Impact of Aldehyde Content on Amphotericin B−Dextran Imine Conjugate Toxicity

Marina Sokolsky-Papkov,[†] Abraham J. Domb,*[,†,§] and Jacob Golenser[‡]

Department of Medicinal Chemistry and Natural Products, School of Pharmacy, and Department of Parasitology, Hadassah Medical School, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

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The biocompatibility of oxidized dextran (40 kDa) was investigated in vitro. The contribution of aldehyde groups to the toxicity of polymer−drug conjugates, such as dextran−amphotericin B (AmB) was evaluated. Oxidized dextran was proved to be toxic against the RAW 264.7 cell line with an IC_{50} of 3 μmol/mL aldehydes. Modification of aldehyde groups and their reaction with ethanolamine reduced the toxicity at least 15-fold. Accordingly, the antifungal and antileishmanial dextran−AmB imine conjugate, which contains unreacted aldehyde groups, was modified with ethanolamine and compared to dextran−AmB amine and imine conjugates. Modification of the imine conjugate with ethanolamine reduced its toxicity toward the RAW cell line by 100%. The effect on *Leishmania major* parasites was 5 times higher than that of the dextran−AmB amine conjugate. The dextran−AmB−ethanolamine conjugate was at least 15 times less hemolytic than free AmB. Stability and drug release profiles in buffer solution were investigated. The imine conjugates released free AmB while the amine conjugate did not. It is concluded that aldehyde groups may contribute to cell toxicity. This toxicity is reduced by converting the aldehyde groups into imine conjugates with ethanolamine. The results have direct implications toward the safety of AmB−polysaccharide conjugates used against fungal and leishmanial infections.

Introduction

Bioactive agents that exhibit limited solubility and stability or possess high toxicity may be conjugated to hydrophilic polymers in order to overcome these limitations.[1] Several conjugates of cytotoxic drugs, to polyacrylamide derivatives or dextran, have been tested in humans or are currently undergoing clinical studies.[2] Amphotericin B (AmB), a polyene antibiotic, is currently recommended as a first-line treatment for systemic fungal infections and a second-line treatment for visceral and mucocutaneous leishmaniases. AmB is a highly toxic water-insoluble compound and therefore it has been delivered as a micellar dispersion of sodium deoxycholate−AmB (Fungizone). However, this dispersion system exhibits therapeutic limitations such as low tolerated dose and toxicity to the kidneys, liver, and central nervous system.[3,4] Other clinically used lipid formulations for AmB include liposomes (Ambisome),[5,6] colloidal dispersion (Amphotec), and lipid complex (Abelcet). Although these formulations reduce the side effects that are exerted by Fungizone, they do not eliminate them completely; in addition, they are too expensive for many customers in need.

Development of water-soluble polymer−drug conjugates may enable drug targeting and reduction of drug toxicity, due to different organ distribution and lower accumulation in the liver and kidneys.[7] In addition, use of a water-soluble polymer as the drug carrier may enhance drug solubility and stability.

In our previous studies, different polysaccharide−AmB conjugates were synthesized and evaluated. Dextran and arabinogalactane, both water-soluble, natural, biocompatible polysaccharides, were used as the polymeric carriers.[8,9] The conjugation process involved oxidation of the polysaccharide to a polyaldehyde, which in turn reacted at large excess with the drug (imine conjugate). The resulting imine conjugate was effective against *Leishmania major* parasites in vitro and in vivo.[10] Still, the imine conjugate showed a slight toxicity compared to the amine conjugate, which does not possess aldehyde groups.

Various publications have shown the toxic effect of low molecular weight aldehydes, such as formaldehyde[11] and glutaraldehyde,[12] as well as of high molecular weight aldehydes, such as lipid peroxidation products.[13] Because of their reactivity, aldehydes are able to interact with electron-rich biological macromolecules and induce adverse health effects, including general toxicity, allergic reactions,[14] mutagenicity, and carcinogenicity.[15] Likewise, dextran polyaldehyde, with a molecular mass of 70 kDa, was considerably cytotoxic toward the keratinocytes and fibroblasts in vitro.[16]

In this study we investigated the hypothesis that the reactive aldehyde groups that remain on the polymer chain are responsible for the extended toxicity of the imine conjugate. Elimination of the aldehyde groups without alteration of the bond between the drug and the polymer may result in both an effective and less toxic conjugate. To evaluate this hypothesis, dextran was oxidized and its toxicity was evaluated in vitro against the RAW 264.7 cell line. Since the oxidized dextran showed considerable toxicity, different approaches to converting the remaining aldehyde groups were evaluated. On the basis of the results, a new imine conjugate with modified aldehyde groups was synthesized. Different aspects of the new conjugate, including in vitro toxicity, efficacy against *L. major* parasites, and hemolysis, were compared to those of previously reported

* Corresponding author: phone 972-2-6757573; fax 972-2-6758959; e-mail adomb@md.huji.ac.il.

† Department of Medicinal Chemistry and Natural Products, School of Pharmacy.

‡ Department of Parasitology, Hadassah Medical School.

§ Leonel Jacobson chair in medicinal chemistry, affiliated with the David R. Bloom Center for Pharmacy and with the Alex Grass Center for Drug Design and Synthesis at the Hebrew University of Jerusalem.

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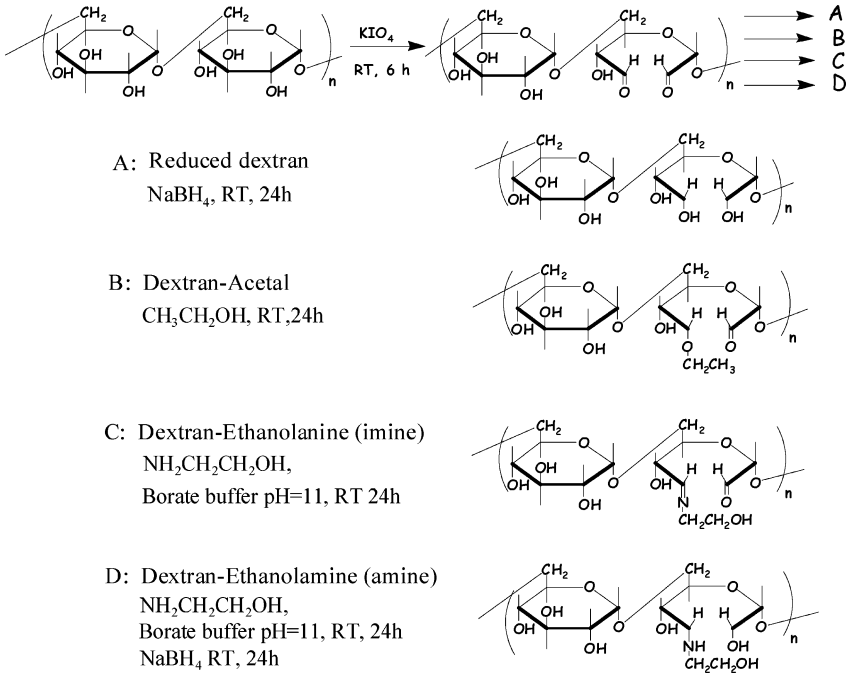
Scheme 1. Oxidation of Dextran and Synthesis of Modified Dextran



conjugates. In addition, the stability and drug release profile of the conjugates, in buffer, were investigated by UV spectroscopy and HPLC.

Here we report on the contribution of aldehyde groups to imine conjugate toxicity and on the synthesis and characterization of a new imine conjugate with modified aldehyde groups. A less toxic amphotericin B conjugate based on this research could improve antifungal and antileishmanial treatment. Both fungal and leishmanial infection are increasingly endangering the global human population.[10,17]

Experimental Section

Materials. AmB powder (clinical grade USP, potency 1050 units/mg) was a gift from Dumex, Copenhagen, Denmark. Dextran with an average molecular mass of 40 kDa was purchased from Sigma Chemical Co. (St. Louis, MO). Potassium periodate (KIO_4), sodium borohydride ($NaBH_4$), ethanolamine, and RPMI 1640 broth medium were purchased from Sigma Chemicals Inc. Ethanol was purchased from Bio-Lab (Jerusalem, Israel). ^{3}H-Thymidine 5Ci/mM, was purchased from Amersham (England). All solvents and reagents were of analytical grade.

Instrumentation. Lyophilization was performed with a ChristAlpha1-4 freeze-drier. Infrared (IR) spectroscopy was performed on KBr pellets in a Perkin-Elmer spectrophotometer FT-IR, model 2000. UV measurements were performed on a Uvikon 930 spectrophotometer.

Molecular weight was determined on a high-pressure gel-permeation chromatography (GPC) system, consisting of a Spectra Physics P1000 pump (Darmstadt, Germany) with UV detection (Applied Bioscience 759A absorbency UV detector) at 405 nm or a refractive index (RI) detector, a Rheodyne (Coatati, CA) injection valve with a 20 mL loop, a Spectra Physics Data Jet integrator, and a WINner/286 computer analyzer. Conjugate purity and free AmB concentrations in buffer solutions were determined by an HPLC (Hewlett-Packard, Waldbronn, Germany) system composed of an HP 1100 pump, HP 1050 UV detector, and HP ChemStation data analysis program.

Synthesis of Dextran Polyaldehyde. Dextran polyaldehyde with a degree of oxidation between 1.5% and 50% (1.5%, 5%, 8%, 15%, 25%, and 50%) was prepared in aqueous solution by the addition of controlled amounts of potassium periodate (0.0836, 0.2875, 0.46, 0.8625, 1.4375, and 2.875 g, respectively) to 1 g of dextran and stirred in a light-protected container at room temperature for 6 h. The resulting polyaldehydes were purified from iodate and unreacted periodate ions by Dowex-1 anion-exchange chromatography (acetate form, pH = 7). Dowex acetate was obtained by pretreatment of the commercial anion exchanger with aqueous 1 M acetic acid. The purified oxidized dextran solution was dialyzed through 3500 molecular weight cutoff dialysis tubing (Membrane Filtration Products Inc., San Antonio, TX) against double distilled water (DDW) (5 L changed 4 times) for 48 h at 4 °C and then lyophilized for 24 h to dryness. Determination of the degree of oxidation was performed as follows: oxidized dextran (0.1 g, 0.625 mmol) was dissolved in 25 mL of 0.25 M hydroxylamine hydrochloride solution, pH 4.0. The solution was stirred for 3 h at room temperature and then titrated with 0.1 M NaOH standard solution. The titration end point was calculated from the graph describing the change in pH per volume (dpH/dV) versus the titration volume (V). Molecular weight was determined by GPC. Samples at a concentration of 10 mg/mL were eluted with 0.05 M sodium nitrate in DDW through a Shodex (KB-803) column at a flow rate of 1 mL/min. The molecular masses of the eluted samples were estimated by use of pullulan standards in the range of 5000−110 000 Da (PSS, Mainz, Germany).

Synthesis of Modified Dextran. *Reduced Dextran*. Oxidized dextran (1 g, 50% oxidation) was dissolved in 100 mL of DDW. $NaBH_4$ (1 g) was added and the reaction mixture was stirred for 24 h. The solution was purified by dialysis and lyophilized (as described under Synthesis of Dextran Polyaldehyde).

Dextran Acetal. Oxidized dextran (1 g, 50% oxidation) was dissolved in 100 mL of ethanol and stirred for 24 h. Dextran acetal was precipitated in DDW and lyophilized (as described under Synthesis of Dextran Polyaldehyde).

Dextran-Ethanolamine Imine/amine. Dextran (2 g, 50% oxidation) was dissolved in 200 mL of borate buffer, pH = 11, and 0.41 mL (1.1 mol equiv) of ethanolamine was added. The reaction mixture was stirred for 24 h, after which a sample of 100 mL was removed, purified by dialysis, and lyophilized to dryness (as described under Synthesis of Dextran Polyaldehyde) to obtain the imine form. To obtain the amine form, 1 g of $NaBH_4$ was added to the remaining 100 mL of reaction solution. The reaction mixture was stirred for 24 h, purified by dialysis, and lyophilized (as described under Synthesis of Dextran Polyaldehyde) (Scheme 1).

Measurement of AmB Content in Conjugates. AmB content in the conjugates was determined by UV absorbance at 410 nm, by use

of dextran−AmB conjugates with known amount of drug as a standard. Purity of the conjugates was determined by HPLC on a C18 reverse-phase column (LichroCart 250-4, Lichrospher 100, 5 μm). A mixture of 70% acetonitrile/27% water/3% acetic acid at a flow rate of 1.8 mL/min was used as eluent. UV detection was at 410 nm. For both tests the conjugate samples were prepared at a concentration of 0.3 mg/mL in DDW.

Dextran Polyaldehyde in Vitro Toxicity. Serial dilutions of dextrans with different degrees of oxidation (1.5%−50% oxidation) were prepared in RPMI 1640 growth medium. The final aldehyde concentrations in the test were 0.01−34 μmol/mL. Oxidized dextran toxicity was compared to glutaric polyaldehyde toxicity, which was added in concentrations between 0.15 and 4.12 μmol/mL aldehyde groups.

The cytotoxicity of dextran derivatives was evaluated in murine RAW 264.7 cells, an internationally recognized cell line for examination of drug effects.[18]

Growth inhibition was estimated by the ^{3}H-thymidine incorporation method.[10] Cells were cultured in flat-bottom flasks at 37 °C. Before each experiment the cells were washed and removed by trypsin treatment or scraped from the flask bottom, and an appropriate volume was centrifuged, resuspended, and diluted in growth medium to the desired cell concentration. The growth medium consisted of RPMI 1640 and 10% fetal calf serum (FCS). By use of an automated dispenser, 200 μL of cell suspension was added to each well of a microtiter plate. After incubation overnight, the appropriate drug concentration, in triplicate, was added to test wells. Drug-free medium was used as control. ^{3}H-Thymidine (0.5 μCi) in 20 μL of medium was added the next day, and the plate was harvested and read by liquid scintillation counter (LKB, Finland) after an additional 24 h. The percent growth inhibition of the cells by the drug tested was calculated as [100 − (count with drug/control count) × 100]. The IC_{50} of the drugs, defined as the concentration that inhibits 50% of the incorporation, was determined graphically from inhibition of incorporation curves.

In Vitro Toxicity of Modified Dextran. Serial dilutions of oxidized dextran and modified dextran were prepared in RPMI 1640 broth medium. The final dextran concentration in the test ranged from 44 to 5555 μg/mL.

To establish that the aldehyde groups are primarily responsible for cytotoxicity, native dextran and dextran with completely eliminated aldehydes (by reduction to hydroxyl) were evaluated. Dextran with 50% oxidation was used as a positive toxicity control. Drug effect and the IC_{50} were defined as previously described (under Dextran Polyaldehyde in Vitro Toxicity).

Dextran−AmB Conjugates in Vitro Toxicity Test. The cytotoxicity test for the conjugates was performed in the same cell system as previously described (under Dextran Polyaldehyde in Vitro Toxicity). Conjugates were prepared in the concentration range in which the carrier (dextran polyaldehyde) had exhibited cytotoxicity. The AmB concentration was similar in all conjugates in order to eliminate the drug influence on conjugate toxicity. Drug effect and the IC_{50} were defined as previously described.

In Vitro Antileishmanial Activity. The in vitro antileishmanial activity was evaluated against *Leishmania donovani* IS promastigotes This strain, isolated from a patient in Sudan, was received from the International Reference Center of the Kuvin Center for Infectious Diseases in the Hebrew University of Jerusalem. Serial dilutions of the tested agents were prepared in RPMI 1640 growth medium. The final AmB concentration in the test ranged from 0.2 to 6 μg/mL. Wells containing drug-free medium served as control. The growth inhibition was estimated by the ^{3}H-thymidine incorporation method. Briefly, 96-well plates were seeded with 60 000 promastigotes/well in 200 μL of medium, and test solutions were added 3 h later. After 24 h of incubation, 0.5 μCi/well ^{3}H-thymidine (in 10% FCS medium) was added, and the cultures were harvested after an additional 24 h. During the experiment the cells were incubated at 25 °C in air. The drug effect and the IC_{50} of the conjugates were estimated as described before (under Dextran Polyaldehyde in Vitro Toxicity).

Table 1. Characterization of Dextrans after Oxidation with Different Molar Ratios of KIO_4

KIO_4/saccharide units (mole ratio)	aldehyde content, %[a]	M_w (GPC)[b]	polydispersity (M_w/M_n)
1:1	52	32 019	2.39
1:2	25	30 520	1.59
1:3	15	31 787	1.56
1:5	8	32 356	1.57
1:10	5	30 491	1.58
1:33	1.5	31 342	1.56

[a] Percent oxidation was determined by the hydroxylamine hydrochloride method.[21] Percent oxidation is the percentage of the saccharide units oxidized to yield two aldehydes per unit. [b] Molecular weight was determined by gel-permeation chromatography.

Conjugate Stability and Drug Release Pattern. Solutions of amine, imine, and ethanolamine AmB−dextran conjugates with about 30% (w/w) AmB content, at a concentration of 0.02 mg of AmB/mL (40% of maximum AmB solubility under given conditions) in phosphate-buffered saline (PBS) + 0.3% SDS, pH 7.4, were incubated at 37 °C in light-protected containers for 7 days. The SDS was added to improve the solubility of AmB in aqueous medium. Samples (2 mL) were taken at 0, 24, 48, and 72 h and 7 days, and the chemical stability of the conjugates was determined by UV spectrum and absorption in the range of 200−600 nm. After UV measurement these same samples were evaluated by HPLC, under the same conditions described under Measurement of AmB Content in Conjugates.

Hemolysis. Sheep blood **(**1.5 mL) was diluted in 28.5 mL of PBS to a concentration of 5% (v/v). Erythrocytes (sheep red blood cells, SRBCs) were precipitated by centrifugation (3000*g* for 10 min), washed twice with fresh PBS buffer, and resuspended in 30 mL of PBS. The hemolysis was detected in glass tubes containing 0.1 mL of the serially diluted drug (2−512 μg of AmB/mL) and 0.9 mL of SRBCs solution for 1 h at 37 °C. Hemolysis values were determined visually as the lowest concentration of the drug resulting in completely clear SRBCs solution.[9]

Results and Discussion

Oxidation of Dextrans to Form Dextran Polyaldehydes. Dextran of $M_w = 40\,000$ was oxidized with different amounts of periodate to form a range of oxidized dextrans with different aldehyde content (Scheme 1). There was a linear correlation between the amount of potassium periodate used for oxidation and the aldehyde content of the oxidized dextran. The degree of oxidation of dextran, after reaction with different molar ratios of periodate (1:1, 1:2, 1:3, 1:5, 1:10, and 1:33 saccharide units: periodate), and the molecular weights of the oxidized dextrans are summarized in Table 1. Excess iodate and periodate ions were removed from the oxidized polysaccharide by ion-exchange chromatography and dialysis. This step was essential because the presence of periodate ions may contribute to cytotoxicity and may oxidaize and degrade AmB. As expected, there was a linear correlation between the amount of potassium periodate used for oxidation and the aldehyde content of oxidized dextran. All oxidized dextrans had a similar average MW of about 32 000 and polydispersity of about 1.6. There was a slight increase in polydespersity value for the highly oxidized dextran ($P = 2.39$), which is related to the large excess of periodate used for oxidation.

Dextran Polyaldehyde in Vitro Cytotoxicity Test. Because of the extensive use of dextran polyaldehyde both as a drug carrier and as a base for tissue scaffold synthesis, the question of biocompatibility and possible cytotoxicity is essential.[16,19−22]

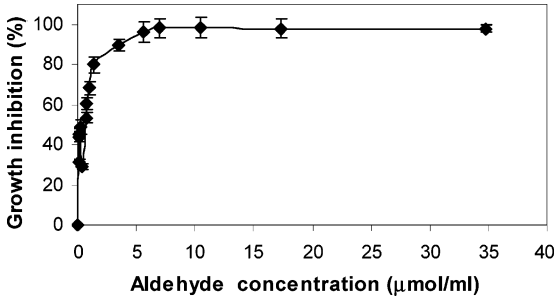


Figure 1. Cytotoxicity of dextran polyaldehyde. The cytotoxicity test was performed by the ^{3}H-thymidine incorporation method in murine RAW 264.7 cells, by application of dextran (40 kDa) with different degrees of oxidation. Each test was performed twice in triplicate. Mean and standard deviation are shown. The aldehyde concentration was calculated as [2(dose weight, g) × (% degree of oxidation)/(saccharide unit weight, 160 g/mol) mL].

Many publications report the toxic effect of low molecular weight aldehydes such as formaldehyde and glutaraldehyde products.[12,15] There is also evidence that high molecular weight dextran polyaldehyde is cytotoxic toward at least two types of cells (human fibroblasts and epithelial cells). It was shown that exposure of keratinocytes to 70 kDa dextran polyaldehyde with a degree of oxidation of 20% at a concentration of 125 μg/mL (0.125% w/w) resulted in complete growth inhibition. Exposure of fibroblasts to similar dextran at a concentration of 250 μg/mL (0.25% w/w) resulted in 85% growth inhibition.[16] In our study, in order to evaluate the cytotoxicity of aldehyde groups, the effect of four different concentrations of dextran polyaldehydes against murine RAW 264.7 cells was examined.

The cytotoxicity experiment was performed by incubating the cells with the same amounts of the oxidized dextrans. A correlation between the aldehyde content in the oxidized dextrans and cell growth inhibition was found (Figure 1). The presence of aldehyde groups caused cytotoxicity, with an IC_{50} of 3 μmol/mL. Exposure of the cells to aldehyde concentration higher than 7 μmol/mL caused complete inhibition. These data are in agreement with previous reports as to the cytotoxic effect of polyaldehydes. It may also be suggested that the molecular weight of the polymer affects toxicity, because dextran with a higher molecular weight showed higher toxicity values[16] compared to the dextran investigated in our study.

Cytotoxicity Evaluation of Modified Dextran Polyaldehyde. The purpose of this experiment was to confirm that the cell growth inhibition described previously was caused only by the aldehyde groups. Therefore, toxic aldehydes were changed to nontoxic groups such as a hydroxyl (end group of ethanolamine) or an aliphatic chain (end group after reaction with ethanol). All modifications were made on dextran polyaldehyde with the highest degree of oxidation (50%) (Scheme 1). The toxicity of the derivative was evaluated in the same cell system (Figure 2). All derivatives depicted dose−response correlations. Oxidized dextran caused almost complete growth inhibition at the lowest tested concentration (130 μg/mL). Modification with ethanol to form hemiacetals reduced the toxicity of the polymer, but still complete growth inhibition was seen at concentrations higher than 1800 μg/mL. Modification with ethanolamine (imine form) reduced the toxicity by 16-fold, and an additional reduction step to form dextran−ethanolamine (amine) further reduced toxicity. The complete elimination of aldehydes (native dextran and reduced dextran) entirely prevented the toxicity in the tested dose range. For easier comparison of the results, IC_{50} values were graphically estimated (Figure 2) and summarized in Table 2.

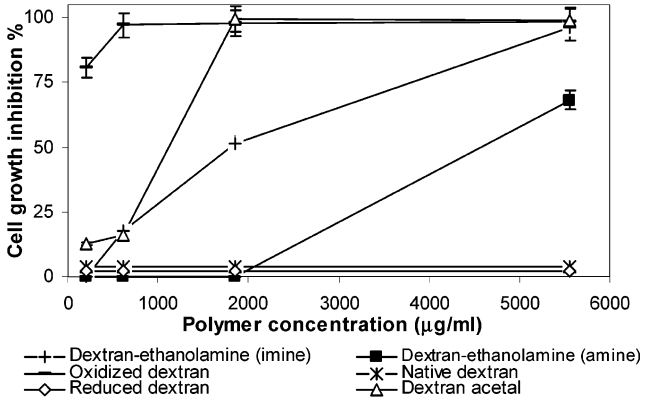


Figure 2. Cytotoxicity of modified dextran polyaldehyde. The cytotoxicity test was performed by the ^{3}H-thymidine incorporation method in murine RAW 264.7 cells, by application of dextran (40 kDa). Each experiment was performed twice in triplicate.

Table 2. In Vitro Cytotoxicity of Modified Dextran as Compared to Oxidized Dextran (50%) and Glutaraldehyde

compound	IC_{50}[a] (μg/mL)
native dextran	>5000
reduced dextran	>5000
dextran−ethanolamine (imine)	2000
dextran−ethanolamine (amine)	4500
dextran acetal	1000
oxidized dextran	130
glutaraldehyde	<0.15

[a] IC_{50} values were determined from in vitro cytotoxicity experiments. The cytotoxicity test for different modifications of dextran (40 kDa) was performed by the measurement of ^{3}H-thymidine incorporation in RAW 264.7 cells. The cytotoxicity was compared to the effect of native dextran and oxidized dextran.

Two reactions (with ethanol and ethanolamine) for aldehyde reduction were proposed. Although the reaction with ethanol was conducted in high excess, the yield of acetal formation was not enough to convert all aldehydes and the IC_{50} was decreased by 10-fold. Because higher amounts of ethanol cannot be used, further improvement of reaction conditions is not possible. This modification is therefore not suitable for further development and new polymer−drug conjugates synthesis. Reaction with ethanolamine at a 1:1.1 aldehyde:ethanolamine molar ratio resulted in a considerable reduction in toxicity, from IC_{50} = 130 to 2000 μg/mL. Moreover, reduction of the imide to an amine bond further improved the toxicity to IC_{50} = 4500 μg/mL (35-fold). Because the excess of ethanolamine was relatively low, additional reduction in toxicity is possible if higher amounts of ethanolamine will be applied. An additional advantage of reaction with ethanolamine is that the reaction conditions are similar to those for coupling of AmB and the polymer and therefore can be applied after drug conjugation without any additional steps.

Another aspect that influences toxicity is the molecular weight of the aldehyde. Glutaraldehyde, a small molecule, is very toxic compared to polyaldehyde, which has a molecular mass of 30 kDa. The permeability of a molecule through the cell membrane increases as the molecular weight decreases. AmB interacts with the cell membranes of fungi and leishmanial parasites. In addition, a polymer−drug conjugate has an increased chance over a small molecule to reach and be released in inflammatory areas.[23]

Conjugate Synthesis. In the first step, oxidized dextran (50% oxidation) was prepared, followed by a second step of conjuga-

Scheme 2. Synthesis of AmB−Dextran Conjugates

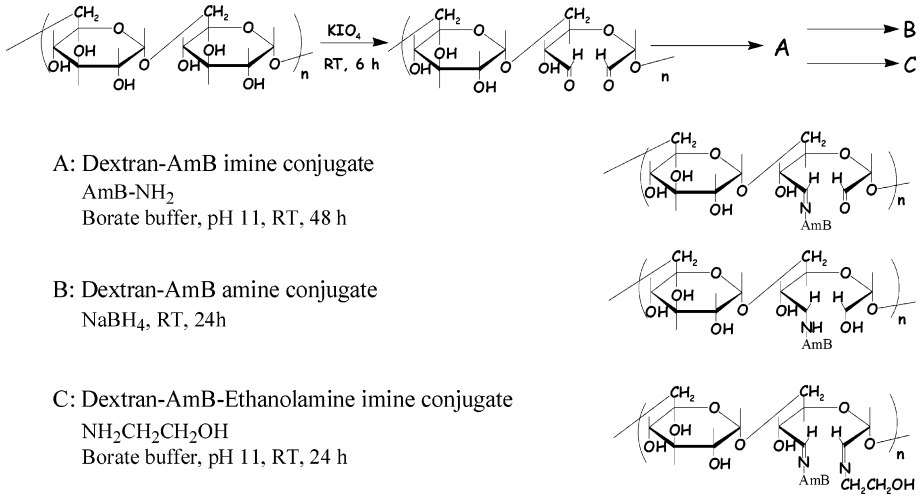


Table 3. In Vitro Activity against *Leishmania donovani*, Cytotoxicity, and Hemolysis of Conjugates

compound	AmB content (% w/w)	antiparasitic activity[a] IC_{50} (μg of AmB/mL)	toxicity[b] IC_{50} (μg of AmB/mL)	hemolysis[c] (μg of AmB/mL)
free AmB	100	0.05	9	16
dextran−AmB (amine)	34.4	1.2	1400	>500
dextran−AmB (imine)	36.6	0.3	200	250
dextran−AmB−ethanolamine (imine)	32.9	0.25	400	>500

[a] IC_{50} values were derived from the activity test of AmB and different dextran−AmB conjugates against *Leishmania donovani*. Parasite growth inhibition was estimated by the ^{3}H-thymidine incorporation method. [b] IC_{50} values were derived from the cytotoxicity test of AmB and different dextran−AmB conjugates against the murine RAW 264.7 cell line. Cell growth inhibition was estimated by the ^{3}H-thymidine incorporation method. Due to chemical limitations, free AmB was not evaluated in this experiment. [c] Hemolysis was evaluated visually after 1 h of incubation at 37 °C with sheep erythrocytes.

tion of the oxidized dextran to AmB (see Scheme 2). In a typical experiment, 1 g of oxidized dextran with a degree of oxidation of 50% of the saccharide units was dissolved in 100 mL of borate buffer, pH = 11. AmB powder (0.25 g) was added, and the mixture was stirred at room temperature in a light-protected container for 48 h. The pH of the reaction mixture was maintained at 11 during the reaction. A clear yellow-orange solution of the imine conjugate was obtained, purified by dialysis, and lyophilized for 24 h (as described under Synthesis of Dextran Polyaldehyde).

Amine conjugate was obtained by addition of $NaBH_4$ to the imine conjugate reaction mixture and continuation of the reaction overnight. During the reduction process, a change of color from yellow-orange to light yellow was observed. The amine conjugate was purified by dialysis and lyophilized (as described under Synthesis of Dextran Polyaldehyde).

Dextran−AmB−ethanolamine (imine) conjugate was prepared by adding (1.1 mol equiv of aldehyde content) of ethanolamine to the imine conjugate mixture and continuing the reaction overnight. The pH of the reaction was maintained at 11. The dextran−AmB−ethanolamine conjugate was purified by dialysis and lyophilized to dryness (as described under Synthesis of Dextran Polyaldehyde) (Scheme 2).

Conjugate Toxicity. After synthesis, the purity of the conjugates was checked by HPLC as described under Measurement of AmB Content in Conjugates. No free drug was detected in any conjugate. The toxicity was evaluated in comparison with the previously described dextran−AmB imine conjugate.[9] In this experiment, AmB−dextran imine conjugates with or without ethanolamine were compared to the AmB−dextran amine conjugate, all containing equivalent AmB amounts, to evaluate the contribution of the remaining aldehyde groups to conjugate toxicity (Figure 3).

It is possible to reduce the toxicity of the dextran−AmB imine conjugate by blocking the aldehyde groups with ethanolamine via an imine bond. The IC_{50} values are summarized in Table 3. Free AmB is extremely toxic to both parasites and cells. The AmB−dextran imine conjugate is preferable as an AmB releasing carrier because the release occurs after hydrolysis of the imine bond between AmB and the dextran carrier. Treatment with ethanolamine makes it possible to maintain this advantage while reducing the toxicity of the imine conjugate. The amine conjugate shows lower toxicity but also lower antiparasitic activity (Table 3).

In Vitro Activity against *Leishmania donovani*. Although the main aim of this study was to examine the contribution of

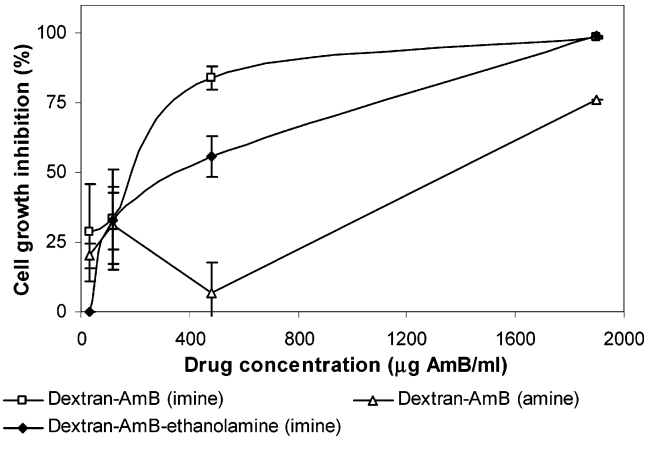


Figure 3. In vitro cytotoxicity of dextran−AmB (imine) and dextran−AmB−ethanolamine conjugates. The cytotoxicity test was performed by the ^{3}H-thymidine incorporation method in RAW 264.7 cells. Conjugates were applied with the same amount of drug. Each experiment was performed twice in triplicate.

Table 4. Decrease in UV Absorption of AmB−Dextran Conjugates and Free AmB at Different Wavelengths after 7 Days[a]

	wavelength					
agent	333 nm	350 nm	363 nm	386 nm	408 nm	411 nm
free AmB no SDS	50.9	NA[b]	NA	97.4	100	NA
free AmB with SDS	NA	87.89	88.9	90.4	NA	91.5
AmB−dextran amine	NA	82	87.7	91.6	NA	94.3
AmB−dextran imine	NA	77.7	75.8	77.5	NA	76.9
AmB−dextran−ethanolamine imine	NA	87.8	87.2	89.9	NA	91.7

[a] Solutions of amine, imine, and ethanolamine AmB−dextran conjugates with about 30% (w/w) AmB content, at a concentration of 0.02 mg of AmB/mL (40% of maximum AmB solubility under given conditions) in phosphate-buffered saline (PBS) + 0.3% SDS, pH 7.4, were incubated at 37 °C for 7 days. The chemical stability of the conjugates was determined by UV absorption in the range of 200−600 nm. The decrease is shown as percent of the initial absorption of AmB conjugates after 7 days of incubation. All AmB conjugates were incubated with SDS. Each data point is an average of two different batches tested. [b] NA, no absorption peak at this wavelength.

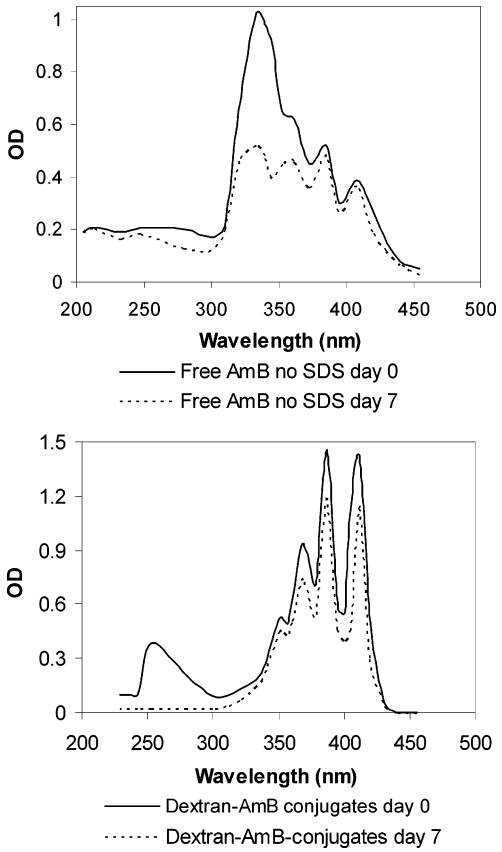


Figure 4. UV spectrum of free AmB and dextran−AmB conjugates incubated in solution at 37 °C at the beginning and after 7 days of incubation.

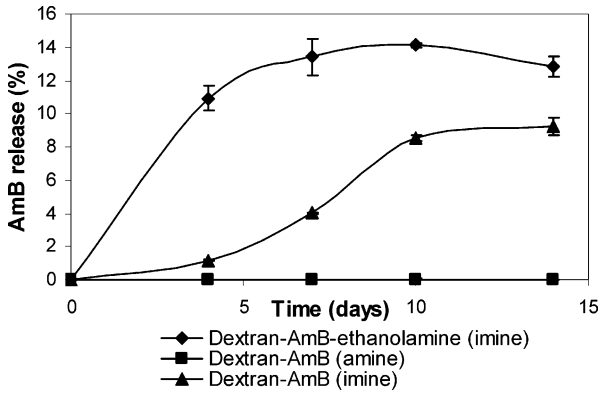


Figure 5. AmB release from dextran−AmB conjugates in solution at 37 °C. AmB release was evaluated by HPLC. Each data point is an average of two different batches.

free aldehyde groups to the cytotoxicity of the imine dextran−AmB conjugate and evaluate the possibility of reducing the toxicity, the purpose of this experiment was to confirm that modification of the imine conjugate does not influence its antiparasitic activity. Both imine conjugates showed higher activity against *Leishmania* parasites relative to the amine conjugates, with an IC_{50} of about 0.3 μg/mL compared to 1.2 μg/mL (Table 3). This may be explained by the fact that the imine bond is more sensitive to hydrolytic degradation.

Stability Pattern. The imine bonds are less stable than amine bonds; therefore the drug is released at a higher rate from the imine conjugate than from the amine conjugate. This may contribute to the toxicity of the imine conjugates. The drug release experiment was intended to establish the drug release profile, which is obtained after the conjugates are incubated in aqueous media for a time period equal to the one used in the toxicity tests. Because in the presence of the cells or parasites the hydrolysis of the bond between the drug and the carrier is quicker then in a noncell system, the drug release was monitored for 2 weeks. The drug release profile was monitored by HPLC as described under Measurement of AmB Content in Conjugates. The drug release profiles are shown in Figure 5. The drug release profile of the dextran−AmB−ethanolamine imine conjugate showed initial quick release of the drug: approximately 11% of the total drug amount was released within the first 4 days of incubation. This was followed by a slow secondary release of an additional 3% to a total of 14% of the drug over the course of 14 days. The dextran−AmB imine conjugate exhibited a slower release profile during the entire incubation period. The total amount of the released drug from the dextran−AmB imine conjugate was 9%. No AmB was released from the dextran−AmB amine conjugate. During the same period, degradation of the conjugates was estimated by evaluation of UV absorbance. Because most of the drug was released during the first week of incubation, the UV absorption and spectrum data were collected for 1 week. Amine, imine, and ethanolamine dextran−AmB conjugates lost only about 15% of their average absorbance during 1 week of incubation. The decrease in UV absorption, at different wavelengths, is shown in Table 4. The decrease in the absorption of the imine conjugates is explained by the detachments of the drug from the polymer carrier as was shown by HPLC. It was also shown that no drug is released from the amine conjugate. Therefore, our hypothesis was that the decrease in UV absorption is obtained because the AmB degrades while still bound to the polymer. To support this hypothesis, the UV spectrum of the free drug incubated without SDS was compared to the spectrum of the free AmB incubated with SDS and to the spectra of all three conjugates in the experiment. The spectrum of free drug incubated with sodium dodecyl sulfate (SDS) and all conjugates (both dextran−AmB imine, dextran−AmB amine, and dextran−AmB−ethanolamine) showed absorption peaks at 350, 363, 386, and 411 nm. The spectrum of

free drug incubated without SDS showed peaks at 333, 386, and 408 nm and an additional peak at 363 nm that appeared during the incubation. For the free AmB incubated without SDS, no changes in absorbance at 386 and 408 nm were noticed, but the absorbance at 333 nm decreased and a new peak at 363 nm appeared (Figure 4). Similar changes of the UV spectrum for free AmB incubated in aqueous media were previously described in the literature.[24,25] The observation that the absorbance of the amine conjugate at 350 and 368 nm decreases faster than at 386 and 411 nm, which is similar to the degradation pattern of the free drug (see Table 4), suggests that in the case of the amine conjugate the decrease in UV absorption is because the drug degrades to subunits similar to free AmB. This experiment shows that although the average stability of the conjugates is similar, the degradation pattern of the amine and imine conjugates is different. The free amphotericin is detached from the polymer carrier by imine hydrolysis in both imine conjugates but not in the amine conjugate. Modification of the imine conjugate with ethanolamine does not affect drug detachment. This drug release might influence the toxicity of the conjugates. However, only a small percentage of the drug is released during the first 48 h, which encompasses the entire time period of the toxicity test. The drug release is quicker from the dextran−AmB−ethanolamine conjugate, which still exhibits lower toxicity than the dextran−AmB imine conjugate, even after consideration of the free AmB contribution.

Hemolysis. Hemolysis is a severe side effect common to primary amine-containing drugs, including amphotericin B. In previous studies it was shown that conjugation of AmB to polymer significantly reduces hemolysis.[9] Free AmB was hemolytic toward sheep erythrocytes at a concentration of 16 μg/mL, while all AmB−dextran conjugates were at least 15 times less hemolytic, as shown in Table 3.

Conclusions

The purpose of this study was to determine whether high molecular weight polyaldehyde possesses any cytotoxicity and, if so, whether it contributes to the toxicity of polymer−drug conjugates, such as dextran−AmB. Oxidation of dextran to a certain degree of aldehyde content proved that there is a correlation between aldehyde content and toxicity. Dextran−AmB−ethanolamine (imine) conjugate free of aldehyde groups showed lower toxicity compared to unmodified imine conjugate. The reaction with ethanolamine did not affect the antileishmanial activity of the dextran−AmB imine conjugate. The stability and toxicity are comparable to that of previously reported dextran−AmB imine conjugates. The results may be applicable for future design of AmB-containing antifungal and antileishmanial drugs.

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