Single-dose treatment for cutaneous leishmaniasis with an easily synthesized chalcone entrapped in polymeric microparticles

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Key Words:	Leishmania, chemotherapy, Drug delivery systems, local treatment, Chalcone
Abstract:	Cutaneous leishmaniasis (CL) is a major health problem in many countries and its current treatment involves multiple parenteral injections with toxic drugs and requires intensive health services. Previously, the efficacy of a single subcutaneous injection with a slow-release formulation consisting of poly(lactide-co-glycolide) (PLGA) microparticles loaded with an antileishmanial 3-nitro-2-hydroxy-4,6-dimethoxychalcone (CH8) was demonstrated in mice model. In the search for more easily synthesized active chalcone derivatives, and improved microparticle loading, CH8 analogues were synthesized and tested for antileishmanial activity in vitro and in vivo. The 3-nitro-2′,4′,6′-trimethoxychalcone (NAT22) analogue was chosen for its higher selectivity against intracellular amastigotes (selectivity index = 1489, as compared with 317 for CH8) and more efficient synthesis (89% yield, as compared with 18% for CH8). NAT22 was loaded into PLGA / polyvinylpyrrolidone (PVP) polymeric blend microspheres (NAT22-PLGAk) with average diameter of 1.9 μm. Although NAT22-PLGAk showed similar activity to free NAT22 in killing intracellular parasites in vitro (IC50 ~ 0.2 μM), in vivo studies in Leishmania amazonensis – infected mice demonstrated the significant superior efficacy of NAT22-PLGAk to reduce the parasite load. A single intralesional injection with NAT22-PLGAk was more effective than eight injections with free NAT22. Together, these results show that NAT22-PLGAk is a promising alternative for single-dose localized treatment of CL.

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1. ABSTRACT

Cutaneous leishmaniasis (CL) is a major health problem in many countries and its current treatment involves multiple parenteral injections with toxic drugs and requires intensive health services. Previously, the efficacy of a single subcutaneous injection with a slow-release formulation consisting of poly(lactide-co-glycolide) (PLGA) microparticles loaded with an antileishmanial 3-nitro-2-hydroxy-4,6-dimethoxychalcone (CH8) was demonstrated in mice model. In the search for more easily synthesized active chalcone derivatives, and improved microparticle loading, CH8 analogues were synthesized and tested for antileishmanial activity *in vitro* and *in vivo*. The 3-nitro-2',4',6'-trimethoxychalcone (NAT22) analogue was chosen for its higher selectivity against intracellular amastigotes (selectivity index = 1489, as compared with 317 for CH8) and more efficient synthesis (89% yield, as compared with 18% for CH8). NAT22 was loaded into PLGA / polyvinylpyrrolidone (PVP) polymeric blend microspheres (NAT22-PLGAk) with average diameter of 1.9 µm. Although NAT22-PLGAk showed similar activity to free NAT22 in killing intracellular parasites *in vitro* (IC_{50} ~ 0.2 µM), *in vivo* studies in *Leishmania amazonensis* – infected mice demonstrated the significant superior efficacy of NAT22-PLGAk to reduce the parasite load. A single intralesional injection with NAT22-PLGAk was more effective than eight injections with free NAT22. Together, these results show that NAT22-PLGAk is a promising alternative for single-dose localized treatment of CL.

Keywords: *Leishmania*; chemotherapy, drug delivery systems, local treatment, chalcone.

2. INTRODUCTION

Leishmaniasis is a neglected disease caused by intracellular protozoans of the genus *Leishmania*. Depending on the parasite species and the patient's immune status, this disease may present a wide spectrum of clinical manifestations ranging from cutaneous leishmaniasis (CL), the most benign form, to life-threatening visceral leishmaniasis (VL). Although not fatal, CL is the most common form of the disease, which is endemic in 87 countries and affects almost 1 million people every year, according to World Health Organization (WHO) estimates (WHO, 2017). Uncomplicated CL is characterized by one or more skin ulcers at the sandfly bite site that evolves for weeks to months, normally leaving a permanent scar. In some individuals the disease may progress to extremely morbid diffuse or mucosal forms (Aronson and Joya, 2019). Currently available treatments for CL are toxic and require intensive health services. For example, first-line therapy uses 20-30 daily intramuscular or intravenous injections with antimonials, pentamidine, or amphotericin B, which produces severe adverse reactions and poor patients' compliance. Liposomal formulations of amphotericin B have appeared as a less toxic alternative treatment, but the high cost and invasive administration have limited their widespread use (Uliana *et al.*, 2017). An ideal CL treatment should not only heal the cutaneous lesions, but also prevent the development of the more morbid forms of disease (Aronson and Joya, 2019).

Chalcones have appeared as a promising new class of antileishmanials (de Mello *et al.*, 2014; Ortalli *et al.*, 2018). They are characterized by the presence of a 1,3-diphenylprop-2-en-1-one scaffold and have, in addition to their antileishmanial effects, a broad spectrum of pharmacological activities. Indeed, chalcones are known to affect bacteria (Kunthalert *et al.*, 2014; Li *et al.*, 2016), fungi (Tiwari *et al.*, 2010; Łacka *et al.*, 2011) and helminths (De Castro *et al.*, 2015), and to have immunosuppressive properties (Luo *et al.*, 2012). We have previously demonstrated the activity *in vitro* and *in vivo* of a natural methoxychalcone and its synthetic derivatives against *Leishmania*

amazonensis (Torres-Santos *et al.*, 1999a; Boeck *et al.*, 2006). Its synthetic analogue 3-nitro-2'-hydro-4',6'-dimethoxychalcone, named CH8, displayed the highest *in vitro* selectivity index against *L. amazonensis* (S.I. = 318 in relation to murine macrophages). Additionally, intralesional injections with CH8 were more effective than the reference drug sodium stibogluconate in a murine model of CL caused by *L. amazonensis* (Boeck *et al.*, 2006). The oral efficacy and safety of CH8 were also demonstrated in BALB/c mouse infections with *L. amazonensis* and *L. infantum* (Sousa-Batista *et al.*, 2018a). More recently, the efficacy of a single injection with CH8 was achieved by loading the drug in biodegradable poly (lactic-co-glycolic acid) (PLGA) microparticles (Sousa-Batista *et al.*, 2018b). The drug encapsulation allows prolonged release in the lesion and, in leishmaniasis, has the advantage of being taken up by phagocytic macrophages (Sousa-Batista and Rossi-Bergmann, 2018). Although CH8 is a promising drug candidate, its burdensome synthesis and purification is a pitfall in pharmaceutical development. Aiming to solve this problem, in the present work we proposed to identify active CH8 analogues with easier purification and better synthesis yield. The ultimate goal of this study was to choose the best analogue to encapsulate in PLGA microparticles and, thus, to develop an optimized delivery system for local and single-dose treatment of CL.

3. METHODOLOGY

3.1. Chalcones

Chalcone CH8 (3-nitro-2-hydroxy-4,6-dimethoxychalcone) was synthesized by aldol condensation as previously described (Boeck *et al.*, 2006). All starting materials were commercially obtained (Merck, Germany). Fifty analogues were produced but only those with acceptable synthesis yield are described here. The chalcone analogue 1 (NAT 1) was prepared by a condensation reaction in which the appropriately substituted acetophenone (1 equiv) was added to an aqueous alcoholic solution (40% v/v EtOH in water) of sodium hydroxide (3% w/v NaOH in water; 3 equiv) with stirring and

with cooling of the reaction mixture in an ice bath during the addition. The mixture was allowed to warm to room temperature (25 ºC), and the appropriately substituted benzaldehyde (1 equiv) was added with vigorous stirring until the product precipitation (Kumar *et al.*, 1985). The analogues NAT22, NAT28, NAT31, and NAT49 were prepared by Claisen-Schmidt condensation (Amslinger *et al.*, 2012) between aromatic acetophenones (1 equiv) and corresponding aldehydes (1 equiv) in methanol- $Ba(OH)_2$ (1 equiv) at 50 ºC with magnetic agitation for 12–48 h. The products were purified by column chromatography or on preparative TLC plates to yield the corresponding chalcone. For the analogues NAT31 and NAT49, a further step of deprotection was done: to each chalcone in dry CH_2Cl_2 was added dropwise a solution of BCl_3 in hexane at –78 °C (Amslinger *et al.*, 2012), after which the solution was stirred for 3 h at 0 °C. All of the synthesized compounds have been previously described, and their spectral data corresponded to that in the literature. Individual yields of the compounds were as follows: NAT1 = 61%; NAT22 = 89%; NAT28 = 81%; NAT31 = 12% and NAT49 = 13%.

3.2. PLGAk and NAT22-PLGAk microparticles

Microparticles were prepared by an emulsion and solvent evaporation process. The polymers Poly(lactide-co-glycolide) 50:50 (PLGA - Purac 504, Purasorb, Corbion, Netherlands) and polyvinylpyrrolidone (PVP - Kolidon K17, BASF, Germany), at final concentration of 90% and 10% were dissolved in dichloromethane (DCM – Vetec, Brazil). NAT22 (10%) was dissolved in this solution and the resulting homogenous mixture injected into an aqueous phase containing Polyvinyl alcohol (PVA - Sigma-Aldrich, EUA), 3% (w/v). The mixture was emulsified by agitation in an Ultra-Turrax T25 Basic (Ika, Germany) for 2 min at 13000 rpm on ice. The organic phase evaporation was performed under reduced pressure using a rotary evaporator (LOGEN Scientific, Brazil). After solvent full evaporation, the obtained microparticles (NAT22-PLGAk) were washed

with water and centrifuged at 9000 rpm at 4 ºC. Finally, the microparticles were dispersed in trehalose 5% and cooled to −18 °C for subsequent freeze-drying (FreeZone 1 lyophilizer, Labconco Corporation, EUA). The freeze-dried microparticles were stored at 4 °C. Empty microparticles (PLGAk) were prepared following the same procedure, but without added NAT22.

Average particle size and its distribution were measured by a laser light scattering analyzer (Zetasizer 3000, Malvern Instruments, Malvern). The particles were dispersed in 0.5% (w/w) Tween-20 solution and subsequently submitted to ultrasound for 20 s.

The drug present in the microparticles was determined directly by measuring the amount of NAT22 entrapped in the microparticles. Briefly, microparticles were accurately weighed and dissolved in 5 mL acetonitrile (Vetec, Brazil) by bath sonication until complete solubilization was achieved. Absorbance of appropriately diluted stock solutions was measured at 320 nm (SpectraMax M5), and the NAT22 concentration was calculated using calibration curves (correlation coefficient > 0.999) over a concentration range of 10 to 60 mg/L NAT22. All samples were measured in triplicate.

The shape and surface of the microparticles was studied using a scanning electron microscope (SEM, JSM-5600LV, JEOL, Japan) operating at an acceleration voltage of 20 kV under nitrogen atmosphere. Before SEM analysis, the dry microparticles were coated by sputtering with gold (JFC-1300, JEOL, Japan).

3.3. *In vitro* antileishmanial activity

Antileishmanial drug activity was determined against both the promastigote and the intracellular amastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa strain).

For antipromastigote studies, 2×10^5 promastigotes/mL of medium 199 supplemented with 5% heat-inactivated fetal bovine serum (HIFBS - Cutilab, Brazil) were first incubated in triplicate with each compound (0, 0.1, 1, 10 and 100 µM) to determine the IC_{50} range, and again with 2- fold dilutions

for a more precise determination. All cultures including controls contained 1% dimethyl sulfoxide (DMSO – Sigma-Aldrich, EUA). After 72 h at 26 °C, cell viability was measured by the MTS Cell Proliferation Colorimetric Assay Kit (Promega, EUA) using a plate-reader spectrometer (SpectraMax M5, Molecular Devices, EUA) at 490 nm. The results were expressed as the drug concentration that inhibited parasite growth by 50% (IC_{50}, calculated as in 3.6 below).

For anti-amastigote activity, 5×10^5 mouse peritoneal macrophages were plated on glass coverslips and infected with 5×10^6 promastigotes (1:10) at 34 °C for 4 h, when non-internalized parasites were washed away with phosphate buffered saline (PBS). After 24 h of incubation in RPMI supplemented with 5% HIFBS at 37°C, the amastigote-infected macrophages were treated with varying concentrations of compounds for 48 h. NAT22-PLGAk concentrations were relative to NAT22 content, while PLGAk concentrations were relative to its content in NAT22-PLGAk. Treatment time in the particle assay was increased to 96 h to allow more time for polymer degradation and drug release (Sousa-Batista *et al.*, 2018c). Then, coverslips were stained with Giemsa for cell counting under microscope (400x). Parasite loads in culture were calculated as total numbers of amastigotes / 200 total macrophages.

3.4. Macrophages viability evaluation

Adherent mouse peritoneal macrophages were cultured in triplicate at 37 °C with two-fold dilutions of compounds for 48 h (analogue assay) or 96 h (microparticle assay). The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was measured using an assay kit (Doles, Brazil) and a plate-reader spectrometer (SpectraMax M5) at 340 nm, as a cytotoxicity indicator. Maximum and minimum release values were obtained with cells cultured with 2% Triton X-100 or medium, respectively, and LDH release was calculated as percent of the positive control. Equation: % specific release = [(test release - spontaneous release) / (maximal release - spontaneous

release)] x 100. Representative results from three independent experiments were expressed as the drug cytotoxic concentration for 50% of cell in culture (CC_{50}).

3.5. *In vivo* antileishmanial activity and ethical standards.

The CL model used was 8-week-old female BALB/c mice weighing about 23 g, as previously approved by the institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol number CAUAP118). To compare the efficacy of CH8 and NAT22 against CL, five BALB/c mice / group were infected in the ear pinna with 10^6 promastigotes of green fluorescence protein GFP- *L. amazonensis* (Costa *et al.*, 2011) at the stationary phase of growth. After 7 days of infection, animals were given local s.c. injections with 1.2 mg/kg of NAT22 (3.3 µg /injection) or 10 µl of the vehicle alone (PBS) twice a week during 4 weeks (Sousa-Batista *et al.*, 2018b). Alternatively, they received a total of 8 injections with NAT22 alone twice a week during 4 weeks. The parasite loads in the individual lesions were expressed as arbitrary fluorescence units (Demicheli *et al.*, 2004). The fluorescence intensity of contralateral uninfected ears was subtracted from that of the treated ears. Alternatively, mice were given a single s.c. injection with 3.3 µg of NAT22 alone or equivalents in NAT22-PLGAk. Controls received the same dose of empty PLGAk, or 10 µl of PBS alone. On day 32 of infection, animals were anesthetized with isoflurane, sacrificed by cervical dislocation, the ears removed, grounded and assayed by limiting dilution assay (LDA) for determination of parasite loads (Lima *et al.*, 1997).

3.6. Statistical analysis

Groups were compared using unpaired Student´s *t* test and considered different when $p < 0.05$. IC_{50} and CC_{50} values were calculated by logarithmic regression analysis from a sigmoidal dose-response

curve. Data was normalized to run from 0% to 100% using a normalized dose-response equation. All analyses were conducted using GraphPad Prism 5 software.

4. RESULTS

The need for CH8 analogues with better yields led us to synthesize approximately 50 molecules, however only the chalcones that could be synthesized more efficiently than CH8 will be shown in this work. Table 1 contains the synthesis efficiency of 5 analogues as well as their antileishmanial activity. The overall yield for each was superior to that obtained with CH8 (18%), with the highest yield (89%) being obtained for NAT22. The inhibitory activity of the analogues was tested against the amastigote and promastigote forms of *L. amazonensis*. CH8 displayed the highest antipromastigote activity (IC_{50} = 0.7 µM), followed by NAT22 (IC_{50} = 1.9 µM). All the compounds displayed potent inhibitory effects against the amastigote form of *L. amazonensis*, with NAT22 displaying the highest activity (IC_{50} = 0.1 µM). None of the analogues had detectable cytotoxicity against murine macrophages, as indicated by the low specific release of LDH enzyme (CC_{50} > 100 µM). As previously noted, the nitro group is important for optimal antiparasitic effect (activity / selectivity), as demonstrated by the superior performance of CH8 *vs.* NAT49 and of NAT22 *vs.* NAT28.

Since NAT22 was the most active compound and its synthesis was simpler and more efficient than that of CH8 (85% and 18%, respectively), this compound was chosen for evaluation of its efficacy in a murine model of CL. Intralesional treatment with NAT22 demonstrated significant efficacy in reducing parasite load when compared to untreated controls. At the same treatment regimen (8 doses of 3.3 µg each), although NAT22 was slightly more effective than CH8 in preventing parasite growth, the difference was not statistically significant ($p>0.05$) (Fig 1).

With the goal of improving its local efficacy in CL, NAT22 was loaded into biodegradable polymeric microparticles. To this end, microparticles composed of a mixture of PLGA and PVP polymers were prepared by a solvent evaporation technique, yielding particles with 9.5% encapsulated NAT22 (NAT22-PLGAk). Empty microparticles were also prepared. The loaded and empty microparticles showed similar topography, with a round smooth surface (Fig 2B) and diameters of 1.90 ± 0.01 µm (dispersion = 2.30 ± 0.07 µm) and 2.60 ± 0.13 µm (dispersion = 3.57 ± 2.10 µm) respectively, as measured by light laser scanner (not shown). Figure 2A shows by SEM the crystalline structures of unencapsulated NAT22. Drug crystals were not seen outside the microparticles allowing a durable effect, suggesting a high drug internalization (Fig 2B).

When NAT22 was tested for inhibition of intracellular amastigotes, entrapment into microparticles did not significantly increase drug activity ($p>0.05$), as shown in Table 2. None of the NAT22 presentations were cytotoxic to macrophages as measured by release of the cytoplasmic enzyme LDH (Table 2). Finally, we tested the efficacy of single-dose subcutaneous treatment with NAT22-PLGAk in CL (Fig. 3). The safety of PLGA s.c. injection was previously demonstrated in mice using PLGA- CH8 formulation (Sousa-Batista *et al.*, 2018b). Mice receiving a single intralesional injection with NAT22-PLGAk had, 25 days later, only 11% of the number of parasites present in animals receiving the PBS vehicle alone (100%). The resulting efficacy was higher than that obtained with a single or even eight doses of unencapsulated NAT22.

5. DISCUSSION

Previously, we have described the promising efficacy of the nitrochalcone CH8 for the intralesional treatment of CL caused by *L. amazonensis* (Boeck *et al.*, 2006). However, the low synthesis yields together with difficult drug purification showed to be a bottleneck in the process scale up due to

212 interconversion of a percentage of resulting CH8 with a flavanone. Five CH8 analogues were then
213 synthesized with fewer purification steps and higher yields.
214 Previous studies have shown that modifications in the positions of substituents in the structure of
215 chalcones can improve their antileishmanial effects (Nielsen *et al.*, 1998; Kayser and Kiderlen, 2001;
216 Boeck *et al.*, 2006). We produced five easily synthesized CH8 analogues bearing modifications in
217 both aromatic rings and observed that the nitro group was important for their ability to inhibit *L.*
218 *amazonensis* growth *in vitro*. The nitro group has also been demonstrated to be important in anti-
219 trypanosome activity (Patterson and Wyllie, 2014). The nitrochalcone NAT22 was selected for
220 further *in vivo* studies due to its high synthesis yield and better antileishmanial activity.
221 A single intralesional injection with NAT22 resulted in 40% reduction in local parasite load
222 measured 3 weeks later. That effect is similar to observed with CH8 (37%), although *in vitro* NAT22
223 demonstrated an anti-amastigote activity greater than CH8 (0.1 and 1.4 μM, respectively). The
224 relative low water solubility and large crystals shown in Fig.2 may have provided a slow dissolving
225 depot in the injection site, providing a longer lasting effect. Although an effective and short
226 treatment for CL is highly desirable, the problem with the crystalline nature is needle clogging and
227 difficult dose reproducibility.
228 To circumvent NAT22's poor water solubility and chemical stability, we attempted to entrap it in
229 PLGA particles, as demonstrated previously with CH8 (Sousa-Batista *et al.*, 2018b). PLGA, a
230 copolymer of lactic acid and glycolic acid, has been shown to be safe and effective for use in
231 sustained-release drug delivery systems, and has also been clinically approved for other uses, such as
232 absorbable suture threads, orthopedic scaffolds, and treatment of localized tumors (Ortega-Oller *et*
233 *al.*, 2015; Wan and Yang, 2015). In order to increase NAT22 loading efficiency, reduce the initial
234 burst release and to improve delivery characteristics, similar to that previously observed for CH8,
235 PLGA was blended with the polymer polyvinylpyrrolidone (PVP) (Sousa-Batista *et al.*, 2018c). This

PVP effect is due to its higher solubility in organic polymer solution that result in extensive diffusion of PVP molecules into the dispersed droplets of polymer solution during microparticle preparation and its high capacity to interact with drug by hydrogens bonds (Meeus *et al.*, 2013, 2015). Thus, the PLGA/PVP polymer blend (PLGAk) was used to improve NAT22-PLGA interaction, and the process was adjusted to provide particles with an average diameter of 1.9 µm, a size large enough to allow phagocytosis by macrophages and yet prevent absorption into blood circulation. Such particle size provides a potential advantage compared to both injected intralesional antimonials that require repeated injections (Oliveira-Neto *et al.*, 1997) and smaller poly-lactide nanoparticles (Torres-Santos *et al.*, 1999b) or larger PLGA microparticles (Sousa-Batista *et al.*, 2018b) that have been used to carry subcutaneous chalcone implants. Additionally, the NAT22-PLGAk formulation is a single dose treatment, meeting the DNDi Target Product Profile for CL that recommends the use of treatments with few injections, local effect and low cost. It is worth noting that despite the technology adding cost, a single dose treatment will have positive impact in reduced hospital and mobility costs.

We found NAT22-PLGAk inhibited parasite growth inside macrophages *in vitro*, but this effect was not superior to unencapsulated NAT22, probably due to the fact that the time employed in the *in vitro* assay was not sufficient to achieve maximum chalcone release from the microparticles, as seen previously with CH8-PLGA (Sousa-Batista *et al.,* 2018b). It is conceivable that PVP has further slowed the intracellular release, and therefore less NAT22 was available to produce its effect on the parasite during the 96-hour assay. Recently, we reported that amphotericin B release from PLGA microparticles is much faster in the subcutaneous ear tissue (30 days) than in saline solution *in vitro* (200 days), possibly because of presence of enzymatic hydrolysis in the tissue (Sousa-Batista *et al.,* 2019). Whether or not NAT22 release from PLGAk microparticles follows the same kinetics remains to be determined. In addition to showing good anti-amastigote activity, NAT22-PLGAk did not induce cytotoxic effects against mammalian macrophages, supporting its safety and promise as a

therapeutic tool *in vivo*. It appears that the anti-amastigote effect of NAT22-PLGAk is due to intracellular drug targeting rather than to macrophage activation of NO production, as this function was not affected in treated macrophages (data not shown). This is in accordance with the results obtained previously with free or encapsulated CH8 (Sousa-Batista *et al.*, 2018b) and with the well-known antioxidant (Sivakumar *et al.*, 2011) and anti-inflammatory (Herencia *et al.*, 1999) properties of chalcones. However, other potential microbicidal mechanisms of NAT22-PLGAk should not be discarded. Since, although to a much lesser extent than NAT22-PLGAk, empty PLGAk alone displayed some inhibitory effect on the intracellular parasites (IC_{50} > 10 µM), suggestive of a macrophage stimulatory effect of PLGA microparticles (Luzardo-Alvarez *et al.*, 2005).

In this work, the use of NAT22 plus the polymer blend (PLGA/PVP) in the microparticle composition allowed an increased drug content (9.5% w/w), in contrast to the previous CH8 plus PLGA which maximally incorporated 7.8% of drug (Sousa-Batista *et al.*, 2018b). More importantly, the easier and more efficient NAT22 synthesis yield (89%) as compared with CH8 (18%) will further reduce production costs. Because the currently available treatments for localized CL are invasive and produce systemic side effects, NAT22-PLGAk appears promising for single-dose local use. The mouse ear model of CL used here appears translatable to the human infection as the drug is discharged in the subcutaneous tissue not on the skin surface where differential skin permeability would be more critical. Future studies on local drug kinetics, evaluation of efficacy over extended time periods, and use of other relevant parasite species like *L. braziliensis* and *L. tropica* will add further insight to this new mode of CL therapy.

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7. CONFLICTS OF INTEREST

None

8. REFERENCES

Amslinger, S., Al-Rifai, N., Winter, K., Wörmann, K., Scholz, R. and Wild, P. B. and M. (2012). Reactivity Assessment of Chalcones by a Kinetic Thiol Assay. *Organic & Biomolecular Chemistry* 2–5. doi:10.1039/b000000x.

Aronson, N. E. and Joya, C. A. (2019). Cutaneous Leishmaniasis: Updates in Diagnosis and Management. *Infectious Disease Clinics of North America* **33**, 101–117. doi:10.1016/j.idc.2018.10.004.

Boeck, P., Bandeira Falcão, C. A., Leal, P. C., Yunes, R. A., Filho, V. C., Torres-Santos, E. C. and Rossi-Bergmann, B. (2006). Synthesis of chalcone analogues with increased antileishmanial activity. *Bioorganic and Medicinal Chemistry* **14**, 1538–1545. doi:10.1016/j.bmc.2005.10.005.

Costa, S.S ; Golim M.A. ; Rossi-Bergmann B. ; Costa, F. T. M. ; Giorgio, S. (2006). Use of in vivo and in vitro systems to select *Leishmania amazonensis* expressing green fluorescent protein. *Korean Journal of Parasitology* **49**, 357-364. doi:10.3347/kjp.2011.49.4.357.

De Castro, C. C. B., Costa, P. S., Laktin, G. T., De Carvalho, P. H. D., Geraldo, R. B., De Moraes, J., Pinto, P. L. S., Couri, M. R. C., Pinto, P. D. F. and Da Silva Filho, A. A. (2015). Cardamonin, a schistosomicidal chalcone from Piper aduncum L. (Piperaceae) that inhibits Schistosoma mansoni ATP diphosphohydrolase. *Phytomedicine* **22**, 921–928. doi:10.1016/j.phymed.2015.06.009.

de Mello, T. F. P., Bitencourt, H. R., Pedroso, R. B., Aristides, S. M. A., Lonardoni, M. V. C. and Silveira, T. G. V. (2014). Leishmanicidal activity of synthetic chalcones in Leishmania (Viannia) braziliensis. *Experimental Parasitology* **136**, 27–34. doi:10.1016/j.exppara.2013.11.003.

Demicheli, C., Ochoa, R., da Silva, J. B. B., Falcão, C. a B., Rossi-Bergmann, B., de Melo, A. L., Sinisterra, R. D. and Frézard, F. (2004). Oral delivery of meglumine antimoniate-beta-cyclodextrin complex for treatment of leishmaniasis. *Antimicrobial Agents and Chemotherapy* **48**,

100–103. doi:10.1128/AAC.48.1.100.

Herencia, F., Ferrándiz, M. L., Ubeda, A., Guillén, I., Dominguez, J. N., Charris, J. E., Lobo, G. M. and Alcaraz, M. J. (1999). Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclooxygenase-2 in mouse peritoneal macrophages. *FEBS Letters* **453**, 129–134. doi:10.1016/S0014-5793(99)00707-3.

Kayser, O. and Kiderlen, A. F. (2001). In vitro leishmanicidal activity of aurones. *Phytotherapy Research* **15**, 148–152. doi:10.1055/s-1999-13993.

Kumar, C. V., Ramaiah, D., Das, P. K. and George, M. V. (1985). Photochemistry of aromatic .alpha.,.beta.-epoxy ketones. Substituent effects on oxirane ring-opening and related ylide behavior. *The Journal of Organic Chemistry* **50**, 2818–2825. doi:10.1021/jo00216a003.

Kunthalert, D., Baothong, S., Khetkam, P., Chokchaisiri, S. and Suksamrarn, A. (2014). A chalcone with potent inhibiting activity against biofilm formation by nontypeable *Haemophilus influenzae*. *Microbiology and Immunology* **58**, 581–589. doi:10.1111/1348-0421.12194.

Łacka, I., Konieczny, M. T., Bułłakowska, A., Rzymowski, T. and Milewski, S. (2011). Antifungal action of the oxathiolone-fused chalcone derivative. *Mycoses* **54**, 407–414. doi:10.1111/j.1439-0507.2010.01936.x.

Li, H., Chen, Y., Zhang, B., Niu, X., Song, M., Luo, Z., Lu, G., Liu, B., Zhao, X., Wang, J. and Deng, X. (2016). Inhibition of sortase A by chalcone prevents Listeria monocytogenes infection. *Biochemical Pharmacology* **106**, 19–29. doi:10.1016/j.bcp.2016.01.018.

Lima, H. C., Bleyenberg, J. A. and Titus, R. G. (1997). A simple method for quantifying Leishmania in tissues of infected animals. *Parasitology Today* **13**, 80–82. doi:10.1016/S0169-4758(96)40010-2.

Luo, Y., Song, R., Li, Y., Zhang, S., Liu, Z.-J., Fu, J. and Zhu, H.-L. (2012). Design, synthesis, and biological evaluation of chalcone oxime derivatives as potential immunosuppressive agents.

Bioorganic & Medicinal Chemistry Letters **22**, 3039–3043. doi:10.1016/j.bmcl.2012.03.080.

Luzardo-Alvarez, A., Blarer, N., Peter, K., Romero, J. F., Reymond, C., Corradin, G. and Gander, B. (2005). Biodegradable microspheres alone do not stimulate murine macrophages in vitro, but prolong antigen presentation by macrophages in vitro and stimulate a solid immune response in mice. *Journal of Controlled Release* **109**, 62–76. doi:10.1016/j.jconrel.2005.09.015.

Meeus, J., Scurr, D. J., Amssoms, K., Davies, M. C., Roberts, C. J. and Van Den Mooter, G. (2013). Surface characteristics of spray-dried microspheres consisting of PLGA and PVP: Relating the influence of heat and humidity to the thermal characteristics of these polymers. *Molecular Pharmaceutics* **10**, 3213–3224. doi:10.1021/mp400263d.

Meeus, J., Scurr, D. J., Appeltans, B., Amssoms, K., Annaert, P., Davies, M. C., Roberts, C. J. and Van Den Mooter, G. (2015). Influence of formulation composition and process on the characteristics and in vitro release from PLGA-based sustained release injectables. *European Journal of Pharmaceutics and Biopharmaceutics* **90**, 22–29. doi:10.1016/j.ejpb.2014.11.009.

Nielsen, S. F., Christensen, S. B., Cruciani, G., Kharazmi, A. and Liljefors, T. (1998). Antileishmanial Chalcones : Statistical Design , Synthesis , and Three-Dimensional Quantitative Structure - Activity Relationship Analysis. *J. Med. Chem.* **41**, 4819–32. doi:https://doi.org/10.1021/jm980410m.

Oliveira-Neto, M. P., Schubach, A., Mattos, M., da Costa, S. C. and Pirmez, C. (1997). Intralesional therapy of American cutaneous leishmaniasis with pentavalent antimony in Rio de Janeiro, Brazil - an area of Leishmania (V.) braziliensis transmission. *International Journal of Dermatology* **36**, 463–468. doi:https://doi.org/10.1046/j.1365-4362.1997.00188.x.

Ortalli, M., Ilari, A., Colotti, G., De Ionna, I., Battista, T., Bisi, A., Gobbi, S., Rampa, A., Di Martino, R. M. ., Gentilomi, G. A., Varani, S. and Belluti, F. (2018). Identification of chalcone-based antileishmanial agents targeting trypanothione reductase. *European Journal of Medicinal*

Chemistry **152**, 527–541. doi:https://doi.org/10.1016/j.ejmech.2018.04.057.

Ortega-Oller, I., Padial-Molina, M., Galindo-Moreno, P., O'Valle, F., Jódar-Reyes, A. B. and Peula-García, J. M. (2015). Bone Regeneration from PLGA Micro-Nanoparticles. *BioMed Research International* **2015**,. doi:10.1155/2015/415289.

Patterson, S. and Wyllie, S. (2014). Nitro drugs for the treatment of trypanosomatid diseases: Past, present, and future prospects. *Trends in Parasitology* **30**, 289–298. doi:10.1016/j.pt.2014.04.003.

Sivakumar, P. M., Prabhakar, P. K. and Doble, M. (2011). Synthesis, antioxidant evaluation, and quantitative structure-activity relationship studies of chalcones. *Medicinal Chemistry Research* **20**, 482–492. doi:10.1007/s00044-010-9342-1.

Sousa-Batista, A. de J. and Rossi-Bergmann, B. (2018). Nanomedicines for Cutaneous Leishmaniasis. In *Leishmaniases as Re-emerging Diseases*, pp. 181–197. doi:10.5772/32009.

Sousa-Batista, A. J., Escrivani-Oliveira, D., Falcão, C. A. B., Da Silva Philipon, C. I. M. and Rossi-Bergmann, B. (2018a). Broad spectrum and safety of oral treatment with a promising nitrosylated chalcone in murine leishmaniasis. *Antimicrobial Agents and Chemotherapy* **62**, 1–5. doi:10.1128/AAC.00792-18.

Sousa-Batista, A. J., Pacienza-Lima, W., Arruda-Costa, N., Falcão, C. A. B. and Rossi-Bergmann, B. (2018b). Depot subcutaneous injection with chalcone CH8-loaded PLGA microspheres aiming at a single-dose treatment of cutaneous leishmaniasis. *Antimicrobial Agents and Chemotherapy* **62**, e01822-17. doi:10.1128/AAC.01822-17.

Sousa-Batista, A. J., Arruda-Costa, N., Rossi-Bergmann, B. and Ré, M. I. (2018c). Improved drug loading via spray drying of a chalcone implant for local treatment of cutaneous leishmaniasis. *Drug Development and Industrial Pharmacy* **18**, 1–8. doi:10.1080/03639045.2018.1461903.

Sousa-Batista, A.J., Pacienza-Lima, W., Ré, M.I., and Rossi-Bergmann B. (2019). Novel and safe single-dose treatment of cutaneous leishmaniasis with implantable amphotericin B-loaded

microspheres. I*nternational Journal for Parasitology: Drugs and Drug Resistance* **11**, 148-155. doi: 10.1016/j.ijpddr.2019.06.001.

Tiwari, B., Pratapwar, A., Tapas, A., Butle, S. and Vatkar, B. (2010). Synthesis and antimicrobial activity of some chalcone derivatives and their copper complexes. *International Journal of ChemTech Research* **2**, 499–503. doi:10.1155/2008/876257.

Torres-Santos, E. C., Moreira, D. L., Kaplan, M. A. C., Meirelles, M. N. and Rossi-Bergmann, B. (1999a). Selective effect of 2',6'-dihydroxy-4'-methoxychalcone isolated from Piper aduncum on Leishmania amazonensis. *Antimicrobial Agents and Chemotherapy* **43**, 1234–1241. doi:10.1128/AAC.43.5.1234.

Torres-Santos, E. C., Rodrigues, J. M., Moreira, D. L., Kaplan, M. A. C. and Rossi-Bergmann, B. (1999b). Improvement of in vitro and in vivo antileishmanial activities of 2',6'- dihydroxy-4'-methoxychalcone by entrapment in poly(D,L-lactide) nanoparticles. *Antimicrobial Agents and Chemotherapy* **43**, 1776–1778. doi:10.1128/AAC.43.7.1776.

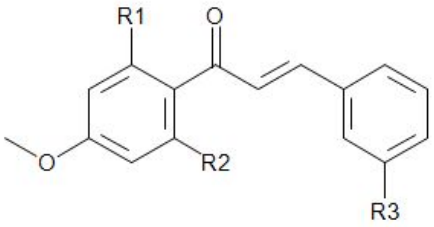
Uliana, S. R. B., Trinconi, C. T. and Coelho, A. C. (2017). Chemotherapy of leishmaniasis: present challenges. *Parasitology* **20**, 1–17. doi:10.1017/S0031182016002523.

Wan, F. and Yang, M. (2015). Design of PLGA-based depot delivery systems for biopharmaceuticals prepared by spray drying. *International Journal of Pharmaceutics* **498**, 82–95. doi:10.1016/j.ijpharm.2015.12.025.

WHO (2017). Global leishmaniasis update, 2006–2015: a turning point in leishmaniasis surveillance. *Weekly epidemiological record* **92**, 557–572.

TABLES

Table 1. Synthesized chalcone analogues and their *in vitro* activity and safety



Analogue	R_1	R_2	R_3	Yield %	Promastigote IC_{50} (µM)	Amastigote IC_{50} (µM)	Macrophage CC_{50} (µM)
CH8	OH	OCH_3	NO_2	18	0.7 ± 0.2	1.4 ± 0.1	>100 (444*)
NAT1	H	H	H	61	24.0 ± 0.1	1.7 ± 0.2	>100 (191*)
NAT22	OCH_3	OCH_3	NO_2	89	1.9 ± 0.1	0.1 ± 0.2	>100 (149*)
NAT28	OCH_3	OCH_3	OCH_3	81	6.7 ± 0.1	1.0 ± 0.1	>100 (165*)
NAT31	OH	OCH_3	OCH_3	12	4.5 ± 0.1	0.7 ± 0.1	>100 (216*)
NAT49	OH	OCH_3	OH	13	3.0 ± 0.1	1.2 ± 0.1	>100 (206*)

Maximum and minimum release optical densities were = 1.824 ± 0.003 and 1.014 ± 0.014. Means ± SD (n = 3). * CC_{50} values as extrapolated from curve fitting.

Table 2: Anti-amastigote activity and cytotoxicity of free and microparticulated NAT22.

Formulations	Amastigotes IC_{50} (µM)	Macrophages CC_{50} (µM)
NAT22	0.2 ± 0.1	>100 (179*)
NAT22-PLGAk	0.3 ± 0.1	>100 (154*)
PLGAk	>100 (130*)	>100 (318*)

Maximum (2 % Triton 100-X) and minimum LDH release values were = 1.824 ± 0.003 and 1.014 ± 0.014, respectively. Means ± SD (n = 3). * CC_{50} values as extrapolated from curve fitting. No concentrations higher than 100 µM were used due to dense particle deposits over cell monolayers.

421 **FIGURE CAPTIONS**

422

423 **Figure 1. Effect of free chalcones in *L. amazonensis*-infected mice.** BALB/c mice were infected
424 with *L. amazonensis*-GFP in the ear. From day 7 of infection they were treated twice a week with
425 CH8 or NAT22 at a total dose of 1.2 mg/kg (3.3 µg/dose/10 µl) or with 10 µl of vehicle alone (PBS)
426 for 4 weeks. Parasite loads were measured on day 32 of infection and expressed as specific
427 fluorescence units (FU). Means ± SD (n = 5), *** p< 0.05 in relation to PBS.

428

429 **Figure 2. Scanning electron microscopy of:** A) Large crystal structures of free NAT22, and B)
430 Microparticle-entrapped NAT22 (NAT22-PLGAk).

431

432 **Figure 3. Efficacy of NAT22-PLGAk in *L. amazonensis*-infected mice.** BALB/c mice were
433 infected in the ear with *L. amazonensis*. On day 7 of infection they were given a single (1X) or eight
434 (8X within 4 weeks), intralesional injections with a total dose of 1.2 mg/kg of NAT22, NAT22-
435 PLGAk, or PLGAk. Parasite loads were measured in the ears on day 32 of infection. Means ± SD (n
436 = 5). ** p<0.01 in relation of PBS group, # p<0.05.

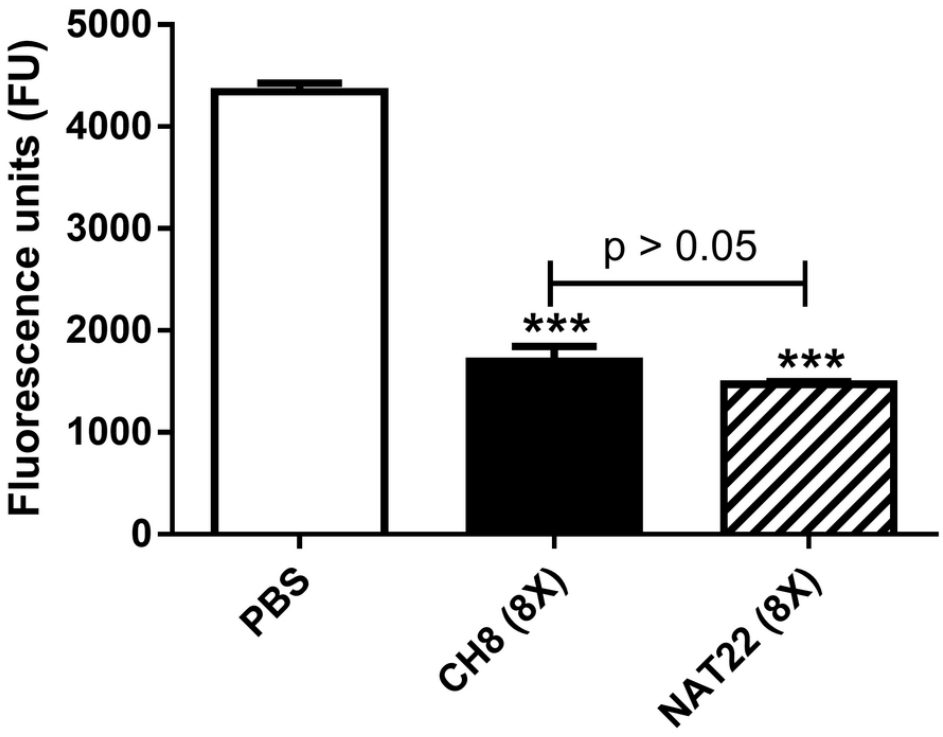


Figure 1. Effect of free chalcones in L. amazonensis-infected mice. BALB/c mice were infected with L. amazonensis-GFP in the ear. From day 7 of infection they were treated twice a week with CH8 or NAT22 at a total dose of 1.2 mg/kg (3.3 µg/dose/10 µl) or with 10 µl of vehicle alone (PBS) for 4 weeks. Parasite loads were measured on day 32 of infection and expressed as specific fluorescence units (FU). Means ± SD (n = 5), *** p< 0.05 in relation to PBS.

79x65mm (300 x 300 DPI)

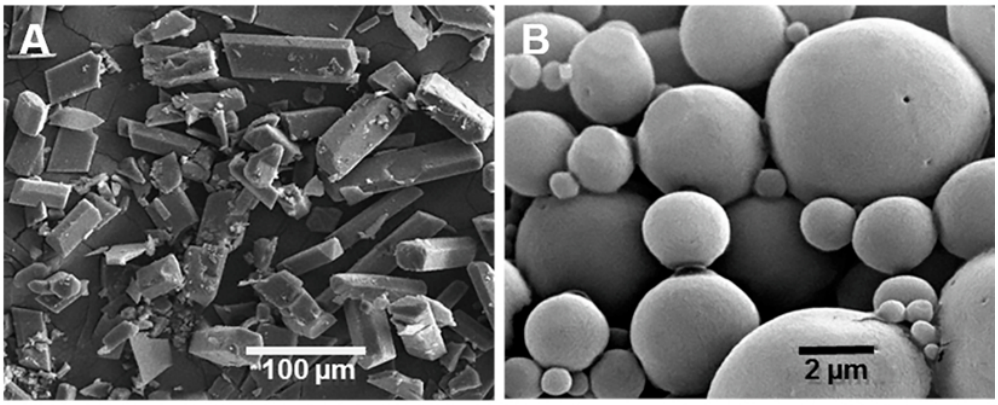


Figure 2. Scanning electron microscopy of: A) Large crystal structures of free NAT22, and B) Microparticle-entrapped NAT22 (NAT22-PLGAk).

139x57mm (150 x 150 DPI)

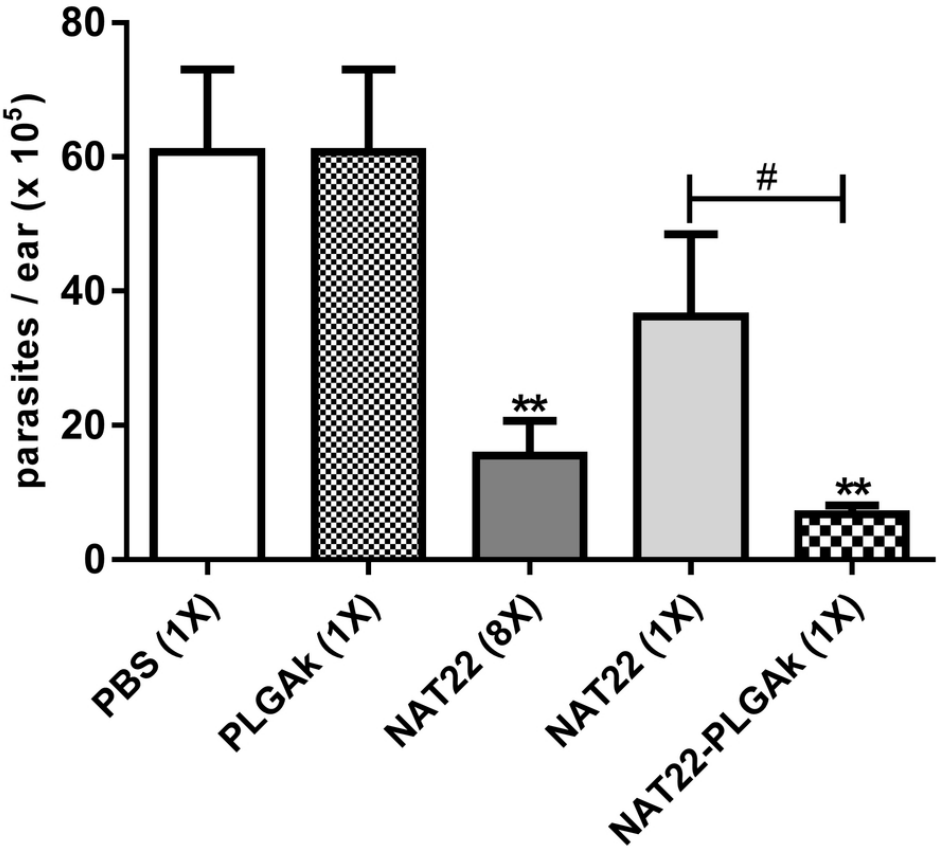


Figure 3. Efficacy of NAT22-PLGAk in L. amazonensis-infected mice. BALB/c mice were infected in the ear with L. amazonensis. On day 7 of infection they were given a single (1X) or eight (8X within 4 weeks), intralesional injections with a total dose of 1.2 mg/kg of NAT22, NAT22-PLGAk, or PLGAk. Parasite loads were measured in the ears on day 32 of infection. Means ± SD (n = 5). ** $p<0.01$ in relation of PBS group, # $p<0.05$.

79x75mm (300 x 300 DPI)

We thank the referees for their time helpful comments which helped us to substantially improved our manuscript.

We have carefully considered all comments and answered to them point by point, indicating the changes made, as below (in blue):

Referee: 1

Comments to the Author:

The results of this new approach are promising and providing excellent prospects for future research. This research brings a perspective for cutaneous leishmaniasis treatment since a single dose of chalcone analogue was effective. Comments and suggestions were done in a PDF file.

AUTHORS RESPONSE: We appreciate your relevant comments and suggestions. Accordingly, all changes were made, and marked in blue font in the reviewed text.

Referee: 2

Comments to the Author:

In this manuscript Sousa-Batista et al. describe new chalcone analogues for the treatment of cutaneous leishmaniasis. A particular difficulty with a previously reported chalcone, CH8, was the challenging synthetic route. A new compound presented here, NAT22, is much more straightforward to synthesize, and when formulated as a slow release microparticle, shows encouraging in vivo activity in a mouse model of CL after a single injection. Overall, this manuscript makes an interesting contribution to the development of new therapeutics for a highly neglected disease and may trigger further development of this approach. The manuscript is of interest to a wide audience of people working on leishmaniasis and drug delivery.

Main comments:

I struggle with the reported cell-assay potency values in the manuscript. My understanding from the methods is that dose response curves were determined using only 4 concentrations using a very wide 10-fold dilution scheme (concentrations tested: 0.1, 1, 10 & 100µM). Using such a scheme it is not possible to determine accurate IC_{50} values. The authors also do not explain how the potencies were determined (e.g. line 101, which equation was used, was normalisation applied, what constraints were in place (top and bottom fixed?)?).

AUTHORS RESPONSE: Thank you for drawing attention to our mistake. Actually, 10-fold dilution curves were always used to determine the IC_{50} range, but subsequent 2-fold dilution curves were used to calculate the IC_{50}. Accordingly, that was corrected in **lines 116-117.**

As to data normalization, the information was provided in **line 164.**

On line 196 the authors state that "entrapment into microparticles increased its IC50 from 0.2 µM (NAT22 alone) to 0.3 µM (NAT22-PLGAk)", it is not possible to differentiate between these two potencies with the dose response method used, it is also not appropriate to return an IC50 of 0.1µM (for NAT22, table 1) when this was the lowest concentration tested – the recommendation for accurate IC50s is that there are at least two concentrations with 0% effect and two with 100% effect (https://onlinelibrary.wiley.com/doi/full/10.1002/pst.426). In my opinion it is impossible to determine potencies with 100nM precision using the 4-point dose response curves used. I recommend that the authors remove all IC50s, and instead report the lowest actual concentration at which >50% (or another threshold) inhibition was seen. Also, in table 1, extrapolated values are shown for the CC50, and these are used to calculate the selectivity index. I cannot see how these extrapolated values can be anywhere near accurate as the only concentration with some cytotoxicity has to be 100µM (as CC50 >100µM). I would prefer to see this data also represented as the concentration at which >50% inhibition was seen, which means it can probably still be presented as >100. There is no value in a selectivity index when accurate IC50s have not been determined so it should be removed (just state that the compounds appear not to have any cytotoxic effects at the concentrations tested).

AUTHORS RESPONSE:

- Accordingly, the sentence in **lines 196-197** now reads: *'...entrapment into microparticles did not significantly increase drug activity ($p>0.05$), as shown in Table 2.'*
- As for promastigotes, curves using 2-fold dilutions that plateaued with the lowest and highest concentrations were used for amastigotes.
- As to CC_{50} extrapolated values, the curve slopes never went below 70% at 100 uM - the maximum drug concentration possible. This is explained in the legend to Table 2, **lines 414-415**.
- As suggested, the selectivity index values were removed both from the main text and Tables 1 and 2.

In the discussion it is worth including the need to test against other strains, in particular *L. tropica*, which can be more difficult to treat.

AUTHORS RESPONSE: We added '*…and use of other relevant parasite species like L. braziliensis and L. tropica will add further insight to this new mode of CL therapy'*. (**lines 274-275**).

In general it would be useful to discuss the Target Product Profile for cutaneous leishmaniasis (https://www.dndi.org/diseases-projects/leishmaniasis/tpp-cl/) and how the microparticle approach fits with the ideal or acceptable profile.

AUTHORS RESPONSE: Thank you for raising this point. The following text was introduced in **lines 242-245**: *'Additionally, the NAT22-PLGAk formulation is a single dose treatment, meeting the DNDi Target Product Profile for CL that recommends the use of treatments with few injections, local effect and low cost. It is worth noting that despite the technology adding cost, a single dose treatment will have positive impact in reduced hospital and mobility costs.'*

I would also encourage the authors to discuss briefly how translatable the mouse ear model is. Are there other models that bear more relevance towards the human condition in which this approach should be tested in the future?

AUTHORS RESPONSE: The CL model of mouse ear infection has been widely accepted

for drug and vaccine tests mainly because of the reproducible measurements of ear thickness as compared with other sites, e.g. dorsum area or footpad thickness, and also because the ears are out of reach of licking by the mouse. Since ours is a subcutaneous, not topical treatment where differential skin permeability would be critical, the results comparing free vs encapsulated drug reported here may be suitable for proof of concept purposes.

In order not to make the Discussion too long, the following was introduced in **Lines 271-273**: *'The mouse ear model of CL used here appears translatable to the human infection as the drug is discharged in the subcutaneous tissue not on the skin surface where differential skin permeability would be more critical.'*

Minor comments:

Line 106: Why different time for compounds +/- microparticles, why not treat for the same amount of time?

AUTHORS RESPONSE: We reported previously that 96 h is a more appropriate time to evaluate the action of microparticles than 48 h, as it allows more time for polymer degradation (Sousa-Batista et al., 2018c) . The text has been modified to make this information clearer **(lines 127-129)**: '*Treatment time in the particle assay was increased to 96 h to allow more time for polymer degradation and drug release (Sousa-Batista et al., 2018c).*'

Line 151: Please specify how treatment was applied: topically, injection,…

AUTHORS RESPONSE: Thanks for noting that. Accordingly, we added to **Line 150**: *'... local s.c. injections'.*

Line 246: This paragraph discusses encapsulated NAT22, and states that due to its slow release there may not have been a full effect in the 48h assay. However, in the methods section it states that assays with microparticles were carried out for 96h. Can the authors please clarify if it is 48h or 96h?

AUTHORS RESPONSE: **Line 251** now reads: *"during the 96-hour assay".*

Please check text for minor typos: line 4: require should be requires, line 34: affect should be affects, line 35: according to the WHO estimates should be according to WHO

estimates, …

AUTHORS RESPONSE: Thanks, the typos have been revised.

Referee: 3

Comments to the Author:

This is a very interesting paper which addresses the treatment of a very neglected tropical disease, cutaneous leishmaniasis.

I recommend that the paper is accepted after minor amendments as follows:

1. The manuscript needs careful proof-reading as there are occasional grammatical errors.

AUTHORS RESPONSE: Thanks, the manuscript has been revised.

2. The authors need to add detail on how many infected macrophages were counted per sample. It would also be useful to see data on the mean number of parasites per infected macrophage, IC_{50} curves and confidence intervals.

AUTHORS RESPONSE: The number of macrophages counted per sample (200 total macrophages) has been added to **Line 130**. We have not recorded the % of infected macrophages because what matters here is the reduction in the numbers of parasites in culture, translatable to parasite loads in the lesions. Statistics is now detailed in **Lines 164-165**.

3. The discussion needs to be expanded regarding the microparticles. Is there any published data on how long these would likely persist at the site of injection and the rate at which drug would disperse?

AUTHORS RESPONSE: Yes, accordingly we have added the following sentence **(Lines 251-255)**: *'Recently, we reported that amphotericin B release from PLGA microparticles is much faster in the ear subcutaneous tissue (30 days) than in saline solution in vitro (200 days), possibly because of presence of enzymatic hydrolysis in the tissue (Sousa-Batista et al., 2019). Whether or not NAT22 release from PLGAk microparticles follows the same kinetics remains to be determined.'*

What are the immunosuppressive effects, and would this potentially be an advantage for the host or parasite?

AUTHORS RESPONSE: PLGA is more likely to be an immunostimulant rather than an immunosuppressive agent.

Were any side effects seen in the treated mice?

AUTHORS RESPONSE: The safety of PLGA formulations for mice was tested in previous studies of our group, with no evidence of toxicity. The text has been modified to show this information.

Lines 199-200: *'The safety of PLGA s.c. injection was previously demonstrated in mice using PLGA- CH8 formulation (Sousa-Batista et al., 2018b).'*