N-Succinyl-(*β*-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin: An Extracellularly Tumor-Activated Prodrug Devoid of Intravenous Acute Toxicity

Anne-Marie Fernandez,*,† Kim Van derpoorten,† Luc Dasnois,† Karim Lebtahi,† Vincent Dubois,† Thomas J. Lobl,‡ Sanjeev Gangwar,‡ Cecilia Oliyai,‡ Evan R. Lewis,‡ Dan Shochat,‡ and André Trouet†

Laboratory of Cell Biology, Université Catholique de Louvain, Place Croix du Sud 5, B-1348 Louvain-la-Neuve, Belgium, and Corixa Corporation, 600 Gateway Boulevard, South San Francisco, California 94080

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Intravenous administration of *N*-(*β*-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin (**4**) induces an acute toxic reaction, killing animals in a few minutes. This results from its positive charge at physiological pH combined with its propensity to form large aggregates in aqueous solutions. Negatively charged *N*-capped versions of **4** such as the succinyl derivative **5** can be administered by the iv route at more than 10 times the LD_{50} of doxorubicin without inducing the acute toxic reaction, and they are active in vivo.

Introduction

The efficacy of cancer chemotherapy is limited by the side effects of the cytotoxic agents used as well as by resistance to treatment.[1] The side effects result from the action of anticancer drugs on normal cells, and it is therefore highly desirable to develop more specific chemotherapeutic agents.[2] Agents specific to tumor cells would allow the use of much higher doses of the drugs and/or more frequent treatments. Besides improving efficacy, this could also allow, at least in certain cases, to overcome resistance to treatment by increasing the tumoral concentration of the drug. We developed an extracellularly tumor-activated prodrug (ETAP) of doxorubicin (**1**), *N*-(*β*-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin (**4**; Scheme 1) that is stable in blood and unable to enter cells as such but is activated by peptidases released by tumor cells into *N*-(L-leucyl)doxorubicin (L-Dox), a metabolite that freely diffuses inside cells where it is activated into doxorubicin.[3,4] In our preliminary studies, **4**, nicknamed "Super-Leu-Dox" (SLD), showed reduced in vivo toxicity with an estimated LD_{50} value for the mouse that is 9 times higher than that of the free drug **1** and displayed increased efficacy as compared to **1** in two human breast tumor xenograft models.[3] In these preliminary in vivo studies, the drugs were administered by the ip route, and the first iv studies revealed a severe acute toxicity of **4** that could be overcome by capping the free amine with negatively charged moieties. Our new lead compound is the succinyl derivative CPI-0004Na (Scheme 1).

Chemistry

N-[*N*-(9-Fluorenylmethoxycarbonyl)-*β*-alanyl-L-leucyl-L-alanyl-L-leucyl]doxorubicin (Fmoc-SLD; **3**) was synthesized using a one-pot procedure (Scheme 1) with in situ activation of the protected tetrapeptide (**2**) in order to reduce C-terminal racemization. While preactivation of **2** systematically led to complete racemization of the leucine 4 residue, this allowed the generation of pure compound **3** (98.5%) with less than 1% racemization. Prodrug **4** was obtained after Fmoc removal as previously described,[5] except that the reaction was quenched using an aqueous buffer that allows the extremely rapid decrease of pH required to preserve the integrity of the anthracycline moiety while directly generating the desired salt (Scheme 1). The 94% pure compound obtained, as assessed by analytical HPLC, was used without further purification.

For the synthesis of the *N*-capped, negatively charged derivatives of prodrug **4**, we developed two procedures, a stepwise and a one-pot, both starting from the Fmoc-protected derivative **3** (Scheme 1). The succinyl derivative of **4** (**5**) was initially obtained from the reaction of the free base of **4** with succinic anhydride, but the preparation of the free base of **4** is a poor yield procedure that always generates impurities. Even when the free base of **4** was prepared in situ prior to the addition of the anhydride, the purity of the final compound **5** was not acceptable, and at least 5% of the starting material was present in the final product. Using succinic anhydride in the quenching step instead of lactate buffer significantly improved the synthesis procedure by allowing quenching of the deprotection reaction and succinylation simultaneously. Both yield and purity were significantly improved (from 65 to 95% for yield from **3**, and from 80 to 91% for the purity). Also, no remaining starting material **3** could be detected, and less than 0.3% unsuccinylated prodrug **4** was present as determined by HPLC analysis. This simple synthetic procedure gave similar results within the scale range of 50 mg−15 g. Prodrug **5** generated this way was purified up to 98.5% by preparative HPLC.

Biological Data

While **4** had proved safe and efficient as a prodrug in animal studies performed by the ip route,[3] we found an unexpected, extremely severe toxicity when the compound was administered iv. Dose levels as low as 34.5 *μ*mol/kg caused death in 100% of the mice within 15 min after administration of the drug, although this dose is below the LD_{50} of **1** in the same strain and sex as determined at 14 days after treatment (42.1 *μ*mol/kg, our unpublished data). This was unanticipated because,

* Corresponding author tel: +32-10-47-3509; fax: +32-10-47-3515; e-mail: fernandez@bani.ucl.ac.be.
† Université Catholique de Louvain.
‡ Corixa Corporation.

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Scheme 1

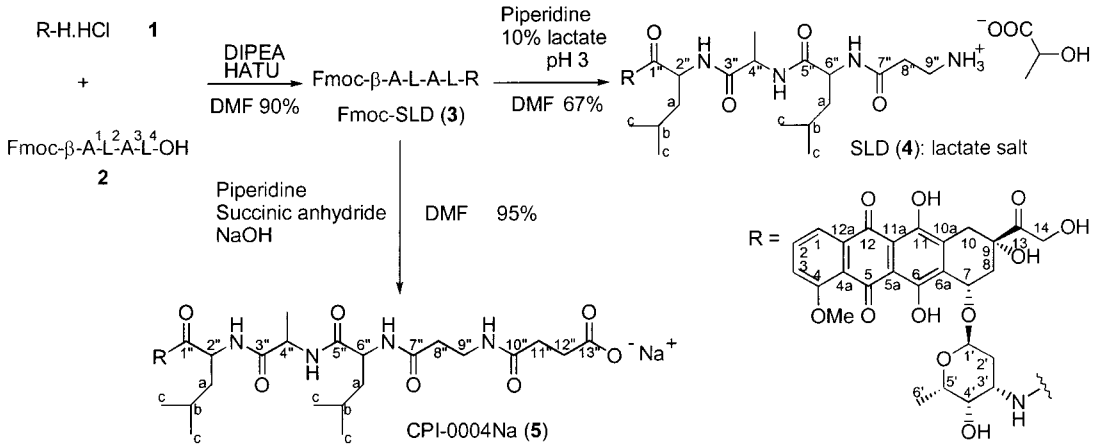

Table 1. Aggregation of Doxorubicin (**1**) and Prodrugs **4** and **5** in Aqueous Solution[a]

compd	concn (mM)	MWCO (Da) 3000 (%)	10 000 (%)	30 000 (%)	50 000 (%)
1	17.24	10	2	nd[b]	0.5
4	17.24	89	76	64	43
4	1.72	78	24	nd	nd
4	0.17	60	28	nd	nd
5	17.24	98	85	nd	nd

[a] Solutions of doxorubicin (**1**), SLD (**4**), and CPI-0004Na (**5**) in water were prepared and filtered through Centricon ultrafiltration units of increasing molecular weight cutoff (MWCO) for the same time and at the same relative centrifugal force. The proportion of compound retained by the filter is presented. [b] nd, not determined.

Table 2. Effect of Heparin Pretreatment on the Acute Toxicity of Prodrugs **4** and **5**[a]

compd	route of pretreatment	heparin dose level (IU)	min survival time (days)	acute toxicity (% of animals)
4	na[b]	0	0	100
4	ip	4000	9	37
4	ip	8000	11	0
4	iv	4000	11	33
5	na	0	9	0

[a] Low molecular weight heparin was administered 1 h prior to the iv injection of SLD (**4**) and CPI-0004Na (**5**) (172.4 μmol/kg). [b] Not applicable (na): no heparin pretreatment.

although a number of peptidic derivatives of **1** were described,[6−8] no in vivo data following iv bolus administration of such compounds had been published.

It has been reported by some investigators that **1** and other anthracyclines self-associate in aqueous solutions as measured by various spectroscopic methods.[9,10] As for the prodrug **4**, it may potentially undergo self-association similar to **1**, and the hypothesis is that **4**, being positively charged at physiological pH, forms cationic polymers in situ as a result of aggregation. The formation of polycations might contribute to the observed toxicity following iv administration of **4** as the symptoms affecting the mice resemble those described for positively charged polymers such as protamines, polylysines, or their aggregates.[11−13]

To investigate the presence of aggregates, ultrafiltration experiments were carried out using membranes of increasing molecular weight cutoff (MWCO). Table 1 shows the results obtained with solutions containing 17.24 mM **1** or **4** in water. There are no visible clumps or precipitates in these solutions by visual inspection. When a membrane MWCO of 3000 is used, 89% of **4** (molecular weight 1002) is retained by the membrane as compared to 10% in the case of the parent drug **1** (molecular weight 580). While only 2% of **1** is retained when the MWCO used is 10 000, 76% of **4** is still retained. The corresponding values obtained with a 50 000 MWCO are 0.5 and 43%, respectively. These data suggest that a relatively large percentage of **4** found in aqueous solutions consists of aggregates larger than 50 kDa (i.e., more than 50 molecules/aggregate). The presence of aggregates of **4** was confirmed by dynamic light scattering analysis. Different populations of aggregates whose mean diameters range from 3 to 32 nm were found (data not shown), corroborating the ultrafiltration data. The acute iv toxicity of **4** presumably results from interactions between the polycations formed by these aggregates and the heparin-like residues present on the luminal surface of blood vessels that can lead to disseminated intravascular blood coagulation.[13] In agreement with this hypothesis, it was observed that pretreatment of the animals with heparin prevents iv acute toxicity (Table 2). For example, when animals were treated by the ip route with 8000 IU of heparin 1 h prior to the iv administration of 172.4 μmol/kg of **4**, a dose more than 4-fold higher than the LD_{50} of doxorubicin (**1**), no acute toxicity was observed, and the animals died after 11 days from usual anthracycline toxicity. In contrast, without heparin pretreatment, all mice died within 15 min after treatment. According to the same hypothesis, a negatively charged compound should not be toxic since it cannot bind heparin-like molecules, and indeed, capping the free amino terminus of **4** with a succinyl moiety (compound **5**) resulted in eradication of acute iv toxicity without heparin pretreatment (Table 2), even at dose levels as high as 431 μmol/kg. Similar results were obtained with other negatively charged derivatives (glutaryl and diglycolyl) of **4**. Since **5** has similar properties as prodrug **4** in that it is also retained by ultrafiltration membranes that have MWCO ratings which exceed its theoretical molecular weight (Table 1), these data strongly suggest that it is not necessarily aggregates as such that mediate the acute toxicity but rather the positive charges they carry. The fact that such an acute toxic reaction was never observed in our in vivo studies of **4** using the ip route[3] could be explained by the observation that aggregation of **4** in aqueous solutions is concentra-

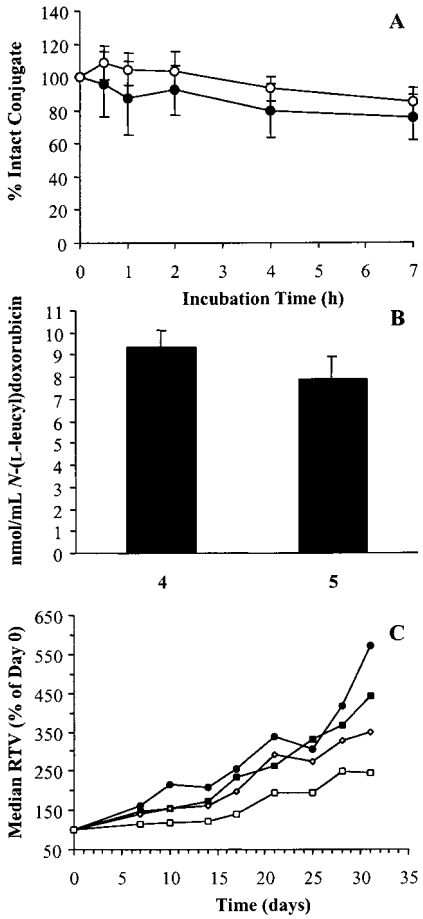

Figure 1. (A) Comparison of the stability of SLD (**4**; ●) and its succinyl derivative (**5**; ○) in whole human blood at 37 °C. Both compounds were used at 17.24 μM and quantified by HPLC analysis after extraction. Combined results from three independent experiments are presented. (B) Comparison of the activation of **4** and **5** by enzymes released by MCF-7/6 human breast cancer cells. The amount of *N*-L-(leucyl)doxorubicin generated from both compounds (17.24 μM) after incubation in conditioned media for 7 h at 37 °C is shown (combined results of seven independent experiments). Quantification was done by HPLC analysis after extraction. (C) In vivo activity of **5** in comparison with **4**. Balb/c *nu-nu* mice bearing established MCF-7/6 human breast tumor xenografts were treated with 5 ip injections of either saline (●), 3.5 μmol/kg of **1** (■), 35 μmol/kg of **4** (◇), or 47.5 μmol/kg of **5** (□) on consecutive days (starting on day 0). The evolution of median relative tumor volumes (RTV) in the different groups is presented.

tion dependent (Table 1). Actual blood concentration of the prodrug is very likely much lower following an ip administration than after an iv bolus injection.

Although *N*-capping solved the acute toxicity issue, allowing iv treatment of animals, we had to check that the critical features of **5** as an ETAP prodrug, such as blood stability and reactivation by peptidases released from cancer cells, were retained. Figure 1A shows the in vitro blood stability of **4** and **5** in comparison. There are no significant differences, and **5** is not less stable than **4** with a mean degradation of 15% after 7 h.

We compared **4** and **5** with regard to their activation by peptidases released by human cancer cells in culture. Figure 1B shows that no significant difference between the two compounds can be observed with regard to the amount of L-Dox generated after a 7-h incubation at 37 °C in MCF-7/6 breast cancer cells conditioned media. This indicates that succinylation of the free amine of **4** does not affect its susceptibility to cleavage by the peptidases released by these cancer cells.

The most important criterion being in vivo activity, nude mice bearing MCF-7/6 human breast tumors were used to compare the efficacy of **4** and **5**. To allow direct comparison and because of the acute toxic reaction inevitably following iv administration of **4**, drugs were administered by the ip route. Based on clinical signs and body weight loss, all drugs were administered close to their maximal tolerated dose. Figure 1C shows changes in tumor volume in the different groups. As expected from the in vitro data, *N*-capping of **4** does not result in its inactivation. In this model, **5** is even more active with a tumor growth inhibition of 57% at the end of the study instead of 39 and 23% in animals treated with **4** and **1**, respectively.

In conclusion, **5**, the succinyl derivative of **4**, appears to be a candidate ETAP prodrug devoid of iv acute toxicity that deserves further development. We are now confirming the increased efficacy of this compound by the iv route and that this is actually the result of an increased uptake of **1** in tumors.

Experimental Section

Doxorubicin hydrochloride was obtained from Meiji Seika Pharma International, Japan. *N*-(9-Fluorenylmethoxycarbonyl)-β-alanyl-L-leucyl-L-alanyl-L-leucine (Fmoc-βALAL) was custom-synthesized by Abbott Laboratories. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and L-lactic acid 85% were from Aldrich. Diisopropylethylamine (DIEA) was obtained from Acros, Belgium; DMF (Uvasol for spectroscopy), MeOH, pyridine, and formic acid were from Merck. Piperidine and Et_2O were from Fluka; succinic anhydride, trifluoroacetic acid (TFA), and low molecular weight heparin were from Sigma. All other solvents were obtained from Labscan, Ireland. HPLC analyses of all synthesized compounds were performed on TSK Super-ODS 4.6 × 100 mm columns (TosoHaas) with 0.1% TFA in water as solvent A, 0.1% TFA in acetonitrile as solvent B, and a flow of 1 mL/min. Gradient elution conditions were as follows: 30−36% of B in 2 min, 36−41% of B in 10 min, 41−90% of B in 3 min, and 5 min at 90% B. Purity of the compounds was assessed as the percentage surface area of the peaks at 254 nm. Preparative purification was achieved using Waters Delta Prep 4000 system with a C-4 column (15 μm, 40 × 100 mm, 30 mL/min) eluted with a 80−100% gradient of MeOH in 70 min. ^{1}H NMR spectra were recorded at the Université Libre de Bruxelles (Belgium) on a 600 MHz spectrometer. Chemical shifts (δ) are reported in ppm, and *J* values are quoted in Hz. ^{1}H assignments were supported by (^{1}H−^{1}H) COSY spectra. Electrospray mass spectra were obtained at the Mass Spectroscopy Laboratory of the Université Catholique de Louvain (Belgium). Elemental analyses were performed at the Université de Liège (Belgium).

***N*-[*N*-(9-Fluorenylmethoxycarbonyl)-β-alanyl-L-leucyl-L-alanyl-L-leucyl]doxorubicin (3).** Doxorubicin hydrochloride (**1**) (8.70 g, 15.00 mmol) and Fmoc-βALAL (**2**) (9.13 g, 15.00 mmol) were dissolved in DMF (700 mL). After DIEA (5216 μL, 30 mmol) was added, the mixture was stirred for 15 min at room temperature (rt; protected from light). A solution of HATU (6.27 g, 16.50 mmol) in DMF (300 mL) was added, and the mixture was stirred for 2 h at rt. Cold water (4 °C, 2 L) was then added. After being stirred for 30 min, the precipitate was filtered on qualitative paper (Whatman No. 1) and washed in turn with water (300 mL), 2% lactate buffer pH 4 (2 × 400 mL), and then water (2 × 400 mL) before being dried under vacuum to give 12.62 g of **3** (90% yield, HPLC purity: 98.5%). *m*/*z* (ESI): 1134 $[M + H]^+$. Anal. ($C_{60}H_{71}N_5O_{17}\cdot H_2O$) C, H, N.

***N*-(β-Alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin Lactate (4).** Piperidine (26 mL, 264 mmol) was added to a solution of **3** (6.00 g, 5.3 mmol) in DMF (265 mL). After being stirred for 5 min at rt, the reaction mixture was placed in an ice−salt bath, and precooled (4 °C) 10% lactate pH 3 buffer (600

mL) was immediately added. The aqueous solution was extracted with DCM (3 × 500 mL) and loaded on preconditioned C18 ODS-A silica gel (120 g, YMC) in a glass frit. After washes with water (2 × 500 mL) and drying, **4** was recovered with MeOH. Solvent was evaporated, and the residue was dissolved in water. This solution was lyophilized to give 3.54 g of **4** (67% yield, HPLC purity: 95.5% at 254 nm; 94.2% at 475 nm). ^{1}H NMR (600 MHz, DMSO-d_6, δ): 0.77−0.89 (4d, 12H, H2″^{c}, H6″^{c}, J = 6.0, 6.3, 6.3, 6.3), 1.22 (d, 3H, H6′, J = 6.7), 1.27 (d, 3H, H4″^{a}, J = 7.4), 1.44−1.55 (m, 6H, H2″^{a},H2″^{b}, H6″^{a}, H6″^{b}), 1.67 (m, 1H, H2′), 1.88 (ddd, 1H, H2′, J = 3.7, 12.6, 12.6), 2.06 (dd, 1H, H8, J = 4.1, 10.6), 2.28 (bd, 1H, H8), 2.29 (m, 2H, H8″), 2.90 (m, 2H, H9″), 2.95 (d, 1H, H10, J_{gem} = 18.0), 3.17 (d, 1H, H10, J_{gem} = 18.0), 3.57 (m, 1H, H4′), 3.96 (m, 1H, H3′), 3.99 (s, 3H, H15), 4.08−4.19 (m, 4H, H5′, H2″, H4″, H6″), 4.58 (s, 2H, H14), 5.19 (s, 1H, H7), 5.40 (d, 1H, H1′, J = 3.7), 7.32 (d, 1H, H1, J = 8.3), 7.70 (dd, 1H, H2, J = 8.0, 8.0), 7.95 (d, 1H, H3, J = 7.7). m/z (ESI): 912 $[M + H]^+$.

***N*-(Succinyl-β-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin Sodium (5).** From **4**: DIEA (417 μL, 2.40 mmol) was added to a solution of **4** (1.200 g, 1.20 mmol) in DMF (35 mL). After stirring for 15 min at rt, succinic anhydride (0.144 g, 1.44 mmol) was added. The mixture was stirred for 2 h, and DMF was removed by rotary evaporation. The residue was dissolved in a mixture of $CHCl_3$ and MeOH (4/1; 6 mL), and a mixture of Et_2O and hexane (1/1; 200 mL) was added. After being stirred for 30 min, the precipitate was filtered on quantitative paper (Whatman No. 42), washed (Et_2O/hexane: 1/1), and air-dried. The filter cake was suspended in water (150 mL), and 1 M NaOH (1.5 mL, 1.5 mmol) was added dropwise until complete dissolution (pH 7.2). The solution was lyophilized to give 1.218 g of **5** (97% yield; HPLC purity: 80.2%, 5% of **4** remaining).

From **3**: Piperidine (21.8 mL, 220.65 mmol) was added to a solution of **3** (5.00 g, 4.41 mmol) in DMF (220 mL). After being stirred for 5 min at rt, the reaction mixture was quickly cooled (−5 °C), and succinic anhydride (22.49 g, 224.91 mmol) was added immediately. The cold bath was removed as soon as the color changed, and the mixture was stirred for 10 min. DMF was removed by rotary evaporation, the residue was dissolved in $CHCl_3$ (125 mL), and Et_2O (1400 mL) was added. The precipitate was stirred for 10 min, filtered on quantitative paper (Whatman No. 42), and washed with Et_2O before being suspended in water (400 mL) and dissolved by dropwise addition of 0.025 M NaOH (215 mL, 5.37 mmol; final pH 6.95). This solution was lyophilized to give 4.33 g of **5** (95% yield, HPLC purity: 91%). Preparative HPLC was used to obtain a 98.5% pure compound. ^{1}H NMR (600 MHz, DMSO-d_6 δ): 0.78−0.84 (4d, 12H, H2″^{c}, H6″^{c}, J = 6.0, 6.0, 6.6, 7.2), 1.12 (d, 3H, H6′, J = 6.5), 1.16 (d, 3H, H4″^{a}, J = 7.1), 1.40 (2dd, 4H, H2″^{a}, H6″^{a}, J = 7.8, 15.0), 1.44 (dd, 1H, H2′, J = 4.1, 12.2), 1.55 (m, 2H, H2″^{b}, H6″^{b}), 1.82 (ddd, 1H, H2′, J = 3.0, 12.6, 12.6), 2.11 (dd, 1H, H8, J = 5.4, 14.4), 2.20 (bd, 1H, H8), 2.27 (m, 4H, H8″, H11′), 2.39 (t, 2H, H12″, J = 7.2), 2.90 (d, 1H, H10, J_{gem} = 18.0), 2.97(d, 1H, H10, J_{gem} = 18.0), 3.20 (m, 2H, H9″), 3.34 (m, 1H, H4′), 3.95 (m, 1H, H3′), 3.97 (s, 3H, H15), 4.17 (m, 1H, H5′), 4.22 (m, 3H, H2″, H4″, H6″), 4.58 (s, 2H, H14), 4.76 (s large, 1H, OH4′), 4.83 (s large, 1H, OH14), 4.92 (t, 1H, H7, J = 4.9), 5.22 (d, 1H, H1′, J = 3.0), 5.41 (s, 1H, OH9), 7.35 (d, 1H, NH3′, J = 8.4), 7.62 (m, 1H, H1), 7.76 (d, 1H, NH4″, J = 7.8), 7.81 (t, 1H, NH9″, J = 5.4), 7.86−7.89 (m, 2H, H2, H3), 7.97−8.00 (2d, 2H, NH2″, NH6″, J = 7.8, 8.4), 12.00 (s large, 1H, OH acid), 13.20 (s, 1H, OH6), 14.00 (s, 1H, OH11). m/z (ESI): 1012 $[M + H]^+$. Anal. ($C_{49}H_{65}N_5O_{18}$·$2H_2O$) C, H, N.

Aggregation of 4 in Aqueous Solution. Ultrafiltration experiments were performed with Centricon filter units (Amicon) of different MWCO. Solutions of drugs (17.24 mM) in water were used, and each filter unit was centrifuged at 1500*g* for 2 h. The proportions of drug filtered and retained were estimated by determination of the absorbance of the solutions at 475 nm. Similar experiments were performed with solutions of varying concentrations.

In Vivo Acute Toxicity of 4. Solutions of heparin and of **4** in 0.9% NaCl were prepared and sterilized by filtration (0.22 μm). Heparin pretreatments (4000 or 8000 IU) were administered to OF-1 male mice by the ip or iv route. After 1 h, **4** was administered iv (10 μL/g body weight) at a dose level of 172.4 μmol/kg. The occurrence of acute toxicity was checked. At least 3 animals were used in each experiment.

Stability of 4 and 5 in Whole Human Blood. Compounds **4** and **5** were added (10 μL/mL) at a final concentration of 17.24 μM to human blood freshly collected from healthy donors and incubated at 37 °C. At selected times, three 25-μL aliquots were removed, and conjugates and their metabolites were extracted as previously described.[3] For the extraction of **5**, a 0.5 M citrate buffer (pH 3.0) was used instead of borate.

Activation of 4 and 5 by Enzymes Released by Cancer Cells. MCF-7/6 human breast cancer cells were used for the preparation of conditioned media as described previously.[3] Compounds **4** and **5** (17.24 μM) were incubated in these media at 37 °C, and the generation of metabolites as a function of time was studied as in the case of blood stability studies.

In Vivo Activity of 5 and 6 As Compared to 4. MCF-7/6 human breast tumors were implanted and grown subcutaneously in both flanks of female Balb/c *nu-nu* mice (6 animals per group as previously described[3]). Treatments were administered ip once a day for five consecutive days, starting when tumors reached a mean diameter of at least 6 mm. Tumor volume was determined twice a week from caliper measurements [length × (width)^2/2]. Treatment toxicity was assessed based on clinical signs and body weight evolution.

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