Bioconjugate Chemistry

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Synthesis and Biological Evaluation of New Bisphosphonate–Dextran Conjugates Targeting Breast Primary Tumor

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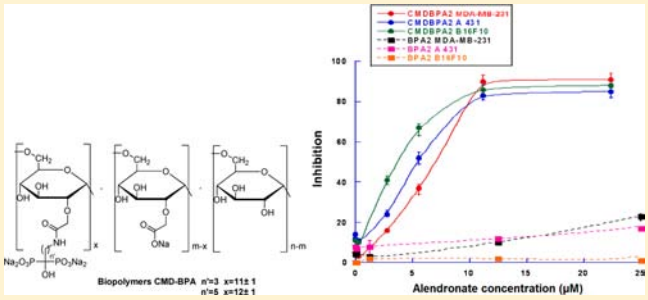
Supporting Information

ABSTRACT: Bisphosphonates (BPs) have interesting antitumor effects as well *in vitro* as *in vivo*, despite their poor bioavailability in the organism after oral ingestion. To overcome this problem and reduce drug doses and secondary effects, we report the chemical synthesis of new bioconjugates. They were built with a nitrogen-containing BP as the drug covalently coupled to the carboxymethyldextran. This polysaccharide was used as a carrier, in order to increase BP lifetime in bloodstream and to target tumor cells which have a strong affinity with dextran. The efficiency of our vectorization system was biologically proved *in vitro* and *in vivo* on mammalian carcinoma models in mice.



INTRODUCTION

Bisphosphonates (BPs) are bone-targeting agents used for decades in the therapy of bone-related diseases such as osteoporosis and Paget's disease. They mainly inhibit osteoclastic action on bone resorption binding to hydroxyapatite crystals, the main component of bones.[1]

They are synthetic analogs of inorganic pyrophosphate, an endogenous regulator of calcium homeostasis, with a P–C–P linkage instead of a P–O–P one. According to the chemical structure of the two side groups bearing by the central carbon atom, BPs can be distinguished in two classes: (i) the non-nitrogen-containing BPs, which induced apoptosis after metabolization in cytotoxic agents, and (ii) the nitrogen-containing BPs, more efficient than the previous ones, which suppress osteoclastic function by inhibition of mevalonate pathway enzymes as farnesyl pyrophosphate synthase.[2] Inhibition of this enzyme prevents the formation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These two isoprenoid ways involved in the cholesterol synthesis are required for the cellular survival and used for the prenylation of proteins as small GTPases including Ras proteins that regulate the proliferation, invasive properties, and proangiogenic activity of human tumor cells.[3]

More recently, BPs have shown interesting anticancer activity,[4] and now, they are commonly administered in the treatment of bone metastasis of various malignancies[5] such as breast, prostate, and colon cancer. Indeed, bone metastases increase osteolytic activity resulting in the release of growth factors that stimulate cancerous cell proliferation, and it is widely admitted that BPs can reduce skeletal morbidity (hypercalcemia, pain, pathologic fractures, surgery) for patients with breast cancer[6] and multiple myeloma,[7] for example.

Moreover, aside from their direct action on osteoclasts, *in vitro* studies have also shown that BPs might directly inhibit the proliferation and induce the cell death of the cancer cells themselves,[8] in particular, in the case of breast tumors[9] as well as in prostate tumor cells,[10] melanoma[11] and epidermoid carcinoma,[12] osteosarcoma,[13] and myeloma.[14] At last, they could inhibit tumor angiogenesis.[15]

Unfortunately, BPs are poorly absorbed by oral pathway and quickly eliminated from plasma after intravenous administration due to renal excretion and accumulation in bone (about 55% of the administered dose in the case of zoledronate, for example).[16] This therapeutic drawback is due to poor lipophilicity, a highly charged nature, and a propensity to chelate divalent cations (Ca^{2+}, Mg^{2+}) in the gastrointestinal tract.

BPs have also shown some side effects that could prevent their oral administration as esophagus inflammation, stomach irritation, or abdominal pain due to gastrointestinal toxicity, as well as their intravenous use inducing bone pain, myalgias and fevers, rare ophthalmologic side effects,[17] renal toxicity,[18] and rare osteonecrosis of the jaw in patients treated in cancer IV therapy.[19]

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In spite of side effects probably due to a dose-dependent toxicity, BPs are potent therapeutic agents and are considered among the most important advances in the treatment of bone metastatic disease. In addition, their therapeutic applications could be extended to a specific delivery to cancer cells localized in soft tissues instead of bone, the usual site of accumulation of these drugs. To this end, different carriers enable this specific delivery as liposomes,[20] micelles,[21] and nanoparticles.[22] On our part, we considered linking BPs to an inert macromolecular polymeric carrier in order to (i) improve their site specific and time-controlled delivery and (ii) decrease the toxicity of these compounds and minimize undesirable side effects by reducing the needed doses.

Polymers for therapeutic use as well as products of their degradation must be nontoxic, biocompatible, and bioremovable by normal metabolic pathways. Macromolecules must be also devoid of antigenicity, immunogenicity, and hemolytic, procoagulant, or cytotoxic activities. In addition, the drug must be released at the target site from the polymeric prodrug in its active form. Moreover, efficient polymeric drug conjugates with a molecular weight of more than 40 kDa usually have a longer $t_{1/2}$ than smaller molecules, are rapidly cleared from kidneys into urine, and exhibit high retention time in the tumor cells due to EPR effect.[23] Then, these macromolecules are known to be taken inside the cell by endocytosis, transported to lysosomes, and the active drug released by proteolytic enzymes at low pH.[23] In this approach, prolonged effective levels of anticancer drugs can be maintained due to the effects of slow release from the polymer. That is why there is a necessity to develop a more efficient release system of anticancer drugs using novel delivery platforms that will improve therapy and reduce side effects.

In our study, a dextran derivative was chosen as the drug carrier and coupled to a BP via an amide bond. Dextrans are widely available bacterial polysaccharides of broad range molecular weights. Dextrans of about 40 kDa to 70 kDa are among the most promising carrier candidates for passive targeting of drugs thanks to their excellent physicochemical properties, hemocompatibility, and physiological acceptance.[24]

In drug delivery strategy, Dang et al.[25] have synthesized methotrexate−dextran conjugates via a covalent short-lived ester bond ($t_{1/2}$ about 3 days in buffered saline) and a longer-lived amide link ($t_{1/2}$ longer than 20 days in buffered saline). They also observed a greater stability in phosphate buffered saline of the methotrexate−amide−dextran conjugate than the ester one, suggesting that the drug dissociation rate *in vivo* will probably be faster than that *in vitro* due to the presence of proteases and esterases.

Various active compounds have been coupled to dextran via a periodate oxidation as daunomycin[26] and methotrexate.[27] About the delivery of BPs, Varghese et al.[28] reported the synthesis of a high molecular weight hyaluronan−BP conjugate as an injectable hydrogel for the cell-specific targeting and the localized sustained release of therapeutic doses of the drug through the gel degradation.

Recently, Holmberg et al.[29] described the coupling of alendronate and aminoguanidine to oxidized dextran with subsequent reductive amination (approximately 8 alendronate and 50 guanidine moieties per conjugate). Bone resorption assay and tumor cell toxicity on prostate and breast cancers were studied and compared to zoledronate (Zometa) used as a positive control. Similar results were observed with this dextran conjugate and zoledronate in bone resorption assay, whereas best results were obtained with the first one in cytotoxicity and apoptosis assays compared to zoledronate. So, this novel poly-BP conjugate appears to be a promising polymer conjugated therapeutic with high dual efficacy in the treatment of osteoporosis and bone metastasis.

Our objective was to improve the biological potency of weakly active N-containing BPs by means of a dextran derivative polysaccharidic carrier. Thus, alendronate and neridronate were considered as good candidates to optimize their efficiency and were attached to a dextran derivative to form a prodrug by direct amide linkage.

EXPERIMENTAL SECTION

Reagents and Instrumentation. Dextran T40 (M_n = 28 800 Da, M_w = 40 400 Da, batch 228 608) was supplied by Pharmacia (St Quentin-en-Yv., France). All "analytical grade" chemicals and solvents were purchased from Sigma Aldrich (St Quentin Fallavier, France), Acros (Noisy-le-Grand, France), and Interchim (Montluçon, France) and were used without furthermore purification. Dialysis was realized with Spectra/Por Cellulose Ester membrane (MWCO: 25 000 Da). Acidimetric titrations of CMD were performed using an Automatic Titrimeter (DL 53 Mettler Toledo) programmed with Lab X software. Molecular weights were obtained after collecting and processing data with Omnisec 4.0 software (Viscotek, Great Britain). NMR spectra were recorded with a Bruker 400 MHz Avance III spectrometer (^{1}H: 400.16 MHz, ^{31}P: 161.98 MHz) in D_2O. Chemical shifts (δ) are given in ppm using residual solvent signal from D_2O as reference in ^{1}H spectra and phosphoric acid as external reference in ^{31}P NMR.

Chemical Procedures. CMD **1** (CarboxyMethyl Dextran)[30] and Bisphosphonates **2**[31] are known compounds and were prepared according to literature procedures. Their physical and spectroscopic data were in agreement with those previously reported.

Synthesis of CMD-BPA2 3a and CMD-BPA3 3b. CMD (300 mg, D.S. = 0.91, 1.16 mmol) in distilled water (30 mL) was introduced in a three-necked round-bottom flask equipped with a thermometer, a condenser, a pH-meter, and a magnetic stirrer. Aqueous HCl solution (1 N) was added dropwise at 0 °C until pH = 4.5. Then, EDC (444 mg, 2.32 mmol) and NHS (267 mg, 2.32 mmol) were successively added to CMD at 0 °C at pH = 4.5 and the reaction mixture was stirred for 30 min in the same conditions. A solution of neridronate (796 mg, 2.32 mmol) or alendronate (642 mg, 2.32 mmol) in distilled water (5 mL) at pH = 11.5 was added to the previously prepared reaction mixture. pH was maintained at 11.5, using if necessary aqueous NaOH solution (1 N). The reaction mixture was stirred at 60 °C 4 h for neridronate and 1 h for alendronate. Then, the reaction was stopped by addition of aqueous HCl solution (1 N) until pH = 6.5. The mixture was concentrated under reduced pressure, precipitated with methanol at 0 °C, and washed three times with iced methanol. Next, the white solid in distilled water (10 mL) was precipitated with ethanol (90 mL) at 0 °C and washed three times again with iced methanol. The recovered powder was then dissolved in milli-Q water (100 mL) and dialyzed against 2 L of distilled water. The water was exchanged 14 times after at least 2 h of dialysis. After lyophilization, a white solid was obtained (172 mg, 11 ± 1 molecules of alendronate grafted on 246 glucose units) or (260 mg, 12 ± 1 molecules of neridronate grafted on 246 glucose units).

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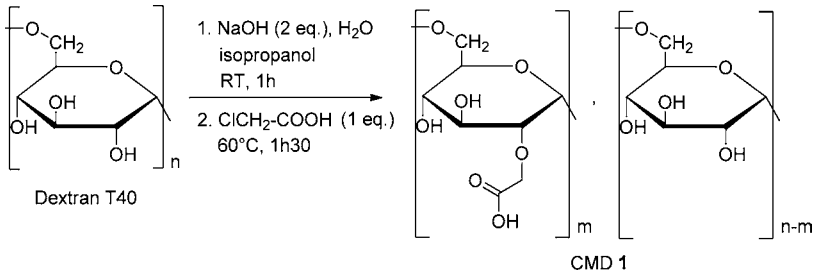
Scheme 1. Synthesis of CMD 1



Determination of the Molecular Weight of Dextran Derivatives. The chromatographic molecular weight of dextran derivative samples was determined by high-performance steric exclusion chromatography in 0.15 M NaCl, 0.05 M NaH_2PO_4 buffered at pH 7, using two columns connected in series (respectively, Licrospher Si 300 diol and Lichrospher Si 100 diol, Merck-Clevenot, Nogent-sur-Marne, France) and a 510 model pump (Waters, St Quentin-en-Yv., France) with an injection loop of 100 μL. The effluent was monitored using a TDA model 301 (refractive index and light scattering; Viscotek, UK). The flow rate was 0.5 mL/min. Average molecular weights were obtained by processing data with Omnisec 3.0 software (Viscotek, UK).

Cell Lines and Cell Culture. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 $U.mL^{-1}$ streptomycin (all obtained Invitrogen Inc.), at 37 °C in a 5% CO_2 humidified atmosphere. Human A431 squamous cell carcinoma (vulvar epidermoid carcinoma), breast carcinoma (MDA-MB-231), and mouse melanoma (B16F10) cells were obtained from the American Type Culture Collection.

Cell Viability Experiments. Cell viability was evaluated using the MTT microculture tetrazolium assay (Mosmann, 1983) based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) into purple formazan crystals. Cells were seeded at a density of 5×10^3, 20×10^3, and 2.5×10^3 cells/well for A431, MDA-MB-231, and B16F10 cells, respectively. Cells were seeded in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 h. Then, the medium was removed and replaced by 10% FCS-medium containing increasing concentrations of alendronate, CMD, and CMD−alendronate varying from 0 to 25 μM. After 72 h incubation for A431, MDA-MB-231, and B16F10 cells, the cells were washed with phosphate-buffered saline (PBS, Life Technologies) and incubated with 0.1 mL of MTT (2 $mg.mL^{-1}$, Sigma-Aldrich) for an additional 4 h at 37 °C. The insoluble product was then dissolved by addition of 200 μL of DMSO (Sigma-Aldrich). The absorbance corresponding to the solubilized formazan pellet (which reflects the relative viable cell number) was measured at 570 nm using a Labsystems Multiskan MS microplate reader. Concentration−response curves were constructed, and IC_{50} values (concentration of the compound inhibiting 50% of cell proliferation) were determined. All *in vitro* cell experiments (viability, migration, and invasion assays) were carried out at 37 °C in a 5% CO_2 incubator.

Cell Migration Assays. The influence of alendronate, CMD, CMD−alendronate on the migration of MDA-MB-231 cells was investigated using Boyden invasion chambers with 8 μm pore size filters coated with 100 μL of fibronectin (100 $\mu g.mL^{-1}$, Santa Cruz Biotechnology, Santa Cruz, CA) and were allowed to stand overnight at 4 °C. Then, 5×10^4 MDA-MB-231 untreated cells or 24 h alendronate, CMD, CMD−alendronate (35 μM or 035 μM) pretreated MDA-MB-231 cells were added to each insert (upper chamber). A strong chemoattractant (10% FCS) for MDA-MB-231 cells was added to the lower chamber. After 24h incubation at 37 °C in a 5% CO_2 incubator, nonmigrated cells were removed by scraping and migrated cells were fixed in methanol and stained with hematoxylin. Cells migrating on the lower surface of the filter were counted in 10 fields using a Zeiss microscope. Results were expressed as a percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

Cell invasion assays. Cell invasion experiments were performed with Boyden chambers as described above. The inserts were coated with Matrigel membrane matrix (Falcon, Becton Dickinson Labware, Bedford, MA). The 5×10^4 MDA-MB-231 cells were seeded in the upper well of the Boyden chamber, and 10% FCS was added to the lower chamber. Before seeding in the upper chamber, the cells were pretreated with alendronate, CMD, CMD-alendronate (35 μM or 0.35 μM). After 24 h at 37 °C in a 5% CO_2 incubator, noninvaded cells in the upper chamber were wiped with a cotton swab and the filters were fixed, stained, and counted. Results were expressed as a percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

***In Vivo* Biological Evaluation: Xenografts in Nude Mice.** MDA-MB-231 cells (2×10^6 cells) were inoculated subcutaneously (s.c.) 4-week-old athymic nude mice (nu/nu, Janvier, France, $n = 40$). Then, mice were arbitrarily placed in control ($n = 9$) and in each bisphosphonate treated group ($n = 8$). The administration of alendronate or CMD-alendronate started one week after cell inoculation when tumor sizes were 30 mm^3. Mice were treated twice a week during four weeks by intravenous injection of 0.1 mL of NaCl 0.9% solution (control group) or containing BPA2 or CMD-BPA2 at 6 or 0.6 $mg.kg^{-1}$/injection. Tumor volume was measured once a week along major axes using calipers. Tumor volume (mm^3) was calculated as follows: $V = 4/3\pi R_1 R_2$ where $R_1 < R_2$. The animal protocol was in accordance with a French ethics committee (N° Ce5/2010/020).

RESULTS AND DISCUSSION

Chemical Synthesis and Characterization of Bioconjugates. In order to link an aminoBP to a 40 kDa dextran (namely, dextran T40, containing about 246 anhydroglucosyl units), we first modified the polymer via a carboxymethylation reaction as described by Huynh et al.[30] The chlorine atom of monochloroacetic acid was replaced in a nucleophilic substitution by the dextran alcoholate. This reaction was

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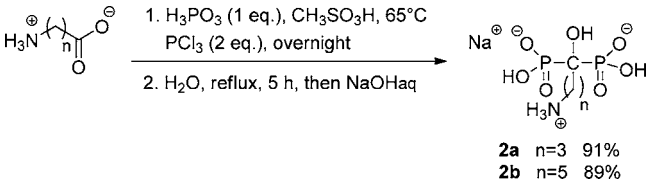
regioselective on the carbon 2 of the polysaccharide, giving CMD **1** (Scheme 1). Experimental conditions were established in order to obtain a degree of substitution (D.S., i.e., the number of carboxymethyl groups (CM) per anhydroglucose unit) of the synthesized CMD of about 1.

After precipitation in methanol and filtration, an additional dialysis furnished the pure desalted CMD **1**. ^{1}H NMR analysis confirmed the synthesis of CMD **1** with the presence of two peaks at 5.2 and 5.3 ppm corresponding to the anomeric proton and a multiplet from 3.5 to 4.5 ppm corresponding to eight protons (methylene of CM group and six sugar protons). In order to determine the degree of substitution of the prepared CMD **1**, acidimetric titrations were carried out in a water/acetone mixture (1:1 v/v). The D.S. was 0.91 ± 0.01.

N-containing BPs, alendronate **2a** (BPA2) and neridronate **2b** (BPA3), were synthesized according to a large-scale one-step procedure reported by Kieczykowski et al.[31] (Scheme 2).

Scheme 2. Synthesis of Alkyl Aminobisphosphonates 2a (Alendronate, BPA2) and 2b (Neridronate, BPA3)



Alkyl aminoBPs **2a** and **2b** were analyzed in ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and obtained analytically pure without further purification in yields of about 90%. Data corroborated with those published by Kieczykowski et al. in particular with a δ_P value at 18.4 and 19.1 ppm, respectively. Moreover, a characteristic signal was assigned for the carbon atom bearing the two phosphonic acid functions about 76 ppm as a triplet with a $^{1}J_{C-P}$ constant of 134 Hz.

The key step was the coupling of the primary amine on the side chain of alendronate **2a** or neridronate **2b** with the carboxylic groups of CMD **1** via the EDC/NHS widely described procedure leading to an amide linkage (Scheme 3).

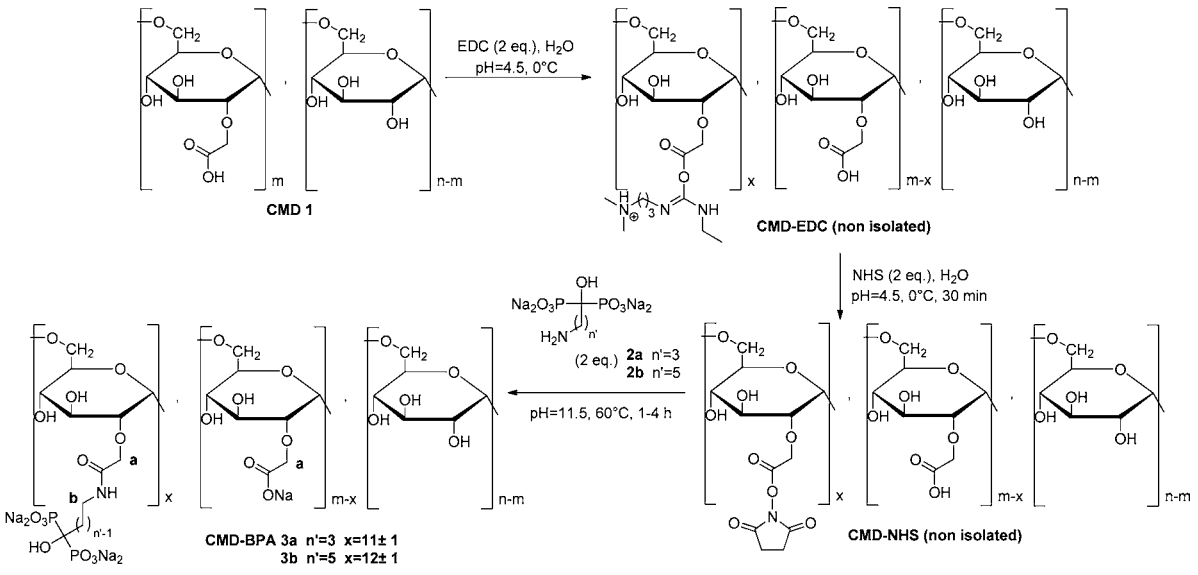
Our synthetic method was based on the selectivity of the coupling reaction between the carboxylic acid group of CMD and the amino group of BP, without protection of hydroxyl groups of dextran and aminoBP. Indeed, the more nucleophilic amino function compared to the alcohol one should be more reactive toward the carboxylic acid.

First, it was necessary to activate the carboxylic acid function of CMD **1**. Carbodiimides are the more commonly used reagents to modify the carboxylic acid function in proteins in mild conditions. Moreover, this polymer and aminoBPs were hydrophilic compounds. So, among the water-soluble activators used in peptidic synthesis at different pH, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) was chosen. However, the intermediate activated ester rapidly lost its efficiency by hydrolysis in acidic aqueous medium and activation had to be carried out at pH = 4.5. So, a transesterification reaction with *N*-hydroxysuccinimide (NHS) at pH = 4.5 and 0 °C for 30 min furnished a more stable active ester at acidic pH bearing a good nucleofuge.

The last step consisted of the reaction between CMD-NHS and BPA2 **2a** or BPA3 **2b** at pH = 11.5. The reaction mixture was heated at 60 °C for one hour for CMD-BP 2 and four hours for CMD-BPA3. The evolution of the reaction was followed by ^{31}P NMR and the pH was controlled and adjusted to 11.5 when necessary. The reaction was quenched to pH = 6.5 with an aqueous hydrochloric acid solution. After evaporation of the reaction mixture, the solid was precipitated in freshly distilled methanol, dialyzed, then precipitated again in a mixture ethanol/water (95:5 v/v) and lyophilized. Qualitative tests with silver nitrate and ninhydrin confirmed the absence of sodium chloride formed during the reaction and of remaining free BPA.

Different experimental conditions have been tested (data not shown) by varying reaction time (from 1 to 24 h) and temperature (RT, 40, and 60 °C). The preliminary best results in cellular proliferation tests were observed using two equivalents of EDC and NHS at pH = 4.5 then two equivalents of BPA at pH = 11.5 and carrying out the coupling at 60 °C for 1 h (for BPA2 **2a**) and 4 h (for BPA3 **2b**).

Scheme 3. Synthesis of CMD-BPA 3



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The synthesis of CMD-BPA **3** was realized several times and was reproducible. The new polymers were characterized in ^{31}P NMR with a chemical shift about 20 ppm. The ^{1}H NMR analysis confirmed the removal of byproducts of the coupling reaction and the effective coupling between CMD **1** and BPA **2** with a signal about 3.2 pm corresponding to the CH_2-NH-C(=O)- of CMD-BPA **3**, whereas the same methylene group in free BPA gave a signal about 3.0 ppm. The degree of substitution in BPA molecules of CMD-BPA **3** was calculated by comparison of peak integration of CH_2-NH-C(=O)- of linked BP and integration of a multiplet between 3.5 and 4.5 ppm corresponding to the CH_2 of CM group and six sugar protons. Thus, CMD-BPA **3a** contained 4.4% of alendronate corresponding to 11 ± 1 molecules of BP on the bioconjugate whereas CMD-BPA **3b** contained 5.0% of neridronate corresponding to 12 ± 1 molecules of BP coupled onto the 246 anhydroglucosyl units of the CMD **1**.

Molecular mass distribution analysis was obtained by high performance size exclusion chromatography with refractometry and multiangle light scattering detection. The number average molecular weight ($\overline{M}_n$), the weight average molecular weight ($\overline{M}_w$), and the polydispersity index ($\overline{M}_w/\overline{M}_n$) of dextran, CMD **1**, and CMD-BPA **3** were measured. Results in Table 1

Table 1. Number Average Molecular Weight, Weight Average Molecular Weight, and Polydispersity Index of Dextran, CMD 1, CMD-BPA 3a, and CMD-BPA 3b

	Dextran[a]	CMD **1**[a]	**3a**[a]	**3b**[a]
$\overline{M}_n$	35 000	43 000	49 000	44 000
$\overline{M}_w$	41 000	48 000	58 000	51 000
$\frac{\overline{M}_w}{\overline{M}_n}$	1.17	1.11	1.18	1.16

[a] ±3000 Da.

showed an increase of $\overline{M}_n$ and $\overline{M}_w$ after carboxymethylation and coupling with the alkyl aminoBPs **2**. The polydispersity index of all polymers was constant at about 1.2 meaning that no degradation of the polysaccharidic backbone occurred during the overall modification process.

***In Vitro* Biological Evaluation of Bioconjugates.** Several biological studies have reported the antitumor effects of BPs on different cancers such as osteosarcoma,[11] breast[9] and prostate[10] cancers, and myeloma.[14]

To increase their known effect, therefore their bioavailability, the antitumor potency of the new generation CMD-BP (alendronate, BPA2, and neridronate, BPA3) was investigated. A preliminary antiproliferative study of BPA2, BPA3, CMD-BPA2, and CMD-BPA3 was carried out on three cell lines: MDA-MB-231 (highly metastatic human breast tumor cells); A431 (epidermoid carcinoma); and B16F10 (melanoma).

In vivo, due to the rapid removal of BP from the blood circulation and its accumulation in bone, millimolar concentrations of drug have to be administered to be efficient. However, micromolar doses are sufficient to inhibit tumor cell proliferation. So, first *in vitro* assays were realized in a concentration scale below 25 μM. CMD-BPA2 **3a** was the only compound showing a total inhibition of cell proliferation on all the cell lines (Figure 1). That is why CMD-BPA2 was chosen to be biologically evaluated *in vivo*, to be compared with free BPA2. Moreover, its antimigrative and anti-invasive effects have also been studied.

In our vectorization strategy using a polysaccharidic carrier, we observed that cell exposure to CMD-BPA2 at a range of 5 μM to 10 μM was sufficient to induce 40−90% of inhibition in the three cell lines, whereas the free alendronate BPA2 was able to inhibit 20% maximum of cell proliferation (Figure 1). This first *in vitro* study after 72 h incubation showed IC_{50} values of CMD-BPA2 of 6.7 μM, 5 μM, and 3.6 μM, respectively, for MDA-MB-231, A431, and B16F10 cells. It was found that this compound caused an irreversible loss of cell viability, inhibiting proliferation in a time- and dose-dependent manner (Figure 1 and data not shown). Besides, CMD alone showed no effect on cell proliferation in the same experimental conditions (concentration, incubation time) on the three tested cell lines (less than 5% inhibition). These results suggest that the conjugation of BPA2 to CMD improves significantly the antiproliferative effect of the BP in the three tested cell lines. Indeed, BPA2 exhibited maximum inhibition of about 20% for MDA-MB-231 and A431 cell lines at 25 μM concentration and no effect on B16F10 cells.

Metastasis is a complication of most cancers and it is also the main failure of their treatment. Because several reports from animal and clinical studies demonstrated that BPs affect the invasive behavior of metastatic cells *in vivo*,[32] the anti-invasive effect of CMD-BPA2 was also investigated.

With the intention of obtaining the most realistic *in vivo* model, we decided to carry out the *in vivo* study on the most

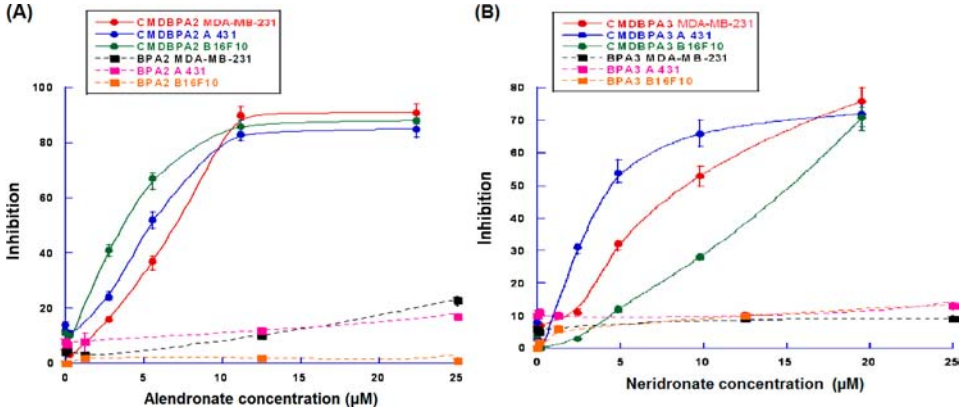

Figure 1. Antiproliferative study of (**A**) CMD-BPA2 and BPA2 and (**B**) CMD-BPA3 and BPA3, on MDA-MB-231, A431, and B16F10 cells.

metastatic MDA-MB-231 cells. So, cell migration and invasion are also reported on this same MDA-MB-231 cell line only.

Preliminary *in vitro* matrigel assays demonstrated that CMD-BPA2 exhibited significant anti-invasive activity on MDA-MB-231 cells. CMD-BPA2 inhibited both cell migration and cell invasion, two different processes that are engaged in tumor growth and metastasis. These results are in accordance with previous studies on the effect of BPs on migration and invasion on different cell types.[12]

Dark histograms represent migration and clear histograms invasion assays (Figure 2). About control cells, 100% of

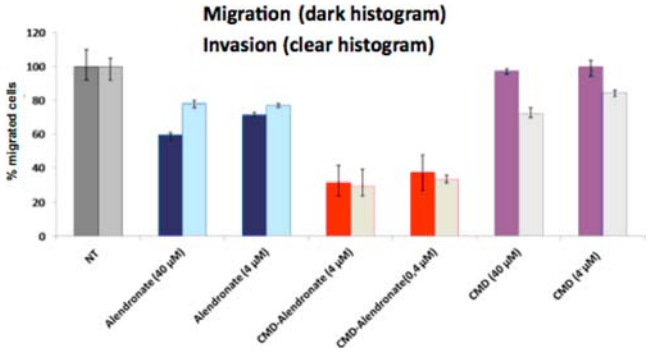

Figure 2. Antimigrative (dark histogram) and anti-invasive (clear histogram) studies of CMD-BPA2, BPA2, and CMD on MDA-MB-231 cells.

nontreated cells migrated. The amount of migrated cells (around 30%) exposed to the biopolymer **3a** (4 μM BPA2 concentration) was half that number observed with free alendronate at the same concentration. Ten times less concentrated CMD-BPA2 (0.4 μM) showed a similar efficiency in migration and invasion processes. As previously known, CMD alone has no effect on cell migration or invasion.

BPAs are weakly efficient drugs due to their strong hydrophilicity and negative charge at physiological pH. This explains why they do not easily penetrate the cell. However, when they were conjugated to a carrier such as CMD, a drastic increase of activity in cell proliferation, migration, and invasion was observed. This shows decreasing hydrophilic character of biopolymers compared to free BPAs (due to the low ratio BPA number/glucosidic unit number) and the biopolymer can mask the negative charges of bisphosphonate. CMD-BPA cell entry by endocytosis may then facilitate the penetration of BPAs in the intracellular medium. Thus, released BPAs could be more effective against tumor cells than free BPAs.

***In Vivo* Biological Evaluation of Bioconjugates.** In order to evaluate the efficiency of our polysaccharidic carrier, the effect of administration of CMD-BPA2 and free BPA2 was studied in nude mice with xenografted tumor models (MDA-MB-231 cells, Figure 3). In order not to sacrifice mice without reason, free CMD was not injected here because it was known to have no effect either *in vitro* than *in vivo*. Because of the heterogeneity in size of the tumors inside each group, a growth rate was calculated as follows: for each tumor, the ratio between the size of the tumor before the resection (end of the experiment) and the size measured at the beginning of the treatment was determined. Then, the mean of growth rates was calculated for each group. Both doses of CMD-BPA2 (0.6 mg/kg and 6 mg/kg) reduced significantly the tumor growth rate after four weeks treatment. Indeed, the growth rate was 4.31 for

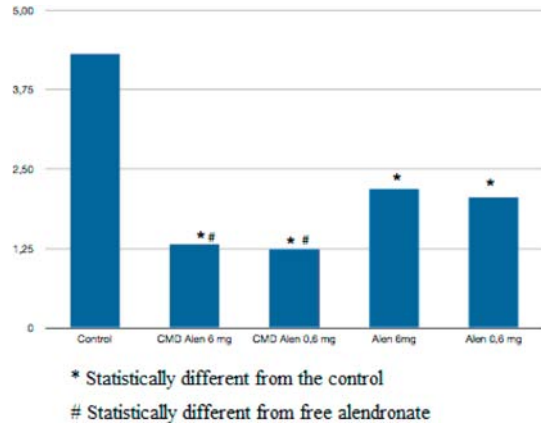

Figure 3. Growth rate of MDA-MB-231 cell tumors in nude mice after four weeks treatment.

the control and, respectively, 1.32 and 1.24 for CMD-BPA2 6 mg/kg and 0.6 mg/kg. This result was approximately twice lower with free alendronate treatment (2.18 and 2.05, respectively). In addition, no effect of dose of both BPA2 and CMD-BPA2 on the *in vivo* activity was observed. This can be explained by the maximal effect of these drugs at 0.6 mg.kg^{-1} concentration. Finally, we showed that CMD-BPA2 was a more potent inhibitor of tumor growth than free BPA2 alone, evidencing the potentiating effect of carboxymethyldextran.

Conclusion. In conclusion, we have synthesized new bioconjugates using a carboxymethyldextran as a carrier in order to vectorize BPs as alendronate. We showed that the amide link between CMD and alendronate does not alter the antitumor effects of the drug both *in vitro* and *in vivo* on MDA-MB-231 cells and can act as direct anticancer drug by increasing its bioavailability in breast cancer carcinomas.

However, further *in vivo* experiments are required to determine if this BP is clinically relevant in the treatment of other aggressive and highly metastatic carcinomas.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Determination of the D.S. of CMD, NMR spectra. 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.

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