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Pharmacokinetics of fungal (1–3)-β-D-glucans following intravenous administration in rats

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Abstract

Glucans are microbial cell wall carbohydrates that are shed into the circulation of patients with infections. Glucans are immunomodulatory and have structures that are influenced by bacterial or fungal species and growth conditions. We developed a method to covalently label carbohydrates with a fluorophore on the reducing terminus, and used the method to study the pharmacokinetics following intravenous administration of three highly purified and characterized glucans (glucan phosphate, laminarin and scleroglucan) that varied according to molecular size, branching frequency and solution conformation. Elimination half-life was longer (3.8 ± 0.8 vs. 2.6 ± 0.2 and 3.1 ± 0.6 h) and volume of distribution lower (350 ± 88 ml/kg vs. 540 ± 146 and 612 ± 154 ml/kg) for glucan phosphate than for laminarin and scleroglucan. Clearance was lower for glucan phosphate (42 ± 6 ml/kg h) than for laminarin (103 ± 17 ml/kg h) and scleroglucan (117 ± 19 ml/kg h). Since plasma levels at steady state are inversely related to clearance, these differences suggest that pharmacokinetics could favor higher blood levels of glucans with certain physicochemical properties.

Keywords: Glucans; Pharmacokinetics; Rat; Intravenous; Immunomodulation; Fungal

1. Introduction

Glucans are (1 → 3)-β-D-linked polymers of glucose that are major components of the outer cell wall of fungi as well as certain bacteria [19]. Glucans are released from the microbial cell wall into the blood of patients with fungal, and to a lesser extent, bacterial infections [4]. Since (1 → 3)-β-D-glucans are evolutionary conserved in microorganisms, and are not produced by higher species, the presence of these carbohydrates in the blood is presumed to be indicative of the presence of an infection [5]. It is not clear what role blood-borne glucans play in the pathogenesis of infection in critically ill patients. Glucans have been demonstrated to stimulate innate immunity [20] and to play a pivotal role in the recognition of and response to fungal pathogens by the innate immune system [2]. Consequently, glucans have been identified as fungal pathogen associated molecular patterns (PAMPs) [2].

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Glucans are bound and internalized by pattern recognition receptors on macrophages, neutrophils, NK cells, T cells, dendritic cells, fibroblasts, vascular endothelial cells and anterior pituitary cells [1]. Surprisingly, there is very little information available on the persistence and/or clearance of glucans from the circulation. We do know that glucan molecules can vary according to degree of polymerization, molecular weight, branching frequency and solution conformation. Mueller et al. [15] have reported that differences in molecular size, branching frequency and solution conformation dramatically alter the affinity with which PRRs interact with glucans. However, there is no information available on the effect of these physicochemical parameters on the pharmacokinetics of glucans.

The purpose of this study was to examine the pharmacokinetics of (1–3)-β-D-glucans. Understanding the pharmacokinetics of glucans and the effect of structure on glucan pharmacokinetics will be essential to interpreting how blood glucan levels might change during infection. We selected laminarin, glucan phosphate and scleroglucan for this study because these glucan polymers showed significant differences with regard to degree of polymerization, molecular weight, molecular size, branching frequency and solution conformation (Table 1). Using these three polymers, we examined glucan pharmacokinetics following intravenous administration in rats.

2. Methods

Adult male virus antigen-free Sprague–Dawley rats (Harlan, Indianapolis, IN) were maintained on standard lab chow and water ad libitum prior to experimentation. Animal procedures were reviewed and approved by the University Committee on Animal Care at East Tennessee State University.

Three water soluble glucan polymers were characterized [17] (Table 1) and used in these experiments. Laminarin was purchased from Sigma (St. Louis, MO). Glucan phosphate was prepared from *S. cerevisiae* and characterized as previously described [22]. Scleroglucan was prepared according to the protocol of Pretus et al. [17].

Glucan polymers were derivatized by the covalent addition of a diaminopropane (DAP) moiety at the reducing terminus as previously described [11]. Fluorescent-labeled glucans were generated by attaching AlexaFluor 488 (Molecular Probes, Eugene, OR) to the DAP moiety at the reducing terminus of the carbohydrates. The glucan preparations were dialyzed to remove unreacted material and stored protected from light at 4 °C in PBS until used.

Rats were briefly sedated with carbon dioxide while approximately 1–2 mm of the distal tail was removed to permit blood sampling and animals were injected (1 ml/kg) via the dorsal penile vein with solutions containing 1–3 mg/kg of carbohydrate. Blood samples were collected from the distal tail and centrifuged in heparinized capillary tubes. Plasma was stored protected from light in pyrogen-free MicroAmp vials (Perkin Elmer, Norwalk, CT) at 4 °C prior to analysis. Our preliminary experiments showed that plasma samples containing fluorescent-tagged glucans would remain stable under these conditions for at least 1 month. Samples were taken prior to injection and 1, 2, 5, 7, 10, 15, 20, 30, 45 min and 1, 2, 3, 4, 5, 6, 8, 12, 24 h following glucan administration.

Detection and quantitation of fluorescent-labeled glucans was performed in a 96-well plate using a FLUOstar* Galaxy Plate Reader and software (Biotechnology and Life Sciences, Maarssen, Netherlands) with excitation of 485 nm and an emission filter of 520 nm. Each assay contained 10 μl plasma or standard and 200 μl water.

Table 1
Physicochemical and pharmacokinetic characteristics of laminarin, glucan phosphate and scleroglucan

Parameter	Laminarin	Glucan phosphate	Scleroglucan
Weight average molecular weight (g/mol)	7700	125,000	1,000,000
Average degree of polymerization	~43	~700	~5600
Degree of side chain branching	1 per 10 glucose subunits	1 per 7 glucose subunits	1 per 3 glucose subunits
Solution conformation	single helix	single helix	triple helix
Polymer charge	neutral polysaccharide	polyelectrolye	neutral polysaccharide

To confirm the presence of glucan in the blood, samples were also analyzed with the Glucatell assay according to manufacturer instructions (Seikagaku/Cape Cod Associates, Falmouth, MA) following pretreatment with blood reagent. Samples were maintained at 4 °C prior to analysis. Standards ranged from 400 to 6.25 pg/ml.

Standards were analyzed by linear regression with sample concentrations determined by interpolation. Pharmacokinetic data were plotted as concentration versus time and were fit to established compartmental models using unweighted nonlinear regression (Prism, Graph Pad Software, San Diego, CA) to determine pharmacokinetic parameters. The $t_{1/2}$ values for each compartment were determined as ln2/k. Best fit for each curve was determined using the sequential F-test; reported values for pharmacokinetic parameters are mean ± S.E.M.

3. Results

The glucan polymers differed in degree of polymerization, molecular weight, polymer size, branching frequency and solution conformation [15] (Table 1). Pharmacokinetics for each of the glucan polymers were described using estimates of compartment size (Volume of distribution, V_D) and rate of change (half-life, $t_{1/2}$, the time it takes for half of the material to exit the compartment).

Intravenous administration of laminarin (1 mg/kg) produced an immediate rise in plasma laminarin levels with an estimated plasma concentration at time = 0 (C_P0) of 10 ± 3 mcg/ml and V_D of 252 ± 99 ml/kg (Table 2 and Fig. 1). Pharmacokinetics for laminarin were best resolved using a two-compartment model corresponding to a rapid distribution phase ($t_{1/2}$ = 3.7 ± 1.3 min) in which the carbohydrate is rapidly dispersed throughout the body, and a slower elimination phase with a $t_{1/2}$ of 2.6 ± 0.2h and a $V_D\beta$ of 540 ± 146 ml/kg, in which the carbohydrate is slowly cleared from the plasma. A distribution phase was seen in four of the six curves following laminarin administration.

Intravenous administration of glucan phosphate produced an immediate rise in plasma glucan phosphate levels with an estimated plasma concentration at time = 0 (C_P0) of 37 ± 8 mcg/ml (equivalent to 12 ± 3 mcg/ml for a 1-mg/kg dose) and $V_D(0)$ of 100 ± 19 ml/kg (Table 2 and Fig. 1). Pharmacokinetics for glucan phosphate was best resolved using a two compartment model corresponding to a rapid distribution phase ($t_{1/2}$ = 4.3 ± 0.7 min) and a slower elimination phase with a half-life of 2.6 ± 0.2 h and a $V_D\beta$ of 350 ± 88 ml/kg.

Intravenous administration produced an immediate rise in plasma scleroglucan levels with an estimated plasma concentration at time = 0 (C_P0) of 2.3 ± 0.4 mcg/ml and V_D of 261 ± 130 ml/kg (Table 2 and Fig. 1). Pharmacokinetics for scleroglucan was resolved using a two-compartment model corresponding to a rapid distribution phase ($t_{1/2}$ = 2.1 ± 0.5 min) and a slower elimination phase with a half-life of 3.1 ± 0.6 h and a $V_D\beta$ of 612 ± 154 ml/kg). A distribution phase was seen in five of the six curves following scleroglucan administration.

Peak plasma glucan concentrations following administration were highest for glucan phosphate, and approximately equal for laminarin and scleroglucan. V_D values at C_P0 were lowest for glucan phosphate (100 ± 19 ml/kg), with similar values found for laminarin, and scleroglucan (261 ± 130 mg/kg). Elimination V_D values were lower for glucan phosphate than

Table 2
Pharmacokinetic characteristics for plasma laminarin, glucan phosphate and scleroglucan following intravenous administration

Parameter	Laminarin	Glucan phosphate	Scleroglucan
$k_{distribution}$ (h^{-1})	15.6 ± 4.3	11.2 ± 2.4	19.2 ± 6.0
Distribution half-life (min)	3.7 ± 1.3	4.3 ± 0.7	2.1 ± 0.5
V_D (ml/kg) elimination	540 ± 146	350 ± 88[a]	612 ± 154
$k_{elimination}$ (h^{-1})	0.27 ± 0.02	0.22 ± 0.03	0.27 ± 0.05
Half-life (h) elimination	2.6 ± 0.2	3.8 ± 0.8[b]	3.1 ± 0.6
Clearance (Total) (ml/kg h)	102.6 ± 17.4	41.9 ± 5.8[a,b]	116.7 ± 18.9

Laminarin and scleroglucan were administered 1 mg/kg; glucan phosphate was administered 3.125 mg/kg.
[a] Significantly different from scleroglucan.
[b] Significantly different from laminarin.

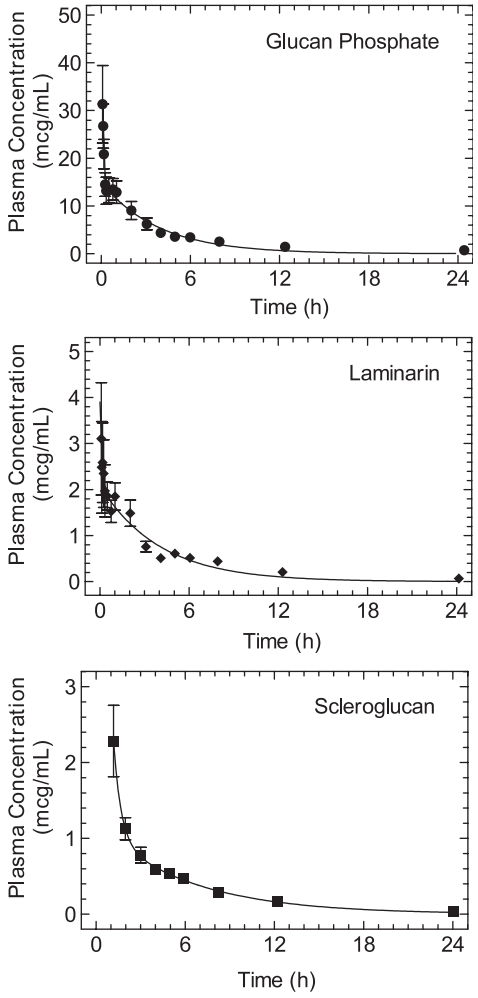

Fig. 1. Plasma concentrations of each carbohydrate were measured following intravenous administration of glucan phosphate (3.125 mg/kg), laminarin (1 mg/kg) and scleroglucan (1 mg/kg). While the carbohydrates vary remarkably in molecular size, side chain branching, and solution conformation, differences in elimination $t_{1/2}$ were subtle, reflecting both a lower clearance and smaller V_D for glucan phosphate. Curve fits are shown for a two compartment model with distribution and elimination phases using mean data ($\pm$ S.E.M.) from five to six animals.

for scleroglucan. Although the difference between laminarin (2.6 h) and glucan phosphate (3.8 h) was statistically significant ($p < 0.05$), this was influenced by a single animal with a prolonged (8.1 h) half-life following glucan phosphate administration. Total clearance was lower for glucan phosphate than for laminarin and scleroglucan (Table 2).

The *Limulus*-based Glucatell assay was used to confirm the presence of measurable plasma glucan levels following intravenous administration. Because of the large sensitivity difference between the assays, (mcg/ml for fluorescence assay versus pg/ml for the Glucatell assay) and the exquisite sensitivity of the Glucatell assay, we were not able to simultaneously analyze samples using the two methodologies. Fluorescent-tagged glucan phosphate was detected using the Glucatell assay (data not shown) following injection of low doses; control animals had undetectable glucan blood levels ($n = 3$).

4. Discussion

Our data indicate that there are significant differences in pharmacokinetics of three glucans which differ markedly in physicochemical properties such as molecular size, branching frequency and solution conformation. The three glucans studied also vary according to polymer charge [18]. Glucan phosphate is a polyelectrolyte, while laminarin and scleroglucan are neutral polysaccharides [18]. While each of the glucans studied is bound and internalized by pattern recognition receptors on a variety of cell types [15], they differ in biological activity. Glucan phosphate and scleroglucan increase immune function [21] , while laminarin does not stimulate innate immunity. We conclude that the in vivo clearance of glucans from the blood is largely independent of molecular size, branching frequency and solution conformation, even though these properties influence biological response to glucans.

Glucans are recognized by several pattern recognition receptors, including dectin, CR3 and scavenger receptors [23]. These pattern recognition receptors respond to varying concentrations of glucan. Dectin appears to be the sentinel receptor for glucan with exceptionally high affinity (< 1 nM) [2,3]. Scavenger receptors interact with varying glucan structures with affinities ranging from 10 nM to 10 μM [18]. Thus, the pharmacokinetic properties of individual glucans can result in glucan blood levels that interact with one or more receptors for glucan and potentially trigger biological responses that vary with concentration or time.

Intravenous doses of the carbohydrates chosen in this study are comparable to those known to have biological activity. Browder et al. [1] achieved favorable effects in trauma patients using daily administration of 50 mg/m^2 glucan phosphate for 7 days. The dose of glucan phosphate used in the present study is equivalent to 22 mg/m^2 [6].

In the present study, glucan phosphate had a smaller volume of distribution as well as lower clearance, resulting in an elimination half-life similar to that of laminarin and scleroglucan. Under conditions of constant administration, steady state plasma glucan levels would be inversely proportional to clearance. As glucans shed from the fungal or bacterial cell wall, a process that is not necessarily occurring at a constant rate, plasma glucan levels would likely be a function of both the rate of release and clearance. The results of the present study suggest that during infection, there could be higher plasma levels of certain glucans based on differences in clearance.

Volume of distribution is the hypothetical volume that links the amount of glucan administered to the plasma concentration observed. For many drugs, V_D provides an indication of the distribution of the drug into plasma (0.40 ml/kg), blood (0.80 ml/kg), extracellular water (0.200 ml/kg), or total body water (0.600 ml/kg). For glucans, however, V_D is influenced by uptake of the carbohydrates into monocytes and macrophages [16], platelets and cells of the liver, spleen and kidney.

There are previous reports concerning the pharmacokinetics of individual glucans [12,25,26]. These reports are sometimes clouded by a failure to fully characterize the carbohydrate used, but nevertheless support the conclusion that intravenously administered glucans have similar half-lives [12,25,26]. It has also been reported that glucans are eventually deposited in the liver and spleen [12,25,26]. Glucan derived from *Grifola frondosa* is reported to have a $t_{1/2}$ of 5.4 and 6.4 h following systemic administration in autoimmune-prone and normal mice, respectively, with about 70% of the glucan recovered in the liver and spleen [12]. Following intravenous administration in rabbits, Yoshida et al. [25,26] reported a distribution $t_{1/2}$ of less than 5 min for a 92-kDa radiolabeled (1−3)-β-D-glucan isolated from *Candida albicans*. Intravascular clearance studies suggested that the rapid distribution phase was followed by a prolonged elimination phase of several hours [26]. Most of the radiolabeled glucan (>97%) was associated with cell-free plasma, while radioactivity associated with blood cells was initially found in platelets and later (2 h) distributed to polymorphonuclear leukocytes and red blood cells [26]. More than 80% of the material was contained in the liver and 10% in the kidney after 24 h.

There is also evidence that uptake into cells may differ not only for individual carbohydrates, but also for various cells or tissues. Substantial differences were reported for accumulation of glucuronoxylmannan from *Cryptococcus neoformans* by macrophages and neutrophils [13]. While neutrophils rapidly ingested carbohydrate, which was expelled or degraded after 1 h, macrophages continued to accumulate glucuronoxylmannan for up to 1 week [13]. This supports the view that various immune cell types may differentially process carbohydrates and/or respond differently to the same carbohydrate.

There is an important caveat to this study. At present, we do not know the precise chemistry of the glucans released from the cell wall of pathogens during infection. Even though the presence of circulating glucans has been proposed as a marker for infection [4,7–10,14,24], it has not been possible as yet to isolate sufficient amounts of these glucans to characterize them. We speculate that the glucans found in the blood exhibit a broad range of physicochemical features depending upon the organism that produced them, growth conditions, and the extracellular milieu into which they are released.

In the present study, we employed fluorescent-labeled glucans. This approach has significant advantages for pharmacokinetic studies. Glucans are abundantly present in the environment [19]. This may result in glucan contamination during pharmacokinetic studies when using the glucan specific *Limulus* assay [5]. Administration and measurement of fluorescent glucans precludes the possibility of contamination during blood collection, and minimizes interference from basal glucan levels. Additionally, this method could be applied to any carbohydrate with a reducing terminus, and therefore is not limited to glucans which trigger the *Limulus* clotting cascade. Importantly, we also employed the Glucatell assay to specifically identify glucans and confirm the presence of glucan in the plasma [14].

In conclusion, we have demonstrated that fluorescent labeling of carbohydrates through DAP derivatization at the reducing terminus provides a means to reproducibly and effectively label carbohydrate polymers and that the labeled polymers can be effectively employed to study the pharmacokinetics of natural product carbohydrates. Physicochemical analysis indicates that addition of DAP at the reducing terminus results in minimal structural changes to the polymers. This method should be readily adaptable to a variety of natural product carbohydrates which have a reducing terminus. Our study demonstrates that three well-defined glucan polymers, laminarin, glucan phosphate and scleroglucan, have differences in pharmacokinetics following intravenous administration in rats. This suggests that the in vivo clearance of glucan polymers may depend on physicochemical properties and that differences in clearance may result in higher plasma levels for particular glucan structures.

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