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Anti-fungal and anti-leishmanial activities of pectin-amphotericin B conjugates

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ABSTRACT

Citrus pectin was oxidized using periodate and amphotericin B (AmB), a water-insoluble polyene antibiotic was conjugated via imine linkage to the oxidized pectin in borate buffer (pH 11) at ~20 wt% concentration. The imine conjugate was reduced using sodium borohydride to give the more stable amine conjugates. The AmB conjugates were evaluated for their anti-fungal activity against *C. albicans and A. fumigatus*. The minimum inhibitory concentration (MIC) against *C. albicans* was 0.19 μg/mL for both pectin-AmB imine and amine conjugates whereas the MIC against *A. fumigatus* was 6.25 μg/mL for imine conjugates and 25 μg/mLfor amine conjugates. The conjugates also exhibited potent anti-leishmanial activity against different strains of *L. donovani* in culture. The IC_{50} (inhibitory concentration 50%) values for the imine and amine conjugates against *L. donovani* LV9 intramacrophage amastigote were 0.25 ± 0.02 μg/mL and 0.13 ± 0.02 μg/mL, respectively in comparison to a value of 0.03 ± 0.01 μg/mL for pure AmB whereas the values were 0.12 ± 0.01 and 1.18 ± 0.11 μg/mL for the imine and amine conjugates against *L. donovani* DD8. While pure AmB produced 71% hemolysis to human blood at a concentration of 10 μg/mL, the pectin-AmB conjugates exhibited negligible hemolytic activity showing only 0.6–0.9% hemolysis at an AmB concentration of 100 μg/mL. The conjugates were soluble in water and were stable during storage in their dry form. The amine conjugate was able to reverse AmB-resistance in *Leishmania*. This promising data and the unexpected observation that the amine conjugate was more active than the imine conjugate requires further mechanistic studies.

1. Introduction

Life threatening opportunistic fungal infections are major causes of morbidity and mortality, especially in immunocompromised patients [1,2]. Amphotericin B (AmB) is the drug of choice for the treatment of systemic fungal infections [3] and is also widely used for the treatment for visceral and mucocutaneous leishmaniasis [4]. However, severe side effects such as nausea, fever and chills and nephrotoxicity accompany the use of AmB [5,6]. AmB is insoluble in water and therefore, the drug is administered as deoxycholate micelles (Fungizone®) or as liposomal formulations. Clinically used liposomal AmB such as AmBisome®, Amphotec®, and Abelcet® are considered less toxic [7,8]. However, the instability of liposomes, its high cost of production and the necessity for continuous intravenous infusions prevent their widespread use.

There has been much interest in the polymeric prodrugs of AmB in recent years. Thus, Domb et al. conjugated AmB to a water-soluble polysaccharide arabinogalactan, which was reported to overcome many limitations of AmB such as its insolubility and toxicity [9–12]. Farber et al. [13] have examined prodrugs of AmB with galactomannan. AmB has been conjugated to ergosterol [14] and poly(vinyl pyrrolidone) [15] and the resulting conjugates were found to be biologically active. AmB has also been conjugated to poly(ethylene glycol) (PEG) [16–18], N-(2-hydroxypropyl) methacrylamide (HPMA) [19], and oxidized dextran [20] and these conjugates exhibited potent anti-fungal activity. A molecular

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umbrella conjugate of AmB has been reported to exhibit high water solubility and low toxicity [21]. We have previously described the synthesis and the anti-fungal and anti-leishmanial properties of AmB-gum Arabic conjugates [22].

Citrus pectin is a branched, polysaccharide extracted from citrus fruits. It is composed of 1,4-linked α-D-galacturonic acid and its methyl ester along with natural sugars such as galactose, glucose, and mannose [23,24]. Pectin has attracted much attention as a drug carrier because of its biocompatibility. Pectin-5-fluorouracil [25] and pectin-4-aminothiophenol conjugates [26] were reported as promising carriers for colon-specific drug delivery. When doxorubicin was coupled onto oxidized pectin, it was found that the released drug retained anticancer activity [27]. It was also observed that oxidized pectin itself had a potency to prevent homotypic cancer cell aggregation. Pectin-cisplatin conjugate was reported to augment the activity of cisplatin resulting in a three-fold reduction in tumour volume in a murine melanoma model [28]. When methotrexate was conjugated to pectin, the cytotoxicity of methotrexate was enhanced indicating improved drug delivery to cancer cells [29].

It was therefore interesting to examine the possibility of synthesizing a pectin-AmB conjugate and to evaluate its potential for anti-fungal and anti-leishmanial activity.

2. Materials and methods

2.1. Materials

Pectin, sodium m-periodate, sodium hydroxide and methyl orange were from SRL Labs, Mumbai, India. Dimethyl sulfoxide (DMSO), disodium hydrogen orthophosphate, disodium tetra borate decahydrate, ethanol, ethanediol, hydrochloric acid, hydroxylamine hydrochloride, potassium iodide, potassium dihydrogen orthophosphate, sodium thiosulfate, sodium borohydride, sodium bicarbonate and Tween 20 were from S. D. Fine Chemicals, Mumbai, India. AmB was from Hi Media Laboratories, Mumbai, India. HEPES, M - 199 medium and FCS were from Life Technologies, Cergy-Pontoise, France. Gentamycin was from Schering-Plough (Levallois-Perret, France). Dialysis tubings of molecular weight cut off (MWCO) of 3500 and 6000–8000 were from Spectrum Laboratories, CA, USA. Pullulan standards were from Jordi Labs, MA, USA. Tissue culture plates were from Nunclon Delta, Strasbourg, France. All other chemicals employed were of analytical or equivalent grade. Promastigotes of *L. donovani* (MHOM/ET/L82/LV9) currently called LV9 from Africa, LV9 (Hexadecylphophocholine-resistant, HePC-R), LV9 (Sitamaquine resistant, Sita-R), DD8 (Wild type, WT) and DD8 (AmB resistant) were employed for testing anti-leishmanial activity. *Candida albicans* (CMP12009) and *Aspergillus fumigatus* (IP 229774) were employed for the anti-fungal studies. Double distilled water was used throughout.

2.2. Instrumentation

Infrared (IR) spectra were recorded on a FT-IR instrument (Perkin Elmer, USA) using KBr pellets. UV-Visible spectra were recorded on a Jasco spectrophotometer (JascoV-650 Series, USA). Elisa plate reader used was from Multiskan MS, Labsystem, Helsinki, Finland. Gel permeation chromatography (GPC) was performed using a HPLC system (Shimadzu, Japan) equipped with 510 pump, R401 refractive index detector and 7725 Rheodyne injector. The column used was Polysep 4000/5000 (Phenomenex, Hyderabad, India). A two compartment diffusion cell (Model Z756830-IEA, Sigma, USA) was used in the in vitro release experiments.

3. Methods

3.1. Oxidation of pectin

The oxidation of pectin was carried out by a procedure as reported earlier for gum arabic [22]. Into 100 mL of a 10% solution of the pectin, 2.5 g of sodium m-periodate was added to obtain 20% oxidation. After stirring the contents magnetically at 20 °C in dark for 6 h, the reaction was arrested by adding a few drops of ethylene glycol. The contents were then dialyzed against distilled water for two days with several changes of water. The solution was then frozen, lyophilized to dryness and stored in the desiccator until use.

3.2. Extent of oxidation

The percentage of oxidation was determined by iodometric titration of the residual periodate present in the reaction mixture [30]. Into a 5 mL aliquot of the reaction mixture, 10 mL of 10% sodium bicarbonate solution was added to neutralize the solution. After the addition of 2 mL 20% potassium iodide solution to liberate iodine, the flask was kept under dark for 15 min and liberated iodine was then titrated with standardized sodium thiosulphate solution using starch as the indicator. Values reported for degree of oxidation are average of a minimum of three oxidation experiments. The aldehyde content was further confirmed by the hydroxylamine hydrochloride method [31].

3.3. Synthesis of Pectin-AmB conjugates

Pectin-AmB conjugates were synthesized as reported earlier for the synthesis of gum arabic-AmB conjugates [22]. AmB was added to a 1% solution of 20% oxidized pectin in 0.1 M borate buffer (pH 11), to obtain a final concentration of 2.5 mg/mL and the solution was stirred magnetically in dark at 40 °C for 24 h. The solution was dialyzed against distilled water with several changes of water for 48 h using a dialysis bag (MWCO 6000–8000), frozen and lyophilized for 48 h to obtain the imine conjugate in ~60% yield.

The imine conjugate was reduced to the amine conjugate with sodium borohydride. Sodium borohydride was added to an aqueous solution of the imine conjugate (1.2 mol $NaBH_4$/mol of saccharide unit) and stirred overnight at room temperature. The amine conjugate thus formed was purified by dialysis in a similar fashion and lyophilized and conjugates were stored in light protected containers at 4 °C. The extent of AmB conjugated to oxidized PG was estimated by measuring the absorption at 405 nm spectrophotometrically. A stock solution of AmB in DMSO was prepared by dissolving 1 mg of AmB per mL of DMSO. The solution was serially diluted and the absorbance of the solutions was measured at 405 nm where AmB shows maximum absorption. A calibration curve of absorbance vs concentration was then constructed and the concentration of AmB in the conjugate was determined by measuring the absorption of the sample at 405 nm. The incorporation efficiency of AmB in the conjugate was over 90% (Theoretical load 20 wt%, actual load 18-19 wt%).

3.4. Stability of the conjugates

Stability of the conjugates in their dry form was determined by keeping them as lyophilized powders at 4 °C in light-protected glass containers for 6 months. Samples were taken at 0, 1, 2, and 4 week intervals and then every month and their absorptions were measured at 405 nm using solutions freshly prepared at a concentration of 0.05 mg/mL in distilled water.