SHORT COMMUNICATION

In vitro and in silico cytotoxicity of hinokinin-loaded PLGA microparticle systems against tumoral SiHa cells

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ABSTRACT
This work aimed to synthesize poly (D, L-lactic-co-glycolic acid) (PLGA) microparticles containing hinokinin (HNK) and to evaluate their cytotoxic activity against tumoral SiHa cells and non-tumoral HaCaT cells. Hinokinin was incorporated into PLGA (PLGA-HNK) with an encapsulation efficiency of 84.18 ± 2.32%. PLGA and PLGA-HNK were characterized by SEM microscopy and showed spherical morphology with an average size of ≈3.33. Encapsulation efficiency was determined by a calibration curve using UV-vis spectroscopy. PLGA-HNK more active inhibiting proliferation of SiHa cells (IC50 = 14.68 μM) than free HNK (IC50 = 225.5 μM). In relation to HaCaT cells, PLGA-HNK showed no significant difference compared to the negative control. These results led to an increase in HNK bioavailability and thereby, biological activity. In silico prediction analysis suggests that HNK is cytotoxic against SiHa cells with E6 and MDM2 inhibition as possible main mechanism of action.

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1. Introduction

Although there is great gene diversity involved in the development of cancer, the suppressor protein p53 works as the main signaling pathway in this process. Its activation can prevent the proliferation of tumor cells by inducing cell growth arrest, apoptosis, tumor stroma modulation, or by inhibiting invasion and metastasis. (Brandão et al. 2018; Pereira et al. 2019). In human cancers, the p53 tumor suppressor pathway is one of the most altered. In half of all cancer types, the p53 pathway (p53 wild type) is inactivated due to overexpression of the main endogenous negative regulator, murine double minute 2 (MDM2). MDM2 binds to p53 by negatively regulating its activity; therefore, compounds that manage to inhibit the interaction of this type of p53 with MDM2 are interesting targets for the development of new drugs for cancer treatment (Brandão et al. 2018).

Currently, several drugs used in chemotherapy treatments cause many serious side effects due to the high dosage required and their low selectivity; that is, they also act on normal cells besides producing high toxic metabolites (Maeda and Khatami 2018). Thus, new compounds that act on more selective targets present in tumor cells are required.

Natural products are an inexhaustible source of compounds with the most varied chemical structures and biological properties. However, many of these active compounds have hydrophobic nature, which decreases absorption and thus bioavailability in the biological environment (Ashraf 2020). Given the high potential of these compounds, several researchers have been exploring new methodologies to incorporate them into nanoparticles/microparticles to optimize their biological use (Huang et al. 2019). Encapsulation avoids decomposition and enhances bioavailability, so that the amount of natural unchanged product that reaches the target (tissues and organs) is greater, as well as the profile release that occurs (Gigliobianco et al. 2018). In this context, nanoparticles/microparticles are considered one of the best drug delivery systems due to their chemical nature and physical properties (Bale et al. 2016; Shen et al. 2020). Therefore, introducing biologically active natural products into nanoparticles/microparticles is a great way to enhance pharmacological activity and minimize side effects, as well as increasing intracellular penetration. However, particle shape and size are crucial for the application type; for example, for systemic (intravascular) application, particles with diameters smaller than 500 nm are required, whereas for intramuscular application or in some cases oral delivery applications, particles greater than 1 μm and smaller than 125 μm can be easily administered. The particle’s shape can significantly modulate their function on cellular uptake by immune cells, biomolecules’ release behavior, and cell targeting (Maghrebi et al. 2020; Shen et al. 2020).

Among the various bioactive natural products available are lignans (Teponno et al. 2016; Santos et al. 2019; Wenfeng et al. 2020) such as hinokinin (HNK). Due to the biological potential of HNK, there is a need to explore how encapsulation in poly(lactic-co-glycolic acid) microparticles (PLGA-MCPs) affects its cytotoxic potential. Thus, this work aimed to study the synthesis, characterization, and stability of PLGA particles containing hinokinin lignan to study the cytotoxic potential in vitro and in silico.
2. Results and discussion

The structure of hinokinin isolated from *Piper cubeba* fruits was confirmed by \(^1\)H and \(^{13}\)C NMR (Figure S1 and Table S1) and agrees with data published in the literature (Desai et al. 2014; Arruda et al. 2018). Through Uv-vis analysis and using the equation S1, the average value of encapsulation efficiency (average from five analyses) was 84.18 ± 2.32\% (Figure S15). The morphological study of PLGA and PLGA-HNK was performed by SEM analysis. As observed in the micrographs, PLGA (Figure S2A) and PLGA-HNK (Figure S2B) have spherical morphology. Regarding PLGA-HNK, better homogeneity in diameter size compared with PLGA was observed (Figure S3).

DLS analysis confirmed the obtained formulations in microscale (~3.0 μm) with lower polydispersion index (PDI) for PLGA-HNK compared with PLGA (Table S2). The numerical value of zeta potential was higher for PLGA-HNK, suggesting more stable particles due to the interaction between polymer chains and HNK molecules. The variation in numerical value of zeta potential for PLGA is dependent on several factors, such as the polymer composition of PLGA, particle size, and drug loaded according to the literature (Operti et al. 2019). PLGA polymers have been observed to produce negative potential systems due to the presence of terminal carboxyl PLGA on the particle surface. The surface charge of PLGA is extremely important, as it can influence their cytological and hematological interactions (Operti et al. 2019).

Results obtained by Yang et al. (2009), using paclitaxel-loaded chitosan-nanoparticles, indicated that pH affects positive particles, therefore affecting their cytotoxicity, while the same is not true for negative particles where cytotoxicity against A549 and CT-26 cells was higher. According to Pillai et al. (2015), the importance of proteins linked to the surface in the recognition and cellular interactions of particles is great and occurs in a greater extent in particles with positive charge. However, negative charge particles also interact with the same proteins at a lesser extent, although with greater interaction than neutral particles. Positive charge induces hemolysis at high concentrations to a greater degree than negative or neutral particles. The surface charge of the particles affects how they interact with proteinase to a greater or lesser extent, but according to the authors, the cytotoxicity of the particles is not affected by the surface charge since the cytotoxic effect is only on the encapsulated drug and not on the particles’ material. Despite several articles regarding the effect of the particle charge on its delivery potential, there is still much to be studied to direct the synthesis of microparticles by choosing their charge according to the delivery location (He et al. 2010; Fröhlich 2012).

Differential scanning calorimetry (DSC) studies were used to obtain more information about possible PLGA-HNK interactions (Figure S4). DSC of HNK showed the endothermic peak at ~70 °C, which corresponds to the melting of HNK. From the DSC curve of PLGA, two characteristics events (endothermic) corresponding to the (i) glass transition (\(T_g\)) of the polymer at ~35 °C and the (ii) melting point of PLGA at ~349 °C were observed. DSC of HNK loaded into PLGA (PLGA-HNK) increases both the glass transition and melting point of the polymer to 48 °C and 355 °C, respectively. These changes evidenced the interactions between PLGA and HNK that lead to an increase of polymer rigidity and stability due to the presence of HNK in PLGA (Figure S4).
**In vitro** dissolution test (Figure S5) results from PLGA-HNK showed that 66% of HNK was released during the first 7 h, and 98% within a total of 30 h. HNK released from PLGA seems to consist of a fast release stage first followed by a slower one. The initial burst release of HNK from the PLGA system can be defined as the quantity of drug that diffuses from the polymer. Burst may be an inherent property of diffusion-controlled drug delivery devices. This stage involves the initial release of HNK by easy access to the particles surface by PBS aqueous medium. Based on the highest regression values (r), the best-fit model for PLGA-HNK was the Higuchi Model (Table S3). The diffusion and dissolution process of HNK from the polymeric matrix is governed by a mechanism that can be explained by the Higuchi model of drug released that describes the mechanism largely based on diffusion. Diffusion also depends on the drug dispersion mode within the particle, which is determined by the drug solubility in the organic solvents used for microparticle fabrication, such as dichloromethane as used in our experiments. HNK is soluble in dichloromethene and is distributed in microparticles as molecular dispersions within the amorphous polymer matrix. Similar results were obtained by Saraiva et al. (2010), where 70% of HNK release occurred within the first 12 h in a pH 7.4 buffer solution. In this study, HNK was released faster compared with the literature (Saraiva et al. 2010), probably due to the microparticles larger size.

### 2.1. Proliferation assay

HNK and podophyllotoxin showed IC₅₀ values of 121.9 μM and 89.62 μM, respectively, against SiHa cells (Figures S6 and S7). According to Kuete and Efferth (2015), the obtained IC₅₀ for these compounds on SiHA cell are within the range considered as weakly active (50 μM < IC₅₀ < 250 μM); however, podophyllotoxin was more active than HNK. The IC₅₀ values for HNK and podophyllotoxin were used for PLGA assays were podophyllotoxin was used as positive control. The concentrations of PLGA-HNK used were 40, 80 and 120 μg/ml, corresponding to 9.5 μM, 19.0 μM and 28.5 μM of HNK, respectively, considering that the efficiency of incorporation was ~80 and that the ratio between HNK and PLGA was 1:10 (w/w).

Although the concentration of HNK in the encapsulated form is below the IC₅₀ determined for the free substance, it is believed that the evaluated concentration range would be enough to demonstrate the result of encapsulating HNK on its cytotoxicity, as PLGA exhibit higher cytotoxicity owing to their higher cellular uptake (Ramasamy et al. 2014). The concentration of PLGA used was 120 g/mL, corresponding to the maximum concentration used for the PLGA-HNK system. In the biological assays, PLGA showed no significant difference (p < 0.05) with the negative control not inhibiting SiHa cell proliferation. Podophyllotoxin lignan, at a concentration of 89.62 μM, and used as positive control, showed no significant difference with HNK at 121.9 μM (Figure S8) (p < 0.05). Encapsulated HNK showed an IC₅₀ of 14.68 μM, confirming that HNK has low availability in an aqueous medium, only when not encapsulated; a large dose is required to promote satisfactory cell proliferation inhibition. An IC₅₀ of 14.60 μM was found for PLGA-HNK, considered within the range of moderate activity (10 μM < IC₅₀ < 50 μM) (Kuete and Efferth 2015).
These results confirm that this formulation contributed very effectively and significantly to increase HNK’s action against tumor SiHa cell proliferation. HNK and podophyllotoxin showed an IC\textsubscript{50} of 131.1 and 296.2 \textmu M on HaCaT cells, respectively (Figure S9 and S10). HNK showed low activity on normal cells with 50\% cell viability inhibition at this concentration compared to the control (p < 0.05). Podophyllotoxin also inhibited around 50\% cell growth at the evaluated concentration, but its IC\textsubscript{50} value indicated inactivity on these cells (Kuete and Efferth 2015). PLGA-HNK and PLGA showed no toxicity on normal cells (Figure S11 and Table S4), showing cell growth when exposed to the systems. In this experiment involving normal cells, it was possible to verify that even though HNK was not highly active in its free form, its encapsulation also provided protection for normal cells; however, the selectivity index for PLGA-HNK could not be calculated, since the IC\textsubscript{50} value was higher than the highest evaluated concentration where no cell growth inhibition of normal HaCaT cells occurred.

The literature has been showing various natural and synthetic compounds encapsulated in nano or microparticles that increase their biological potential while lowering their toxicity, such as paclitaxel, docetaxel, and podophyllotoxin (Yadav et al. 2014; Wu et al. 2018; Iliev et al. 2019). These results corroborate with this study, whose main finding was the use of PLGA as a tool that should be studied even with low active substances such as HNK, since most of these substances have reduced cytotoxic potential due to poor solubility, resulting in low bioavailability in an aqueous medium.

### 2.2. Molecular docking

Since HNK and podophyllotoxin inhibited cell proliferation of tumoral SiHa cells, the interactions between HNK and podophyllotoxin with the amino acids of E6, MDM2, and p53 proteins were evaluated using the molecular docking calculations. Recent studies have shown that the interaction of E6 with known inhibitors occurs through hydrophobic interactions and/or hydrogen bonding with the amino acids Cys51, Leu50, Arg102, Arg131, Leu67, Val62, and Gln107, that are of great importance for protein inhibition. HNK interacts with E6 through hydrophobic interactions with the amino acids Val31, Leu50, Val53, and Val62 (Figure S12A and Table S5) and two hydrogen bonds with the amino acids Ser74 and Gln107. Among these interactions, three of them are with the amino acids cited by Kolluru et al. (2019). In relation to podophyllotoxin, the interaction with E6 protein occurs mainly via hydrophobic interactions with the amino acids Leu50, Cys51, and Val53 (Figure S12 B and Table S5). Two hydrogen bonds are visualized between podophyllotoxin and the E6 protein with the amino acids Cys51 and Gln107.

These interactions are also relevant for E6 inhibition. Studies with MDM2 protein showed that its inhibition occurs from interaction of the inhibitors with the key residues Leu54, Leu57, Gly58, Ile61, Met62, Val93, His96, Ile99, and Tyr100 (Atatreh et al. 2018). HNK interacts with MDM2 through hydrophobic interactions with the amino acids Leu54, Ile61, Met62, Try67, Val93, Ile99, and Tyr100, which are among the most relevant amino acids involved in protein inhibition. One hydrogen bond between HNK and Leu54 is also observed (Figure S13 and Table S6).
The interaction between the HNK compound and p53 protein is related to two hydrophobic interactions and one hydrogen bond. The HNK molecule interacts with the amino acids Arg248 and Arg273, from hydrophobic interactions (Figure S14A and Table S7). Besides, there is a hydrogen bond between HNK and Arg248. The podophyllotoxin compound interacts with p53 protein through one hydrophobic interaction, one π interaction, and three hydrogen bonds. The first two interactions are related to amino acid Arg248 (Figure 14B and Table S7). The hydrogen bonds between the podophyllotoxin and p53 protein involve the amino acids Arg248 and Arg273. Importantly, the interaction with the amino acid Arg248 appears essential since it is the most frequently mutated residue of p53 in human cancers (Kamaraj and Bogaerts 2015).

Overall, it is important to highlight that the total free energy change (dG) related to podophyllotoxin binding and, mainly, HNK structure to E6 and MDM2 proteins, are more favorable relative to p53 protein binding. Furthermore, HNK is preferably stabilized when interacting with E6 rather than the MDM2 protein. On the other hand, podophyllotoxin shows similar values of dG relative to E6 and MDM2 protein binding. Importantly, HNK is largely stabilized when it interacts with E6 and MDM2 when compared to the podophyllotoxin molecule. In addition, in the interaction between podophyllotoxin and p53, larger stabilization of the ligand structure compared to the bond between HNK and p53 exists. The results from the molecular docking calculations indicate that HNK showed potential against tumoral cells. These data agree with the experimental data, where the free HNK compound promotes lower SiHa cell proliferation in comparison with podophyllotoxin and that the encapsulation in PLGA-HNK could be a promising way to potentiate HNK effects.

3. Conclusions

The encapsulation of HKN into PLGA was successfully obtained. Spherical morphology of both PLGA and PLGA-HNK formulations was demonstrated. The presence of HNK in the PLGA system led to an increase of glass transition temperature and melting point of the polymer, suggesting interactions of HNK and the PLGA system. HNK encapsulation in PLGA generated stable particles that increased HNK cytotoxicity against SiHa cells compared to free HNK. The possible action mechanism of HNK activity against SiHa cells may be through bonding with E6 and MDM2 proteins. These results, although preliminary, are promising and open possibilities for future evaluations of this formulation in other in vitro and in vivo studies.

Disclosure statement

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