontaneously under physiological conditions, prodrug **1** and human serum albumin (HSA) were incubated together in phosphate buffer (pH 7) at 37 °C and the evolution of the mixture over time was monitored by HPLC. As shown in Figure 1a, the peaks corresponding to the two diastereoisomers of **1** disappeared almost completely after 5 h of incubation. In contrast, when the experiment was conducted with HMR 1826, a glucuronide prodrug of doxorubicin that does not contain a maleimide functional group (for the structure of HMR 1826 see Supporting Information), the mixture appeared perfectly stable with time (Figure 1b). As demonstrated previously by Kratz and co-workers with other albumin binding prodrugs,[8] this indicated that prodrug **1** reacts with HSA through its maleimide-containing side chain. Furthermore, since more than 90% of **1** reacted with HSA in 2 h, it may be anticipated that the kinetics of formation of the albumin conjugate **2** is compatible with the plasmatic half-life of glucuronide prodrugs such as HMR 1826.[14] The subsequent addition of *β*-

dx.doi.org/10.1021/jm300348r | J. Med. Chem. 2012, 55, 4516−4520

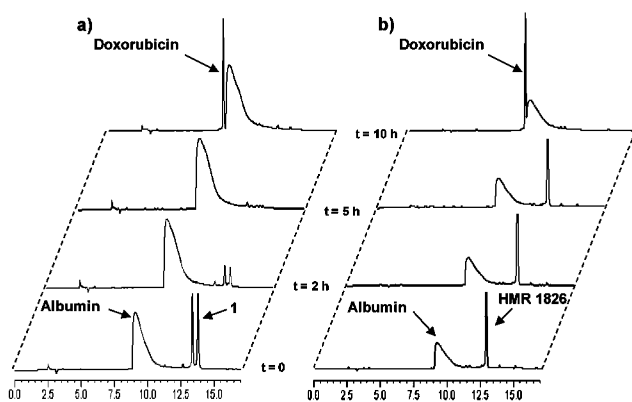


Figure 1. Chromatograms of HSA incubated with prodrug **1** (a) or HMR 1826 (b) in a 0.02 phosphate buffer (pH 7, 37 °C). β-Glucuronidase was added at t = 5 h.

glucuronidase in the media (t = 5 h, Figure 1) resulted in the clean release of doxorubicin which was fully completed 5 h after the incubation of the activating enzyme (t = 10 h, Figure 1). Taken together, these results demonstrated that prodrug **1** possesses the capacities to bind to HSA and to release the free drug upon enzymatic activation.

Prodrug **1** was then evaluated for its antiproliferative activity against human H290, MDA-MB-231, U87-MG, and murine LLC tumor cell lines after 48 h of treatment (Table 1). When

Table 1. IC_{50} (nM) of Doxorubicin and Prodrug 1 with or without β-Glucuronidase Determined by Cell Viability Assays[a]

agent	H290	MDA-MB-231	U87-MG	LLC
doxorubicin	320 ± 24	340 ± 26	790 ± 171	207 ± 34
1	na	na	na	na
1 + β-Glu	310 ± 62	320 ± 65	850 ± 34	173 ± 36

[a]na: no activity.

incubated alone, glucuronide **1** did not affect viability of cells at the highest tested dose of 2 μM whereas the free drug was highly toxic. This result indicated that derivatization of doxorubicin in the form of prodrug **1** markedly reduced its cytotoxicity. On the other hand, addition of β-glucuronidase in the culture medium induced a dramatic antiproliferative effect similar to that recorded for doxorubicin. As expected, enzymatic activation of the glucuronide trigger led to the efficient release of the drug, thereby restoring its initial activity. Further experiments conducted on LLC cells showed that prodrug **1** is 4-fold less toxic than HMR 1826 in the absence of β-glucuronidase (IC_{50} of 14 and 3.5 μM, respectively) while these two targeting devices exhibit the same antiproliferative activities when incubated with the activating enzyme (IC_{50} of 173 and 199 nM, respectively). This finding suggests that the additional hydrophilicity imparted by the polyethylene glycol-containing side chain limits passive cellular uptake and further intracellular activation of **1** by lysosomal β-glucuronidase.

The in vivo efficacy of prodrug **1** was assessed in a subcutaneous murine Lewis lung carcinoma (LLC) implanted in C57BL/6 mice. The animals received two iv injections of 43.6 μmol/kg of prodrug **1** or HMR 1826 at days 4 and 11 after transplantation. Doxorubicin was tested at its maximal tolerate dose (2 × 13.7 μmol/kg) following the same therapeutic protocol (Figure 2).

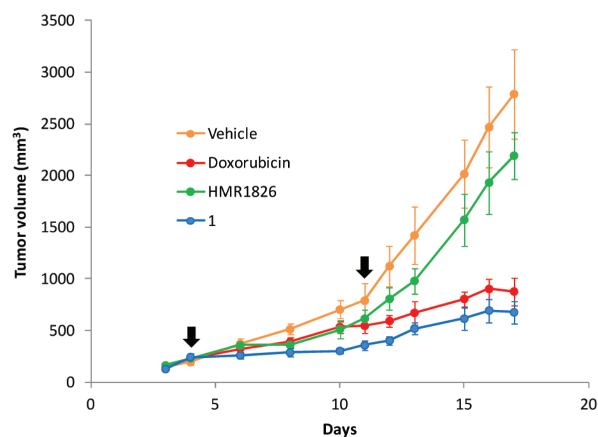


Figure 2. Tumor growth inhibition of subcutaneous murine LLC xenografts under therapy with doxorubicin, HMR 1826, and prodrug **1** administered intravenously at days 4 and 11.

As shown in Table 2, HMR 1826 was poorly active at the tested dose (T/C = 69%). In contrast, prodrug **1** produced a

Table 2. Antitumor Activity of Doxorubicin, HMR 1826, and Prodrug 1 against Murine LLC Xenografts in Vivo

agent	dose [μmol/kg]	mortality	body weight change [%][a]	T/C [%] (day)[b]
doxorubicin	2 × 13.7	1/8	−21	31 (17)
HMR 1826	2 × 43.6	0/8	+15	69 (13)
1	2 × 43.6	0/8	+8	24 (17)

[a]Measured at day 17. [b]T/C = [mean relative tumor volume of treated group]/[mean relative tumor volume of control group]. As a parameter for maximum efficacy, the minimal T/C was given on the day it was obtained.

good antitumor response (T/C = 24%) which was comparable to that obtained with doxorubicin (T/C = 31%). The higher activity of **1** compared to HMR 1826 could be explained by the formation of the macromolecular drug carrier **2** in the bloodstream leading to enhanced permeability and retention in the tumor combined with prolonged half-life. Moreover, the free doxorubicin induced a body weight loss of 21%, whereas prodrug **1** was well tolerated without any sign of overt toxicity.

As nephrotoxicity is one of the important side effects of anthracycline antibiotics, histopathological examination of kidneys was undertaken. This investigation revealed atrophy of the renal pericapsular tissues in the group treated with doxorubicin which correlates with the decreased body weight observed clinically in these animals (Figure 3). On the other hand, kidneys of mice that received iv injection of glucuronide **1** did not exhibit microscopic evidence of such toxicity. Overall, these in vivo experiments demonstrated that the albumin-binding prodrug **1** is a promising candidate for selective treatment of solid tumors in vivo.

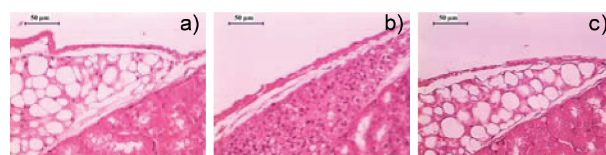


Figure 3. Sections of the kidney stained with hematoxylin eosin: (a) untreated; (b) treated with doxorubicin; (c) treated with prodrug **1**.

dx.doi.org/10.1021/jm300348r | J. Med. Chem. 2012, 55, 4516−4520

■ CONCLUSION

In summary, we prepared a glucuronide prodrug of doxorubicin bearing a maleimide-containing side chain which allows its binding to plasmatic albumin through Michael addition under physiological conditions. This β-glucuronidase-responsive targeting system is more efficient than its analogue HMR 1826 when administrated at the same dose in mice. Furthermore, our albumin-binding prodrug exhibits an antitumor efficacy similar to that of doxorubicin while avoiding side effects induced by the free drug. This finding could be of interest in the search for efficient anticancer prodrug for selective chemotherapy of solid tumors.

■ EXPERIMENTAL SECTION

General Method. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz at ambient temperature in the indicated solvent. High-resolution ESI mass spectra were obtained using a Waters MicrO-Tof-Q2 mass spectrometer. Analytical thin layer chromatography was performed using 0.2 mm silica gel 60-F plates. Flash chromatography was carried out with silica gel 60 (15−40 μm) as the stationary phase. Purity of compound **1** was determined to be >95% by HPLC.

Synthesis of 5. To a solution of doxorubicin hydrochloride (207 mg, 0.36 mmol) in DMF (3.8 mL) was added Et_3N (49 μL, 0.36 mmol). The mixture was stirred at room temperature for 30 min. HOBt (48 mg, 0.36 mmol) and a solution of **4** (300 mg, 0.36 mmol) in DMF (2.5 mL) were added. The mixture was stirred at room temperature for 7 h and concentrated in vacuo. The crude product was purified by column chromatography over silica gel (CH_2Cl_2/MeOH, 99/1, 98/2) to afford **5** (364 mg, 83%) as a mixture of two diastereoisomers (red solid). Mp = 140−150 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (m, 3H), 1.81 (m, 3H), 1.97 (m, 1H), 2.09−2.51 (m, 4H), 2.66 (m, 2H), 2.91 (m, 1H), 3.19 (m, 1H), 3.59 (s, 0.5H), 3.67 (s, 0.5H), 3.81 (m, 1H), 4.09 (m, 4H), 4.30 (m, 1H), 4.56−4.72 (m, 10H), 5.21−5.36 (m, 13H), 5.48 (m, 1H), 5.65 (m, 1H), 5.80−5.93 (m, 4H), 7.27 (m, 2H), 7.37 (m, 1H), 7.47 (m, 1H), 7.77 (m, 2H), 7.98 (m, 1H), 13.15 (2s, 1H), 13.91 (2s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.9, 26.4 and 26.5, 30.1 and 30.2, 34.0, 35.7, 47.2 and 47.3, 56.8, 65.6, 67.0, 67.4, 69.3, 69.4, 69.5, 69.6 and 69.7, 69.8, 71.9 and 72.0, 72.3, 72.4, 72.5 and 72.6, 73.9 and 74.0, 75.0 and 75.1, 76.7, 78.6, 99.4 and 99.6, 100.7 and 100.8, 111.4, 111.6, 118.6 and 118.7, 118.8, 119.1, 119.4, 119.5, 119.9, 120.8, 123.4, 123.6, 130.9, 131.0, 131.1, 131.3, 132.3 and 132.4, 133.5 and 133.6, 133.6 and 133.7, 135.4 and 135.5, 135.9, 140.6 and 140.8, 149.0, 153.5 and 153.6, 154.0, 154.3, 155.6, 156.2, 161.1, 165.6 and 165.7, 186.6 and 186.7, 187.0 and 187.1, 213.9. HRMS (ESI) $[M + Na]^+$ m/z 1267.3222 (calcd for $C_{59}H_{60}N_2O_{28}Na$, 1267.32248), $[M + K]^+$ 1283.3005 (calcd for $C_{59}H_{60}N_2O_{28}K$, 1283.296 42).

Synthesis of 8. To a solution of **5** (186 mg, 0.149 mmol) and *O*-(2-aminoethyl)-*O*′-(2-azidoethyl)nonaethylene glycol (102 mg, 0.149 mmol) in CH_2Cl_2 (7 mL) was added $Cu(MeCN)_4PF_6$ (78 mg, 0.209 mmol). The mixture was stirred at room temperature for 3 h, and a solution of disodium EDTA (956 mg, 2.568 mmol) in 0,1 M phosphate buffer (14 mL) was added. The resulting mixture was stirred for 5 h and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude product was dissolved in DMSO (3 mL), and **7** (58 mg, 0.194 mmol) was added. The mixture was stirred at room temperature for 16 h, and CH_2Cl_2 (50 mL) was added. The organic layer was washed with water (5 × 50 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by column chromatography over silica gel (CH_2Cl_2/MeOH, 95/5, 92/8) to afford **8** (166 mg, 57%) as a mixture of two diastereoisomers (red solid). Mp = 140−150 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21−1.33 (m, 5H), 1.54−1.76 (m, 5H), 1.82−1.90 (m, 1H), 2.15 (m, 3H), 2.31 (d, 1H, J = 14.1 Hz), 2.98 (m, 1H), 3.22 (m, 3H), 3.44−3.82 (m, 46H), 4.07 (m, 4H), 4.30 (m, 1H), 4.47−4.73 (m, 12H), 5.19−5.36 (m, 13H), 5.48 (m, 1H), 5.73−6.06 (m, 5H), 6.26 (bs, 1H), 6.69 (s, 2H), 7.27 (m, 2H), 7.41 (m, 2H), 7.67 (m, 2H), 7.78 (m, 1H), 8.02 (m, 1H), 13.22 (2s, 1H), 13.95 (2s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.0, 25.2, 26.5, 28.4, 29.8, 30.2, 32.9, 34.0, 35.7, 36.4, 37.8, 39.2, 47.3, 50.2, 53.5, 56.8, 65.6, 66.9, 67.4, 67.7, 68.6, 69.2, 69.3, 69.4, 69.5, 69.7, 70.0, 70.3, 70.4−70.7, 72.2, 72.4, 73.6, 73.9 and 74.0, 75.1, 76.7, 77.9, 99.5, 100.9, 111.4, 111.6, 118.6, 119.1, 119.3, 119.4, 119.5, 120.0, 121.0, 122.9, 123.2, 123.9, 130.9, 131.0, 131.1, 131.3, 132.1, 132.5, 133.7, 134.2, 135.6, 135.9, 140.7, 140.9, 142.4, 148.7, 153.5, 153.6, 154.0, 154.6, 154.7, 155.7, 155.8, 156.3, 161.2, 165.6, 165.7, 170.9, 172.9, 186.8, 187.2, 214.0. HRMS (ESI) $[M + Na]^+$ m/z 1986.7184 (calcd for $C_{91}H_{117}N_7O_{41}Na$, 1986.71777), $[M + 2Na]^{2+}$ m/z 1004.8535 (calcd for $C_{91}H_{117}N_7O_{41}Na_2$, 1004.8535), $[M + Na + K]^{2+}$ m/z 1012.8404 (calcd for $C_{91}H_{117}N_7O_{41}NaK$, 1012.840 47).

Synthesis of Prodrug 1. To a solution of **8** (166 mg, 0.084 mmol) in THF/H_2O, 9/1 (2 mL), were added $Pd(PPh_3)_4$ (14.6 mg, 15 mol %) and aniline (19 μL, 0.211 mmol). Total deprotection was achieved after stirring at room temperature for 4 h. Solvents were removed under reduced pressure. High degree of purity for **1** was obtained using preparative-reverse phase HPLC (56 mg, 40%, purity >95%). Mp = 140−150 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14 (m, 5H), 1.46 (m, 4H), 1.83 (m, 1H), 2.01−2.19 (m, 4H), 2.94 (m, 2H), 3.14−3.75 (m, 49H, partially masked by H_2O residual peak), 3.88−3.99 (m, 4H), 4.12 (m, 1H), 4.37−4.44 (m, 2H), 4.55 (s, 2H), 4.91 (m, 1H), 5.17−5.47 (m, 4H), 5.76 (m, 1H), 7.0 (s, 2H), 7.10 and 7.23 (2s, 1H), 7.32 (m, 1H), 7.47−7.55 (m, 1H), 7.66 (m, 1H), 7.75−7.83 (m, 2H), 7.91 (m, 2H), 13.26 (1s, 1H), 14.05 (1s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.0, 24.8, 25.8, 27.8, 32.11, 35.1, 37.0, 38.3 and 38.4, 47.1, 49.3, 52.3, 54.9, 56.6, 63.7, 66.7, 67.9, 68.8, 69.2, 69.6−69.9, 71.1, 72.7, 74.9, 75.3, 75.8, 99.8, 100.1, 110.7, 110.8, 112.0, 115.7, 115.8, 116.5 and 116.8, 117.7, 118.6, 118.8, 119.0, 119.8, 120.0, 122.7, 123.5 and 123.6, 128.9, 130.2, 130.3, 132.0, 133.7 and 133.8, 134.1, 134.4, 134.7, 135.5, 136.3, 139.5, 139.7, 141,8 and 141,9, 148.5, 154.4 and 154.5, 156.0 and 156.1, 157.6, 157.9, 160.8, 170.0, 171.1, 172.0, 186,5 and 186.6, 213.8. HRMS (ESI) $[M - H]^-$ m/z 1670.6264 (calcd for $C_{76}H_{100}N_7O_{35}$, 1670.626 58).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Structure of HMR 1826, ^{1}H NMR and ^{13}C NMR spectra of **5**, **8**, and **1**, HPLC conditions, cell viability assays, curves depicting body weight change. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank CNRS and La Ligue Nationale contre le Cancer (Comité Charente-Maritime) for financial support of this study.

■ ABBREVIATIONS USED

EPR, enhanced permeability and retention; rt, room temperature; HOBt, *N*-hydroxybenzotriazole; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; HSA, human serum albumin; β-Glu, β-glucuronidase; iv, intravenous

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