Activity of chitosan and its derivatives against *Leishmania major and L. mexicana in vitro*.

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Running Head: Anti-leishmanial activity of chitosan

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Abstract

There is an urgent need for safe, efficacious, affordable and field-adapted drugs for the treatment of cutaneous leishmaniasis which affects around 1.5 million new people worldwide annually. Chitosan, a biodegradable cationic polysaccharide, has previously been reported to have antimicrobial, anti-leishmanial and immunostimulatory activities. We investigated the *in vitro* activity of chitosan and several of its derivatives and showed that pH of the culture medium plays a critical role on anti-leishmanial activity of chitosan against both extracellular promastigotes and intracellular amastigotes of *Leishmania major* and *Leishmania mexicana*. Chitosan and its derivatives were approximately 7-20 times more active at pH 6.5 than at pH 7.5 with high molecular weight chitosan being the most potent. High molecular weight chitosan stimulated the production of nitric oxide and reactive oxygen species by uninfected and *Leishmania* infected macrophages in a time and dose dependent manner at pH 6.5. Despite the *in vitro* activation of bone marrow macrophages by chitosan to produce nitric oxide and reactive oxygen species, we showed that the anti-leishmanial activity of chitosan was not mediated by these metabolites. Finally, we showed that rhodamine-labelled chitosan is taken up by pinocytosis and accumulates in the parasitophorous vacuole of *Leishmania* infected macrophages.

KEYWORDS: Cutaneous leishmaniasis, *Leishmania major*, *Leishmania mexicana*, chitosan, macrophage uptake.

Introduction

Leishmaniasis is an infectious disease caused by protozoan parasites belonging to the genus *Leishmania*. The parasite is transmitted between humans and mammalian reservoirs (e.g. dogs and rodents) through the bite of a female phlebotomine sandfly (1). There are two main clinical forms, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL), with CL being the most common (2). In addition to “simple” CL, there are other complex cutaneous manifestations including mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (RL) and post-kala-azar dermal leishmaniasis (PKDL) (3, 4).

CL is caused mainly by *Leishmania tropica, Leishmania major* and *Leishmania aethiopica* in the Old World and by *Leishmania braziliensis, Leishmania guyanensis, Leishmania mexicana* and *Leishmania amazonensis* in the New World(5). Of the 88 countries where CL occurs, 90% of the cases are in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (1). In the mammalian host, the parasite survives and multiplies within macrophages. The cellular immune responses in CL play a critical role in the control and progress of the disease, which include two main mechanisms of macrophage activation: (i) the classical pathway (M1 macrophages) in which Th1 and NK cells produce cytokines (such as IFN-γ) which stimulate the production of nitric oxide (NO) and reactive oxygen species (ROS) and the activation of other lysosomal anti-microbial activities which are responsible for killing the *Leishmania* parasites and (ii) the alternative pathway mediated by Th2 cytokines, such as IL-4 and IL-13 in the early stages of infection forming a favourable environment for *Leishmania* proliferation (6, 7).

Pentavalent antimonial compounds, sodium stibogluconate (Pentostam ®) and meglumine antimoniate (Glucantime®), have been the standard treatment for CL for the past 70 years (8). These drugs have several limitations including difficulty of administration, toxicity of the drug and variable sensitivity among *Leishmania* species (9). Second-line treatments include the polyene antifungal amphotericin B which also suffers from toxicity, the oral phospholipid miltefosine, the use of which is limited by teratogenicity, and the aminoglycoside antibiotic paromomycin (PM) which has low cure rates for certain *Leishmania* species (10, 11, 12). Treatment with intravenous AmBisome® (liposomal amphotericin B) is safe and has achieved clinical success at a dose of 3 mg/kg daily for 7 days against CL(13, 14) but the high cost of this formulation limits its use (15). Two Cochrane analyses have clearly shown clinical deficiencies of most drugs. There is an urgent need for new treatments which can eliminate the parasites, improve the healing process, are safe, reliable and also field-adaptable for use in diverse health care systems (16, 17).

Chitosan is a biodegradable, biocompatible, positively charged non-toxic muco-adhesive biopolymer produced by the deacetylation of chitin. Chitosan has a pKa of approximately 6.3, is insoluble at alkaline pH but soluble in weak acidic solvents like acetic acid where the amino groups become protonated. Many reports have described the antimicrobial activity of chitosan but the actual mechanism of action has not been fully elucidated (18) although three direct mechanisms have been suggested. The first is the interaction between the protonated NH3+ groups of chitosan and the negative cell membrane of microbes. This interaction changes the permeability of the microbial cell membrane, causing osmotic imbalances, and consequently killing them (18, 19). The

second suggested mechanism is that chitosan binds to microbial DNA and inhibits DNA transcription, assuming that chitosan penetrates the microbial cell membrane and reaches the DNA (19, 20). The third mechanism is via the chitosan chelation of metals and the binding of basic nutrients essential for microbial growth (19). An indirect mechanism of action may be related to the known pro-inflammatory effect of chitosan on macrophages. This involves stimulation of tumour necrosis factor (TNF-α), interleukin 6 (IL-6), NO, ROS and interferon gamma (IFN-γ) which play a critical roles in the proinflammatory response against intracellular microbes (by enhancing the production of microbicidal reactive nitrogen species) (21, 22, 23, 24, 25). Chitosan activates polymorphonuclear leukocytes, macrophages and fibroblasts and these properties promote wound healing (18, 26).

The poor solubility of chitosan and the loss of the cationic charge at neutral and alkaline environments are two of the major obstacles to the consideration of chitosan as a useful antimicrobial. Recently, the chemical modification of chitosan to produce various derivatives to improve its solubility and widen its application has gained attention (27) (28). Chitosan and its derivatives have been shown to have *in vitro* anti-leishmanial activity with EC_{50} values (50% effective concentration) ranging from 70 to 240 µg/ml against *L. infantum*, *L. amazonensis* and *L. chagasi* promastigotes and amastigotes (29, 30, 31, 32, 33, 34). All this makes chitosan an appropriate candidate for further studies to evaluate its suitability for the treatment of CL.

The aim of our work was to: (i) determine the *in vitro* anti-leishmanial activity of chitosan and its derivatives against *L. major* and *L. mexicana* promastigotes and intracellular amastigotes at two different pH values (the culture medium pH of 7.5 and a lower pH of

6.5, which are both suitable for macrophage and parasite growth(35, 36, 37), (ii) to evaluate the *in vitro* role of chitosan in the activation of macrophage M1 proinflammatory phenotype, via the measurement of NO ,ROS and TNF-α production by host cells and by measuring parasite survival, and (iii) investigate chitosan uptake by macrophages to explain its activity against intracellular amastigotes.

115 **Results**

116

117 ***In vitro* activities of chitosan and derivatives against *L. major* and *L. mexicana*.**

118 Anti-leishmanial activity (against promastigotes and amastigotes) of high, medium and
119 low molecular weight (HMW, MMW and LMW respectively) chitosan and its derivatives
120 (a total of 11) was tested. Dose dependent activity (Fig S1 and S2) against *Leishmania*
121 promastigotes and amastigotes was observed for chitosan and its' derivatives except for
122 carboxymethyl chitosan which showed no activity against either parasite stage within
123 the experimental parameters tested (pH 7.5 or 6.5 and concentrations up to 400 µg/ml).
124 In the 72 h assays, chitosan and its derivatives (except carboxymethyl chitosan) were 7-
125 20 times more active against *L. major* and *L. mexicana* promastigotes and intracellular
126 amastigotes (infecting peritoneal mouse macrophages (PEMs)) in culture medium at
127 pH=6.5 than at pH=7.5 ($p<0.05$ by t-test) (Tables 1 and 2). HMW, MMW and LMW
128 chitosan, from both crustacean and fungal sources, exhibited significantly higher
129 activities against promastigotes and intracellular amastigotes ($EC_{50} \approx 6$ µg/ml against *L.*
130 *major* promastigotes and 10 µg/ml against *L. mexicana* promastigotes; $EC_{50} \approx 12$ µg/ml
131 against *L. major* amastigotes and 16 µg/ml against *L. mexicana* amastigotes) than the
132 derivatives at pH= 6.5 (Tables 1 and 2) ($p<0.05$ by an extra sum-of-squares F test).
133 Additionally, *L. major* promastigotes and amastigotes were significantly more sensitive
134 to chitosan and its derivatives than *L. mexicana* promastigotes and amastigotes
135 (approx. 1.5 to 2 times, $p<0.05$ by an extra sum-of-squares F test).

136 To allow like-for-like comparison, EC_{50} values were recalculated in terms of molarity
137 using estimated molecular weights (HMW: MW= 342.5 KDa, MMW: MW=250 KDa,

LMW: MW= 120 KDa and fungal chitosan MW=130 KDa) at pH = 6.5. Based on molarity (Table S4 and S5), HMW chitosan was significantly more active against *L. major* and *L. mexicana* promastigotes and amastigotes and hence used in all subsequent studies.

Host cell dependence of the anti-leishmanial activity of HMW chitosan at pH 6.5

We aimed to assess the host cell dependence of the anti-leishmanial activity of HMW chitosan and Fungizone by evaluating the *in vitro* activity against *L. major* amastigotes in three different macrophage type; EC_{50} and EC_{90} values in the three different macrophage populations are summarized in Table 3. There was a significant difference in the activity of HMW chitosan depending on the type of macrophage; PEMs, bone marrow-derived macrophages (BMMs) or human leukaemic monocytes-like derived cell line (THP-1)) ($p<0.05$ by an extra sum-of-squares F test). HMW chitosan was significantly more active against intracellular amastigotes in PEMs and BMMs compared to differentiated THP-1 cells.

Effects of HMW chitosan on the production of TNF-α by uninfected or *L. major* infected BMMs at pH = 6.5

The activation of M1 macrophages by Th1 lymphocyte plays an important role in the control of CL (6, 38, 39). Therefore, we measured TNF-α production by BMMs stimulated by HMW chitosan. Following exposure to HMW chitosan, the TNF-α production by BMMs was found to be dose-dependent, in a bell-shaped manner, in both *Leishmania*-infected and uninfected cells as shown in Fig. 1. After 24 h, the levels of TNF-α in the culture fluid of BMMs exposed to HMW chitosan (at concentrations 14.8,

161 44.4 and 133.3 µg/ml) was significantly higher than BMMs (infected and uninfected),
162 that had not been exposed to chitosan with TNF- α being highest at 44.4 µg/ml chitosan.
163 While at other concentrations (1.64, 4.9 and 400 µg/ml), HMW chitosan did not
164 stimulate BMMs to produce TNF-α ($p < 0.05$ by t-test).

165 HMW chitosan at concentrations 14.8, 44.4 and 133.3 µg/mL stimulated BMMs to
166 produce TNF-α with 87± 4.5 - 712± 9 - 48±3 pg/ml respectively in uninfected BMMs and
167 56± 3.5 - 464± 10 - 32±4 pg/ml respectively in *L. major* infected BMMs. Less TNF-α was
168 generated when the chitosan concentration was increased to 133.3 µg/ml and above.
169 Lipopolysaccharides from *Escherichia coli* O26:B6 (LPS; positive control) stimulated
170 TNF-α production in both uninfected and infected BMMs after a 24 h incubation period
171 at a significantly higher level than chitosan ($p < 0.05$ by t-test). Our results indicated that
172 HMW chitosan activated M1 macrophages.

173

174 **Effects of HMW chitosan on the production of ROS by BMMs at pH = 6.5**

175 ROS plays an important role in the killing of intracellular amastigotes (6, 38, 39)
176 therefore, we measured ROS production by BMMs stimulated by HMW chitosan. HMW
177 chitosan (at concentrations 14.8, 44.4 and 133.3 µg/ml) increased the production of
178 ROS (indicated by H2DCFDA fluorescence) after 4 h of incubation but did not stimulate
179 ROS after 8 h of incubation (Table S1). Other concentrations of HMW chitosan (1.64,
180 4.9 and 400 µg/ml) did not stimulate BMMs to produce ROS after 4 h or 8 h of
181 incubation.

182 The ROS dose response in both uninfected and infected BMMs was bell-shaped –
183 similar to that seen with TNF-α. Increasing chitosan concentration from 14.8 to 44.4
184 µg/mL increased ROS production, after which further increase concentration reduced
185 ROS production. In addition, ROS production by BMMs was significantly decreased ($p < 0.05$ by t-test) by infecting the cells with *L. major* as shown in Fig. 2.

187 We found that HMW chitosan had an *in vitro* stimulatory effect on BMMs ROS
188 production after 4h of incubation. We therefore investigated whether this ROS plays any
189 role in the activity of HMW chitosan against intracellular amastigotes. For these
190 experiments, the 4 h post treatment time point was taken because ROS peaked at this
191 point in BMMs in response to chitosan treatment at a time when chitosan does not
192 induce NO in BMMs (*ibid*). Scavenging of ROS by the ROS scavenger, 5mM N-acetyl-
193 L-cysteine (NAC), had no significant impact on the activity of chitosan against
194 intracellular amastigotes ($p > 0.05$ by t-test) – see Fig. 3. The ROS scavenger caused a
195 complete scavenging of ROS production after 4 h (Table S2) and had no cytotoxicity
196 against KB cells or leishmanicidal activity against *L. major* amastigotes (data not
197 shown). Even though chitosan stimulated ROS production it did not play a role in the
198 anti-leishmanial activity of chitosan.

199

200 **Effects of HMW chitosan on the production of NO by BMMs at pH = 6.5**

201 NO plays an important role in the killing of intracellular amastigotes (6, 38, 39) therefore,
202 we measured NO production by BMMs stimulated by HMW chitosan. We showed that
203 chitosan did not have a stimulatory effect on BMM NO production after 4 h of incubation

(Table S3). However, after a 24 h incubation, HMW chitosan at pH=6.5 had a stimulatory effect on BMMs NO production in a clear bell-shaped dose dependent manner (Figure 4). HMW chitosan at concentrations of 14.8, 44.4 and 133.3 µg/mL induced both uninfected and infected BMMs to produce NO (at 14.9± 0.3, 34±1.2 and 11±1 µM respectively in uninfected BMMs and 11 ±1, 26 ± 2.5 and 8 ± 1.2 µM respectively in infected BMMs), NO being highest at 44.4 µg/ml. While other concentrations of HMW chitosan (1.64, 4.9 and 400 µg/ml) did not stimulate BMMs to produce NO after 24 h of incubation.

LPS caused significantly higher NO production compared to HMW chitosan ($p < 0.05$ by t-test) in both uninfected and infected BMMs. The levels of NO produced by *L. major* infected BMMs exposed to LPS (positive control) or HMW chitosan were significantly lower than levels produced by uninfected BMMs ($p < 0.05$ by t-test) (Fig 4).

As HMW chitosan had an *in vitro* stimulatory effect on BMM NO production after 24h of incubation, we investigated further whether NO has any role in the activity of HMW chitosan against intracellular amastigotes. Inhibition of NO production by the NO inhibitor NG-methyl-L-arginine acetate salt (L-NMMA) at 0.4mM, had no significant influence on the activity of chitosan against intracellular amastigotes ($p > 0.05$ by t-test) (Fig. 5), although the NO inhibitor did cause a complete inhibition of NO production (Table S2) after 24 h and had no cytotoxicity effects against KB cells and no leishmanicidal activity against intracellular *L. major* amastigotes (data not shown). Even though chitosan stimulated NO production it did not play a role in the anti-leishmanial activity of chitosan.

Cellular uptake of HMW chitosan and inhibition of endocytosis

We found that the activation of M1 macrophages by HMW chitosan did not play a role in its activity against intracellular amastigotes. Therefore, we investigated whether the anti-leishmanial effects of HMW chitosan against intracellular amastigotes after 4 h and 24 h exposure were dependent on the direct activity of chitosan following its entry into the macrophages at pH 6.5. No significant difference was observed in the activity of chitosan against intracellular amastigotes when it was added after prior phagocytosis inhibition with cytochalasin D (Figure 6, $p > 0.05$ by t-test). In contrast, dynasore (an inhibitor of pinocytosis, a clathrin-mediated endocytosis (CME) inhibitor) did significantly affect chitosan mediated parasite killing at pH = 6.5 (Fig. 6, $p< 0.05$ by t-test). The same activity was seen at pH 7.5. – see Fig 6, panel C. The two inhibitors had no cytotoxicity against KB-cells or activity against intracellular *L. major* amastigotes at the concentrations used. Pinocytosis (CME) played a critical role in the efficacy of HMW chitosan against intracellular amastigotes.

Fluorescence microscopy of the uptake of chitosan by macrophages

Rhodamine-labelled chitosan was used to track the delivery of chitosan to the parasitophorous vacuole (PV) of *Leishmania* infected macrophages. Fig. 7 illustrates the cellular uptake of chitosan by *L. major*-GFP- or *L. mexicana*-GFP- infected BMMs after 4 h and 24 h rhodamine-labelled chitosan exposure. There was co-localization of chitosan and intracellular amastigotes after 4 h and 24 h with nMDP colour index 0.7 and 1 respectively (see nMDP material and methods). The uptake of chitosan increased

in a time-dependent manner. Fig 7 (Panels D and E) shows this uptake after 4 h and 24 h respectively, and the accumulation of chitosan in PVs (shown as yellow that indicates co-localization of rhodamine and GFP). Fig 7 (Panel F) also shows that the inhibition of pinocytosis (CME) with dynasore prevented the uptake of chitosan with a negative nMDP colour index that represents no co-localization of chitosan and amastigotes. This is also supporting evidence for the uptake by pinocytosis as seen in Fig 6.

Discussion

The literature on the anti-leishmanial activity of chitosan and its derivatives is limited, especially pertaining to its mechanism(s) of action (19, 40, 41). In this study, we assessed the anti-leishmanial activity of various forms of chitosan, including low, medium and high molecular weight chitosan, and chitosan derivatives. Chitosan derivatives are generally produced by chemical modification of the amino or hydroxyl groups of chitosan for the optimization of the physicochemical properties. We found that chitosan and its derivatives had minimal cytotoxicity against KB-cells with LD_{50} values ≥750 µg/ml in RPMI 1640 at pH 7.5 or 6.5. This data supports previous reports of chitosan's low cytotoxicity against CCRF-CEM (human lymphoblastic leukaemia) and L132 (human embryonic lung) cells with similar LD_{50} values (42, 43).

We determined that a lower pH 6.5, compared to pH 7.5, enhanced, by 7-20, times the anti-leishmanial activity of chitosan and its derivatives against *L. major* and *L. mexicana* promastigotes and amastigotes. This higher activity of chitosan at the lower pH 6.5 could be due to its greater ionisation (protonation of the amino groups; pKa of

chitosan≈6.3). The greater positive charge could increase the chitosan antimicrobial activity by interacting with the negatively charged microbial membrane – in accordance with the first postulated mechanism of antimicrobial activity described in the Introduction (18, 19). A higher chitosan activity at lower pH (pH ≈ 5) has previously been reported against *Escherichia coli* and *Salmonella typhimurium* (44, 45).

Our study is the first to show the pH dependence of the anti-leishmanial activity of chitosan and its derivatives and could explain why literature reports of the anti-leishmanial activity of chitosan have shown such variability, with EC_{50} values ranging from 70 to 240 µg/ml against *L. infantum*, *L. amazonensis* and *L. chagasi* promastigotes and amastigotes (29, 30, 31, 32, 33, 34). For example, in one study, the EC_{50} of chitosan against *L. infantum* amastigotes (in PEMs) in RPMI 1640 medium was 100.81 µg/ml, but the pH at which the experiment was conducted was not mentioned (29). Influence of pH was also seen when the anti-leishmanial activity of chitosan (of the different molecular weights) and chitosan derivatives were compared. While the different chitosans and derivatives showed minor differences in their anti-leishmanial activity at pH 7.5, the derivatives were 3 to 5 times less active than the HMW, MMW, LMW and fungal chitosan at lower pH 6.5. This reduced activity could be due to the lower number of amino groups on the chitosan derivatives (see Fig 8). These derivatives are more soluble at a higher pH and have similar activity to chitosan, but at a lower pH the higher protonation of the chitosan improves the anti-leishmanial activity significantly (46, 47). Carboxymethyl chitosan had no anti-leishmanial activity - most of the amino groups on this derivative have been substituted by carboxymethyl moieties making the molecule negatively charged (48) .

294 The higher anti-leishmanial activity of HMW chitosan compared to MMW and LMW
295 chitosan mirrors its greater antibacterial activity in another study against *Escherichia*
296 *coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (49). HMW has a long
297 chain, and therefore more glucosamine units, and possesses more amino groups (Fig 8)
298 resulting in more protonated groups (-NH3+) than MMW and LMW(49) which could
299 explain its greater potency.

300 We also showed that the anti-leishmanial activity of chitosan is significantly greater
301 against *L. major* infected PEMs or BMMs compared to differentiated THP-1 cells in the
302 order PEMs>BMMs>THP-1 cells underlining the need to take the host cell into
303 consideration when conducting similar experiments(50).

304 In order to understand the potential anti-amastigote mechanism(s) of chitosan, we
305 investigated whether the activity of HMW chitosan against the intracellular amastigotes
306 was via direct uptake into the host cell and localisation in the parasitophorous vacuole
307 or indirectly via the activation of M1 macrophages, given that the cellular immune
308 responses in cutaneous leishmaniasis play a critical role in self-cure (51, 52).

309 The activation of M1 macrophages by Th1 lymphocyte subpopulation, which produces
310 different cytokines, primarily IFN-γ and TNF-α, is crucial for the killing of the intracellular
311 *Leishmania* via the triggering of an oxidative burst and therefore, the host cells increase
312 the production of ROS and NO which are responsible for killing of the parasite (38, 39).
313 We found that HMW chitosan stimulated TNF-α production by macrophages and this
314 would be expected to be an indicator of an M1 macrophage that would have greater
315 leishmanicidal activity. Our results show that chitosan stimulated BMMs ROS production
316 with a peak after 4 h and led to a significant increase in the TNF-α and NO production

after 24 h in a bell-shaped response. Similar findings have been reported showing that HMW chitosan had *in vitro* stimulatory effect on NO production in PEMs (from male rats) (25) and LMW chitosan stimulated RAW264.7 macrophage TNF- α production (24). Another study demonstrated that LMW chitosan induced ROS production in an epithelial, human breast cancer cell line (53). The bell-shaped responses are consistent with a study that showed that chitosan stimulated NO and TNF-α production in peritoneal macrophages in a dose-dependent manner and their levels tended to decrease at higher concentrations of chitosan (320μg/ml)(54). This type of response has also been reported previously for tucaresol for both, its immunomodulatory and activity against experimental *L. donovani* infections, albeit at lower doses (55). Despite the observed chitosan-induced ROS and NO production, there was no evidence that this contributed to the anti-leishmanial activity in our study – the inhibitors that we used to suppress their production had no effect on the ability of chitosan to kill intracellular *Leishmania* amastigotes (Figs 3 and 5). This led us to investigate the cellular uptake of HMW chitosan and its relationship to the anti-leishmanial activity.

The uptake of the large charged molecule HMW chitosan has not been systematically studied before and there is no clear evidence of its penetration of cell membranes or of its uptake mechanism. Macrophages are known to take up extracellular materials and plasma by endocytosis. Endocytosis mainly occurs via two different cellular uptake mechanisms: pinocytosis or phagocytosis, where pinocytosis is fluid-phase endocytosis and phagocytosis is the process of engulfing large particles (56). Inhibition of pinocytosis (CME) significantly reduced the anti-leishmanial activity of HMW chitosan. Therefore, in our study pinocytosis (CME) was considered to be the main mechanism

for the uptake of HMW chitosan by BMMs, indicating a direct anti-leishmanial effect of this molecule against amastigotes. Other studies have previously reported pinocytosis as the pathway for the uptake of chitosan of different molecular weights by HEK293 epithelial cells (57). The fluorescence imaging in our study showed that in BMMs HMW chitosan is taken up into the parasitophorous vacuole (PV) where the *Leishmania* parasites reside, with the labelled chitosan being internalized within 4 h and increasing up to 24 h later. This is consistent with another study where rhodamine isothiocyanate-chitosan (RITC-chitosan 98-10 K) was found to be directly delivered to the U937 macrophage lysosome after 24 h (58). The accumulation of chitosan in the PV might be due to chitosan's relatively high pKa of 6.3, making it more soluble and protonated in the acidic contents of the vacuole. This is consistent with a study using bafilomycin to inhibit acidification and prevent chitosan accumulation within macrophages (58).

In summary, our studies indicate that chitosan and its water-soluble derivatives showed anti-leishmanial activity against both *L. major* and *L. mexicana* promastigotes and amastigotes in a pH dependent manner. At pH 6.5 HMW chitosan is more active than MMW and LMW chitosan and chitosan derivatives, in particular those where the amino groups are substituted. In addition, HMW chitosan activated M1 macrophages, stimulating them to produce NO and ROS. However, the anti-leishmanial activity of chitosan was not due to such immune activation, as an NO inhibitor and a ROS scavenger failed to reduce the anti-leishmanial activity. Instead, the anti-leishmanial activity was related to direct uptake of chitosan into the parasitophorous vacuole by pinocytosis (CME). HMW chitosan demonstrated effective *in vitro* anti-leishmanial

activity with minimal cytotoxicity and future work will focus on *in vivo* studies, formulations and routes of administration.

Materials and methods

(i) Drugs and chemicals

Stocks of amphotericin B deoxycholate (5.2 mM [aq]) (Fungizone; Gibco, UK) were prepared, aliquoted, and kept at -20°C until use. Chitosan with three different molecular weights and its derivatives were used and are summarised in Table 1 (28, 59, 60, 61). Solutions of chitosan and derivatives were prepared by dissolving 1 g in 100 ml of 1% (v/v) acetic acid solution at room temperature with continuous stirring for 24 h until a clear solution was obtained. The pH of the solution was adjusted to approximately 6 by adding sodium hydroxide 2N (NaOH, Sigma, UK) solution with a pH meter (Orion Model 420A). The chitosan solutions were autoclaved (121 °C; 15 mins). Phosphorylcholine substituted chitosan was kindly provided by Prof F Winnik (Montreal University, Canada) generated through reductive amination of PC-glyceraldehyde with primary amines of deacetylated chitosan (57kD). Percentage of substitution was controlled and determined by NMR (28). Chitosan pKa is approximately 6.3 and therefore, the approximate ionisation degree of chitosan is a 61% and 6% at pH 6.5 and 7.5 respectively.

382 **(ii) Ethics statement.**

383 All animal work is carried out under a UK Home Office project licence according to the
384 Animal (Scientific Procedures) Act 1986 and the new European Directive 2010/63/EU.
385 The Project Licence (70/8427) has been reviewed by LSHTM Animal Welfare & Ethical
386 Review Board prior to submission and consequent approval by the UK Home Office.

387 **(iii) Cell lines**

388 **Preparation of macrophages**

389 - Peritoneal mouse macrophages (PEMs) were obtained from 8-12 week old
390 female CD1-mice (Charles River Ltd, UK). Two ml of a 2% (w/v) starch solution in
391 phosphate buffered saline (PBS, Sigma, UK) was injected intraperitoneally (IP).
392 After 24 h, the animal was sacrificed and the PEMs were harvested by peritoneal
393 lavage with cold RPMI 1640 medium (Sigma, UK) containing 200 units penicillin
394 and 0.2 mg streptomycin/mL (PenStrep; Sigma, UK). Subsequently, PEMs were
395 centrifuged at 450 g at 4°C for 15 min and then the pellet was resuspended in
396 RPMI 1640 with 10% (v/v) heat-inactivated fetal calf serum (HiFCS; Gibco, UK).
397 - Bone marrow-derived macrophages (BMMs) were obtained from femurs of 8-12
398 week old female BALB/c mice (Charles River Ltd). Briefly, the bone marrow cells
399 were carefully flushed from the bone with Dulbecco's Modified Eagle's Medium
400 (DMEM; Thermofisher, UK) with 10% (v/v) HiFCS, 100 U/mL penicillin and 100
401 mg/mL streptomycin (Sigma, UK). Cells were pelleted by centrifugation (450 g,
402 10 min) and re-suspended in 10ml DMEM with 10% (v/v) HiFCS and human
403 macrophage colony stimulating factor 50ng/ml (HM-CSF; Thermofisher, UK).

After plating out in T175 flasks (Greiner Bio-One, Stonehouse, UK), BMMs were kept at 37°C, 5% CO_2 for 7-10 days after which they were harvested, counted and used.

- THP-1 cell is a human leukemic monocyte-like derived cell line. THP-1 cells were cultured in RPMI 1640 medium supplemented with L-glutamine and 10% HiFCS. THP-1 cells were incubated in RPMI 1640 plus 10% (v/v) HiFCS and 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, UK) at 37°C and 5% CO_2 for 72 h to induce maturation transformation of these monocytes into adherent macrophages (50).

Human squamous carcinoma (KB) cells are adherent cells derived from epidermal carcinoma from the mouth. KB cells were cultured in RPMI 1640 medium 10% HiFCS.

The number of cells and macrophages was estimated by counting with a Neubauer haemocytometer by light microscopy (x 400 total magnification).

(iv) Parasites

Four *Leishmania* species; two GFP labelled species (*L. major* (MHOM/SU/73/5ASKH) and *L. mexicana* (MNYC/BZ/62/M379), kindly donated by Dr. G Getti (University of Greenwich, UK) were used for the fluorescence microscope study. They were cultured in Schneider's insect medium (Sigma, UK) with 23% (v/v) HiFCS, 1× penicillin-streptomycin-glutamine (Gibco-Invitrogen) and supplemented with 700 µg/mL G418 (an aminoglycoside antibiotic, Sigma, UK). *L. major* (MHOM/SA/85/JISH118) and *L. mexicana* (MNYC/BZ/62/M379) were used for other experiments as described, minus the G418. Promastigotes were incubated at 26°C, maximum passage number used = 7.

(v) *In vitro* cytotoxicity assays

Re-suspended KB cells (4 x 10^4 /100uL) were allowed to adhere to the bottom of 96-well plate overnight and then exposed to specific concentrations of the compounds for 72 h at 37°C and 5% CO_2 incubator. Podophyllotoxin (Sigma, UK) was included as a positive control at a starting concentration of 0.05 µM. Cytotoxicity was evaluated by a cell viability assay using the resazurin sodium salt solution (AlamarBlue, Sigma, UK) which was prepared according to the manufacturer's instructions. 20µL of the resazurin solution was added to each well of the plates and fluorescence (cell viability(62)) was measured over a period of 1 to 24 h using a Spectramax M3 plate reader (EX/EM 530 / 580 nm and 550 nm cut off). Results were expressed as percentage inhibition = (100 – x)% viability (means ± standard deviation σ). Cytotoxicity was evaluated in RPMI 1640 at two pH values (at normal pH of RPMI 7.5 and at a lower pH 6.5). The pH of RPMI 1640 was reduced from 7.5 to 6.5 by adding 0.05M acidic buffer, 2-N-morpholino ethanesulfonic acid (MES, Sigma, UK). RPMI 1640 plus MES (0.05M) at pH=6.5 did not show any cytotoxicity to KB-cells.

(vi) *In vitro* 72 h activity of chitosan and its derivatives against extracellular *L. major and L. mexicana* promastigotes

Promastigotes in RPMI 1640 medium were tested while in the exponential growth phase. The promastigotes were diluted to a density of 5x10^6 promastigotes/ml and then exposed to different concentrations of (HMW, MMW, and LMW) chitosan, chitosan derivatives and Fungizone (positive control) in sterile 96-well flat bottom culture plates for 72 h at 26°C. The activity of the compounds against promastigotes was evaluated using the Alamar Blue™ assay as previously described. pH plays a critical role in the

449 solubility and protonation of chitosan, so the activity against promastigotes was
450 evaluated at two different pH values (pH=7.5 and a lower pH of 6.5 by adding MES).
451 Results were expressed as percentage inhibition= 100% - x% viability (means ± SD).

452 **(vii) *In vitro* 72- hour activity of chitosan and its derivatives against**
453 **intracellular amastigotes of *L. major and L. mexicana***

454 100uL of PEMs culture at 4 $_x$ 10^5 cells/mL, dispensed into each well of a 16-well LabTek
455 tissue culture slide (Thermo Fisher, UK) at pH 7.5 or pH 6.5 and incubated for 24 h at
456 37 °C in 5 % CO_2. After 24 h, the wells were washed with fresh culture medium to
457 remove non-adherent cells. Stationary phase, low-passage-number *Leishmania*
458 promastigotes were then added at a ratio of 5 :1 PEM. This infection ratio was
459 previously found to give sufficiently high and reproducible infection levels. Slides were
460 incubated for another 24h at 34 °C to mimic dermal temperatures in 5 % CO_2. Any free,
461 extracellular parasites were removed by washing the wells with cold culture medium.
462 One slide was fixed with 100 % methanol for 2 min and stained with 10 % Giemsa for 5
463 minutes. The number of PEMs infected with *Leishmania* amastigotes per 100
464 macrophages was microscopically counted. All the experiments were conducted at
465 macrophages infection levels above 80% prior to addition of chitosan. Chitosan, its
466 derivatives and Fungizone© solutions at a range of concentrations (in quadruplicate)
467 were added to the wells (100µl) and the slides were incubated for 72 h at 34 °C in 5 %
468 CO_2. After 72 h, the slides were fixed with 100% methanol for 2 min and stained with
469 10% Giemsa for 5 min. The slides were examined and the % of macrophages infected
470 was counted. The anti-leishmanial activity of compounds was expressed as percentage

reduction in infected macrophages compared to untreated control wells (63). RPMI 1640 plus MES (0.05M) with pH=6.5 had no activity against *Leishmania* amastigotes.

(viii) Influence of the origin of the host cell on the *in vitro* activity of HMW chitosan against *L. major* amastigotes

A further two host cell types, THP-1 and BMMs were infected with *Leishmania major* and the activity of HMW chitosan was assessed. THP-1 cells (cultured in RPMI 1640 + 10% HiFCS) and BMMs (cultured in DMEM + 10% HiFCS) were used to assess the host cell dependence of the anti-leishmanial activity of HMW chitosan(50). The experiment was conducted as described in section (vii) at pH 6.5.

(ix) The role of HMW chitosan on BMMs activation

We chose BMMs to evaluate the activation effects of HMW chitosan and to study the cell uptake of chitosan as this macrophage population is more homogenous than PEMs and THP-1 cells (64); both PEMs and BMMs have been reported to have a similar acidic pH ≈ 5.5 of parasitophorous vacuoles of *L. amazonensis* infected PEMs and BMMs (65, 66, 67). 100uL of BMMs (4 x 10^5/ml) in DMEM at pH=6.5 were dispensed into each well of 96 well plates (standard clear plates for nitric oxide assay and black wall/clear bottom plates for ROS and TNF-α assay) and incubated for 24 h at 37 °C in 5 % CO_2. Plates were washed with DMEM to remove non-adherent macrophages. *L. major* at 1:5 ratio (5 parasites per host cell) was then added to the wells and the plates were incubated for 24 h at 34 °C in 5 % CO_2 to allow infection of the adherent macrophages. After 24 h incubation with macrophages, infection rate more than 80%. The effects of HMW

chitosan on BMMs activation was determined by quantifying the release of TNF-α, ROS and NO, as described below at pH 6.5.

A. Measurement of TNF-α

HMW chitosan at concentrations of 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml was added to infected and uninfected macrophages (section x) and the plates were incubated for 4, 24 h at 34°C in 5% CO_2 . Lipopolysaccharides from *Escherichia coli* O26:B6 (LPS, 100ng/ml; Sigma, UK) was used as a positive control and inducer. TNF-α release by the BMMs was measured using a mouse TNF-α ELISA kit (ab208348, abcam, UK) according to the manufacturer's instructions using a Spectramax M3 microplate reader (wavelength 450 nm) .

B. Measurement of ROS

ROS was measured using a 2′,7′–dichlorofluorescein diacetate (DCFDA, cellular reactive oxygen species detection assay kit, abcam, UK). Uninfected and infected macrophages were treated with 25 µM DCFDA in PBS for 45 min at 37°C and then washed once in the buffer. The cells were cultured at 34°C in 5% CO_2 for 0.5, 1, 2, 4,8 and 24 h, with 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml of HMW chitosan or in the presence of H_2O_2 (25mM) (Thermofisher, UK) as a positive control in DMEM + 10% HiFCS (pH=6.5) in quadruplicate wells. In some experiments, cells were pre-treated with a selective inhibitor of ROS, N-acetyl-L-cysteine (NAC, 5mM; Sigma, UK), for 2 h before the addition of the inducer or chitosan. At 0.5, 1, 2, 4, 8 and 24 h the plates were read, using a Spectramax M3 microplate reader (Ex=485nm, Em=535nm).

C. Measurement of NO

514 NO was measured using Griess reagent (Thermofisher, UK). HMW chitosan at
515 concentrations of 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml was added to infected and
516 uninfected macrophages and the plates were incubated at 4, 24 h at 34°C in 5% CO_2.
517 LPS (100ng/ml) was used as a positive control. In some experiments, cells were pre-
518 treated with selective inhibitor of nitric oxide with NG-methyl-L-arginine acetate salt (0.4
519 mM, L-NMMA; Sigma, UK) for 2 h before the addition of LPS. NO was quantified
520 according to the kit protocol, Briefly, 150µl of the cell culture supernatants (particulates
521 were removed by centrifugation) was mixed gently with 150µl of the Griess reagent in a
522 96 well plates and the mixture was incubated for 30 minutes at room temperature. The
523 absorbance was measured using a Spectramax M3 plate reader (wavelength 548 nm).
524 Sodium nitrite (Sigma, UK) at different concentrations was used to create a standard
525 curve(68).

526 **(x) Uptake of chitosan by macrophages**

527 The uptake of HMW chitosan was evaluated using two methods. The first method used
528 two endocytosis inhibitors; cytochalasin D (1µg/ml , Sigma, UK) which is a phagocytosis
529 inhibitor and dynasore (30 µg/ml, Sigma, UK) which inhibits pinocytosis (clathrin-
530 mediated endocytosis (CME) by blocking GTPase activity of dynamin) (69, 70, 71) . The
531 second method used dynasore and rhodamine-labelled chitosan (MW 200 kDa,
532 Creative PEGWorks, USA) to track cellular uptake of chitosan over time by fluorescence
533 microscopy.

534

535

536 **A. Activity of chitosan after inhibition of the endocytic pathway of BMMs**

537 100uL of BMMs culture (4 x 10^5/ml) in DMEM at pH 6.5 or pH=7.5 were dispensed into
538 each well of 16-well LabTek™ culture slides and were infected with stationary phase *L.*
539 *major* promastigotes. Some of the infected BMMs were pretreated with dynasore (30
540 µg/ml) or cytochalasin D (1µg/ml) for two hours. Subsequently, HMW chitosan was
541 added to each well at concentrations of 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml and
542 macrophages were incubated for 4 or 24 h at 34 °C in 5 % CO_2. After each point, the
543 slides were examined as described in section (vii).The inhibition activity of the uptake
544 (phagocytosis or pinocytosis) of the two inhibitor was evaluated by using a fluorescence
545 plate reader, by using fluorescent latex beads and pHrodo™ Red dextran (72). We
546 showed that cytochalasin caused 94 and 84% phagocytosis inhibition of fluorescent
547 latex beads (Sigma-Aldrich, UK) after 4 h and 24 h respectively and dynasore caused
548 95 and 90% pinocytosis inhibition of pHrodo™ Red dextran (Mw= 10,000 MW, Thermo
549 Fisher, UK) after 4h and 24h respectively (Table S6).

550 **B. Microscopic imaging of the cellular uptake of rhodamine-labelled chitosan**

551 The qualitative characterisation of chitosan uptake of cells was carried out by wide field
552 microscopy (Nikon Ti-E inverted microscope). Briefly, after deriving BMMs, 500µl of the
553 BMMs (in DMEM plus 10% HiFCS at pH 6.5, 4 x 10^4 macrophages per ml) was seeded
554 on each well of a 4-well LabTek tissue culture slide (Thermo Fisher, UK) and incubated
555 for 24h at 37°C in 5% CO_2. Subsequently, 5 µg/mL Hoechst 33342 stain (Ex/Em =
556 350/461 nm, Thermofisher, UK) as a nuclear dye was added and the slides were
557 incubated for 30 min at 37°C in 5% CO_2. The macrophages were washed with PBS, *L.*
558 *major*-GFP of *L. mexicana*-GFP was then added, at a ratio of 10:1 and further incubated

for 24h at 34°C in 5% CO_2 (We used 10:1 ratio not 5:1 as previously as at this experiment different species of *L. major-GFP* and *L. mexicana-GFP* were used and the ratio 10:1 was sufficient to obtain a high infection rate). Macrophages were then washed with PBS and 500 µl of LysoTracker® far Red (50 nM, Ex/Em;647/668nm; Thermo Fisher, UK) was added to each well. The labelled, infected macrophages were then exposed to 30 µg/ml rhodamine-labelled chitosan (MW 200kDa, Creative PEGWorks, USA) in 500 µl of fresh DMEM plus 10% HiFCS pH 6.5 and incubated for 4 h and 24h at 37°C with live imaging at each time point. In some experiments, infected BMMs were pre-incubated with dynasore 30 µg/ml for 2 h before adding rhodamine-labelled chitosan. All the images were collected using a Nikon Ti-E inverted microscope equipped with (63x objective) using Nikon Elements software. Three images for each experiment were then analysed using ImageJ software. The degree of correlation between pixels in the red and green channels was assessed by the Colocalization Colormap plugin in the ImageJ software. This plugin enables quantitative visualisation of colocalization by calculating the normalized mean deviation product (nMDP) in a colour nMDP scale (from -1 to 1): negative refers (cold colours) to no colocalization while indexes more than 0 (hot colours) display colocalization and the higher number refers to more colocalization (73, 74).

(xi) Statistical analysis.

Dose-response curves and EC_{50} values were calculated using GraphPad Prism© version 7.02 software and the corresponding sigmoidal dose-response curves were established by using a nonlinear fit with variable slope models. Results represent means ± SD. EC_{50} values were compared by using extra-sum-of-squares F tests. t test was

used to compare differences between means of two or more groups respectively and p values of 0.05 were considered statistically significant.

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819 **Tables:**

TABLE 1 *In vitro* activity of chitosan and its derivatives against promastigotes at two pH values

Compound	pH=7.5 *				pH=6.5*,**			
	L. major		*L. mexicana*		*L. major*		*L. mexicana*	
	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml
Fungizone	0.05± 0.01	0.2± 0.02	0.14± 0.01	0.3± 0.03	0.07± 0.02	0.3± 0.1	0.13± 0.07	0.3± 0.02
HMW chitosan	105± 12	1549± 525	140± 12	2187± 928	5.9± 0.5	37± 9	10.4± 1.6	98± 33
MMW chitosan	113± 9	1277± 580	150± 12	2223± 681	6.2± 0.3	43± 8	10.9± 1.4	96± 27
LMW chitosan	118± 11	1238± 582	157± 13	2225± 723	6.7± 0.3	40± 8	10.2± 1.5	84± 28
Fungal chitosan	118± 11	1228± 560	150± 13	1991± 580	6.2± 0.3	42± 6	10.5± 1.3	61± 17
Chitosan Oligosaccharide	153± 15	1680± 506	190± 20	2366± 461	62.5± 4	446± 92	77± 2.7	452± 36
Chitosan Oligosaccharide- lactate	98± 9	1226± 130	125± 14	765± 83	14± 0.1	135± 2	23± 1.4	311± 25
Chitosan HCL	96± 7	1189± 211	110± 24	746± 169	13.2± 1	118± 34	20.8± 2.4	264± 61
PC1-CH(Phosphorylcholine substituted chitosan)	111± 20	1875± 230	176± 14	2832± 412	19.9± 2.8	187± 90	32± 2.2	328± 48
PC2-CH	104± 6	1485± 259	170± 8	2744± 377	16.5± 2.7	138± 49	28± 2.4	296± 53
PC3-CH	119± 19	1860± 365	187± 16	3175± 580	23.3± 2.5	218± 44	37± 2.5	442± 65
Carboxymethyl chitosan	No activity up to 400 µg/ml							

Experiments were conducted in triplicate cultures, data expressed as mean +/- SD (experiment was reproduced further two times with confirmed similar data not shown). *Statistically significant differences were found for the EC_{50} values of chitosan and its derivatives at pH=6.5 and pH=7.5 ($p<0.05$ by using t-test). ** *L. major* promastigotes were significantly more susceptible to chitosan and derivatives than *L. mexicana* (($p<0.05$ by an extra sum-of-squares F test)). Amphotericin B deoxycholate (Fungizone) was used as a positive control. Both RPMI alone pH 6.5 and chitosan solvent did not show any activity against promastigotes.

TABLE 2 *In vitro* activity of chitosan and its derivatives against amastigotes infecting PEMs and their cytotoxicity

Compound	pH 7.5*				pH 6.5*				pH 6.5**	
	L. major		*L. mexicana*		*L. major*		*L. mexicana*		KB cells	
	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µg/ml	EC_{90} µg/ml	LD_{50} µg/ml	LD_{90} µg/ml
Fungizone	0.07± 0.01	0.13± 0.05	0.19± 0.05	1.5± 0.2	0.06± 0.01	0.11± 0.06	0.18± 0.06	1.7± 0.3	58± 8	190± 9
HMW chitosan	98± 6	1635 ± 245	119 ± 9	1804 ± 304	11.4± 1	69± 18	15.4±2	103± 28	752± 90	3022± 366
MMW chitosan	103± 8	1652 ± 287	125± 10	1793 ± 323	12.9± 1	81± 18	16.3±2	122± 34	758± 89	3019± 400
LMW chitosan	102 ± 7	1651 ± 282	125± 10	1795 ± 320	12.1± 1	74± 14	16.1±2	116.6± 33	803± 90	3088± 420
Fungal chitosan	102 ± 7	1650± 276	124 ± 9	1796 ± 316	12.6±3	92± 27	16.9 ±2	144± 44	759± 91	3134± 380
Chitosan Oligosaccharide	145 ± 12	2473 ± 500	175 ± 14	2543 ± 505	73 ± 4	260± 32	86.2±6	288±39	765± 93	3232± 400
Chitosan Oligosaccharide-lactate	93 ± 7	1957 ± 174	120 ± 9	2365 ± 239	39± 1	201± 16	47±2	245± 23	754± 92	3058± 390
chitosan HCl	97 ± 11	2080± 516	121 ± 15	2402 ± 667	40± 2	210± 23	47.9±3	243± 33	781± 92	3589± 405
PC1-CH	144 ± 10	1292 ± 217	169 ± 12	1365 ± 212	68± 3	246± 26	81.7±6	274±38	756± 93	3364± 398
PC2-CH	133 ± 6	1005 ± 194	159 ± 6	1705 ± 170	60± 3	202± 22	71.9±5	237±36	800± 92	3709± 410
PC3-CH	163 ± 11	1052 ± 144	187± 10	1107 ± 142	71± 4	251± 30	83.5±6	286± 41	786± 93	3719± 378
Carboxymethyl chitosan	No activity up to 400 µg/ml								1184± 99	3999± 500

Experiments were conducted in quadruplicate cultures, data expressed as mean +/- SD (experiment was reproduced further two times with confirmed similar data and data not shown). *Statistically significant differences were found between the EC_{50} values of chitosan and its derivatives at pH=6.5 and pH=7.5 ($p<0.05$ by using t-test). Chitosan and its derivatives had a low cytotoxicity at both pH values (6.5 and 7.5) toward KB-cells and there was no significant difference in the cytotoxicity at these two pH values ($p <0.05$ by t-test). ** No statistically significant difference was found in LD_{50} (50% lethal dose) values between three types of chitosan and other derivatives against KB-cells (except carboxymethyl chitosan which is the least toxic) ($p>0.05$ by an extra sum-of-squares F test). Both RPMI alone pH 6.5 and chitosan solvent did not show any activity against amastigotes.

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TABLE 3 HMW chitosan activity against *L. major* amastigotes in three different macrophage cultures after 72 h at pH 6.5

	HMW chitosan		Fungizone	
Host cell / infection rate % at 24 h	EC_{50} µg/ml	EC_{90} µg/ml	EC_{50} µM	EC_{90} µM
PEMs / > 80%	10.31 ± 1.22*	89.07 ± 20.46	0.02 ± 0.004**	0.27 ± 0.07
BMMs / > 80%	14.60 ± 1.79*	145.7 ± 36.2	0.04 ± 0.005**	0.43 ± 0.1
THP-1/ > 80%	24.28 ± 2.87*	200.1 ± 48.8	0.08 ± 0.006**	1.15 ± 0.37

Experiments were conducted in quadruplicate cultures, data expressed as mean +/- SD (experiment was reproduced further two times with confirmed similar data and data not shown)*,** statistically significant difference in EC_{50} values between the three types of cells (chitosan and Fungizone were significantly more active in PEMs and BMMs compared with THP-1 cells) ($p<0.05$ by an extra sum-of-squares F test). % infection rate gives the percentage of infected macrophages. Both RPMI and DMEM alone pH 6.5 and chitosan solvent did not show any activity against amastigotes.

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TABLE 4 Details of chitosan and its derivatives used in the study

Compounds	Properties	Supplier
HMW (source: crustacean shells)	MW=310-375 KDa	Sigma, UK
MMW (source: crustacean shells)	MW=190-310 KDa	Sigma, UK
LMW (source: crustacean shells)	MW=50-190 KDa	Sigma, UK
Fungal chitosan (white mushroom)	MW=110-150 KDa	Dr. S Somavarapu
Chitosan oligosaccharide	MW=≤ 5KDa	Dr. S Somavarapu
Chitosan oligosaccharide lactate	MW=average Mn 5, oligosaccharide 60%	Dr. S Somavarapu
Chitosan- HCl	MW= 47 - 65 KDa	Dr. S Somavarapu

Carboxymethyl chitosan	MW=543.519 Da, level of substitution is 95%	Dr. S Somavarapu
PC1-CH (Phosphorylcholine substituted chitosan)	MW=33 KDa, PC(mol%)= 30	Prof F Winnik
PC2-CH	MW=108 KDa, PC(mol%)= 20	Prof F Winnik
PC3-CH	MW=109 KDa, PC(mol%)= 30	Prof F Winnik

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824 **Figures**

Fig 1 TNF-α production in uninfected and *L. major* infected BMMs after 24 h of exposure to 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml of chitosan at pH 6.5. The dose response in both uninfected and *L. major* infected BMMs was bell-shaped. TNF- α production was significantly decreased ($p < 0.05$ by t-test) by infecting the cells with *L. major.* Experiments were conducted in quadruplicate, data is expressed as mean +/- SD (experiment was reproduced further two times with confirmed similar data and data not shown). Positive control= BMMs treated with LPS 10 µg/ml. Negative control = BMMs not exposed to chitosan. *Initial macrophage infection rate was >80% after 24 h. Chitosan solvent did not cause any TNF-α production.

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Fig 2 ROS production in uninfected and *L. major* infected BMMs after 4 h of exposure to 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml of HMW chitosan at pH=6.5. High levels of ROS were induced by both uninfected and *L. major* infected BMMs exposed to HMW chitosan compared to those that were not ($P < 0.05$ by t-test). Maximum production of ROS occurred at 44.4 µg/mL of chitosan. ROS production by *L. major* infected BMMs was significantly lower compared to uninfected cells ($p < 0.05$ by t-test). Experiments were conducted in quadruplicate, data is expressed as mean +/- SD (experiment was reproduced a further two times with confirmed similar data (not shown). Positive control = BMMs treated with H_2O_2 25 mM (a known ROS inducer). Negative control = BMMs not exposed to chitosan. *Initial macrophage infection rate was >80% after 24 h. Chitosan solvent alone did not cause any ROS production.

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Fig 3 Activity of HMW chitosan against *L. major* amastigotes in BMMs* after 4 h, with and without ROS scavenger at pH = 6.5. Infected macrophages were pre-incubated with 5 mM NAC for 2 h, after which HMW chitosan at concentrations 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml was added and the cells were incubated for a further 4 h. Chitosan activity against intracellular amastigotes was evaluated as described in section (vii). Values are expressed as % inhibition of infection relative to untreated controls. After 4 h, there was no significant difference in the anti-leishmanial activity of chitosan after scavenging of ROS ($p > 0.05$ by t-test). Experiments were conducted in quadruplicate, data is expressed as mean +/- SD. Experiment was reproduced further two times with confirmed similar data (not shown). *Initial macrophage infection rate was >80% after 24 h.

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Fig 4 NO production in uninfected and *L. major* infected BMMs after 24 h of exposure to 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml of chitosan at pH = 6.5. The response in both uninfected and infected BMMS was bell-shaped in relation to chitosan concentration. Maximal production of NO was stimulated by 44.4 µg/mL of chitosan. NO production was significantly decreased ($p < 0.05$ by t-test) when the cells had been infected with *L. major.* Experiment was conducted in quadruplicate cultures, data expressed as mean +/- SD (experiment was reproduced a further two times with confirmed similar data and data not shown). Positive control = BMMs treated with LPS 10 µg/ml. Negative control = BMMs not exposed to chitosan. *Initial macrophage infection rate was >80% after 24 h. Chitosan solvent alone did not cause any NO production.

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Fig 5 Activity of HMW chitosan against *L. major* -infected BMMs* after 24 h in the presence or absence of an NO inhibitor at pH = 6.5. Infected macrophages were pre-incubated with the NO inhibitor L-NMMA (0.4 mM) for 2 h, following which HMW chitosan at concentrations 1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml was added and the cells were incubated for a further 24h. Chitosan activity against intracellular amastigotes was evaluated as described in section (vii). Values are expressed as % inhibition of infection relative to untreated controls. After 24h, there was no significant difference in the activity of chitosan after inhibition of NO ($p > 0.05$ by t-test). Experiment was conducted in quadruplicate cultures, data expressed as mean +/- SD. Experiment was reproduced a further two times and confirmed the results (data not shown). *Initial macrophage infection rate was >80% after 24 h.

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Fig 6 Activity of HMW chitosan against *L. major* infected BMMs* after 4 h, pH=6.5 (A), 24 h, pH=6.5 (B) and at 24h, pH=7.5 with or without phagocytosis inhibitor or pinocytosis (CME) inhibitor. We found that chitosan requires pinocytosis (CME) not phagocytosis by BMMs for killing of *L. major* amastigotes at pH = 6.5 and 7.5. BMMs were infected with stationary-phase promastigotes. Some of the infected macrophages were pre-incubated with cytochalasin D (phagocytosis inhibitor) or dynasore (pinocytosis (CME) inhibitor) and exposed to various concentrations (1.64, 4.9,14.8, 44.4, 133.3 and 400 µg/ml) of chitosan for 4 h and 24 h, followed by microscopic counting of the number of infected macrophages. There was no

significant difference in the activity of HMW chitosan after inhibition of phagocytosis ($p > 0.05$ by t-test). In contrast, a significant inhibition of chitosan-mediated parasite killing occurred in the presence of dynasore at two pH values ($p < 0.05$ by t-test). Values are expressed as % inhibition of infection relative to untreated controls. Experiment was conducted in quadruplicate cultures, data expressed as mean +/- SD>. Experiment was reproduced a further two times and confirmed the results (data not shown). *Initial macrophage infection rate was >80% after 24 h.

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Fig 7 Fluorescence microscopy images of the cellular uptake of rhodamine-labelled chitosan at 4h and 24 h at pH=6.5 by BMMs infected with *L. major*-GFP (XA) or with *L. mexicana*-GFP (XB). Blue represents the nuclei of BMMs. Green represents intracellular amastigotes, red represents labelled chitosan and yellow represents merged red chitosan and green *Leishmania*. Panels A-F represent the following: Infected BMMs unexposed to chitosan after 4 h (panel A) or 24 h (panel B); Infected BMMs exposed to chitosan after 4h (panel D) or 24 h (panel E); Infected BMMs unexposed to chitosan after 24 h (panel C) and Infected BMMs exposed to chitosan and pinocytosis inhibitor (dynasore) after 24 h (panel F)

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Fig 8 The structure of chitosan (60) and its derivatives, (chitosan HCl, carboxymethyl chitosan (61), chitosan oligosaccharide (60), PC-CH (reprinted with permission from reference 28) and chitosan oligosaccharide lactate (59))

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