Evaluation of lignan-loaded poly(ε-caprolactone) nanoparticles: synthesis, characterization, in vivo and in silico schistosomicidal activity

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ABSTRACT
Lignan dinitrohinokinin displays important biological activities, which led to the preparation of its poly-ε-caprolactone nanoparticles. Kinetics analysis revealed initially slow drug release followed by a prolonged, moderate release 6 h later due to DNHK diffusion through the polymeric matrix. Molecular dynamics simulations show that DNHK molecules that interact stronger with other DNHK molecules near the PCL/DNHK surface are more difficult to dissociate from the nanoparticle. The smaller diameter nanocapsules with negative surface charge conferred good colloidal stability. The formulations showed a size distribution with monodisperse systems formation. In vivo evaluation of schistosomicidal activity against Schistosoma mansoni showed that DNHK, when incorporated into nanoparticles, caused egg number reduction of 4.2% and 28.1% at 40 mg/kg and 94.2% and 84.4% at 400 mg/kg in the liver and the spleen, respectively. The PCL nanoparticles were stable in aqueous dispersion and could be optimized to be used as a promising lignan release agent.

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1. Introduction

Nanotechnology and nanoscience are currently considered to be fascinating advances in Natural products chemistry and constitute one of the leading research focus, development, and innovation activities in all developed countries. Some of the reasons for conventional therapy failure include insufficient drug absorption, rapid metabolism, elimination, and distribution of the drug in non-target tissues with high toxicity and low solubility, excluding administration of aqueous solutions (Magalhães et al. 2001). Thus, the search for new alternatives has offered novel tools for treating neglected diseases, particularly schistosomiasis. Schistosomiasis is an infectious parasitic disease caused by worms of the *Schistosoma* genus. Although treatment with praziquantel (PZQ) effectively controls schistosomiasis, less susceptible strains to PZQ reinforce the need to develop safer and more effective schistosomicidal drugs. The dinitrohinokinin (DNHK) is a dibenzylbutyrolactone lignan compound with *in vitro* and *in vivo* moderate schistosomicidal activity (Magalhães et al. 2001; Lima et al. 2017). In this context, the present study aimed to generate nanoparticle formulations with DNHK incorporation in poly-ε-caprolactone (PCL). The PCL was chosen due to its properties of high permeability, lack of toxicity, biocompatibility, and bioavailability, which is suitable for the controlled release of drugs. *In vitro* and *in vivo* analyses, interactions between the PCL matrix, and DNHK molecular dynamics simulations were also performed.

2. Results and discussion

2.1. Analytical method

The elution time of the compounds was detected in 5.1 min (Figure S3). The analytic curves to be linear over the proposed range (1.00–20.00 μg.mL⁻¹). The determination coefficient (r²) obtained was 0.9994; thus, making it possible to attest to the linearity of the method. The limits of detection (LOD) are 0.300 μg mL⁻¹ and quantification (LOQ) are 4.090 μg mL⁻¹. The relative standards deviation (RSD%) to intra-day and inter-day precision was found to be all lower than 2.0%. The method’s accuracy was
assessed by adding high, medium, and low concentrations of DNHK. The developed method displayed good accuracy, exhibiting good recovery (between 91.17% and 113.7%). The robustness of the method was analyzed by examining the effects of variations on the rrt (relative retention time) and concentration to calculate effects (Ex) that were converted to RSD%. The RSD% was lower than 20% when all factors were considered showing that all factors influence the method equally. The results obtained allowed us to consider that the developed method is reliable for the quantification of DNHK compounds in nanoparticle formulations.

2.2. Characterization of the PCL obtained formulations

Unloaded PCL and PCL containing DNHK (40 and 400 mg) nanoparticles were characterized by scanning electron microscopy (SEM) for morphological analysis (Figure S4). PCL nanoparticles were found in sphere-like shape (agglomerates) with ~18 nm mean size (Figure S4A). The presence of low DNHK concentration (40 mg) in the PCL nanoparticles showed no significant changes in the polymer morphology (Figure S4B). The particle-size distribution spectrum of PCL particles is presented in Figure S5A, showing that PCL had a narrow particle-size distribution. The zeta-potential distribution spectrum of PCL had a negative surface charge (Figure S5B) close to $\pm 32.68$ mV. These values follow previous reports where negative values were attributed to PCL due to the presence of terminal carboxyl groups. (Muller et al. 2001; Mora-Huertas et al. 2010) Zeta Potential (ZP) can influence the release kinetics, and the biological fate of the nanotransporters as positively charged particles are attracted to the negative charges of biological components and surfaces and vice versa (Tamjidi et al. 2013). The PCL nanoparticles produced in this study presented negative ZP due to a polyester-type polymer, PCL, in the formulations. Typically, nanoparticles, including nanocapsules, with ZP values close to $\pm 30$ mV have good colloidal stability in the solution (Melo et al. 2010). Recovery percentages varied between 77.4% (PCL-1) and 99.8% (PCL-2). After filtration and centrifugation, the rates of DNHK association to the nanoparticles were 82.4% and 99.6% for PCL-1 and PCL-2, when quantification by HPLC, respectively. PCL was suitable for DNHK incorporation due to its permeability between the oily nucleus and the external aqueous phase, that better interaction between the active compound and the polymer.

The in vitro release behavior of PCL-DNHK-encapsulated in phosphate-buffered saline (pH 7.4) was studied (Figure S6). A linear release of DNHK was observed in the PCL with low DNHK content in the first 6h of assay (Figure S6 A), whereas high DNHK content in the PCL polymer resulted in an exponential release with the same time of assay (Figure S6 B). After 6h, a similar amount of DNHK (~35%) was released by the PCL polymer with low and high DNHK content (Figures S6 A and S6 B), while after 8h, both materials reached 100% of DNHK release in the aqueous medium. DNHK release profile differences by PCL (first 6h) could be explained mainly by DNHK presence in the surface/bulk of the polymer nanoparticles. The low amount of DNHK (40 mg, Figure S6 A) in PCL leads to a fast-initial release, suggesting dissolution of DNHK present on the polymer’s surface. However, slow DNHK release (Figure S6 B) might be due to structural change of the PCL particles (observed by SEM image – Figure S4 C),
which hinders DNHK diffusion into the aqueous medium in the first hours. The in vitro results presented here indicate that DNHK can be released slowly or fast from the PCL polymer reaching 100% release after 8 h (Calvo et al. 1996) and suggested that the polymeric wall of the nanocapsules does not influence the release process (Calvo et al. 1996; Schaffazick et al. 2003).

In silico calculations were performed to understand the interaction between DNHK and PCL. In this sense, the dynamics of model nanoparticle formation from the solution of solvated PCL$_{10}$ and DNHK molecules were investigated by MD (molecular dynamics) in fully solvated systems. The design of system models with shorter x and y dimensions relative to z allows the formation of a nanoparticle with a periodic surface on the xy plane to avoid the artifacts of finite small system boundaries. Additionally, the larger z dimension was sufficient to displace DNHK molecules involved in SMD (Steered Molecular Dynamics) calculations. Along with the dynamics, the formation of a phase containing only PCL$_{10}$/DNHK molecules with the previously reported structural characteristics at the final stage of MD simulation was observed (Figure S7A–C). The dynamics of the phase formation monitored by the profiles of the averaged gyration radius around the x and y-axis showed similar behavior along time for both systems. These results showed a similar behavior despite differences between the gyration radius values and the systems because of different dimensions of these systems imposed in their construction. They showed initial values around 4 nm to 10 ns with collapse initiation for nanoparticle formation after this time with a rapid decrease of the gyration radius around these axes. After 25 ns, the nanoparticles started the organization process and reached the organized structure observed at the end of MD, as indicated by the decreasing variations of the gyration.

Dissociation of DNHK molecules at the surface of PCL$_{10}$/DNHK nanoparticles formed at the final snapshots of the MD trajectory by SMD allow both determination of the force strength and the energetic components related to it. Because of the heterogeneous environment, different orientation, and surface exposure of each DNHK molecule, the pull force necessary to dissociate from the nanoparticle are dependent of these factors, thereby showing different profiles (Figure S7C). This heterogeneity is correlated with different behavior for the total interaction energies ($E_{\text{total}}$) and their decomposition due to the contribution for interaction with PCL$_{10}$ molecules ($E_{\text{PCL}}$) and the remaining DNKH molecules ($E_{\text{DNKh}}$) (Figure S7C). The visual comparison among these profiles, looking for extreme values, indicate an interesting pattern, where, i.e., for DNHK9, the more attractive $E_{\text{DNKh}}$ near $-100 \text{kJ.mol}^{-1}$, lead to the strongest pulling force, around of 350 kJ.mol$^{-1}$.nm$^{-1}$, with $E_{\text{PCL}}$ near $-130 \text{kJ.mol}^{-1}$. This indicates that a more attractive $E_{\text{DNKh}}$ leads to a stronger pull force for dissociation and can be related with experimental observations related to drug liberation.

To understand the effect of different environments to dissociate the studied DNHK molecules, maximum force ($F_{\text{max}}$), solvent accessible surface (SAS) and more attractive $E_{\text{total}}$ ($E_{\text{total,min}}$), $E_{\text{PCL}}$ ($E_{\text{PCL,min}}$) and $E_{\text{DNKh}}$ ($E_{\text{DNKh,min}}$) parameters were calculated for each DNHK molecule under SMD (Table S4). To evaluate the early described effect of DNHK9 concerning other molecules, the Pearson correlation coefficient ($R$) between $F_{\text{max}}$ and $E_{\text{DNKh,min}}$, excluding some DNHK molecules with $E_{\text{DNKh,min}} < -5 \text{kJ.mol}^{-1}$ and with small SAS, revealed a negative correlation with $R$ equal to $-0.91$ (p-value < 0.01),
with an explanation of 0.81 (p-value < 0.01), in the variation considering the linear regression (Figure S8). No significant correlation was observed between $E_{\text{total, min}}$ or $E_{\text{PCL, min}}$ with $F_{\text{max}}$. From data in Table S3 and Figure S7, the excluded DNHK molecules 1, 7, 10 and 11 show a different behavior with no association pattern of $E_{\text{DNHK, min}}$ with $F_{\text{max}}$. The $F_{\text{max}}$ observed for these molecules are distributed along the value ranges observed for other molecules with DNHK11, presenting the strongest $F_{\text{max}} = 399.7 \text{ kJ.mol}^{-1}.\text{nm}^{-1}$. This behavior resulted from a deeper insertion of these molecules in the nanoparticles and to different orientation and local environments that modulate the nanoparticles’ dissociation. However, the present results indicate that DNHK molecules that interact stronger with other DNHK molecules near the surface are more difficult to dissociate from the nanoparticle, as early observed for DNHK9.

### 2.3. Effects of DNHK-loaded PCL in animals infected with S. mansoni

The present study investigated the effects of DNHK-PCL nanoparticles using the experimental model of schistosomiasis. For that, parameters such as the number of worms and eggs in the liver and granuloma formation were evaluated. Oral treatment with DNHK at a 400 mg/kg concentration significantly reduced the number of total worms compared to the control diluent with a 54% reduction of adult worms after treatment with DNHK. The DNHK nanoparticle formulations (Tables S2 and S4) also caused a significant reduction in the number of worms, with 31% and 21%, 21% and 31% reduction at 40 and 400 mg/kg dosages, respectively, when compared to diluent 2 (poly-ε-caprolactone). In contrast, PZQ, at a dose of 400 mg/kg, caused an 85% reduction of adult worms’ number. The reduction of adult worms values of 31% using DNHK formulation dose of 40 mg/kg compared with 21% in high dosage (400 mg/kg) suggests that the release of DNHK by the formulation presents a vital role in these results. The low dosage formulation (40 mg/kg) showed a fast release of DNHK by the PCL, while with a high dosage (400 mg/kg) the percentage of DNHK released was slow.

Oral treatment with nonincorporated DNHK (400 mg/kg) did not significantly reduce (16.1%) the number of eggs in the liver compared to diluent 1 (Saline:EtOH:DMSO 50:25:25). Likewise, encapsulated DNHK at doses of 40 and 400 mg/kg did not significantly reduce (4.20% and 28.13%, respectively) the number of eggs in the liver compared to treatment with diluent 2 (poly-ε-caprolactone). Treatment with PZQ (400 mg/kg) reduced the number of eggs in the liver by 24%.

### 2.4. In vivo evaluation of histopathological aspects during experimentally induced schistosomiasis

The histopathological evaluation of animals experimentally infected with S. mansoni was performed by measuring the areas of hepatic granulomas after treatment. Treatment with 400 mg/kg of nonencapsulated DNHK reduced the area of hepatic granulomas compared to treatment with diluent 1 (Saline:EtOH:DMSO 50:25:25), but the encapsulation did not cause a significant reduction. Treatment with PZQ also reduced the area of hepatic granulomas compared to treatment with either diluent 1
or 2 (Figures S9 and S10), reduction in the number of eggs in the liver is extremely important because the egg is responsible for major pathogenesis during the severe phase of schistosomiasis (Gryseels et al. 2006; Pereira et al. 2015). Treatment against schistosomiasis is typically done with PZQ. However, there has been increasing concern with parasitic resistance, and the drug’s disseminated use has stimulated the search for novel drugs. (Gryseels et al. 2006; Pereira et al. 2015). Additionally, it is possible that treatment could induce the regulation of the immune response allowing for granuloma formation less aggressively (Gryseels et al. 2006; Pereira et al. 2015).

3. Conclusions

The dispersion of nanoparticles containing DNHK indicates the homogeneity of size distribution, and the colloidal dispersions were composed of homogeneous nanoparticles. The nanospheres displayed two-step mechanisms on the release kinetics – slow initial release followed by a rapid release reaching 60% of DNHK release. Anti-inflammatory activity was evidenced by a decrease in both the number and area of granulomas in the liver. The obtained nanoparticles are stable in aqueous dispersion and can be optimized to be used as a promising agent for lignans release. This would allow the development of new formulations that could enhance the efficacy of drugs, in addition to the investigation of the mechanism of action of both their anti-inflammatory and schistosomicidal activities.

Disclosure statement

The authors declare no conflict of interest.

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